JOURNAL OF CHROMATOGRAPHY LIBRARY - volume 69B

chromatography 6th edition

fundamentals and applications of chromatography and related differential migration methods

part B: applications

edited by
E. Heftmann

JOURNAL OF CHROMATOGRAPHY LIBRARY — volume 69B

chromatography 6th edition

fundamentals and applications of chromatography and related differential migration methods

part B: applications

This page is intentionally left blank

JOURNAL OF CHROMATOGRAPHY LIBRARY — volume 69B

chromatography 6th edition

fundamentals and applications of chromatography and related differential migration methods

part B: applications

edited by

E. Heftmann

2004

ELSEVIER

Amsterdam – Boston – Heidelberg – London – New York – Oxford – Paris
San Diego – San Francisco – Singapore – Sydney – Tokyo

ELSEVIER B.V.	ELSEVIER Inc.	ELSEVIER Ltd	ELSEVIER Ltd
Sara Burgerhartstraat 25	525 B Street, Suite 1900	The Boulevard, Langford Lane	84 Theobalds Road
P.O. Box 211, 1000 AE Amsterdam	San Diego, CA 92101-4495	Kidlington, Oxford OX5 1GB	London WC1X 8RR
The Netherlands	USA	UK	UK

First edition 2004

Library of Congress Cataloging in Publication Data
A catalog record is available from the Library of Congress.

British Library Cataloguing in Publication Data
A catalogue record is available from the British Library.

ISBN: 0-444-51108-3
Set ISBN: 0-444-51106-7
ISSN: 0-301-4770

♾ The paper used in this publication meets the requirements of ANSI/NISO Z39.48-1992 (Permanence of Paper). Printed in The Netherlands.

TO BIBI

This page is intentionally left blank

CONTENTS

This page is intentionally left blank

List of Authors

Francesca Antonucci, Department of Agricultural & Industrial Biotechnologies, Università di Verona, Strada le Grazie 15, I-37134 Verona, Italy, Fax: (39-458)027-901, e-mail: antonucci@sci.univr.it

Yoshinobu Baba, Department of Medicinal Chemistry, Faculty of Pharmaceutical Chemistry, University of Tokushima, School of Medicine, 78-1 Shomachi, Tokushima 770-8505, Japan, Fax: (81-88)633-9507, e-mail: ymttbaba@ph.tokushima-u.ac.jp

Damià Barceló, Environmental Chemistry Department, IIQAB-CSIC, c/Jordi Girona, 18-26, E-08034 Barcelona, Spain, Fax: (34-93)204-5904, e-mail: dbcqam@cid.csic.es

Maciej J. Bogusz, Department of Pathology & Laboratory Medicine, King Faisal Specialist Hospital and Research Center, P. O. Box 3354, MBC #10, Riyadh 11211, SAUDI ARABIA, Fax: (966-1)442-4280, e-mail: mbogusz@web.de

Günther K. Bonn, Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria, Fax: (43-512)507-2943, e-mail: guenther.bonn@uibk.ac.at

Alessandra Bossi, Department of Agricultural & Industrial Biotechnologies, Università di Verona, Strada le Grazie, Cà Vignal, I-37134 Verona, Italy, Fax: (39-045)802-7929, e-mail: bossi@sci.univr.it

Angel Carracedo, Institute of Legal Medicine, University of Santiago de Compostela, E-15705 Santiago de Compostela, Spain, Fax: (34-981)580-336, e-mail: apimlang@usc.es

Massimo Castagnola, Istituto di Biochimica e Biochimica Clinica, Facoltà di Medicina e Chirurgia, Università Cattolica del Sacro Cuore, Largo F. Vito 1, I-00168 Rome, Italy, Fax: (39-06)305-3598, e-mail: massimo.castagnola@icrm.cnr.it

Shirley C. Churms, Department of Chemistry, University of Cape Town, Private Bag, Rondebosch 7701, South Africa, Fax: (21)689-7499, e-mail: churms@science.uct.ac.za

Ethel Eljarrat, Environmental Chemistry Department, IIQAB-CSIC, c/Jordi Girona 18-26, E-08034 Barcelona, Spain, Fax: (34-93)204-5904, e-mail: eeeqam@cid.csic.es

Paul R. Haddad, School of Chemistry, University of Tasmania, Private Bag 75, Hobart, Tasmania 7001, Australia, Fax: (61-3)6226-2858, e-mail: paul.haddad@utas.edu.au

Mahmoud Hamdan, Computational, Analytical and Structural Sciences, Centro Ricerche Glaxo S.p.A., Via Fleming 4, I-37135 Verona, Italy, Fax: (39-45)921-8196, e-mail: mh12706@gsk.com

Erich Heftmann, P.O. Box 928, Orinda, CA 94563, United States, Fax: (925)254-7754, e-mail: chromatography@comcast.net

Alois Jungbauer, Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria, Fax: (43-1)369-7615, e-mail: alois.jungbauer@boku.ac.at

Adamantia Kampioti, Environmental Chemistry Department, IIQAB-CSIC, c/Jordi Girona 18-26, E-08034 Barcelona, Spain, Fax: (34-93)204-5904, email: akaqam@cid.csic.es

Agnieszka Kraj, Faculty of Chemistry, Jagellonian University, Ingardena 3, P-30-060 Kraków, Poland, Fax: (48-12)292-7949, e-mail: sciubisz@chemia.uj.edu.pl

Arnis Kuksis, Banting & Best Department of Medicinal Research, University of Toronto, 112 College Street, Toronto Ont. M5G IL6, Canada, Fax: (416)978-8528, e-mail: arnis.kuksis@utoronto.ca

Silvio Lippa, Istituto di Biochimica e Biochimica Clinica, Facoltà di Medicina e Chirurgia, Università Cattolica del Sacro Cuore, Largo F. Vito 1, I-00168 Rome, Italy, Fax: (39-06)305-3598, e-mail: s.lippa@rm.unicatt.it

Maria José Lopez de Alda, Department of Environmental Chemistry, IIQAB-CSIC, c/Jordi Girona, 18-26, E-08034 Barcelona, Spain, Fax: (34-93)204-5904, e-mail: mlaqam@cid.csic.es

Christine Machold, Department of Biotechnology, University of Natural Resources and Applied Life Sciences, Muthgasse 18, 1190 Vienna, Austria, Fax: (43-1)369-7615, e-mail: c.machold@iarn.boku.ac.at

Irene Messana, Department of Sciences Applied to Biosystems, Cagliary University, Complesso Universitario di Monserrato, I-09042 Cagliary, Italy, Fax: (39-070)675-4523, e-mail: i.messana@ unica.it

Mira Petrovic, Department of Environmental Chemistry, IIQAB-CSIC, c/Jordi Girona, 18-26, E-08034 Barcelona, Spain, Fax: (34-93)204-5904, e-mail: mpeqam@cid.csic.es

Pier Giorgio Righetti, Department of Agricultural & Industrial Biotechnologies, Università di Verona, Strada le Grazie, Cà Vignal, I-37134 Verona, Italy, Fax: (39-045)802-7929, e-mail: piergiorgio.righetti@univr.it

Kevin Robards, School of Science and Technology, Charles Sturt University, Locked Bag 588, Wagga Wagga, NSW 2678, Australia, Fax: (61-2)6933-2737, e-mail: krobards@csu.edu.au

Jerzy Silberring, Faculty of Chemistry, Jagellonian University, Ingardena 3, P-30-060 Kraków, Poland, Fax: (48-12) 292-7949, e-mail: silber@chemia.uj.edu.pl

Günther Stecher, Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University Innsbruck, Innrain 52a, A-6020 Innsbruck, Austria, Fax: (43-512)507-2943, e-mail: Guenther.Stecher@uibk.ac.at

Barbara Verzola, Department of Agricultural & Industrial Biotechnologies, Università di Verona, Strada le Grazie 15, I-37134 Verona, Italy, Fax: (39-045)802-7929, e-mail: verzola@sci.univr.it

Ian D. Wilson, Department of Drug Metabolism & Pharmacokinetics, AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, Great Britain, Fax: (44-1625)583-074, e-mail: ian.wilson@astrazeneca.com

Lihua Zhang, National Chromatographic R & D Centre, Dalian Institute of Chem. Physics, Chinese Academy of Sciences, 161 Zhongshan Road, 116011 Dalian, Liaoning, China, Fax: (86-411)369-3459, e-mail: lihuazhang@ms.dicp.ac.cn

Cecilia Zuppi, Istituto di Biochimica e Biochimica Clinica, Facoltà di Medicina e Chirurgia, Università Cattolica del Sacro Cuore, Largo F. Vito 1, I-00168 Rome, Italy, Fax: (39-06)305-3598, e-mail: c.zuppi@rm.unicatt.it

This page is intentionally left blank

List of Abbreviations

A

A	ampere, adenine, amphetamine
Å	ångstrom = 10^{-8} cm
AA	amino acids, arachidonic acid
AAEE	acryloylaminoethoxyethanol
AAS	atomic absorption spectrometry
ABEE	4-aminobenzoic acid ethyl ester
AC	alternating current, acetylcodeine, affinity chromatography
Ac	acetyl
AcP	acyl phosphatase
ADCC	antibody-dependent cellular cytotoxicity
ADME	absorption, distribution, metabolism, and excretion
AED	atomic-emission detector
AEDA	aroma extract-dilution analysis
AEO	alcohol ethoxylates
AEOC	2-(9-anthryl)ethyl chloroformate
AES	atomic emission spectrometry
AFS	atomic fluorescence spectrometry
ag	attogram = 10^{-18} g
AGP	acid glycoprotein
AIA	Analytical Instrument Association
Ala	alanine
AMAC	2-aminoacridine
AMD	automated multiple development
amol	attomol = 10^{-18} mol
AMS	accelerator mass spectrometer
amu	atomic mass units
ANTS	aminonaphthalene-1,3,6-trisulfonic acid
ANDI	analytical-data interchange
AP	alkylphenols
APCI	atmospheric-pressure chemical ionization
APD	avalanche photodiode
APEC	alkylphenoxy carboxylates
APEO	alkylphenol ethoxylates
API	atmospheric-pressure ionization
APOC	1-(9-anthryl)-2-propyl chloroformate
aq.	aqueous
AQC	6-aminoquinolyl-*N*-hydroxy-succinimidyl carbamate
ARC	acridone-*N*-acetyl

Arg	arginine
ASB	amidosufobetaine
ASE	accelerated solvent extraction
Asn	asparagine
Asp	aspartate
atm	atmosphere = 1 bar = 760 torr = *ca*. 14.7 psi = 10^5 Pa
AUC	area under the curve

B

bar	atmosphere = *ca*. 14.7 psi
BBP	butylbenzyl phthalate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BDB	benzoxodioxoazolylbutanamine
BDE	brominated diphenyl ethers
BGE	background electrolyte
BHT	2,6-di-*t*-butyl-*p*-cresol (butylated hydroxytoluene)
BN-chamber	Brenner-Niederwieser chamber
BP	buprenorphine
bp	base pair
BPA	bisphenol A
BrNP	brominated nonylphenol
BrNPEC	brominated nonylphenol carboxylates
BrNPEO	brominated nonylphenol ethoxylates
BSA	bovine serum albumin
BTEX	benzene, toluene, ethylbenzene and xylenes
Bu	butyl
BZE	benzoylecgonine
BZITC	benzyl isothiocyanate

C

C	centigrade, celsius, cytosine, codeine
CA	carrier ampholyte
CAD	computer-assisted design
CAE	capillary array electrophoresis
CAGE	capillary affinity gel electrophoresis
cap	capillary
CAPEC	dicarboxylated alkylphenol ethoxylate
CB	chlorinated biphenyls
CBD	cannabidiol
CBN	cannabinol
CBQ	3-(*p*-carboxybenzoyl)quinoline 2-carboxaldehyde
CBQCA	3-(4-carboxybenzoyl)-2-quinoline carboxyaldehyde
CCD	chemical composition distribution
CCD	charge-coupled device
CCLC	column/column liquid chromatography
CD	cyclodextrin
CD	circular dichroism
CD	continuous development
CDEA	coconut diethanolamide

CDICT	(1*R*,2*R*)-*N*-[(2-isothiocyanato)cyclo-hexyl]-6-methoxy-4-quinolinylamide
CE	capillary electrophoresis, cholesterol esters
CEC	capillary electro(kinetic)chromatography
CFD	computational fluid dynamics
CFLSI-MS	continuous-flow-liquid secondary-ion mass spectrometry
CGE	capillary gel electrophoresis
CHARM	combined hedonic and response measurement
CHO	Chinese hamster ovarian (cells)
CI	chemical ionization
CID	collision-induced dissociation
cIEF	capillary isoelectric focusing
CINP	chlorinated nonylphenol
CINPEC	chlorinated nonylphenol carboxylates
CINPEO	chlorinated nonylphenol ethoxylates
CIS	coordinated ion spray
CLC	column liquid chromatography, conjoint liquid chromatography
CLEC	chiral ligand exchange chromatography
CLND	chemiluminescent nitrogen detector
CM	carboxymethyl
cm	centimeter $= 10^{-2}$ m
CMA	carbazole-*N*-(2-methyl)acetyl
CMC	critical micelle concentration
COC	cocaine
conc.	concentrated
CRA	carbazole-9-acetyl
CRF	charge-remote fragmentation
CRMV	collagenase-released matrix vesicle
CRP	carbazole-9-propionyl
CSF	cerebrospinal fluid
CSGE	conformation-sensitive gel electrophoresis
CSP	chiral stationary phase
CTAB	cetyltrimethylammonium bromide
CTAC	cetyltrimethylammonium chloride
Cys	cysteine
CZE	capillary zone electrophoresis

D

2-D	2-dimensional
Da	dalton
dabsyl	4-dimethylaminoazobenzene 4′-sulfonyl chloride
DABTH	4,4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate
DAD	diode-array detector
DAG	diacylglycerols
DAM	diacetylmorphine
DANI	1,3-diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate
DAR	digital autoradiography
DAT	diacetyl-L-tartaric anhydride
DATS	dialkyltetralinesulfonate
DBD	degree-of-branching distribution

DBP	dibutylphthalate
DBT	dibenzoyl-L-tartaric anhydride
DC	direct current
DCCC	droplet counter-current chromatography
DCM	dichloromethane
DCP	direct-current plasma
DDT	dichlorodiphenyltrichloroethane
DEAE	diethylaminoethyl
DEHP	di(2-ethylhexyl)phthalate
DEP	diethylphthalate
DEP	di-electrophoresis
des	desamido
DGGE	denaturing gradient gel electrophoresis
DHA	docosahexaenoic acid
DHB	2,5-dihydroxybenzoic acid
DHC	dihydrocodeine
DHET	dihydroxyeicosatrienoic acid
DHM	dihydromorphine
DHPLC	denaturing HPLC
disc	discontinuous
DLS	dynamic light scattering
DMA	dimethylaniline
DMALS	depolarization multi-angle light scattering
DMOX	2-alkenyl-4,4-dimethyloxazolines
DMP	dimethylphthalate
DMSO	dimethylsulfoxide
DMT	dimethoxytrityl
DNA	deoxyribonucleic acid
DNB	dinitrobenzoyl
DnOP	di-*n*-octylphthalate
DNP	dinitrophenyl
DNPH	dinitrophenylhydrazine
DNPU	dinitrophenylurethane
DNS	5-dimethylaminonaphthalene-1-sulfonyl (dansyl)
DNT	dinitrotoluene
dNTP	deoxynucleoside triphosphate
DOC	dissolved organic carbon
DP	degree of polymerization
dpm	disintegrations per minute
DPPP	diphenyl-1-pyrenylphosphine
DRIFT	diffuse-reflectance Fourier transform
dsDNA	double-stranded DNA
DTAB	dodecyltrimethylammonium bromide
DTDP	dithiodipyridine
DTDP	3-(4,6-dichloro-1,3,5-triazinylamino)-7-dimethylamino-2-methylphenazine
DTE	dithioerythrol
DTPA	diethylenetriaminepentaacetic acid
DTT	dithiothreitol
DVB	divinylbenzene

E

EAD	electro-antennographic detection
EAG	electro-antennograph
EC	electrokinetic chromatography
ECD	electron-capture detector
ECG	ecgonine
ECN	equivalent carbon number
EDC	endocrine-disrupting compounds
EDDP	2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine
EDTA	ethylenediaminetetraacetic acid
ee	enantiomeric excess
EGDN	ethyleneglycol dinitrate
EI	electron ionization, electron impact
EIC	electrostatic ion chromatography
EKC	electrokinetic capillary chromatography
ekd	electrokinetically driven
ELISA	enzyme-linked immunosorbent assay
ELSD	evaporative light-scattering detector
em	emission
EMDP	2-ethyl-5-methyl-3,3-diphenyl-1-pyrrolidine
EME	electrostatic/magnetic/electrostatic geometry
EME	ecgonine methyl ester
EOF	electro-osmotic flow
EPA	eicosapentaenoic acid
EPA	Environmental Protection Agency
EPC	electro planar chromatography
EPF	zero electro-osmotic flow
EPO	erythropoietin
EpPUFA	epoxypolyunsaturated fatty acids
Eqn.	Equation
ERIN	electrochemically regenerated ion neutralizer
ESI	electrospray ionization
ESR	electron-spin resonance
Et	ethyl
ET	electrothermal
ET	energy transfer
EU	endotoxin unit
eV	electron volt
EVB	ethylvinylbenzene
ex	excitation

F

FVIII	blood coagulation Factor VIII
FA	fatty acids
FAAS	flame atomic absorption spectrometry
FAB	fast atom bombardment
FACE	fluorophore-assisted carbohydrate electrophoresis
FACS	fluorescence-activated cell sorting

FAD	full adsorption/desorption
FAME	fatty acid methyl esters
FCSE	fully concurrent solvent evaporation
FD	field desorption
FDA	Food & Drug Administration
FDAA	1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent)
FFA	free fatty acids
FFPC	forced-flow planar chromatography
fg	femtogram $= 10^{-15}$ g
FIA	flow injection analysis
FID	flame-ionization detector
Fig.	Figure
FLD	fluorescence detection
FLEC	1-(9-fluorenyl)-ethyl chloroformate
FMOC	9-fluorenylmetyl chloroformate
fmol	femtomol $= 10^{-15}$ mol
FPD	flame-photometric detector
FPLC	Fast Protein Liquid Chromatography
FRES	forward recoil spectrometry
FS	fused silica
FT	Fourier transform
ft.	foot = 30.48 cm
FTH	fluorescein isothiocyanate
FT-ICR-MS	Fourier-transform ion-cyclotron resonance mass spectrometry
FTID	flame-thermionic ionization detector

G

g	gram
G	guanine
GABA	gamma-aminobutyric acid
GBL	gamma-butyrolactone
GC	gas chromatography
GC × GC	two-dimensional gas chromatography
GD	glow discharge
GH	growth hormone
GHB	gamma-hydroxybutyrate
GITC	2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranosyl isothiocyanate
GLC	gas/liquid chromatography
Gln	glutamine
Glu	glutamate
Gly	glycine
GM	glycidylmethacrylate
GPC	gel permeation chromatography
GPEC	gradient polymer elution chromatography
GPL	glycerophospholipid

H

h	hour(s)
HA	heteroduplex analysis
Hb	hemoglobin
HDC	hydrodynamic chromatography
HDL	high-density lipoproteins
HEC	hydroxyethylcellulose
HEDTC	*bis*(2-hydroxyethyl)dithiocarbamate
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid
HETP	height equivalent to a theoretical plate
HFB	hexafluorobenzoyl
HFBA	heptafluorobutyric acid
HFBAA	heptafluorobutyric anhydride
hGH	human growth hormone
HGP	human genome project
HIBA	α-hydroxyisobutyric acid
HIC	hydrophobic-interaction chromatography
HILIC	hydrophilic-interaction chromatography
His	histidine
HMA	hexamethonium
HPAEC	high-performance anion-exchange chromatography
HPC	hydroxypropylcellulose
HPCE	high-performance capillary electrophoresis
HPLC	high-performance liquid chromatography
HPMC	hydroxypropylmethylcellulose
HPODE	hydroperoxyoctadecadienoic acid
HPSEC	high-performance size-exclusion chromatography
HPTLC	high-performance thin-layer chromatography
HQS	8-hydroxyquinolinesulfonate
HRGC	high-resolution gas chromatography
HRMS	high-resolution mass spectrometry
HS	headspace
HSA	human serum albumin
HSGC	high-speed gas chromatography
HTA	hexdecyltrimethylammonium
HTS	high-throughput screening
Hyp	hydroxyproline
Hz	hertz

I

IC	ion chromatography
ICAT	isotope-coded affinity tag
ICP	inductively coupled plasma
ICR	ion cyclotron resonance
ID	internal diameter
IDA	iminodiacetic acid
IDA	1-methoxycarbonylindolizine 3,5-dicarbaldehyde
IEC	ion-exchange chromatography

IEF	isoelectric focusing
IgG	immunoglobulin G
Ile	isoleucine
IMAC	immobilized-metal-ion affinity chromatography
IMP	ion-moderated partitioning
in.	inch = 2.54 cm
InsP	inositol phosphate
IP	ion pair
IPC	isopycnic centrifugation
IPG	immobilized pH gradient
IR	infrared, isotope ratio
ISEC	inverse size-exclusion chromatography
ITMS	ion-trap mass spectrometry
ITP	isotachophoresis

K

K	kelvin
kbp	kilobase pair = 10^3 base pairs
kDa	kilodalton = 10^3 daltons
KDO	3-deoxy-D-*manno*-octulosonic acid
kPa	kilopascal = 10^3 pascal

L

L	liter, lambert
LALLS	low-angle laser-light scattering
LAS	linear alklbenzenesulfonate
LC	liquid chromatography
LC-CAP	liquid chromatography at the critical adsorption point
LC-CC	liquid chromatography at the critical condition
LCM	laser capture microdissection
LCR	ligase chain reaction
LDL	low-density lipoproteins
LEC	ligand-exchange chromatography
Leu	leucine
LIF	laser-induced fluorescence
LIMS	laboratory information management system
LINAC	linear accelerating high-pressure collision cell
L/L	liquid/liquid
LLC	liquid/liquid chromatography
LLE	liquid/liquid extraction
LOD	limit of detection
LOQ	limit of quantification
LOSI	limit of spectroscopic identification
LPA	linear polyacrylamide
LPA	lysophosphatidic acid
LRMS	low-resolution mass spectrometry
LSC	liquid/solid chromatography
LSD	light-scattering detector

LSIMS	liquid secondary-ion mass spectrometry
Lys	lysine

M

M	molar
m	meter
μ	micro
μLC	micro liquid chromatography
MA	methamphetamine
mA	milliampere $= 10^{-3}$ A
μA	microampere $= 10^{-6}$ A
MACS	magnetic-activated cell sorting
MADGE	microplate-array diagonal gel electrophoresis
MAE	microwave-assisted extraction
MAG	monoacylglycerol
MALDI	matrix-assisted laser desorption ionization
MALS	multi-angle light scattering
MAM	monoacetylmorphine
MBBr	monobromobimane
MBDB	*N*-methylbenzoxodialolylbutanamine
Mbp	megabase pair $= 10^6$ bp
M-chamber	micro-chamber
MC	methylcellulose
MCA	metal chelate affinity
MCIC	metal chelate interaction chromatography
MCT	mercury-cadmium telluride
MD	multi-dimensional
MDA	methylenedioxyamphetamine
MDEA	methylenedioxyethylamphetamine
MDGC	multi-dimensional gas chromatography
MDMA	methylenedioxymethamphetamine
MDMAES	mono(dimethylaminoethyl)succinyl
Me	methyl
MEC	micellar electrochromatography
MEEKC	micro-emulsion electrokinetic chromatography
MEKC	micellar electrokinetic capillary chromatography
MEMS	micro-electromechanical system
MEP	4-mercaptoethylpyridine
MES	morpholinoethane sulfonate
Met	methionine
meq	milliequivalent $= 10^{-3}$ equivalent
μeq	microequivalent $= 10^{-6}$ equivalent
MG	morphine glucuronide
MIMS	membrane-introduction mass spectrometry
min	minutes
MIP	microwave-induced plasma, molecularly imprinted polymer
MISPE	molecular imprint solid-phase extraction
mg	milligram $= 10^{-3}$ g
mL	milliliter $= 10^{-3}$ L

μL	microliter = 10^{-6} L
mm	millimeter 10^{-3} M
μm	micrometer = 10^{-6} m
m*M*	millimolar 10^{-3} *M*
μmol	micromol = 10^{-6} mol
MMS	micromembrane suppressor
MOPS	3-(*N*-morpholino)propanesulfonic acid
MP	medium pressure
MPa	megapascal = 10^{6} Pa
MP	methyl prednisolone
MPS	methyl prednisolone hemisuccinate
MRA	mass-rate attenuator
MRM	multiple-reaction monitoring
mRNA	messenger RNA
MS	mass spectrometry
MS^n	multiple mass spectrometry
MSA	methanesulfonic acid
msec	millisecond = 10^{-3} sec
MS/MS	tandem mass spectrometry
MS^n	multiple mass spectrometry
MSPD	matrix solid-phase dispersion
μTAS	micro total analysis system
MTBA	methyl tetrabutyl ether
MUX	multiplex
mV	millivolt = 10^{-3} V
mW	milliwatt = 10^{-3} W
mw	molecular weight
MWD	molecular mass distribution
mu	mass units

N

N	normal
nA	nanoampere = 10^{-9} ampere
NADP	nicotinamide adenine dinucleotide phosphate
NBP	norbuprenorphine
NC	norcodeine
NCA	*N*-carboxyanhydride
N-chamber	normal chamber
NCI	negative chemical ionization
NDA	naphthalene 2,3-dicarboxaldehyde
NEFA	nonesterified fatty acids
NG	nitroglycerin
ng	nanogram = 10^{-9} g
NICI	negative-ion chemical ionization
nL	nanoliter = 10^{-9} L
nm	nanometer = 10^{-9} m
NM	normorphine
NMIFA	non-methylene-interrupted fatty acids
nmol	nanomol = 10^{-9} mol

NMR	nuclear magnetic resonance
NOM	natural organic matter
NP	nonylphenol
NP	normal-phase
NPC	normal-phase chromatography
NPEC	nonylphenol carboxylates
NPEO	nonylphenol ethoxylates
NPD	nitrogen/phosphorus detector
NPLC	normal-phase liquid chromatography
NSAID	non-steroidal anti-inflammatory drugs
NSIC	non-suppressed ion chromatography
NTA	nitrilotriacetic acid

O

O	olfactometry
oaTOF-MS	orthogonal-acceleration time-of-flight mass spectrometry
OCEC	open-channel electrochromatography
OD	outside diameter
OP	octylphenol
OPA	*o*-phthaldialdehyde
OPEC	octylphenol carboxylates
OPEO	octylphenol ethoxylates
OPLC	overpressured-layer chromatography
OQ	operational qualification
ORM	overlapping resolution mapping
OT	open-tubular
OVM	ovomucoid

P

Pa	pascal $= 10^{-5}$ bar
pA	picoampere $= 10^{-12}$ A
PAD	pulsed-amperometric detector
PAE	phthalate esters
PAF	platelet-activating factor
PAGE	polyacrylamide gel electrophoresis
PAS	photoacoustic spectrometry
PAH	polycyclic aromatic hydrocarbons
PAN	4-(2-pyridylazo)naphthol
PANI	polyaniline
PAR	4-(2-pyridylazo)resorcinol
PATRIC	position- and time-resolved ion counting
PB	particle beam
PBA	phenylboronic acid
PBD	poly(butadiene)
PBDE	polybrominated diphenyl ethers
PBS	phosphate-buffered saline
PC	planar chromatography, personal computer, poly(bisphenol A carbonate)
PCA	principal component analysis
PCB	poly(chlorinated biphenyls)

PCDD	poly(chlorinated dibenzo-*p*-dioxins)
PCDF	poly(chlorinated dibenzofurans)
PCI	positive chemical ionization
PCR	post-column reagent, polymerase chain reaction
PCS	photon correlation spectroscopy
PCSE	partially concurrent solvent evaporation
PD	polydispersity
pd	pressure-driven
PDA	photodiode array
PDD	pulse-discharge detector
PDDAC	poly(diallyldimethylammonium) chloride
PDECD	pulsed-discharge electron-capture detector
PDMA	poly(*N,N*-dimethylacrylamide)
PDMS	poly(dimethylsiloxane)
PDPID	pulsed-discharge photoionization detector
PEC	pressurized capillary electrochromatography
PED	pulsed-electrochemical detector
PEEK	poly(ether ethyl ketone)
PEG	poly(ethylene glycol)
PEI	poly(ethylene imine)
PEMA	poly(ethylmethacrylate)
PEO	poly(ethylene oxide)
PETN	pentaerythritol tetranitrate
PFB	pentafluorobenzoyl
PFE	pressurized-fluid extraction
PFPD	pulsed flame-photometric detector
pg	picogram $= 10^{-12}$ g
PGC	porous graphitic carbon
Ph	phenyl
PHB	poly(3-hydroxybutyrate)
PHBV	poly(3-hydroxybutyrate-co-3-hydroxyvalerate)
Phe	phenylalanine
PI	polyisoprene
pI	isoelectric point
PIBM	poly(isobutylmethacrylate)
PICES	passive *in situ* concentration/extraction sampler
PICI	positive-ion chemical ionization
PID	photoionization detector
pL	picoliter $= 10^{-12}$ L
PLE	pressurized-liquid extraction
PLOT	porous-layer open-tubular
PMA	poly(methacrylate)
PMD	programmed multiple development
PMMA	poly(methylmethacrylate)
pmol	picomol $= 10^{-12}$ mol
PMP	1-phenyl-3-methyl-5-pyrazolone
PMT	photomultiplier tube
PNB	*p*-nitrobenzylhydroxylamine
POP	persistent organic pollutants

ppb	parts per billion $= 10^{-9}$ parts
PPCP	pharmaceuticals and personal-care products
ppm	parts per million $= 10^{-6}$ parts
ppt	parts per trillion $= 10^{-12}$ parts
PPO	poly(propylene oxide)
ppq	parts per quadrillion $= 10^{-15}$ parts
Pr	propyl
Pro	proline
PrP	prion protein
PS	polystyrene
Ps	phosphatides
psi	pounds per square inch = 51.77 torr
PtdCho	phosphatidylcholine
PtdIns	phosphatidylinositols
PTFE	poly(tetrafluoroethylene)
PTH	phenylisothiocyanate
PTV	programmed-temperature vaporizer
PUFA	polyunsaturated fatty acids
PVA	poly(vinyl alcohol)
PVAc	poly(vinyl acetate)
PVC	poly(vinyl chloride)
PVDF	poly(vinylidene fluoride)
PVP	poly(vinylpyrrolidone)

Q

QAP	quaternary ammonium phosphates
QqQ	triple quadrupole
QSAR	quantitative structure/activity relationships
QTOF-MS	quadrupole time-of-flight mass spectrometry

R

r	recombinant
RAM	restricted access medium
Ref.	Reference
RF	radiofrequency
RFLP	restriction-fragment-length polymorphism
RI	refractive index
RIA	radio-immuno assay
RID	refractive-index detector
RNA	ribonucleic acid
ROMP	ring-opening metathesis polymerization
RP	reversed-phase
RPC	reversed-phase chromatography, rotation planar chromatography
rpm	rotations per minute
RRA	radio-receptor assay
RRF	relative response factor
RSD	relative standard deviation
RT	retention time
RT-PCR	real-time polymerase chain reaction

S

S	siemens
SAMBI	α-methylbenzyl isothiocyanate
satd.	saturated
SAX	strong-anion exchange
SB	short-bed
SB	sulfobetaine
SBSE	stir-bar sorptive extraction
S-chamber	sandwich chamber
SCD	sulfur chemiluminescence detector
SDE	simultaneous steam distillation/solvent extraction
SCAN	sample concentrator and neutralizer
SCOT	support-coated open-tubular
SCX	strong-cation exchange
SD	standard deviation
SDA	strand displacement amplification
SDM	stoichiometric displacement model
SDS	sodium dodecyl sulfate
sec	seconds
SEC	size-exclusion chromatography
SEEC	size-exclusion electrochromatography
SELDI	surface-enhanced laser-desorption ionization
Ser	serine
SERS	surface-enhanced Raman spectroscopy
SFC	supercritical-fluid chromatography
SFE	supercritical-fluid extraction
SGC	solvating gas chromatography
SIC	self-interaction chromatography
SIC	suppressed-ion chromatography
SID	surface-ionization detection
SIM	single-ion monitoring, selected-ion monitoring
SIMS	secondary-ion mass spectrometry
SLAB	inter-laboratory standard deviation
SLE	solid/liquid extraction
SLM	supported-liquid membrane
SM	sphingomyelin
SMA	steric mass action
S/N ratio	signal-to-noise ratio
SNEIT	1-(1-naphtyl)-ethyl isothiocyanate
SNP	single-nucleotide polymorphism
SPC	sulfophenylcarboxylate
SPE	solid-phase extraction
SPMD	semi-permeable membrane device
SPME	solid-phase micro-extraction
SPR	surface plasmon resonance
sq.	square
SRM	selective reaction monitoring
SRS	self-regenerating suppressor
SSCP	single-strand conformation polymorphism

SSDNA	single-stranded DNA
SSO	sequence-specific oligonucleotide
STP	sewage treatment plant
STR	short tandem repeats

T

T	thymine
TAG	triacylglycerol
TAPS	3-*tris*[(hydroxymethyl)methylamino] 1-propanesulfate
TAS	total analysis system
TBA	tetrabutylammonium
TBMB	(*S*)-(+)-2-*tert*-butyl-2-methyl-1,3-benzodioxole
TBP	tributyl phosphine
TBQCA	3-(4-tetrazolbenzoyl) 2-quinolinecarboxyaldehyde
TBE	Tris/borate/EDTA
t-BOC	*N*-*tert*-butyloxycarbonyl
tert-BOOH	*tert*-butyl hydroperoxide
TCA	trichloroacetic acid
TCD	thermal conductivity detector
TCDD	tetrachlorodibenzo-*p*-dioxin
TEA	thermal-energy analyzer
TEAA	triethylammonium acetate
TEAF	triethylammonium formate
TEAP	triethylammonium phosphate
TED	triscarboxymethyl ethylenediamine
TEMED	*N,N,N′,N′*-tetramethylethylenediamine
temp.	temperature
TEPA	tetraethylenepentamine
TFA	trifluoroacetic acid
TFC	turbulent-flow chromatography
TFE	trifluoroethanol
TGGE	temperature-gradient gel electrophoresis
TGIC	temperature-gradient interaction chromatography
THC	tetrahydrocannabinol
THCA	tetrahydrocannabinolic acid
THCCOOH	11-nor-Δ^9-tetrahydrocannabinol 9-carboxylic acid
THF	tetrahydrofuran
Thr	threonine
TIC	total-ion chromatogram
TIE	toxicity identification evaluation
TID	thermionic ionization detector
TLC	thin-layer chromatography
TMAE	trimethylaminoethyl
TMAOH	trimethylammonium hydroxide
TMS	trimethylsilyl
TMSO	trimethylsiloxy
TNP	trinitrophenyl
TNT	trinitrotoluene
TOF-MS	time-of-flight mass spectrometry

TPA	tetrapentylammonium 3-[*tris*(hydroxymethyl) methylamino] 1-propanesulfate
Tris	tris(hydroxymetyl)aminomethane
TRITC	tetramethylrhodamine isothiocyanate
Trp	tryptophan
TSI	thermospray ionization
TTA	tetradecyltrimethylammonium
Tyr	tyrosine

U

U	uracil
UCC	universal calibration curve
U-chamber	ultra-micro chamber
UV	ultraviolet

V

V	volt
Val	valine
VBC	vinylbenzyl chloride
vis.	visible range
VNTR	variable number of tandem repeats
vol.	volume
2VP	2-vinylpyridine
v/v	volume-by-volume
vWF	Van Willebrand Factor

W

W	watt
WCOT	wall-coated open-tubular
w/w	weight-by-weight
WWCOT	whiskered-wall-coated open-tubular

X

XNP	halogenated nonylphenols

List of Italic Symbols

(Subscripts and superscripts are not always listed)

A	
a	activity
A_s	surface area of the stationary phase
$A^{(k)}$	surface area of phase k
B	
B_0	chromatographic permeability
b	ion-binding coefficient
C	
C	concentration
C_i	counter-ion concentration
C_m, C_M	solute concentration in the mobile phase
C_s, C_S	solute concentration in the stationary phase
COF	chromatographic optimization function
CRF	chromatographic response function
CRS	chromatographic resolution statistic
D	
D	diffusion coefficient, diffusivity, displacing ion
D_{hb}	energy of the hydrogen bond
D_M	diffusion coefficient in the mobile (gas) phase
d	dimension
d_c	diameter of the channel
d_{col}	column diameter
d_{conn}	connector diameter
d_{det}	flow-cell diameter
d_f	stationary-phase film thickness
d_p	particle diameter
E	
E	electric field strength
e	charge on an electron
F	
F	Faraday constant, net flow-rate
f/f_0	frictional ratio
G	
G	Gibbs free energy
ΔG	change in free energy
g	surface tension, activity coefficient
$g(r)$	pair distribution function
H	
H	column efficiency, plate height, enthalpy

H_{min}	minimum plate height
h	reduced plate height
I	
I	ionic strength, ionization energy
I_0	zero-order modified Bessel function
ISR	inverse sum of resolutions
J	
J	mass flux
K	
K	equilibrium constant
K_A	affinity constant
K_B	binding constant
K_D	distribution coefficient, dissociation constant
K_s	salting-out constant
k'	capacity factor (obsolete)
k	retention factor, Debye screening parameter
k_B	Boltzmann constant
k_d	rate constant for desorption
k_i	distribution coefficient
k_{sm}	rate constant for transfer from stationary to mobile phase
L	
L	length, distance
L_{col}	column length
L_{conn}	connector length
L_{det}	flow-cell length
M	
M,m	molecular mass
$M(r)$	molecular mass
M_η	viscosity-average molecular weight
M_n	number-average molecular weight
M_r	molecular radius
M_w	weight-average molecular weight
M_z	z-average molecular weight
m/z	mass-to-charge ratio
m_s	molality of the salt
m(subscript)	mobile phase
N	
N	plate number, Avogadro's number
n	number, mole number, peak capacity, molecular area
NPR	normalized product of resolutions, non-porus resin
nR_S	number of peaks resolved
P	
P,p	pressure
P'	solvent polarity parameter
p_c	critical pressure
pH_f	eluting pH
Q	
Q	ion-exchange capacity, polydispersity
Q_I	group adsorption energy
q	volume ratio
q_{chr}	net charge of a protein

R

R	radius, gas constant, response, coefficient of correlation
$R\eta$	viscosity-based Stokes radius
R_F	relative rate of migration
Rg	radius of gyration
R_S	resolution, Stokes radius (frictional coefficient)
Re	Reynolds number
RI	retention index
r	radius, radial coordinate, mass, separation distance
r(subscript)	resin phase
r_g	radius of gyration
r_{hb}	separation distance of the hydrogen bond
r_p	particle radius

S

S	sensitivity, solubility, entropy
s, S(subscript)	stationary phase
Sig	signal
S_G	saturation grade
s	molal surface tension
s_i	solvent strength
s_v	selectivity value

T

T	absolute temperature
T_C	compensation temperature, critical temperature
t	time
t_0	time zero, elution time of nonretained solute
t_{mc}	time due to micellar electrokinetic chromatography
t_R	retention time

U

U	molecular inhomogeneity, voltage, internal or potential energy
$U(r)$	sum of the internal energies
U_d	internal energy of dispersion forces
U_{hb}	internal energy of hydrogen-bonding forces
U_i	internal energy of dipole induction forces
U_{II}	internal energy of ion–ion forces
U_{Ii}	internal energy of ion-induced dipole forces
U_{Io}	internal energy of ion–dipole orientation forces
U_o	internal energy of dipole orientation forces
u	solvent velocity
$\bar{u}$	carrier flow-rate, linear velocity
u_{opt}	optimum flow-rate
$u(r)$	radial velocity

V

V	molar volume, molal volume
V_{det}	detection volume
V_{inj}	injection volume
V_m, V_M	volume of the mobile phase
V_o	interstitial volume, void volume
V_P	pore volume
V_R	retention volume
V_s, V_S	volume of the stationary phase

V_t	total volume
v	velocity, molar volume
v_{eo}	velocity due to electro-osmosis
v_{ep}	velocity due to electrode potential
v_i	total velocity
v_{mc}	velocity due to micellar electrokinetic chromatography
W	
w	weight, water solubility, zone width
w_b	width at the base
$w_{1/2}$	width at half-height
X	
X	mole fraction
x	distance
Z	
Z, z	charge, charged species
Z_e	electronic charge
Z_f	solvent front travel

List of Greek Symbols

(Subscripts and superscripts are not always listed)

α	selectivity factor, degree of dissociation, polarizability
β	phase ratio, buffering power
Δ	difference
δ	Hildebrand solubility parameter
δ_i	overall solubility polarity
δ_M	mobile-phase strength
δ_S	stationary-phase solubility parameter
ε	permittivity
ε_0	permittivity of vacuum
ε_i	intra-particle porosity, interstitial porosity
ε_r	relative permittivity, dielectric constant
ε_t	total porosity
ε_u	inter-particle porosity
ε^o	solvent strength
Φ	phase ratio
ϕ	volume fraction
θ^2	fraction of extra-column variance
Γ	preferential-interaction parameter
γ	activity coefficient, tortuosity factor
η	viscosity
φ	fraction of modifier in the mobile phase
κ	solvent velocity, Debye length
κ_o	conductance of an open capillary
κ_p	conductance of a packed capillary
κ^{-1}	Debye length
Λ	salting-in coefficient, binding capacity
λ	ionic equivalent conductance, mean free path, packing-structure constant
μ	mobility, chemical potential, dipole moment, ionic strength
μ_0	standard-state chemical potential
μ_{ep}	electrophoretic mobility
ρ	density
ρ_c	critical density
ρ_r	reduced density
σ	surface charge density, surface-tension increment
σ^2	variance
σ_i	steric factor, standard deviation
σ_P	charge density on the protein surface
σ_v	standard deviation in volume units
$\Omega\sigma$	salting-out coefficient

τ	exponential time constant
Ψ	shape factor
Ψ_B	solvent association factor
ζ	zeta potential

Foreword

A prerequisite for a meaningful study of the chemical and biological properties of a given molecule or particle is that the sample is homogeneous. If it is not, one can only determine the properties of the *mixture*, which may be of little interest. Therefore, the importance of high-resolution separation methods for both analytical and (micro)preparative purposes is obvious and, accordingly, books like this have a place in life science, pharmaceutical, medical and many other types of laboratories.

In general, forewords are focused on the importance of the particular subject the books deal with. However, chromatography is a very well-known and established separation technique and, therefore, it might be superfluous to give examples of its enormous potential. Instead, I will take another approach; namely, to emphasize the *analogies* between chromatography and other separation methods, such as electrophoresis and centrifugation, rather than to discuss the dissimilarities. A common feature of these three methods is that the separations are based on differences in the migration velocity, v, of the sample constituents. Therefore, one can understand intuitively that there exists an equation (or more) which is valid for all of these methods, provided that the migration velocity is chosen as the independent variable. One of these equations is:

$$c_j^{\alpha} \cdot v_j^{\alpha} - c_j^{\beta} \cdot v_j^{\beta} = v^{\alpha\beta}(c_j^{\alpha} - c_j^{\beta})$$

where α and β are two phases separated by a moving boundary; c_j^{α} and c_j^{β} are the concentrations of the ion j in the α and β phases, respectively; v_j^{α} and v_j^{β} are the velocities of the ion j in the α and β phases, respectively; and $v^{\alpha\beta}$ is the velocity of the moving boundary.

From the mere existence of an equation which is universal for chromatography, electrophoresis and centrifugation one can conclude that any characteristic feature of one of these methods (say electrophoresis) has a counterpart in the other methods (chromatography, centrifugation). This finding is of fundamental importance, because as soon as a new technique has been developed in one of these differential velocity-based separation methods, one should start to develop the corresponding technique for the other analogous separation methods. The more methods that are available, the easier and faster one can purify, say, a protein, which is very important in proteomics studies. Similarly, any new phenomenon observed in one of these separation methods has its analog in the other methods.

Ignorance of the analogy between different separation techniques has, unfortunately, delayed the progress of separation science and, thereby, also of life sciences and related areas. For instance, isoelectric focusing and indirect detection in electrophoresis were introduced as early as 1961 and 1967, respectively, but the chromatographic counterparts did not appear until 1977 and 1979.

Another example of lack of thinking about the analogy is the unfortunate exchange of the term displacement electrophoresis for isotachophoresis, which made Professor A.J.P. Martin, Nobel Prize Laureate and one of the pioneers in this field, dismayed. Perhaps commercial interests played also a role in this case, as they did when companies exchanged the term "continuous beds" for "monoliths". A representative of a company admitted that this new notation is linguistically not correct, but "sells" better. Another reason for the misuse of terms is the lack of theoretical knowledge. Nor is it uncommon that a new term is introduced for an already existing technique, which may mislead the reader of the paper to believe that the author is the inventor. For instance, the well-known zone sharpening in electrophoresis (enrichment of a sample by transferring it into a dilute buffer) is often erroneously called "stacking" (enrichment by displacement electrophoresis). I believe that this approach is seldom used consciously by the author to become recognized – rather it is likely that he was not aware that the method had been described earlier. However, it is common that this mode of action is consciously employed in commercial advertisements – ethically dubious tactics.

Electrochromatography has gained much in popularity since the previous edition of this series was published. One reason might be that it is often wrongly stated that the electroosmosis in a packed bed generates a plug flow and, therefore, that the resolution is considerably higher than that in conventional chromatography. A higher resolution can be obtained if the pores in the beads are large enough to create an electroosmotic flow which transports the solutes through the beads faster than does diffusion. However, the resolution cannot be higher than that obtained in beds packed with nonporous beads. Homogeneous gels yield a perfect plug flow and are from that point of view the ideal electrochromatographic medium. Electrochromatography in such gels might be the only chromatographic method that can give a resolution comparable to that obtained in electrophoresis and should, therefore, be used more frequently. However, for project-scale separations chromatography is far superior to electrophoresis.

Much effort is being devoted to improving the resolution of chromatographic media and, in some cases, one has succeeded in approaching the theoretical limit, *i.e.*, the method cannot be improved much more. In such cases one should intensify the research on *selective* chromatographic techniques, which have the advantage to permit very fast purification of both low- and high-molecular-weight substances, as well as viruses and cells, such as bacteria, also for complex samples. The recently introduced "artificial antibodies" in the form of highly selective gel granules will, no doubt, play an important role for rapid one-step isolation of a given substance or bioparticle (by affinity chromatography), as well as for clinical diagnosis, for instance for detection of biomarkers for different diseases and as the sensing element in biosensors.

With the introduction of the continuous beds (monoliths) and the homogeneous gels the prerequisite for high resolving chromatographic and electrochromatographic experiments in microchips was fulfilled. There are, accordingly, no restrictions on the choice of appropriate chromatographic, electrochromatographic or electrophoretic media. The crucial point is rather the design and the cost of the microchip. It will be interesting to see whether the hybrid microdevice will become an attractive alternative.

This year it is one hundred years since Mikhail Semenovich Tswett presented at the Meeting of Warsaw Society of Natural Scientists his famous lecture "On a New Category

of Adsorption Phenomena and Its Application in Biochemical Analysis". During the intervening years the chromatographic technique has continuously been refined and the resolution in analytical experiments is, in some modes, close to what is theoretically possible (see above), *i.e.*, the performance of the column cannot be improved significantly. The situation resembles that in electrophoresis. Therefore, to attain an ultra-high resolution one can expect a trend toward appropriate combinations of these two methods or – as discussed above – toward the use of selective adsorption.

STELLAN HJERTÉN

November 2003 *Uppsala*

This page is intentionally left blank

Preface

Although the fifth edition of this textbook, published in 1992, is still being bought, it is high time to bring out a completely revised new edition. Techniques, such as paper chromatography, countercurrent chromatography, and field-flow fractionation, have been eclipsed by more convenient, faster, more specific and sensitive methods of differential migration, and electronic technology has revolutionized every aspect of chromatography and electrophoresis. This will be the last edition of CHROMATOGRAPHY, not only because of my age, but because hard-copy books cannot compete with their electronic counterparts, which can be continuously updated and rapidly accessed.

Again, I am proud to present a star-studded cast of authors, offering their extensive knowledge of specialized areas in analytical chemistry and ample leads for further reading. I have modified their contributions to avoid overlap as much as possible and to create a more uniform style. I have deleted such expressions as "see also, *e.g.*, the reviews XY and references therein", because most readers will realize that the authors have cited only a selection of references, some of which are review articles. As an editor of the *Journal of Chromatography* I have long fought a losing battle against lab slang, *e.g.*, "hyphenated methods", but I have not yielded to widely practiced abuses of grammar, such as changing transitive verbs to intransitive forms and *vice versa* (*e.g.*, "to elute" and "to adsorb") and confusing misnomers, such as "support", or fuzzy terms, such as "phase". Combined methods are not "hyphenated"; ion-exchange chromatography and size-exclusion chromatography are hyphenated.

Each chapter has been refereed by another author of this book, and authors have checked the edited versions of their chapters, but I am ultimately responsible for the accuracy of the contents of the entire book. This includes lists of abbreviations and symbols and the subject index for the entire book. In the acronyms for the combination of methods ("hyphenation") I have used slashes instead of hyphens. All chapters were up to date in 2003, and some of the references will undoubtedly soon be outdated. Each chapter is either new or completely revised. Only five authors of the fifth edition have survived in the sixth edition. The chapters on Electrochromatography, Combined Techniques, Microfluidics, Instrumentation, Phytochemical Analysis, Forensic Applications, and Computer Resources are entirely new. Proteins are now treated in two separate chapters, one on chromatography and one on electrophoresis.

As before, this book is divided into two parts: Part A deals with fundamentals and techniques of chromatography and electrophoresis, and Part B with their applications. The lists of abbreviation and symbols and the subject index are the same in both parts. I hope this textbook will serve the novice as an introduction to the vast literature in this branch of

analytical chemistry and give the experienced research scientist a perspective of activities outside his/her own laboratory. The last chapter in each part will probably be the first to become obsolete, but these chapters are, in my opinion, the most useful introductions to the practice of chromatography and electrophoresis.

ERICH HEFTMANN
Orinda, California

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 13

Inorganic species

PAUL R. HADDAD and KEVIN ROBARDS

CONTENTS

13.1 GAS CHROMATOGRAPHY

This section examines recent developments and applications in inorganic gas chromatography (GC), mainly for the period 1992–2002. Aspects dealing with elements, binary compounds, co-ordination compounds, anions, and organometallics are covered. The reader is also referred to the previous edition of this monograph [1] which contains much information that retains its relevancy. Developments in inorganic GC generally parallel progress in column technology, detection systems, and new methods of derivatization. There have been significant shifts in emphasis since the previous monograph [1] together with a decline in the number of relevant comprehensive general reviews [2–4] and specialized reviews dealing with applications [5–7]. Reviews worthy of particular note deal with the chromatographic characteristics of aqueous solutions of inorganic electrolytes as stationary liquid phases for vapor-phase GC [8] and element-selective chromatographic detectors [9].

Gas chromatography coupled to highly selective detection systems, based on atomic (or molecular) spectrometry or mass spectrometry, provides very sensitive methods of quantitation, even for elements that are difficult to determine, such as H, C, O, Cl, Br, I, S, P, B, and Si. The advantages of these techniques are the provision of element-selective data plus time-based monitoring that allows speciation studies [9–12]. Guidelines for the definition of concepts related to speciation of elements and, more particularly, speciation analysis of chemical compounds have been provided [13].

Sanz-Medel [14] and Lobinski [15] agree that speciation is a complex and challenging problem but differ in their assessment of the state of maturity of the field. "An analysis of speciation-relevant issues leads to the conclusion that, despite the rapidly increasing number of reports, the field has reached a level of virtual stagnation in terms of research originality and market perspectives. A breakthrough is in sight but requires an advanced interdisciplinary collaboration of chemical-analysts with clinicians, ecotoxicologists, and nutritionists" [15]. Sanz-Medel [14] observes that the current state of real-life analytical speciation involves use of hybrid techniques, combining an adequate separation technique for physical species separation with element-specific detectors, such as those based on atomic spectrometry. The present demand is for "innovative chemical speciation strategies and analytical technologies." Sanz-Medel also noted the urgent plea for quality assurance in non-routine analysis.

An early review of coupled element-selective techniques by Ebdon *et al.* [16] covered the period to 1985 and contains information that remains relevant. Several more recent reviews emphasize various aspects of element-selective detection and speciation. The principles and theory of coupling of (multicapillary-column) GC [17] with microwave-induced plasma atomic-emission spectrometry (MIP-AES) [17], inductively coupled plasma mass spectrometry (ICP-MS) [17,18] and microwave-induced plasma mass spectrometry [18] have been included in a number of reviews. Alternatively, applications to speciated elements [19,20], considering environmental, petrochemical, geochemical, agricultural, and chemical fields [9] have been emphasized in reviews of element-specific detectors, such as flame atomic absorption spectrometry (F-AAS) [9,19], electrothermal atomic absorption spectrometry (ET-AAS) [9,19], MIP-AES [7,9,21], and ICP-MS [19,20].

Of the various combinations, GC/AAS [22] and GC with variants of flame photometry [1] remain the simplest to assemble but have limited application. On the other hand, MIP-AES is highly compatible with GC and, in 1990, GC/MIP-AES was the most common combination technique for the selective quantitation of inorganic and organometallic compounds [1]. The high specificity of such spectrometric detectors and their ability to operate in combination with capillary columns meant that very complex mixtures of organic, organometallic [23,24], and inorganic compounds could be monitored for a number of elements simultaneously. These properties have ensured the continued popularity of AES as a detection system [25–29]. Examples of its use are in the (simultaneous) speciation analysis of mercury, tin, and lead, an area that has attracted considerable attention [30]. The influence of interferences in such analyses, particularly those arising from matrix co-extractives and reagent impurities, has been investigated [31]. Other examples are provided by use of radiofrequency (RF) and direct-current (DC) glow discharge (GD), with AES, as detectors in capillary GC for the speciation of low levels of methylmercury, ethylmercury, and inorganic mercury [32]. GC/DC-GD-AES was applied

successfully to the determination of methylmercury in certified fish-tissue reference material after extraction with sodium diethyldithiocarbamate and Grignard derivatization.

The most significant shift in element-specific detection systems over the last decade has been the maturation of suitable "inorganic" mass-spectrometric detectors, which has facilitated various applications. The direct interfacing of a capillary gas chromatograph with a high-power MIP torch and quadrupole mass spectrometer produced an instrument capable of providing quantitative information about several elements (including C, halogens, P, S, and many transition elements) of geochemical interest [33]. Nevertheless, it is capillary GC/ICP-MS that now provides unsurpassed sensitivities for metal speciation [34–37], as seen in the detection limits for alkyltin compounds as low as 15–35 fg Sn [34]. The performance of four element-specific detectors, namely, a flame-photometric detector (FPD), a pulsed flame-photometric detector (PFPD), MIP-AES, and ICP-MS was evaluated for the speciation of butyl- and phenyltin compounds after SPME and GC separation [38]. ICP-MS was the most sensitive but PFPD, the cheapest, is also of significant interest. The analytical procedure was applied to the determination of organotin compounds in sediment and fish tissue. The combination of GC/ICP-MS with hydride generation provided an improved speciation technique for the identification and quantification of species from twelve elements: Ge, As, Se, Mo, Sn, Sb, Te, I, W, Hg, Pb, and Bi [39] with detection limits below 0.7 pg. An interesting application was the determination of dimethylselenium in human breath in the range of 80 to 980 ng m^{-3} [40].

The majority of applications of combination techniques deal with either organic compounds containing P, S, Si, B, or halogens [41] or with organometallic compounds of Hg, As, Se, Pb, and Sn. For the latter group, speciation and analyses of a broad range of sample types, including biological and environmental materials, air, water, fuels, and standard reference materials, have been carried out. Volatile compounds can be determined directly, following extraction, clean-up, and pre-concentration. Analysis by these techniques is particularly suitable for complex, volatile mixtures, *e.g.*, pyrolyzates or synthetic fuels originating from coal or shale oil, containing compounds of S, P, Se, As, and Si. Nonvolatile species can be converted to volatile alkyl, silyl, or hydride derivatives prior to analysis. Alternatively, such samples (*e.g.*, oil shales for the determination of geopophyrins) can be analyzed by combination with HPLC or SFC [42]. Purely inorganic compounds or metal chelates have been studied to a much lesser degree. Indeed, where total-element determinations are required, there may be no advantage in GC separation, as determinations can be effected spectroscopically. Thus, speciation of tin in poisoned human organs was measured by GC/AES (flame photometry) [43] while major trace metal elements in the same sample were measured directly by ICP-MS.

13.1.1 Elemental analysis

Elemental analysis for carbon, hydrogen, nitrogen, etc., by reaction GC [44] is of interest to inorganic as well as organic chemists [1]. Elemental composition obtained by GC/AES [45] provides empirical formulas with errors of a few percent, if the calibrating reference substance is closely related – by structure, elemental composition, molecular weight, and amount – to the compounds to be identified. Selectivity and sensitivity are improved [46] by using the cyanogen molecular band at 388 nm instead of the 174 nm

atomic emission line. However, response factors depend on the concentration of elements, and this affects the accuracy of the determination of empirical formulas. PFPD [47] has brought within reach the possibility of universal heteroatom-selective detection and, hence, empirical formula determination. The system exploits the dependence of the flame-chemiluminescence emission time.

Gas chromatography/elemental analysis is also used for total-element determination in a sample. For example, total sulfur was determined in a wide range of solid and liquid samples [48] following combustion and chromatographic separation by FPD. Limits of detection were, for solids, 1.2 ng S and, for liquids, 0.2 ng S. Alternatively, the GC determination of an element in its elemental form(s) represents a particular case of element speciation. Thus, the determination of residual chlorine in water [1] requires the determination of Cl_2, as distinct from aqueous chloride. Krylov *et al*. [49] reported the simultaneous determination of hydrogen, argon, oxygen, and nitrogen in volatile inorganic hydrides, following cryogenic pre-concentration. The limits of detection of these gases in hydrides (silane, germane, arsine, phosphine, hydrogen sulfide, hydrogen selenide, and ammonia) were $2 \times 10^{-6} - 3 \times 10^{-5}$ mol%. Other elements determined by GC (apart from the element gases) include P, I [1], and S. For instance, sulfur can be determined directly by GC [50] in environmental samples, coal or pyrites, although a number of peaks attributable to the molecular species $S_2 - S_{16}$ may also be observed in the chromatogram [1].

Other elements, with the exception of Hg, may be too reactive or insufficiently volatile for conventional GC. However, a variety of metallic elements (Na, K, Cs, Ba, Rh, Pd, Pt, Os, Eu, Yb, Tm, TI, Pb, Bi, Pc, Am, Cf, Fm, Md, Lr, etc.) [1,51] can be chromatographed by thermochromatography at 600–1400 K. Thermochromatography includes any GC technique that operates above the maximum temperature of conventional GC (*ca*. 400°C). Typically, this will involve the use of tube furnaces with packed or non-packed quartz, titanium, or graphite tubing as the chromatographic column. Although this technique remains unsuitable for analytical element separations, it has been useful for the characterization of heavy actinide elements [52] and does allow the volatilization and adsorption behavior of metallic elements to be studied [53]. For example, vacuum thermochromatography has provided a fast separation of short-lived nuclides of volatile elements on clean metal surfaces [54]. Using this approach, the adsorption properties of radon were investigated on polycrystalline surfaces of the transition metals Cu, Ag, Au, Ni, and Pd, and adsorption enthalpies were quantified. Moreover, the adsorption enthalpies of the super-heavy elements 112 and 114 were predicted. From the adsorption behavior of radon at zero coverage on the surface of laboratory-made ice [55] it was concluded that radon is adsorbed on the ice surface as a free atom.

13.1.2 Binary compounds

The main groups to be considered here comprise monomeric hydrides and halides sufficiently volatile to be determined by conventional GC or by thermochromatography. The gaseous species, CH_4, CO, CO_2, NH_3, and SO_2 are not discussed.

13.1.2.1 Hydrides

GC is a very selective and sensitive method for the determination of water, as previously described [1]. Such methods involve either direct measurement, usually based on chromatographic separation on porous polymers or open-tubular columns with thermal-conductivity detection, or indirect measurement by reaction GC. In the latter, water is converted to hydrogen, methane, acetylene, or other organic compounds with the advantage that more sensitive detectors, such as the FID can be used. The most widely favored [1] indirect method requires the conversion of water to acetylene [56] according to Eqn. 13.1.

$$CaC_2 + H_2O \rightarrow CaO + C_2H_2 \quad (13.1)$$

Recent applications of GC to the determination of water include the measurement of a thin film of water, deposited on an aluminum surface [57]; use of GC as a reference method for moisture determination [58]; the determination of water in solid chemicals [59] and ammonia [56]; and the simultaneous determination of dissolved gases and moisture in insulating mineral oils [60]. Other hydrides studied by GC include those of boron and elements of groups IV A, V A, VI A, and VII A. Compounds of group VII A, *i.e.*, hydrogen halides, are highly reactive and corrosive and are best converted to suitable derivatives prior to GC, although hydrogen chloride, chlorine, and sulfur dioxide have been determined in thionyl chloride by GC, using headspace analysis and direct injection into a capillary column with a thermal conductivity detector [61]. The hydrides of C, Si, Ge, and B produce extensive homologous series: alkanes, silanes, germanes, boranes, carboranes, carbosilanes, etc. Their GC characterization is exceedingly useful but is not detailed here. It is sufficient to state that such compounds can be chromatographed on conventional nonpolar or porous polymer columns at 50–300°C without difficulty. For example, the manufacture and application of organosilicon compounds, especially silanes, have increased dramatically during recent decades. This has led to a need for analytical methods in occupational exposure assessment. Thus, silanes were diluted in heptane and analyzed by capillary column GC, using flame-ionization detection with peak identity confirmation by GC/MS [62].

Hydrogen sulfide, trapped in alkaline solution, was determined by GC/FPD [63]. Phosphine finds a number of industrial uses, and methods have been described for its determination in biogas and sludge [64], stored products and processed foods [65] by methods that differed in extraction procedure and detector. Phosphine was also measured in the determination of zinc phosphide in the gastrointestinal tracts of squirrels [66]. Tissue extracts, containing zinc phosphide, were acid-hydrolyzed to produce phosphine, which was analyzed by headspace GC/FPD.

The increased interest in metalloid hydrides stems from development of the hydride generation technique and its utilization in trace analysis and speciation studies. In this technique, hydrides of inorganic or organic compounds of As, Sb, Bi, S, Se, Te, Ge, and Sn are generated in aqueous solution by treatment with sodium or potassium borohydride. The volatile hydrides so generated can be readily removed from the bulk matrix. This results in the isolation of the analyte from interferents and gives species

which can be readily concentrated by means of cryogenic trapping or sorbents and separated from other species. Careful control of reaction conditions permits simple speciation studies (*e.g.*, arsenite *vs.* arsenate) [67]. The isolated hydride can be measured spectroscopically or by GC with detection based on AAS, AES, AFS, or MS [68]. Alternatively, Tanaka *et al.* [69] developed a procedure based on the PID for simultaneously determining P, S, and As in steel with detection limits of 2, 280, and 4 ng, respectively. The volatile hydrides were collected in a liquid-nitrogen cold trap. The trap was warmed at 80°C, and the three gases were then separated by GC on a Porapak QS column. Liquid-nitrogen trapping was also used for collecting diborane, generated from boron by treatment with $LiAlH_4$ [70]. The diborane was analyzed by GC, and the method was applied to two types of eye treatment solutions and one type of contact-lens washing solution.

13.1.2.2 Halides

Halides studied by GC include those of element groups III A, IV A, V A, VI A, the interhalogens ClF_2, ClF_3, BrF_3, BrF_5, etc., and the halides of transition metals, such as Ti, V, Mo, and W [1]. Many reactive halides are utilized industrially on a large scale and, despite some problems, GC is usually the most suitable method of analysis [1]. Not all halides are highly reactive. Compounds, such as CF_4, CCl_4, C_2Cl_6, C_3Cl_8, C_4Cl_6, C_6Cl_6, and SF_6 (but not other sulfur halides) are quite stable and can be chromatographed on conventional columns without special precautions [71]. Sulfur hexafluoride is, in fact, so stable that its industrial use (*e.g.*, as a gas tracer) [72] is resulting in a steady environmental buildup that mandates the availability of reliable analytical methods for its determination. A highly sensitive GC method for determining SF_6 in air [1] was enhanced by using Fourier transformation of the data [73]. Methods for the simultaneous measurement of SF_6, O, N, CO_2, CO, and H impurities in tungsten hexafluoride [74] as well as SF_6 and chlorofluorocarbons [75] have been described. The authors inferred that SF_6 was useful for the dating of recently ventilated waters.

Halides of low volatility, including those of Zn, Cd, Mo, W, Np, Tc, Nb, Zr, Re, Os, Hg, Ir, U [1,76], and the lanthanides [1], have been analyzed at temperatures up to 1200°C by thermochromatography. This is carried out in quartz [76], graphite, or silica columns with or without suitable alkali metal halides as stationary phase, as previously described [1]. Related compounds, such as the oxochlorides of Mo, W, and Element 106 [77] and metallic oxides of Cm, Am, Pu, Np, U, Ir, Pt, Re, Tc, and Os have also been chromatographed by thermochromatography on metallic surfaces [1,78]. The sublimation behavior of the oxides was found to be strongly affected by the nature of the metal surface. Heats of adsorption and some separations were reported. GC studies [79] have provided information about thermodynamic properties (*e.g.*, adsorption enthalpies) for compounds of elements, such as the transactinides, which can only be produced on an atom-at-a-time basis. Data were also obtained on the nuclear decay properties of the isotopes of these elements.

13.1.2.3 Other binary compounds

Apart from common gases, such as CO, CO_2, N_2O, NO_2, and SO_2, other binary compounds sufficiently volatile for GC comprise mainly an odd collection of oxides (N_2O_3, N_2O_5, P_2O_3, P_2O_5, SeO_2, SeO_3, TeO_2, As_2O_3, and MoO_3), sulfides (*e.g.*, CS_2, SiS_2, N_4S_4, AsS_2, P_2S_3, P_2S_5), and selenides (C_2Se_4, Se_4S_4, Se_2S_6). Of these, only CS_2 is of common interest and has been determined at ppt levels in air by GC/MS [1].

Methods have been reported for the GC determination of related nonbinary compounds, such as mixed phosphoric acid halides in the air of working premises [80] and the simultaneous determination of cyanogen chloride and cyanogen bromide in treated water [81]. The latter involved headspace SPME, followed by GC/electron capture detection (ECD). The method gave good agreement with results from liquid/liquid micro-extraction (US EPA Method 551.1) for the analysis of spiked ultrapure and granular activated-carbon-filtered water samples.

13.1.3 Co-ordination compounds

The GC of co-ordination complexes encompasses trace determinations of metals, physicochemical measurements, the study of on-column reactions, the separation of isomers, and the utilization of complexes as components of the stationary phase for enhancing selectivity [1].

13.1.3.1 Trace determination of metals

This application area has changed little since the earlier monograph [1]. It remains true that the determination of metal complexes by GC, in the broadest sense, is difficult and often frustrating. Specific papers worth noting include the separation of the isotopes of uranium by thermochromatography of their hexafluoroacetylacetonates in the presence of ligand vapors [82]. However, GC procedures for the trace determination of entire metal groups, such as the groups I A, II A, lanthanides, and actinide elements are, simply, still nonexistent. On the other hand, elements, such as Be, Al, Cr, Co, Rh, Cu, Ni, Pd, and V can be determined individually or in small groups at concentrations which compare favorably with other sensitive methods of metal determination. A list of ligand types examined to date and the applicability of these in trace determinations is given in Table 13.1 and discussed more fully in the earlier monograph [1].

The β-diketones and their analogs (Fig. 13.1) comprise the largest and most extensively studied GC reagents for metal ions. The parent β-diketones are broad-spectrum reagents, forming complexes with nearly all metallic ions [83]. Unfortunately, methods for trace determinations based on these reagents are limited essentially to the ions in Table 13.1. Other ligand analogs of the β-diketones, such as those represented by Structures II–IV and VII, are more selective and have been applied only to the trace determination of Ni(II) [1].

Tetradentate ligands of the type represented in Structure V, due to the greater stability of the complexes, have been more successful [84]. Trace determinations with fluorinated reagents of this type have been developed for Cu(II), Ni(II), Co(II), Pd(II), and V(IV)O.

TABLE 13.1

DERIVATIZING REAGENTS FOR THE GAS CHROMATOGRAPHY OF METAL IONS

Ligand	Structure	Selectivity	Fluorinated derivatives studied	Ions detected at pg level	References
β-Diketone	I	Broad spectrum	Yes	Be(II), Al(III), Cr(III), Cr(VI), Rh(III)	[1,83]
Monothio-β-diketone	II	Class b ions	Yes	Ni(II), Pd(II)	[1]
Dithio-β-diketone	III	Class b ions	Yes		[1]
β-Ketoenamine	IV	Cu(II), Ni(II), Pd(II)	Yes		[1]
β-Ketoenamine (tetradentate)	V	Bivalent ions of co-ordination no. 4	Yes	Ni(II), Cu(II), Pd(II), Co(II), V(IV)O	[1,84]
β-Ketoenamine (hexadentate)	VI	Trivalent ions of co-ordination no. 6	No		[1]
β-Thionoenamine	VII	Class b ions	Yes	Ni(II)	[1]
β-Thionoenamine (tetradentate)	VIII	Class b bivalent ions of co-ordination no. 4	Yes		[1]
N,*N*-Dialkyldithio-carbamate	IX	Mainly class b ions	Yes	Ir(III), Ni(II), Rh(III), Co(III), Pd(II), Cr(III), Cr(VI), Pt(II), Pb(II), Hg(II)	[1,85]
O,*O*′-Dialkyldithio-phosphate	X	Class b ions	No	Zn(II), Ni(II), Pd(II)	[1]
Dialkyldithio-phosphinate	XI	Class b ions	No		[1]
Porphyrin		Bivalent ions of co-ordination no. 4	No		[1]

The ligand bis(acetylpivalylmethane)ethylenediimine [86] has been used for the determination of vanadium in rock samples. The vanadium was complexed with ligand, extracted, and separated on a capillary column from free ligand, Cu(II), Ni(II), Pd(II), and Pt(II) chelates. However, high concentrations of Fe(II) suppressed the oxovanadium(IV) response. Similarly, platinum(II) from cisplatin was complexed with

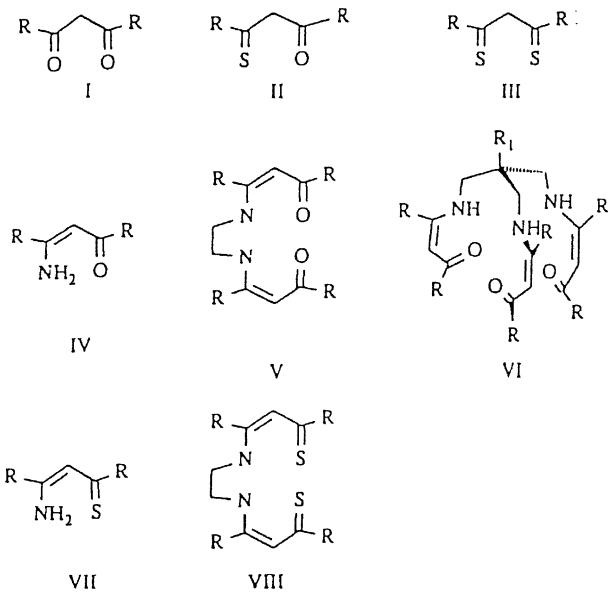


Fig. 13.1. β-Diketones and their analogs.

bis(isovalerylacetone)ethylenediimine [87] and determined in a pharmaceutical preparation and blood samples. However, complexation with the corresponding β-thionoenamines, represented by Structure VIII, and of the hexadentate Schiff bases, indicated in Structure VI, has been disappointing [1].

Another important class of derivatizing reagents comprises the sulfur donor ligands, *N*,*N*-dialkyldithiocarbamates, *O*,*O'*-dialkyldithiophosphates, and dialkyldithiophosphinates, as represented in Structures IX, X, and XI, respectively (Fig. 13.2). Complexes of related ligands, such as xanthates, thioxanthates, and dithioalkylates may also exhibit favorable GC properties. These reagents differ from the β-diketone group in the size of the chelate rings (4- rather than 6-membered). The stability of their complexes is probably due to strong $d\pi/d\pi$ metal → sulfur back-bonding. Fluorinated complexes of the dialkyldithiocarbamates (IX) have been studied by GC [88]. They exhibit greatly enhanced volatility and ECD response relative to the corresponding

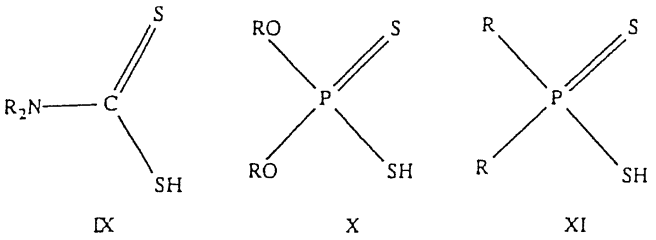


Fig. 13.2. Some derivatizing agents for GC of metal ions.

nonfluorinated complexes. Detection, at sub-nanogram levels, of the complexes of ligands IX to XI can be effected with the ECD or FPD (sulfur mode) [85]. Applications of these reagents in trace determinations include the determination of metal ions as the diethyldithiocarbamate complexes in pharmaceutical preparations [85]. On-column inter-element effects, reminiscent of the β-diketonates, are operative, and this may limit the wider application of these reagents. For instance, Te in urine was complexed by a fluorinated analog of IX but was converted by Grignard reaction to an organometallic derivative prior to GC/MS [89].

The porphyrins are an example of macrocyclic reagents that have been utilized in GC [90]. Derivatives of these reagents (metalloporphyrins) can be satisfactorily chromatographed at elevated temperatures (300–400°C) on fused-silica columns. However, studies of such complexes seem to be aimed chiefly at identifying natural metalloporphyrins in crude petroleum oils [1]. The broader application of co-ordination complexes for the trace determination of metallic species by GC is largely limited by the adverse on-column behavior exhibited by many metal complexes, even on fused-silica capillary columns. Nonideal behavior includes elevated baselines, peak-broadening and tailing, reversible and irreversible adsorption, and displacement effects. These are, in turn, attributed to a variety of factors, including homolytic and heterolytic dissociation, adsorption, chemisorption, oxidation, hydrolysis, dehydrogenation, and catalytic decomposition. These aspects were discussed in the previous monograph [1].

13.1.3.2 Co-ordination complexes as components of the stationary phase

Co-ordination complexes, when incorporated in the stationary phase, can alter the selectivity by virtue of their Lewis acidity. Such complexes can therefore be very useful for selective GC separations. The complexes can be incorporated by adsorption or chemical bonding to the support [91], they can be dispersed or dissolved in the stationary phase [92] or used as polymeric sorbents [93]. In all cases, the special interactions exploited are Lewis acid/base interactions, with the metal ion acting as the Lewis acid. In a broader sense, such specific interactions are the basis of complexation GC [1]. A number of papers describing applications of co-ordination complexes [91–96] have appeared. For instance, copper(II) chelates of Structure V have high potential for the separation of alcohols, ketones, and heteroaromatics [92]. Polysiloxane phases, modified by bonding transition-metal chlorides with cyano ($CuCl_2$ or $CoCl_2$) and thiol groups ($NiCl_2$ or $CoCl_2$), were examined in order to determine their applicability to the analysis of ethers, thioethers, and ketones [96]. Specific interactions were characterized between the bonded metal and the compound analyzed.

These stationary phase additives have also been useful for separating mixtures of racemic Lewis bases, such as ethers, ketones, and alcohols, where the center of asymmetry in the molecules is close to a Lewis base site. An example of the routine separation is provided by the separation of the eight stereoisomers of *sec*-butyloxirane, containing three chiral centres [97]. A GC phase containing the chiral chelates of europium exhibited high selectivity for nucleophilic solutes [98] and provided successful enantioseparations of selected alcohols and ketones but no separation of chiral compounds containing double

bonds and chloro-aliphatic compounds. Chiral separation was also achieved with a polysiloxane phase containing a chemically bonded chiral metal complex, derived from nickel(II) *bis*[(3-heptafluorobutanoyl)-10-methylene-(1*R*)-camphorate] [99]. A further example is the enantioseparation of chiral nucleophiles [100] on a stationary phase containing a chiral substituted tris-β-diketonate of europium(III). The contribution to solute retention was attributed to electron donation toward a metal ion and steric interactions between solute molecules and chiral ligands of the europium species. Stationary phases containing *tris*{3-[(trifluoromethyl)hydroxymethylene]camphorato}-derivatives of lanthanides were characterized [94] using the solvation parameter model. Stationary phase descriptors related to 'hydrogen-bond basicity' and 'hydrogen-bond acidity' were distinctive. The selectivity of these phases was rationalized in terms of opposing electronic and steric effects of the Lewis acid/base interactions between the chelates and chiral alcohols and ketones [101]. Separation of selected racemic alcohols and ketones was achieved, and the determined values of thermodynamic enantioselectivity were correlated with the molecular structure of the solutes studied.

13.1.3.3 Physicochemical aspects

Gas-chromatographic data for the heats of solution of volatile complexes [83] can be utilized to determine the nature of intermolecular forces experienced by co-ordination compounds in the stationary phase, as previously described [1]. Isomerization, decomposition and other reactions of volatile complexes can be studied by GC [1,102]. Among the β-diketonates, where geometrical isomerism and optical isomerism are both possible, separation of isomeric species has been reported only for the substitution-inert complexes of Cr(III), Rh(III), and Mo(III) [1]. However, even these complexes exhibit on-column isomerization at the temperatures (100–200°C) required for elution. Complexes of quadridentate Schiff bases indicated in Structure V exhibit ligand isomerism which does not result in on-column isomerization, even at elevated temperatures, and isomers of these complexes can readily be separated [84]. For example, two pairs of enantiomeric oxovanadium(IV) complexes of the fluorinated Schiff base 1,2-propylene-*bis*(trifluoroacetylacetoneimine) were separated chromatographically, and the absolute configurations were tentatively assigned [102]. The complexes were found to be stable to diastereoisomeric conversion about the carbon chiral center at 200°C but were sensitive to such conversion about the oxovanadium(IV) chiral center at considerably lower temperatures. Moisture and traces of free ligand appeared to hasten this process.

13.1.4 Anions

The determination of inorganic anions by GC can be considered to be complementary to that by ion chromatography and conventional HPLC. The basic requirement for anion determination by GC is the facile conversion of an anion to a neutral, volatile derivative, which is usually produced by the formation of a relatively stable covalent bond. Typically, the latter is selected to have favorable detection properties as well. In the simplest case, anions of weak acids are converted by acidification to the corresponding conjugate acids or

acid anhydrides, and the gaseous products (*e.g.*, CO_2, H_2S, HCN, and SO_2) are determined. A more general procedure involves nucleophilic displacement, where the analyte anion (A^-) displaces a ready leaving group, L^-, from the neutral reagent, *RL* (Eqn. 13.2), to give the derivative *AR*.

$$A^- + R{-}L \rightarrow A{-}R + L^- \tag{13.2}$$

Other derivatization reactions include electrophilic substitution, condensations, and 1,2- or 1,4-additions. For derivatization, either broad-spectrum reagents or nearly specific reagents can be used. Broad-spectrum reagents include silylating reagents [1] and alkylating reagents [1,103]. Whereas the former generally give hydrolytically unstable derivatives, alkylating reagents have been used successfully with aqueous systems involving phase-transfer catalysis to facilitate extraction and derivatization. For example, nitrite and nitrate have been determined in urine, plasma [104], and whole blood [105] as pentafluorobenzyl derivatives, using GC/MS and selected-ion monitoring [104] or ECD [105]. Nevertheless, broad-spectrum reagents, despite their obvious appeal, have not been widely applied in actual analytical procedures. Derivatizing reagents and typical applications in GC analyses were summarized previously [1], and only more recent examples are described.

Anions for which highly selective procedures have been developed and utilized include SeO_3^{2-}, NO_3^-, NO_2^-, F^-, CN^-, Cl^-, Br^-, and I^-. The determination of selenium by GC is very selective and can be used to determine SeO_3^{2-}, SeO_4^{2-}, or total selenium by control of reaction conditions. Many derivatives have been prepared [106] but the most common are the piazselenols, which are easily synthesized from the corresponding phenylenediamines in acid medium. The most suitable reagents at present appear to be 4-nitro-1,2-diaminobenzene, 4-trifluoromethyl-1,2-diaminobenzene [1], and 3-bromo-5-trifluoromethyl-1,2-diaminobenzene [107]. For the analysis, conventional nonpolar columns and electron-capture detection or, more specific element-specific detection, are used [107]. Much of the background chemistry and earlier work have been reviewed [1], including problems encountered.

Methods for halide ions are based on a variety of derivatizing reactions. Fluoride has been determined in a variety of samples [1], following the reaction of fluoride with trimethylsilanol. Chloride has been determined in tap water [108] and adipose tissue [1] after conversion to the phenylchloromercury(II) derivative, while bromide and iodide have been determined in various samples [1]. Methods have also been described for the simultaneous GC determination of chloride, bromide, and iodide [109]; chloride and bromide in mushroom and in soil samples [110]; and iodine, iodide, and iodate in aqueous solution by controlling reaction conditions [111]. Thus, iodide was oxidized by 2-iodosobenzoate, while iodate was reduced with ascorbic acid to iodine. The latter was derivatized to 4-iodo-2,6-dimethylphenol and measured by GC/MS with selected-ion monitoring. A similar approach, but involving derivatization to 4-iodo-*N*,*N*-dimethylaniline, was used for measurement of iodate in iodized table salt and free iodide and total iodine in sea water [112]. Bromate was determined in bread [113] and drinking water [114] by GC/MS, following derivatization.

13.1.5 Organometallics

Organometallics studied to date by GC include various alkyl, aryl, vinyl, and silyl compounds of Be and the III A, IV A, V A, II B element groups, Si compounds (silanes, chloroalkyl and chloroaryl silanes, silatranes), carbonyl, arylcarbonyl, and trifluorophosphinocarbonyl complexes of transition metals, and metallocenes and their substituted derivatives. The GC separation of organometallics is usually not problematic, but difficulties may arise due to limited stability with respect to one or more of thermolysis, hydrolysis, oxidative or photo-oxidative decomposition, and catalytic decomposition [1]. For the less stable compounds, great care in all aspects of their GC, including sampling, transfer, injection, column selection, and deactivation is required. Solvents should be de-aerated, nonreactive toward the solute, and free of reactive impurities, such as peroxides. Where organometallics are involatile or highly reactive, derivatization by alkylation [115, 116], silylation, hydridization [117], co-ordination, or other reactions can be utilized. Novel reagents that extend the application of such derivatizations continue to be reported [118].

Both packed and capillary columns have been used successfully. An interesting development is the application of microcolumn multi-capillary GC/ICP-MS [119] to the analysis of environmental samples. Absolute detection limits were 0.1 pg for Hg, 0.05 pg for Sn, and 0.03 pg for Pb. Separations on multi-capillary columns were approximately 10 times faster than those on conventional columns without sacrifice of resolution or sample load [120]. Examples of the separations achieved with microcolumn GC are shown in Fig. 13.3.

A FID is suitable for the detection of organometallic mixtures or reaction products at levels of *ca.* 1% or higher in solution, while more selective and sensitive detection is based on the ECD or element-selective detection, *viz.* AAS [121], AFS, AES [122], or ICP-MS [34,39,40,123]. The latter offers detection limits in the pg to fg range and is increasing in popularity. An added advantage of using a MS detector is the ability to employ isotope dilution for quantitation [116]. The use of the latter enhanced accuracy by a factor of 30 over the calibration-curve approach and by a factor of 14 over the standard-addition approach [124].

13.1.5.1 Environmental organometallics

The organometallics as a class have progressed from the status of "laboratory curiosities" [1] to important industrial chemicals. An increasing number, mainly certain compounds of Pb, Hg, Sn, As, and Sb can now be determined at low concentrations in natural waters and sediments [35,40]. Research into biomethylation has also led to the realization that some organometallics, *e.g.*, those of Pb, Hg, As, Sn, Se, Tl, and Sb, can originate from biochemical conversions of inorganic substrates [117]. The speciation and determination of organometallics is usually carried out by GC after selective extraction, derivatization, and pre-concentration. The selectivity of SPME is being exploited in an increasing number of applications [122] and may be combined with the derivatization step to eliminate the need for extraction with organic solvents. Derivatization may be important, since a variety of species of a particular element can be determined

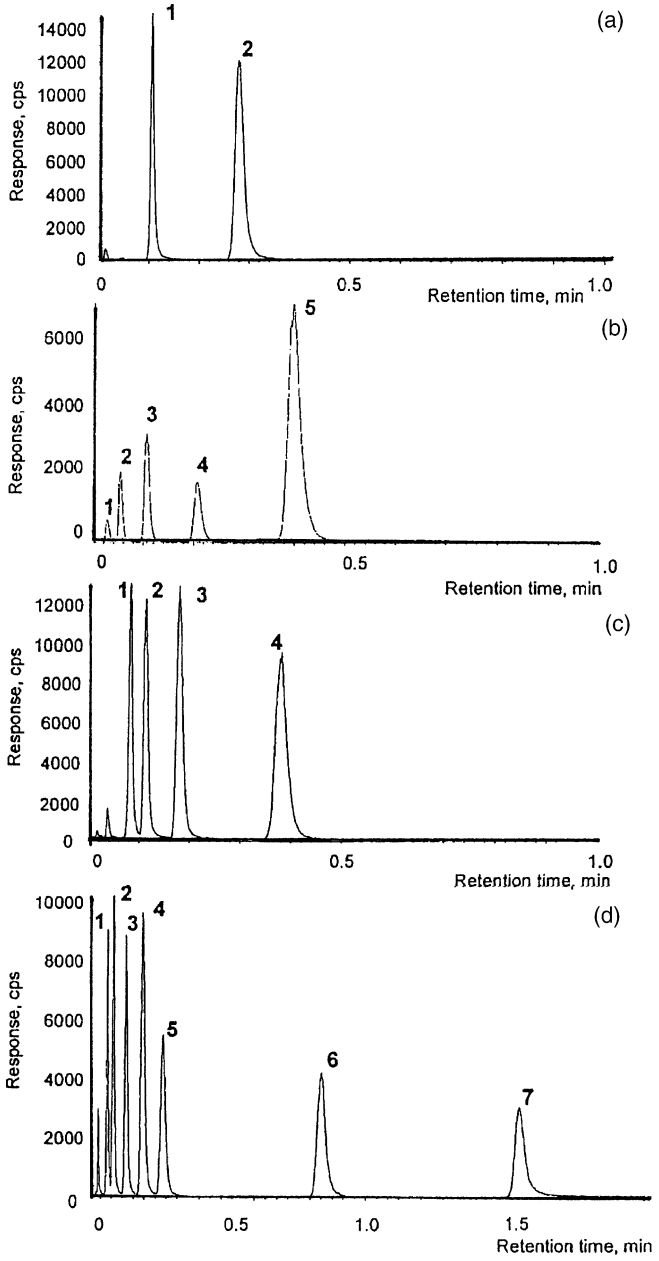


Fig. 13.3. Separation of organometallic compounds on a 20-cm microcolumn with isothermal (a, b, c) or temperature-programmed operation (c). Compounds: (a) Organomercury standards: 1, MeEtHg and 2, Et_2Hg. (b) alkyllead in gasoline 1, Me_4Pb; 2, Me_3EtPb; 3, Me_2Et_2Pb; 4, $MeEt_3Pb$; 5, Et_4Pb. (c) Organotin standards 1, $BuSn_3Et$; 2, Pr_3SnEt; 3, Bu_2SnEt_2; 4, Bu_3SnEt. (d) Organotin standards 1, $BuSn_3Et$; 2, Pr_3SnEt; 3, Bu_2SnEt_2; 4, $PhSnEt_3$; 5, Bu_3SnEt; 6, Ph_2SnEt_2; 7, Ph_3SnEt (reproduced from Ref. 119 with permission).

simultaneously [125]. Usually, the species of interest represent various stages of alkylation or dealkylation of the original organometallic compounds due to weathering or biological transformation. The simultaneous determination of such species is of value in environmental studies. Surprisingly, many environmental organometallics of Pb, Hg, and Sn can be determined at ng- to pg-levels without problems of decomposition, irreversible adsorption, or alkyl-group exchange, a fact that reflects the relatively high stability of these species.

13.1.5.2 Organolead species

Although organolead species can be formed by biomethylation [1,126], the major environmental source of such species is anthropogenic. The species, originating mainly from leaded gasoline, are Me_4Pb, Et_4Pb, Et_3MePb, Me_3EtPb, and Et_2Me_2Pb. These can be determined directly by GC after extraction and pre-concentraton. Methods have been described for the separation and trace determination of these and related species in air [1] and particulates [127], water [128], sediments, and biological material [129] by GC with ECD, AAS [130], AES [131] or ICP-MS [127,132] as detectors. The ECD is characteristically less selective than other detectors, but it is possible to determine co-extractives, such as dichloroethane, at the same time. Applications of ICP-MS include the differentiation between Pb isotope ratios to ascertain sources of lead contamination [127]. This application area requires high precision in isotope ratio measurements [133].

For solids and liquids, extraction with hexane [36] is sufficient, and the extract can be analyzed without pre-concentration. The analysis of biological material is usually the most difficult, due to the poor recoveries (typically $<75\%$) of tetraalkyllead species [1]. Since tetraalkylleads are volatile, samples should be extracted immediately after collection [1]. The determination of alkyllead and other volatile compounds in air involves cryogenic trapping [134] or adsorption on porous polymer [130]. Extraction by liquid-phase [131] and headspace SPME is now routinely exploited as a preliminary to GC separation. Liquid-phase SPME recovery often combines derivatization and extraction in a single step [131]. Other advantages are elimination of organic solvents and, in the case of headspace SPME, reduction in the number of interfering matrix components.

13.1.5.3 Organomercury species

Of the various forms of mercury frequently determined (total, elemental, and organomercury) only organomercury, mainly methylmercury and ethylmercury, is commonly determined by GC, although all three forms can be so determined [124,135]. The methodology for extracting organomercury is well established [1], although new developments are being reported. For instance, inorganic mercury, methylmercury chloride, and ethylmercury chloride were concentrated on dithizone-anchored microbeads [136], achieving a 120-fold decrease in detection limits by capillary GC/AAS. In one procedure, methylmercury and ethylmercury were derivatized with either sodium tetraethylborate or sodium tetraphenylborate [137] prior to GC/AFS and GC/AES. Phenylation was more favorable because of its capability of distinguishing between

ethylmercury and inorganic mercury. However, the tetraethylborate procedure is widely used and was validated [116] by isotope-dilution MS, although various transformation processes occurred during derivatization. This is a general problem with mercury speciation involving derivatization [138], but it was not observed with propylation [116]. In another example, three derivatization approaches were optimized and compared for mercury speciation in fish liver by GC/ICP-MS [139]. The absence of transmethylation during sample preparation was checked, using an enriched ^{202}Hg standard. In contrast, reaction byproducts, eluted near the methylbutylmercury peak, were observed when butylmagnesium chloride was used to convert methyl- and inorganic mercury to nonpolar butyl derivatives [140]. Furthermore, replacement of indigenous methyl groups with butyl groups was demonstrated on prolonged contact of samples containing methyl- and dimethylmercury with butylmagnesium chloride. Similar transalkylation reactions occur naturally and are responsible for some of the observed organomercury levels in environmental samples [141].

As in other areas, headspace and liquid-phase SPME [142] are being used increasingly, as in the example of organomercuric halides from soils, following their conversion to hydrides [143]. Other new extraction methods involve supercritical-fluid extraction of methylmercury from sediments [144]. The extracted species are then quantified by GC. In the case of methylmercury chloride [145], halide exchange from chloride to bromide proceeded during separation. Separations are successfully performed with traditional packed-column technology [146], although the majority of separations currently involve capillary columns or multi-capillary columns [28]. Chromatographic and detection parameters, such as the stationary phase and film thickness, can significantly affect analyte response [147]. Column deactivation [148] and maintenance [149] and the avoidance of metal columns are essential in trace determinations.

Detectors have included the ECD [145,148], AAS [136,140,143], AFS [137], AES [137,140,149] or, increasingly, ICP-MS [116,150]. The latter is particularly powerful and is able to simultaneously determine minute concentrations of methylmercury as well as the degree of methylation of inorganic mercury and de-methylation of methylmercury in brackish water sediments [151]. Other applications of the determination of methylmercury are described for biological material [146,152], including fish tissue [150], body fluids [142], hair [153], atmospheric and natural gas [154], soils and sediments [151,155], and natural waters [116]. In most cases, the organomercury species comprise the bulk of total mercury [152,153]. An interesting comparison of experimental methods and calculation procedures for percent methylmercury versus total mercury has appeared [156].

13.1.5.4 Organotin species

Organotin compounds of types R_4Sn, R_3SnX, R_2SnX_2, and R_3SnX are found in the environment largely as a result of their use in agriculture, wood preservation, and marine antifouling paints [1]. They are analogous to the organolead species, but are considerably more stable, both chromatographically and environmentally. In particular, $RSnX_3$ species are stable under ambient conditions, whereas the corresponding $RPbX_3$ species are not. Although alkyltin halides can be determined directly by GC [157] following extraction,

current practice involves their conversion to either alkyl [158–160] or hydride derivatives [161]. A significant feature of these derivatization reactions is that they are facile and occur without displacement of the original alkyl groups. GC conditions for such derivatives do not appear to be critical, although metal columns and metal-lined injection systems should be avoided. Columns of methyl- or methylphenylpolysiloxanes are commonly used with detection by ECD [1], FPD [43,159,161], AAS [162], or MS [158–160]. The ECD has adequate sensitivity to both alkyl and hydride species and is convenient [1], but poor specificity may require clean-up of extracts to remove interfering electron-capturing organic compounds. Element-selective detectors, on the other hand, are both sensitive and selective. As with previous applications, the use of MS detection is increasing.

The general method for recovering organotins involves either purge-and-trap, in the case of alkyltin hydrides, or extraction with hexane [160], which can be utilized for both the tetraalkyl and hydride derivatives. As previously noted, headspace SPME is assuming increased popularity for more volatile species, such as the alkyltin hydrides [161], while solid-phase extraction has been applied to field sampling [163]. A method for the simultaneous extraction of both organic and inorganic tin from aqueous solution prior to derivatization involves extraction with a benzene solution of tropolone [1]. Sensitivity in such procedures can usually be improved by pre-concentrating extracts to a small volume in, *e.g.*, a Kuderna–Danish extractor. Recent applications involve methods for the determination of organotins in fruit [164], fish tissue [163], body fluids and tissues of poison victims [43], water [163], environmental samples [157], and landfill gas [165].

13.1.5.5 Other organometallic species

Of the other organometallics, compounds of arsenic [166], selenium [40], antimony [167], and germanium [168] have been analyzed by GC. The pattern of analytes and derivatizations [169], extraction procedures including SPME [170], columns, and detectors used for organometallics of other elements are applied again. Thus, capillary GC with AAS, FPD [170], or ICP-MS has been used to study speciation of organoarsenicals [171] in environmental samples. Not surprisingly, the arsenic speciation underwent a slight evolution as a function of sample-storage time [171]. Of more significance was the observation that spiking with standards changed the equilibrium in the system.

13.2 LIQUID CHROMATOGRAPHY

13.2.1 Introduction

Prior to 1980, the development of liquid chromatographic (LC) methods for the separation and determination of inorganic species was much less rapid than for the application of these techniques to organic species. The advent of ion chromatography (IC) in 1975 and its subsequent widespread application provided the stimulus for an acceleration of interest in LC methods for inorganic species. This section focuses

primarily on literature published since the appearance of the 5th Edition of this text (*i.e.*, 1990–2002), during which more than 5,000 papers have appeared on this topic. Because this volume of literature precludes comprehensive coverage, this section will focus on some of the more significant developments and applications, with further representative examples of the main approaches being provided in tabular form. Further detail may be found in the 5th edition [1], as well as in other review articles on specific aspects of LC of inorganic species, and these will be mentioned as each topic is discussed.

LC methods will be considered here to include only modern, high-performance techniques, such as IC (which, for simplicity, will be interpreted here to represent *only* ion-exchange methods), HPLC (both normal- and reversed-phase), ion-pair chromatography and ion-exclusion chromatography. Detailed descriptions of the operating principles of these techniques may be found in Part A, particularly Chaps. 2 and 4. In the interests of brevity, TLC techniques will not be discussed. Further, it will be convenient to subdivide the subject by considering separately each broad class of solute species, namely inorganic anions, inorganic cations, organometallic species, and co-ordination compounds.

Sec. 13.1 has demonstrated the widespread ultility of GC for inorganic analysis. However, the use of GC requires that the solute be volatile and thermally stable. Most inorganic species do not meet these criteria, unless they are bound to an organic moiety. Ionic solutes, such as free inorganic anions and cations, must therefore be derivatized to produce volatile compounds, and this process may perturb the distribution of solute species in a sample. In contrast, LC methods permit the direct determination of these ionic solutes, without the requirement for derivatization. Moreover, many of the volatile organometallic species analyzed by GC are also suitable for direct determination by LC. Detection of eluted solutes is often a problem in GC, where detection must normally be based on a measurable property of the derivatizing agent, rather than a property of the inorganic solute itself. The existence of sensitive general-purpose and selective detectors for LC, especially conductivity detectors, means that detection of inorganic solutes in LC is often relatively straightforward. However, it is also fair to say that the detectors used in LC lack the sensitivity exhibited by many GC detectors.

13.2.2 Inorganic anions

13.2.2.1 Ion chromatography

Ion chromatography on columns of low-capacity, high-efficiency ion-exchange materials and with the use of conductivity detection has provided the mainstay for inorganic anion determinations. IC can be performed either in the suppressed or nonsuppressed modes (see Chap. 4). Suppressed IC involves passage of the eluent and solute anions through a suitable ion-exchange column and thence through a suppressor device in order to reduce the background conductance of the eluent. In most cases, this reduction is achieved in the suppressor by dynamic exchange of eluent cations for hydrogen ions, with subsequent protonation of weak-acid eluent anions. This process does not affect those solute anions which are the conjugates of strong acids. On the other hand, for non-suppressed IC a very dilute eluent is normally used with a column of low

ion-exchange capacity (typically 10–100 μequiv/g), so that the eluted solutes can be detected readily without the requirement for a suppressor.

13.2.2.1.1 Stationary phases and eluents

The special ion-exchange stationary phases used in IC have undergone remarkable development over the past ten years. The agglomerated resins used for suppressed IC, which are formed by electrostatic binding of aminated latex particles onto a sulfonated core particle, have shown a trend towards smaller particle sizes for both the core particle and the latex particles. Modern materials are now typically produced from highly cross-linked (typically 55%) 8–10-μm microporous polystyrenedivinylbenzene (PSDVB) as the core particles, with functionalized latex particles in the size range 60–300 nm serving as the actual ion-exchange sites. The selectivity of the resultant anion exchanger is determined largely by the chemistry of the quaternary ammonium functional groups on the latex [172]. More recently, the functional groups have been grafted to a base ethylvinylbenzene/divinylbenzene polymer to give a grafted layer thickness of 1–5 nm and increased separation efficiency. A further development has been the production of anion-exchange resins with high capacity through the use of a macroporous resin with a sufficiently large pore size as the support particle in order to allow the latex layer to be coated on both the exterior and interior surfaces of the resin. The use of such a superporous resin (2000 Å pore size) provides a simple way to increase the resin capacity 5- to10-fold by using a standard-diameter latex while maintaining the high chromatographic efficiency typically associated with pellicular materials. The increased column capacity is of particular benefit for the analysis of samples containing disparate levels of ions. The use of an efficient, high-capacity column allows the injection of a larger sample volume in order to increase the sensitivity for trace ions while still maintaining resolution of the sample peaks. Reviews on the control of separation selectivity in IC through manipulation of the stationary-phase composition are available [172,173].

Stationary phases for non-suppressed IC have undergone less spectacular development than is the case for suppressed IC. The emphasis has been on resin-based materials in which the quaternary ammonium functionality is bonded directly to the surface of a base polymer, usually either PS/DVB or polymethacrylate. The latter polymer, which is hydrophilic in nature, has the advantage that fouling of the column by organic sample components is reduced, whereas the PS/DVB materials offer the advantages of pressure stability and tolerance towards organic solvents. The resultant surface-functionalized anion exchangers are durable and relatively efficient materials, which continue to find widespread use.

In the case of suppressed IC, there has been a very strong tendency towards the use of hydroxide eluents, and these have, to a large extent, replaced the traditional carbonate/bicarbonate eluents. This trend has been facilitated by two developments: the first is the advent of new resins in which the functional group is selective towards hydroxide, so that analyte anions can be eluted effectively with relatively low concentrations of hydroxide. The second is the development of electrolytic hydroxide generators which enable hydroxide to be produced directly in the eluent at reproducible concentrations and without contamination from carbonate. This approach also allows the

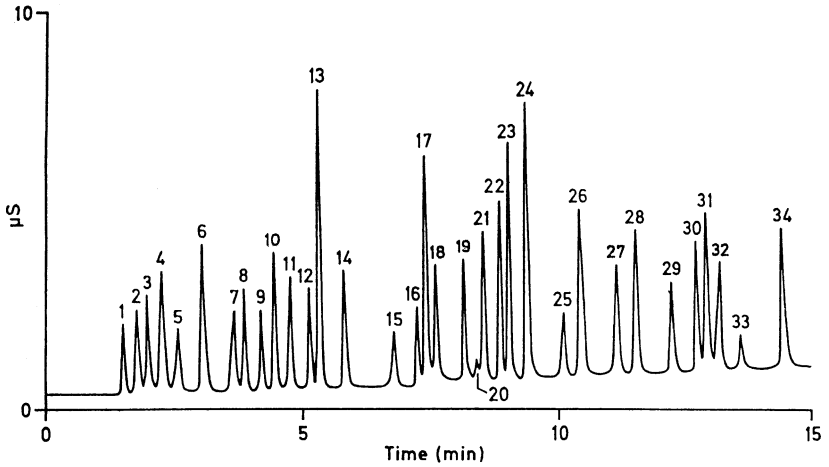


Fig. 13.4. Gradient separation by IC with hydroxide eluent generation. A Dionex IonPac AS11 column with a mobile-phase gradient of water and NaOH. Detection, conductivity in the suppressed mode. Solute identities: 1, *iso*-propylethylphosphonic acid; 2, quinate; 3, fluoride; 4, acetate; 5, propionate; 6, formate; 7, methylsulfonate; 8, pyruvate; 9, chlorite; 10, valerate; 11, monochloroacetate; 12, bromate; 13, chloride; 14, nitrite; 15, trifluoroacetate; 16, bromide; 17, nitrate; 18, chlorate; 19, selenite; 20, carbonate; 21, malonate; 22, maleate; 23, sulfate; 24, oxalate; 25, ketomalonate; 26, tungstate; 27, phthalate; 28; phosphate; 29, chromate; 30, citrate; 31, tricarballylate; 32, isocitrate; 33, *cis*-aconitate; 34, *trans*-aconitate (reproduced from Ref. 174 with permission).

generation of hydroxide gradients, and the most common applications of modern IC now frequently use gradient elution. As a result, very impressive separations can be achieved, such as that shown in Fig.13.4. Eluents for non-suppressed IC continue to be composed of aliphatic or aromatic carboxylate anions (such as citrate, tartrate, phthalate, trimesate, and pyromellitate), aliphatic and aromatic sulfonates (such as methanesulfonate and *p*-toluenesulfonate), and complexes of borate with polyhydroxy compounds (such as gluconate or mannitol).

13.2.2.1.2 Detection

Conductivity detection remains the most widely employed detection method for IC of anions. Its utility for suppressed IC has been enhanced considerably by the introduction of highly efficient electrolytic suppressors, which use electric currents to produce the hydronium ions needed for suppression and also for continuous regeneration of the suppressor [175]. Direct spectrophotometric detection at wavelengths in the range 200–220 nm is suitable for a wide range of anions, particularly nitrate, nitrite, bromide, bromate, iodate, thiocyanate, and thiosulfate. Moreover, use of a UV-absorbing eluent, such as phthalate, permits the indirect detection of anions which by themselves do not absorb at the detection wavelength. This technique, called *indirect photometric chromatography*, is applied widely, aromatic carboxylate or aromatic sulfonate anions being suitable eluent-competing ions.

Electrochemical detection methods, such as amperometry and potentiometry have found increasing use in recent years. Amperometric detection provides sensitive and selective detection for a limited range of anions, such as cyanide, sulfide, bromide, iodide, nitrite, sulfite, and thiosulfate. Platinum, glassy carbon and silver are typically used as the working electrode. Detection limits are usually in the sub-μg/l range. Potentiometric detection is normally performed with an ion-selective electrode of some kind, typically a solid-state or coated wire electrode. This approach is generally used when high detection selectivity is required, rather than good detection sensitivity.

Coupling of IC with atomic spectroscopic detection, such as atomic absorption spectroscopy (AAS) and inductively coupled plasma atomic-emission spectroscopy (ICP-AES) has become increasingly important. The chief disadvantage of flame AAS is that common nebulizers require flow-rates that are higher by a factor of two to three than the chromatographic flow-rates. This mismatch can lead to poor detection limits. However, ICP-AES is well suited to interfacing with an IC instrument, since the flow-rate of pneumatic nebulizers and spray chambers are similar to IC flow-rates. A combination of IC with ICP-AES is desirable for two main reasons: first, spectral interferences from matrix elements can be avoided when a separation step is included. Second, speciation analysis is possible if the different forms of the same element can be separated by IC. Typical applications of IC/ICP-AES are the determination of anions, such as arsenite and arsenate [176], and selenite and selenate [176]. Recent interest has focused on coupling of IC with mass spectrometry or with inductively coupled plasma mass spectrometry (ICP-MS). IC-MS with mass spectrometers adapted to perform well for low-mass ions, has been applied to the determination of oxohalides in water [177]. IC/ICP-MS finds use for very low levels of analytes, usually in complex matrices.

13.2.2.1.3 Sample handling

One of the chief challenges faced in applying IC methods is to implement them successfully for complex samples. Typically, such samples are those having high ionic strength (*e.g.*, brines, concentrated acids, or concentrated alkalis), those that are not readily extracted by aqueous solutions (such as liquid fuels and rocks), and samples containing very low levels of analytes (such as high-purity boiler-water). Some of the more recent approaches to the application of IC to samples of these types are discussed briefly below. Samples having high ionic strength are problematic in that the dominant matrix ion often exerts an ion-exchange displacing effect, leading to band-broadening and poor separation, or where the extreme pH of the sample causes profound disturbances to the critical acid/base equilibria existing in the eluent. A widely used approach for high-ionic-strength samples is to minimize the amount of sample injected into the column so that the dominant matrix ion no longer exerts a deleterious effect on the separation, and to employ a selective detection method. The sample load can be reduced either by diluting the sample or through the use of a small sample volume.

Strongly alkaline samples usually produce chromatograms with uneven baselines because of the destabilizing effect of the injected hydroxide ions on the eluent/stationary-phase equilibria. Simple neutralization of the sample with acid is unsuitable because of the resultant contamination of the sample by the acid anion. In such cases, pH adjustment can

be performed by batchwise addition of a cation-exchange resin in the hydrogen form. Alternatively, ion-exchange replacement of sodium ions in the sample with hydrogen ions can be accomplished by Donnan dialysis. This involves the diffusion of ions of a specified charge across an oppositely charged membrane and is particularly useful for the clean-up of sample solutions at extreme pH. A typical dialytic apparatus consists of a length of cation-exchange membrane fiber immersed in a suitable hydrogen-ion-donating medium. The membrane is usually functionalized with sulfonic acid groups to impart cation-exchange characteristics, and Donnan dialysis occurs between the sample solution on one side of the membrane and an acidic solution on the other side. The sample can be introduced by means of a syringe or pump, and the effluent can be either collected or transferred directly to the injection loop of an IC instrument. As the sample is passed through the fiber, sodium ions in the sample are exchanged for hydrogen ions from the hydrogen-ion-donating medium. Analyte anions are prevented from passing out of the fiber due to the repulsion of the cation-exchange (negatively charged) membrane. This process can be made more efficient by using flat sheets of membrane to increase the surface area, and even greater efficiency is achieved if an electric field is applied across the membrane. This latter process, termed *electrodialysis*, can be performed on-line, using apparatus such as that shown in Fig. 13.5. This device has been shown to permit the direct injection of 1 *M* NaOH, which was effectively neutralized prior to reaching the IC column. In a similar manner, electrodialysis has also been used to neutralize highly acidic (1 *N*) solutions prior to the determination of $Mg^{2+}$and Ca^{2+} [179].

Solid-phase extraction devices for IC have been used for many years, but a new on-line device for the automation of SPE procedures has been reported recently for treating acidic

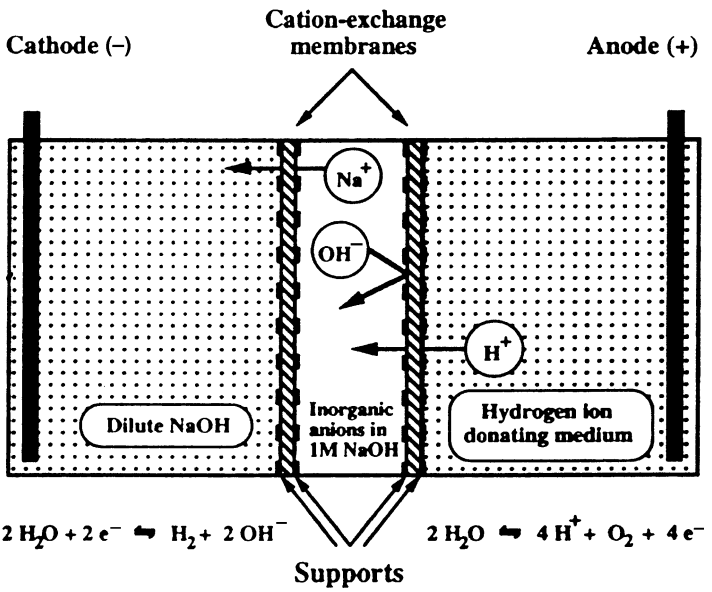


Fig. 13.5. Schematic diagram of an electrodialysis device used for neutralization of alkaline samples prior to IC analysis (reproduced from Ref. 178 with permission).

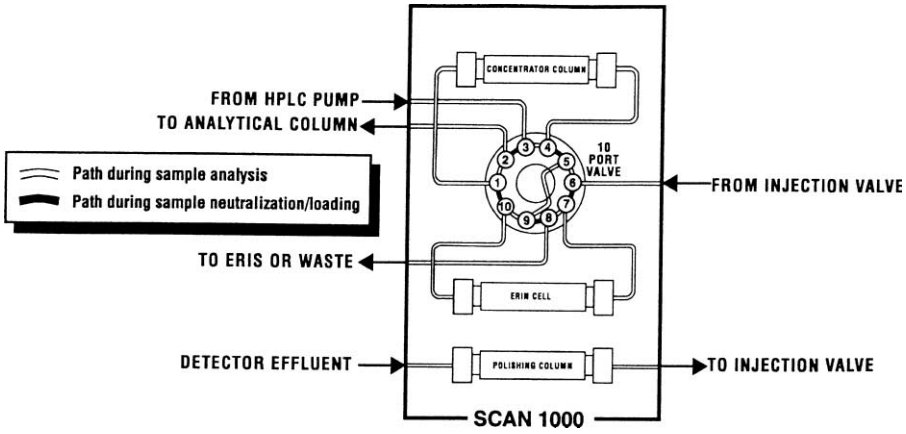


Fig. 13.6. The valve configuration for the SCAN 1000 sample processor. The shaded portion of the valve diagram represents the liquid flow-path during sample neutralization/concentration. The unshaded portion shows the liquid flow-path during sample analysis (reproduced from Ref. 180 with permission).

or basic samples [180]. This device (shown in Fig. 13.6) is termed a "Sample Concentrator and Neutralizer (SCAN) Processor" (Alltech Associates). As the name suggests, it is designed for the on-line neutralization of acidic or basic samples and also for the simultaneous pre-concentration of the analyte anions. The main components of the system are an ion-exchange concentrator column and an electrochemically regenerated ion neutralizer (ERIN) column. The ERIN column operates on the principle of an electrically polarized ion-exchange resin, wherein an electrolysis reaction is used to generate the hydroxide or hydronium ions necessary to regenerate the anion- or cation-exchange resin in the cell. The effluent from the detector is recirculated through the system and carries the injected sample through the ERIN cell where neutralization takes place, and thence to the concentrator column where the analyte anions are retained. Rotation of the valve results in the analytes being eluted from the concentrator column to the analytical column.

Samples such as liquid fuels and rocks are best handled by using combustion techniques in which heteroatoms can be converted to ionic species suitable for determination by IC. Normally, the sample is combusted in oxygen, when some nonmetallic elements are converted to gaseous compounds, which are collected in a suitable absorbing solution for analysis by IC. Several experimental configurations have been applied, but usually in an off-line mode. However, Andrew and co-workers [181] have reported an on-line combustion IC technique for the determination of sub-ppm levels of sulfur and chlorine in liquid hydrocarbon samples. A twin-tube furnace is used, and the oxygen for the combustion is first purified by passage through the furnace and then through three scrubber solutions. The sample is injected into a helium stream before being mixed with oxygen and combusted in the furnace. The gaseous products are then trapped in a bubbler, and the absorbing solution is passed through a concentrator column for enrichment prior to IC analysis. Recoveries of chlorine and sulfur from a range of liquid hydrocarbons were close

to 100%, and good agreement was found between results obtained by the combustion/IC method and microcoulometry.

Routine detection limits for IC with conductivity measurement fall in the 500-ppb to 1-ppm range for most anions when an injection volume of 100 μl is used. These detection limits may be decreased by increasing the injection volume (up to 50 ml), but the limitation to this procedure is the size of the resultant solvent peak, which may eventually obscure the solute peaks. It is interesting to note that there is little significant increase in peak-width for eluted anions when the injection volume is increased in this way. The reason for this is that solute anions in the sample become bound at the head of the column during sample injection and do not begin to traverse the column until contacted by eluent ions following the injected sample. Trace enrichment of anions is also commonly achieved in an anion-exchange pre-column (*concentrator column*), through which the sample is pumped. Solute ions become trapped in that column and, at the conclusion of sample loading, are transferred to the IC column for separation and detection. This process is attractive, because it is simple and convenient to apply, amenable to automation, offers high enrichment factors, and is less prone to sample contamination effects than alternative methods.

IC has become a popular method for the determination of airborne pollutants (gases, aerosols, and particulates) in environmental analysis and occupational hygiene. The sample can be collected in a combination of a filter medium (to trap particulates), a system of denuders (to trap gases), or an impregnated filter (to trap aerosols). Alternatively, impingers or adsorption columns can be employed for sample collection. In each of these approaches, the sample components are ultimately converted to inorganic anions, which are then determined by IC. Further aspects of sample handling in IC are discussed in a recent review [182].

13.2.2.1.4 Applications

IC has found extensive applications over a wide range of areas. Examination of the literature suggests that environmental analysis, particularly of waters, is the most commonly used application of IC, followed by industrial applications, food and plant analysis, and clinical and pharmaceutical analysis. Table 13.2 lists some of these applications, the examples being selected to provide an insight into the types of samples analyzed, the separation and detection methods used, and the detection limits achievable. It should be stressed that Table 13.2 provides only a partial listing of the IC applications published over the period covered by this Chapter. Specific reviews of the application of IC to environmental samples [183–185], clinical samples [186,187], biotechnology samples [188], foods [189], and industrial samples [190,191] are available. Finally, it should be noted that new superporous-column technology has proven to be of great utility for environmental analysis, where regulated analytes often need to be analyzed at low-μg/l levels in the presence of high-mg/l levels of potentially interfering ions. The determination of trace levels of bromate and perchlorate in drinking water are two important examples of the application of superporous, agglomerated ion-exchange columns. Further applications of IC to the determination of anions can be found in a recent review by López-Ruiz [192]; this article also contains a compilation of further review articles on many aspects of IC.

TABLE 13.2

EXAMPLES OF LIQUID-CHROMATOGRAPHIC SEPARATIONS OF ANIONS

Analytes	Column	Eluent	Detection	Method	Sample	Reference
Fluoride, chloride, monofluorophosphate, sulfate, orthophosphate	Dionex AS-15 + AG-15	Gradient, 80 m*M* NaOH (A), water (B), 0 min 40%A, 9.5 min 90%A, 12.5 min 40% A	Suppressed conductivity	SIC	Dental cream	[193]
Chloride, phosphate, sulfate, oxalate	Alltech All-Sep column	0.85 m*M* bicarbonate, 0.9 m*M* carbonate	Suppressed conductivity	SIC	Potato	[194]
Chloride, nitrite, dichloroacetate, nitrate, carbonate, sulfate, trichloroaceate	Dionex AS-11 + AG-11	Gradient, A: 5 m*M* NaOH, B: Water, 0–9 min 10% A, 15–30 min 100% A	Suppressed conductivity	SIC	Chlorinated water	[195]
Phosphates and polyphosphates (P1–P7)	Dionex AS-11 + AG-11	Gradient 30–200 m*M* NaOH over 30 min	Suppressed conductivity	SIC	Ham, fish cheese	[196]
Chloride, nitrite, nitrate, phosphate, sulfate	Metrohm Anion Dual 2	1.3 m*M* carbonate, 2 m*M* bicarbonate	Suppressed conductivity	SIC	Olive oil	[197]
Bicarbonate, chloride, nitrate, sulfate	Capcellpak C18 AG-120A	1.5 m*M* sodium phthalate (pH 6.8)	Indirect UV at 277 nm	NSIC	Pond water	[198]
Chloride, nitrite, chlorate, nitrate, sulfate, thiocyanate, perchlorate	Waters IC-Pak A HR	2.8 m*M* boric acid, 0.4 m*M* D-gluconic acid, 1.1 m*M* LiOH, 1.3 m*M* glycerol, 5.5 m*M* octanesulfonate, 5% MeCN (pH 8.5)	Conductivity	NSIC	Explosives	[199]

Thiocarbamide, sulfate	Polyspher IC AN-1	0.415 g phthalic acid, (pH 4.2) Tris	ICP-AES	NSIC	Wheat	[200]
Nitrite, nitrate	Supelcosil LC 18 T	10 m*M* *n*-octylamine, 1 m*M* H_2SO_4, pH 6	UV at 220 nm	IPC	Rat cortex micro-dialysate	[201]
Chloride, bromide, iodide	Novapak C18	0.1 *M* KNO_3	Ion-selective electrode	IPC	Urine	[202]
Citric, malic, fumaric, acetic, gallic, phthalic, protocatechuic, gentisic, *p*-hydroxybenzoic, coumaric, ferulic acids	BioRad HPX 87 column	5 m*M* H_2SO_4, 7.5% MeCN	UV at 200 nm	IEC	Plant samples	[203]
Strong acid anions, maleic, pyruvic, citric, malic, formic, succinic acids	IonPac ICE-AS6	0.8 m*M* heptafluorobutyric acid	Suppressed conductivity	IEC	Carbonated apple juice	[204]
Citric, tartaric, malic, lactic, acetic acids	Aminex HPX 87 H	Aqueous H_2SO_4, (pH 1)	UV at 214 nm	IEC	Vinegar	[205]
o-Dihydroxybenzoate, *p*-hydroxybenzoate	Tosoh TSK Gel SCX	1 *mN* H_2SO_4, 1 m*M* KCl, 0.25 m*M* EDTA, 13% MeCN	Amperometry	IEC	Rat hippocampus	[206]

SIC = suppressed IC, NSIC = non-suppressed IC, IPC = ion-pair chromatography.

13.2.2.2 Ion-pair chromatography

Ion-pair or paired-ion chromatography, also known as *ion-interaction chromatography*, offers a useful alternative to ion-exchange for the separation of inorganic anions. This approach is especially attractive to those users wishing to extend the capabilities of a conventional HPLC instrument to include anion analysis. When applied to this task, ion-pair chromatography has been utilized in two distinct modes: the first of these involves the addition to the eluent of a relatively lipophilic cationic species (such as tetrabutylammonium), called the *ion-pairing reagent*, which is maintained as an eluent component throughout the chromatographic analysis. This method, known as *dynamic coating ion-pair chromatography*, parallels the conventional use of this method for the separation of organic anions. Retention of solute anions is considered to arise from the formation of an electrical double-layer of the ion-pairing reagent and its counter-cation at the stationary-phase surface. The second operational mode of ion-pair chromatography involves preliminary treatment of a reversed-phase column with a dilute solution of a very lipophilic ion-pairing reagent (such as tridodecylmethylammonium), generally dissolved in a mixed water/organic solvent, the reagent being omitted from the eluent in the subsequent separation of anions. This approach is known as *permanent-coating ion-pair chromatography*, since the reversed-phase column is considered to retain a stable layer of the lipophilic ion-pairing reagent cation, thereby converting it effectively into an anion exchange material. Separations can then be performed in the same manner as used in ion-exchange applications, but permanent-coating ion-pair chromatography has the advantage that the effective ion-exchange capacity of the column can be regulated easily by controlling the amount of adsorbed ion-pairing reagent.

13.2.2.2.1 Stationary phases and eluents

Ion-pair chromatography of anions has been successfully performed on a wide range of stationary phases, including neutral PS/DVB polymers and bonded silica materials with C_{18}, C_8, phenyl, and cyano groups as the chemically bound functionality. Each of these stationary phases gives satisfactory retention of anionic solutes, provided the eluent composition is such that an appropriate amount of the ion-pairing reagent is adsorbed. The choice between stationary phases is usually based on such considerations as chromatographic efficiency, pH stability, and particle size, rather than on differences in chromatographic selectivity. Further factors to be considered in the selection of stationary phases for ion-pair chromatography are specific interactions existing between the stationary phase and either the reagent or the solutes, and the role of residual silanol groups on silica-based stationary phases. Some solutes (*e.g.*, iodide) show particularly strong adsorption on PS/DVB stationary phases, and this may be attributed to the occurrence of π/π interactions with the aromatic moiety of the polymer. The majority of ion-pair separations of inorganic anions are performed on conventional C_{18} silica-based reversed-phase materials or on neutral PS/DVB polymers (such as Hamilton PRP-1, Rohm & Haas XAD-2, or Dionex MPIC columns).

The most important component of the eluent in ion-pair chromatography is the ion-pairing reagent itself. In dynamic coating, the lipophilicity of the reagent governs the

degree to which it is adsorbed on the stationary phase and this, in turn, controls the density of the electrical double-layer and, thus, the retention of solute anions. Similarly, the lipophilicity of the reagent in the permanent-coating method influences the ultimate ion-exchange capacity of the coated column. Moreover, the amount of organic modifier present in the eluent can also be used to vary the amount of adsorbed reagent in both the dynamic- and permanent-coating methods. It may be noted here that the eluents used in permanent-coating ion-pair chromatography (and indeed the detection methods also) are identical to those discussed earlier for use with fixed-site, quaternary ammonium ion exchangers and therefore need not be considered further. The counter-ion plays a very important role in dynamic-coating ion-pair chromatography of anionic solutes. This counter-ion usually acts as an ion-exchange competing anion and is responsible for the elution (and in many cases also the detection) of the solute anions. Typical counter-ions are: hydroxide, fluoride, chloride, perchlorate, bromide, phthalate, citrate, and salicylate. The nature of the counter-ion determines the type of separation achieved.

13.2.2.2.2 Detection

The counter-ion of the ion-pairing reagent also influences the detection modes applicable to a particular separation. This occurs in exactly the same manner as in ion-exchange chromatography with fixed-site exchangers. Thus, counter-ions, such as citrate, phthalate, and hydroxide are suitable for conductivity detection; hydroxide, fluoride, and chloride are suitable for direct spectrophotometric detection; and phthalate is suitable for indirect spectrophotometric detection. The majority of dynamic-coating ion-pair chromatographic separations of anions utilize direct spectrophotometric detection and, thus, they are applied to anions such as nitrate, nitrite, and iodide. Indirect spectrophotometric detection is also possible if the counterion is strongly absorbing (or if such a species is added separately to the eluent as a competing anion) or the ion-pairing reagent itself is strongly absorbing.

13.2.2.2.3 Applications

Conceptually, ion-pair chromatography should offer a very similar range of applications to that of IC, given that separation is achieved by essentially the same mechanism in each case. The two techniques are therefore competitive, and it is fair to say that conventional IC is the more widely used. However, ion-pair chromatography is often preferred when direct UV detection is to be used and in cases where method development is assisted by the ability to alter easily the ion-exchange capacity of the separation column. Examples of such applications are the determination of metallo-cyanides and free cyanide (the latter after post-column derivatization) in cyanidation leach liquors produced in gold processing, and the separation of oxo-sulfur species in thiosulfate leach liquors (Fig. 13.7), also in gold processing. In each of these examples, ion-pair chromatography was found to give better results than IC. Some further applications representative of typical stationary phases, eluents, and sample types are included in Table 13.2.

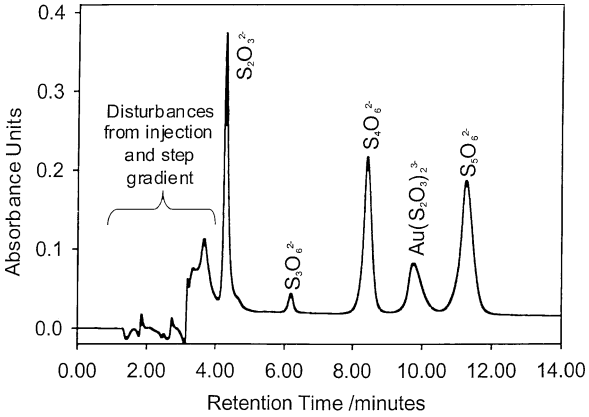


Fig. 13.7. Chromatogram of an optimized separation of polythionates and gold thiosulfate. Column: Dionex NS1 5 μm particle size. Acetonitrile step gradient from 15% v/v to 28% v/v, 3 m*M* TBAOH, 2.5 m*M* sodium carbonate, UV detection, sample composition: 20 mg/L thiosulfate, 40 mg/L trithionate, 21 mg/L tetrathionate, 20 mg/L gold (as thiosulfate complex), 21 mg/L pentathionate. (Reproduced from Ref. 207 with permission.)

13.2.2.3 Ion-exclusion chromatography

Ion-exclusion chromatography involves the separation of weak-acid anions on a column packed with sulfonated cation-exchange resin and with an acid solution as eluent. The acid concentration in the eluent determines the effective charge on the solute. Several factors are known to contribute to the retention of solutes. Neutral species can undergo liquid/liquid partitioning between the eluent and occluded water, trapped within the resin bead, while anionic species are excluded from the resin due to electrostatic repulsion from the sulfonate functional groups on the resin surface. Hydrophobic adsorption on the non-functionalized areas of the stationary-phase resin may also contribute to the overall retention mechanism. Ion-exclusion chromatography has been used chiefly for the separation of organic acids, and it is only in recent years that it has also been applied to inorganic anions.

13.2.2.3.1 Stationary phases and eluents

The most commonly used packing material for ion-exclusion chromatography of inorganic anions is a microporous or gel-type, fully sulfonated cation exchanger in the hydrogen form. A typical column, packed with resin of this type, is the Bio-Rad HPX-87H. More rigid, cross-linked, macro-porous cation exchangers have also been used, because they have not only greater pressure stability than the micro-porous material, but also show stronger hydrophobic interactions with solutes. Typical eluents for ion-exclusion chromatography are dilute solutions of mineral acids, especially sulfuric acid. If conductivity detection is to be used, a less conductive species, such as a carboxylic acid, may be substituted.

13.2.2.3.2 Detection

Direct spectrophotometric detection is the normal operating mode for ion-exclusion chromatography, but this has very limited applicability for the detection of inorganic anions, since very few of the UV-absorbing anions fall into the category of weak acids suitable for separation by ion-exclusion chromatography. As stated above, direct conductivity detection is suitable only if the background conductance of the eluent is kept low through the use of a weakly conducting acid. Alternatively, the background conductance of a strong-acid eluent can be lowered with a membrane suppressor, with tetrabutylammonium hydroxide as the scavenger solution. For example, passage of a hydrochloric acid eluent through such a suppressor results in an exchange of hydrogen ions in the eluent for tetrabutylammonium ions and leads to the formation of weakly conducting tetrabutylammonium chloride.

Since solutes are chromatographed in ion-exclusion chromatography as partially ionized species, their conductivity measurements are often rather insensitive. One means of overcoming this difficulty is the use of an *enhancement column* to increase the conductance of the sample band. An example of this approach is the conductivity detection of carbonate after separation by ion-exclusion chromatography with water as eluent. The eluted sample zone is passed through a cation-exchange enhancement column in the potassium form, and then through an anion-exchange enhancement column in the hydroxide form. The outcome of these steps is that the weakly conducting carbonic acid, eluted from the ion-exclusion column, is converted into the highly conducting potassium hydroxide. An alternative approach is to increase the conductance of the sample by adding a suitable reagent to the eluent; *e.g.*, the addition of mannitol or fructose to the eluent aids in the conductivity detection of boric acid and germanic acid, due to the formation of conducting complexes. Amperometry has also been used extensively with ion-exclusion chromatography, especially for the detection of sulfite and sulfide.

13.2.2.3.3 Applications

Ion-exclusion chromatography is particularly suited to the determination of inorganic weak acid anions, such as sulfite, sulfide, and borate, in very complex samples. The reason for this is that strong acid anions are generally unretained on an ion-exclusion column and are eluted as a band with the void volume, so that little interference with retained solutes results. Thus, ion-exclusion chromatography has been applied to the determination of ions such as bicarbonate, sulfite, and borate.

Tanaka and co-workers [208–214] have made two important extensions to the use of ion-exclusion chromatography: first, they have shown that strong acid anions, such as nitrate, chloride, and sulfate, can be separated using a weak-acid (carboxylate) cation-exchange stationary phase. Second, they have shown that the same stationary phase can be used to separate cations when a carboxylic acid, such as tartartic acid, is used as eluent. The outcome of these developments is that a simultaneous separation of inorganic anions and cations can be achieved. Anions are detected by direct conductivity while cations are detected by indirect conductivity (due to the presence of hydronium ions in the eluent), as shown in Fig. 13.8. This approach has been applied successfully to the determination of

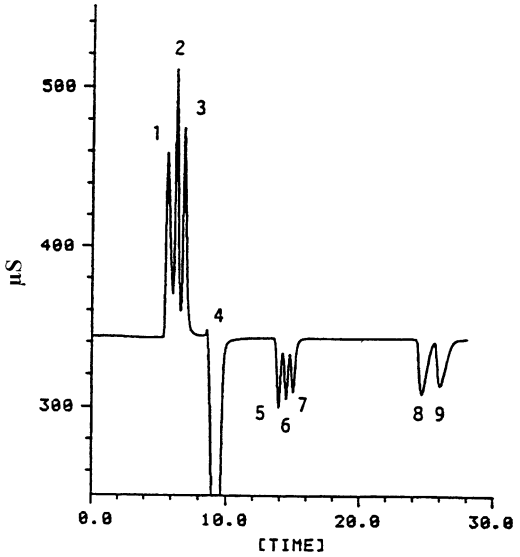


Fig. 13.8. Simultaneous separation of anions and cations by ion-exclusion/cation-exchange chromatography on a TSKgel OA-PAK column by elution with a 5 m*M* tartaric acid/7.5% aq. methanol. Eluent background conductivity, 537 *mS* cm^{-1}; peaks: 1 = SO_4^{2-}; 2 = Cl^-; 3 = NO_3^-; 4 = eluent-dip; 5 = Na^+; 6 = NH_4^+; 7 = K^+; 8 = Mg^{2+}; 9 = Ca^{2+}. Chromatographic conditions: eluent flow rate, 1.2 mL/min; column temp., 26°C; detector sensitivity, 100 mS cm^{-1}; injection volume, 0.1 mL, sample, mixture of 0.1 m*M* each of HNO_3, KCl, NH_4NO_3, $(NH_4)_2SO_4$, $CaCl_2$, and $MgCl_2$. (Reproduced from Ref. 208 with permission.)

anions and cations in acid rain and has been incorporated into a remote, automated monitoring system for acid rain. Further representative applications of the determination of inorganic anions by ion-exclusion chromatography are included in Table 13.2.

13.2.3 Inorganic cations

13.2.3.1 Ion chromatography

Both suppressed and non-suppressed IC are applied extensively to the separation of inorganic cations, especially alkali-metal and alkaline-earth cations. Many of the developments discussed earlier for IC of anions also apply to cation determinations and will not be reiterated, but it is fair to say that the rate of progress of IC has been somewhat slower for cations than for anions. The underlying reason for this is that IC stands alone as the premier method for anion analysis, but represents just one of a number of useful techniques for cation analysis. Nevertheless, the ability of IC to provide rapid and sensitive determination of multiple solutes in a single chromatogram will ensure its continued application in cation analysis.

13.2.3.1.1 Detection

The most significant developments in detection of cations in IC have been in the areas of post-column reactions and the use of spectroscopic detection methods. Post-column reactions continue to be the most widely used detection approach for all classes of cations except the alkali metals, for which conductivity detection is preferred. Arsenazo III and 4-(2-pyridylazo)resorcinol (PAR) are employed commonly as the post-column reagent (PCR) when spectrophotometric detection is used. However, fluorescence and chemiluminescence detection after post-column reactions have added greatly to the utility of this approach. In the case of fluorescence detection, PCRs such as 8-hydroxyquinolinesulfonate (HQS) respond selectively to metals ions, such as Mg^{2+}, and Ca^{2+} [215], $Zn^{2+}$and Cd^{2+} [216], Al^{3+} [216,217], Ga^{3+} [218], and In^{3+} [216,218]. A more universal response with HQS can be obtained, if a suitable displacement equilibrium reaction is included in the PCR system. For example, if $Mg(EDTA)^{2-}$ is added, eluted metal ions can displace Mg^{2+}, which then reacts with HQS to give a fluorescence response [219,220]. PCR chemiluminescence reactions are frequently based on a luminol system, which involves the alkaline oxidation of luminol in the presence of a catalyst [221]. Trace analysis of metals like Cr^{3+}, Co^{3+}, or Cu^{2+} is possible due to their catalytic effect on the oxidation of luminol. Indirect detection can also be applied to species that complex Cu(II). Detection limits for both fluorescence and chemiluminescence PCR are usually in the low-μg/L range. Atomic spectroscopic detection techniques, applied to IC of cations, include AAS and ICP-AES. With AAS, most of its common modes, such as flame, graphite furnace, and hydride generation, have been utilized. IC-ICPAES and IC-ICPMS have become increasingly commonplace, especially for elements such as Cr and V [222], where detection limits at the sub-μg/L level are achieved.

13.2.3.1.2 Applications

As indicated above, the prime application of IC in inorganic cation analysis is the determination of monovalent cations, for which it provides a rapid and sensitive determination. Ion exchange does not offer exceptionally high separation efficiencies for transition metals, unless a pellicular type of ion exchanger (*e.g.*, agglomerated resins or polymer-coated silica) is employed. Table 13.3 shows some representative applications of IC for the determination of inorganic cations.

13.2.3.2 Ion-pair chromatography

Ion-pair chromatography has been used extensively for the separation of transition metals and lanthanides, the latter of which are separated with unrivalled efficiency. The dynamic-coating method is the preferred approach, and the ion-pairing reagent is usually octanesulfonate [234]. The choice of the counter-ion is of much less importance than in the case of ion-pair chromatography of anions, since ion exchange plays only a minor role in the elution process for cations. Solute elution is dominated by complexation effects due to eluent ligands, such as tartrate or α-hydroxyisobutyric acid (HIBA). Therefore, the ligand concentration and the eluent pH are of particular importance in the control of retention.

TABLE 13.3

EXAMPLES OF LIQUID-CHROMATOGRAPHIC SEPARATIONS OF CATIONS

Analytes	Column	Eluent	Detection	Method	Sample	Ref.
Sodium, ammonium, magnesium, calcium, potassium	Dionex IonPac CS-15 + CG-15	5 m*M* H_2SO_4, 9% MeCN	Suppressed conductivity	SIC	Milk	[223]
Sodium, ammonium, potassium, methylamine, ethylamine, trimethylamine	Dionex IonPac CG-12A + CG-10 + CS-10	40 m*M* methanesulfonic acid	Suppressed conductivity	SIC	Hydrogen peroxide	[224]
Lithium, sodium, potassium	Dionex Ionpac CS-12A	25 m*M* H_2SO_4	Suppressed conductivity	SIC	Sea-water	[225]
Copper, nickel, zinc, cobalt, manganese, iron(III), lead	Dionex Ionpac CS-5A + CG-5A 5	28 m*M* oxalic acid, 250 m*M* $NaNO_3$	PCR with PAR at 530 nm	NSIC	Standards	[226]
Creatine, 3-methyl-L-histidine, creatinine, L-tyrosine, L-phenylalanine	Shiseido Capcellpak UG-80	5 m*M* ethylenediamine, 15 m*M* H_3PO_4 (pH 2.5)	UV at 210 nm	NSIC	Urine	[227]
Copper, zinc, manganese	Dionex CS-5A + CG-5A	3 m*M* dipicolinic acid, 4.2 m*M* LiOH, 3 m*M* Na_2CrO_4, 2 m*M* Na_2SO_4 (pH 5.1)	PCR with 5-Br-PADAP at 565 nm	NSIC	Rice powder	[228]

Magnesium	Shimadzu Shim-pack IC-C1	4 m*M* tartrate, 2 m*M* ethylenediamine	Bulk acoustic wave	NSIC	Culture medium	[229]
Copper, lead, zinc, nickel, cadmium, iron(II), manganese	BT TM-S + BT V-U guard	0.1 *M* tartaric acid (pH 2.9)	PCR with PAR at 520 nm	NSIC	Vodka	[230]
Neodymium, praseodymium, cerium, lanthanum	Supelco LC-18 column	0.30 *M* hydroxyisobutyric acid, 7.5 m*M* octanesulfonate (pH 3.75)	PCR with Arsenazo III at 658 nm	IPC	Magnesium alloy	[231]
Nickel, copper	Lichrospher 100 RP 8	53% aq. MeOH, 40 m*M* acetate buffer, 25 m*M* tetrabutylammonium, 25 m*M* NaCl, 0.5 μ*M* Calcion	510 nm photometry	IPC	Tap water	[232]
Zinc, nickel, cobalt, calcium, magnesium, iron	Techsphere 5 ODS	2 m*M* octanesulfonate, 50 m*M* tartrate, 5% MeCN (pH 3.2)	PCR with PAR at 510 nm	IPC	River-water	[233]

SIC = suppressed IC, NSIC = non-suppressed IC, IPC = ion-pair chromatography, PCR = post-column reaction, 5-Br-PADAP = 2-(5-bromo-2-pyridylazo)-5-(diethylamino)phenol.

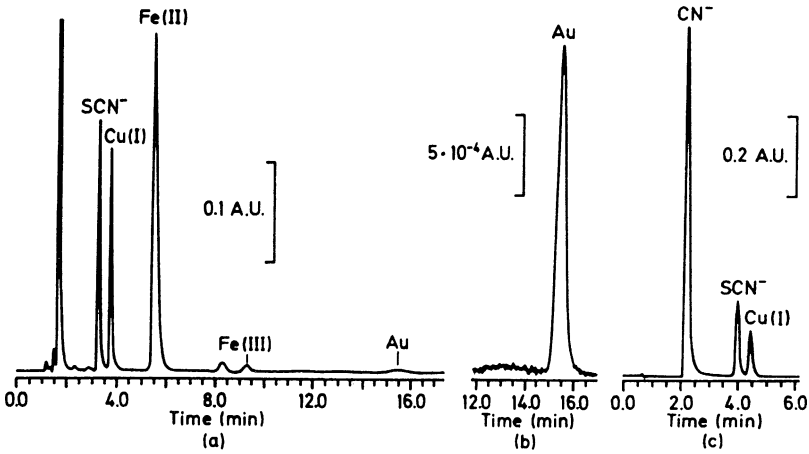


Fig. 13.9. Separation of cyanide species from a cyanidation leach liquor. (a) and (b) Direct UV detection, (c) detection at 500 nm after post-column reaction. A Nova-Pak C_{18} column was used with 25% aq. acetonitrile containing 5 m*M* tetrabutylammonium hydroxide as eluent (reproduced from Ref. 235 with permission).

It should also be noted that anionic complexes of metal ions with suitable ligands (especially cyanide) are readily separated by ion-pair chromatography with chromatographic efficiencies well in excess of those attainable by anion exchange. In this application, direct spectrophotometric detection at 214 nm is suitable. A separation of metallocyanides is shown as Fig. 13.9.

Post-column reaction is the most sensitive detection method, and the combination of dynamic-coating ion-pair chromatography with this mode of detection yields some excellent analytical results for metal ions. Gradient elution by changing the concentration of the eluent ligand is again applicable. These techniques yield optimal results when applied to the determination of lanthanides, so that ion-pair chromatography represents an outstanding analytical method for these species. Fig. 13.10 shows the separation of 14 lanthanides with an isocratic eluent and Eriochrome Black T as the PCR. Ion-pair chromatography has been used for the determination of lanthanides in such complex samples as superconductors [236], leach solutions [237], and rare-earth concentrates [238]. Further applications of this method to inorganic cations are included in Table 13.3.

13.2.3.3 Chromatography with immobilized ligands

An increasingly popular approach to the separation of metal ions involves the use of stationary phases on which a suitable ligand has been immobilized, either by adsorption or by chemical bonding, to a support material (usually silica or a suitable polymer, such as PS/DVB). Both approaches are often referred to as *chelation ion chromatography*. In the case of adsorbed ligands, the most common approach has been to use a chelating dye, adsorbed on either a reversed-phase material or an ion exchanger. Typical dyes used for this purpose are: xylenol orange [239–241], methyl

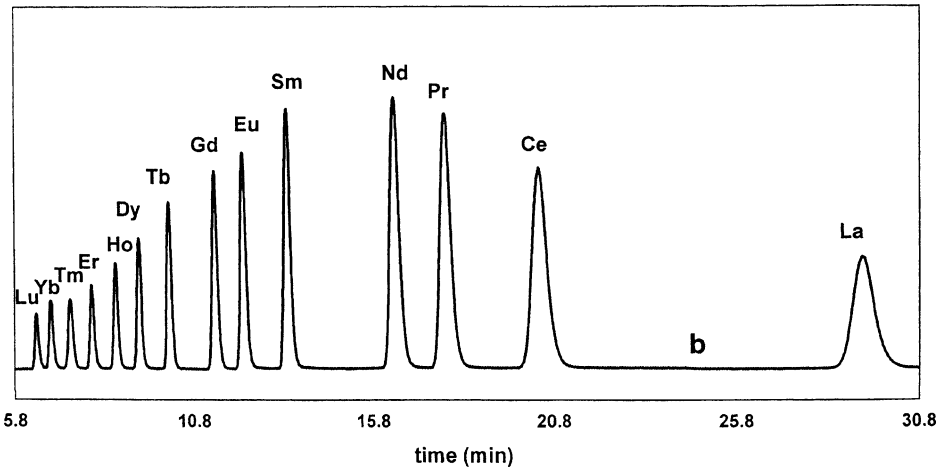


Fig. 13.10. Separation of lanthanides by dynamic ion-pairing chromatography on a reversed-phase column by gradient elution with hydroxyisobutyric acid/octanesulfonate at pH 3.8. Detection by post-column reaction with Eriochrome Black T (reproduced from Ref. 234 with permission).

thymol blue [242–244], and *o*-cresolphthalein complexone [245,246], all of which contain the iminodiacetate functionality. The coated column is then used with a mobile phase which usually also contains a small concentration of the dye for stabilizing the coated layer, to act as a competing ligand by controlling retention of the analytes, and to facilitate detection by virtue of a change in color when the dye is complexed by the analyte. Recent reviews on this approach are available [247,248] and show that, while a wide range of metals can be determined, the chromatographic efficiencies of the coated columns are generally quite modest. However, this approach has the advantage that the stationary phases are inexpensive and easily prepared.

Chemically bonded stationary phases are also used widely and have been the subject of several reviews [249–251]. Typical ligands include: 8-hydroxyquinoline, β-diketones, and azoresorcinols. These materials are more difficult to prepare and characterize, but they are generally more stable than coated materials and tend to show higher separation efficiencies. An extensive listing of bonded chelating stationary phases can be found in Ref. [248].

13.2.4 Co-ordination compounds and organometallics

13.2.4.1 Co-ordination compounds

Many inorganic cations can be determined by LC as their co-ordination compounds. There have been more than 300 publications since 1990 on liquid chromatography of metal complexes, and several recent reviews are available [249,251–255]. The presence

of a suitable ligand in the complex permits separation to be achieved by conventional reversed-phase or normal-phase HPLC methods, and it often also assists in the detection of the eluted metal complex. The ligand and chelate should have a number of properties, including the following:

(a) The ligand should form neutral complexes with a large number of metals, prepared by relatively simple methods.
(b) The complexes formed should be co-ordination-saturated, since this gives the greatest probability of separation of complexes formed from different metals. Moreover, the ligand should not be too large, so that specific properties of the central metal atom preponderate.
(c) The donor atoms in the ligand should have a low total electronegativity to minimize adsorption effects on silica-based reversed phases. Preferred donor atoms are N, O, and S.
(d) Ligand substituents should not have large induction or steric effects, and electronegative atoms should preferably exist in close proximity to the chelate ring to increase separation selectivity.
(e) The complexes should have high stability, good detectability, and high solubility in nonpolar organic solvents.

Many ligands have found application in LC of metal chelates. These ligands include: dithiocarbamates, 8-hydroxyquinoline, β-diketones, 4-(2-pyridylazo)naphthol (PAN), PAR, dialkyldithio-phosphates, xanthates, 2,3-diaminonaphthalene, pyrazolones, and hydrazones. No single ligand is suitable for all metal ions, and typically, only a few metals are determined in a single chromatographic separation. In most cases, water-insoluble chelates are formed, and these must be extracted into a suitable organic solvent prior to chromatographic separation. This sometimes involves extraction with solvents that cannot be injected directly into a reversed-phase HPLC system, so that evaporation and re-dissolution become necessary. Alternatively, complexes can be formed *in situ* by injecting metal ions into a mobile phase containing the ligand and an appropriate buffer, or through the use of a solid-phase reaction on a suitable pre-column. The stability of the metal complexes is also of great importance, because these complexes are generally injected at very low concentrations and are therefore prone to dissociation as they traverse the chromatographic system. This is particularly true of complexes that may undergo ligand-exchange reactions at the surfaces of metallic chromatographic components, such as the injector, interconnecting tubing and the inlet and outlet frits in the column. Kinetic stability is of more importance than thermodynamic stability, since kinetically inert complexes are more likely to pass intact through the chromatographic system. Again, the large volume of literature on HPLC of metal chelates precludes a comprehensive discussion of this topic. Table 13.4 provides a selected listing of some applications of HPLC of metal chelates.

13.2.4.2 Organometallic compounds

One of the factors limiting the applicability of HPLC analysis of metal chelates is the need for forming the chelate. This limitation does not exist for many of the organometallic

TABLE 13.4

EXAMPLES OF LIQUID-CHROMATOGRAPHIC SEPARATIONS OF ORGANOMETALLIC SPECIES AND CO-ORDINATION COMPOUNDS

Analytes	Column	Eluent	Detection	Method	Sample	Ref.
Methylmercury, ethylmercury		0.05 *M* KNO_3, in 75% aq. MeOH	Amperometry on C electrode at +1.15 V *vs.* Ag/AgCl	NSIC	River-water	[256]
Arsenic(III), arsenic(V), monomethylarsonic acid, dimethylarsinic acid, arsenobetaine, tetramethylarsonium, trimethylarsine oxide, arsenocholine	Hamilton PRP-X200 separator and guard	5 m*M* 3-carboxy-4-hydroxybenzene-sulfonic acid (pH 1.9)	AFS after post-column photochemical oxidation and hydride generation	NSIC	Standards	[257]
Arsenic(III), dimethylarsinic acid, arsenic(V) arseno-betaine, arsenocholine	Dionex Ionpac AS-7	0.5–50 m*M* HNO_3 gradient over 20 min	ICP-MS	NSIC	Fish	[258]
Antimony(V), trimethyl-stilboxide, antimony(III)	Hamilton PRP 100 X	2 m*M* phthalate (pH 5), 2% acetone	ICP-MS	NSIC	Soil	[259]
Selenomethionine, selenite, selenate, selenocystine	Dionex Ionpac AS-10	15 m*M* carbonate	Hydride-generation AAS	NSIC	Plant sample	[260]
Selenite, selenate, selenomethionine, trimethylselenonium	Dionex Ionpac CS-5	10 m*M* oxalate, 20 m*M* K_2SO_4 (pH 3), 2% MeOH	ICP-MS	NSIC	Urine	[261]
Thorium(IV), uranyl	Waters Nova-pak C-18	200 m*M* hydroxyiso-butyric acid in 5% MeOH (pH 4.0)	PCR with Arsenazo III at 658 nm	RPLC	Standard mixture	[262]

(*Continued on next page*)

TABLE 13.4 (continued)

Analytes	Column	Eluent	Detection	Method	Sample	Ref.
Selenocystine, methyl-DL-selenocysteine, selenomethionine, selenoethionine	Zorbax SB-C8	98% MeOH, 0.1% trifluoroacetic acid	ICP-MS	RPLC	Yeast extract	[263]
Tributyltin, triphenyltin	Whatman Partisil SCX	0.15 *M* NH_4 acetate, 80% aq. MeOH	Fluorimetry after PCR with fisetin	RPLC	Mollusc extract	[264]
Vanadium(V), tantalum(V), niobium(V), vanadium(IV), cobalt(II), iron(III)	Waters Novapak C18	32% aq. MeOH, 5 m*M* acetate, 5 m*M* TBA, 5 m*M* citrate (pH 6.5)	Direct photometry at 540 nm	IPC	Rocks	[265]

NSIC = non-suppressed IC, IPC = ion-pair chromatography, AFS = atomic fluorescence spectroscopy, TBA = tetrabutylammonium.

species, which can be chromatographed directly. The more important organometallic species that can be analyzed by HPLC are alkyllead, alkylmercury, alkylarsenic, and alkyltin compounds. Of these, the organoarsenic species are the most widely studied. Monomethylarsonate, dimethylarsinate, and phenylarsonate (as well as the inorganic ions arsenate and arsenite) are formed by the action of many common yeasts, fungi, and bacteria on arsenic present in soils. The high toxicities of these compounds necessitate their accurate determination, especially in water samples. Separation is usually accomplished by anion-exchange chromatography, but ion-pair chromatography in both the dynamic-coating and permanent-coating modes has also been employed.

In some cases, metal chelates and organometallic compounds may be detected spectrophotometrically. However, when this detection mode is applied to metal chelates, it is often complicated by the presence of excess ligand in the sample. This may require the use of a wavelength at which detection sensitivity is diminished. For this reason, atomic spectroscopic detection is very attractive, and considerable effort has been expended in successfully coupling HPLC instruments with atomic-absorption spectrometers, or direct-current plasma (DCP) and inductively coupled plasma (ICP) atomic-emission spectrometers. Table 13.4 lists some chromatographic details and applications of HPLC to organometallic compounds.

13.3 ELECTROSEPARATION METHODS

13.3.1 Introduction

Electroseparation methods, especially capillary zone electrophoresis (CZE) and capillary electrochromatography (CEC) have been studied extensively over the past 10 years as separation tools for inorganic species. Since the general theory of these techniques is discussed in Chaps. 7 and 9, this Section will focus only on the special features that apply to inorganic analysis.

13.3.2 Inorganic anions

13.3.2.1 Separation strategy

In all CZE separations there are several important operational variables which must be selected when setting up the separation method. The major variables to be considered are the polarity of the electrodes used to supply the electric field which generates the separation, and also the direction of the electro-osmotic flow (EOF). The most rapid separations are achieved when the electrophoretic migration of the analytes is from the sample introduction end of the capillary towards the detection end, and when the EOF also moves in the same direction. This is termed a *co-EOF* separation and is the preferred approach for inorganic anions. Thus, the normal instrumental arrangement is to assign the negative side of the separation voltage to the electrode at the injection end, so that the electrophoretic migration of the analyte anions is

towards the positive electrode at the detection end. However, the normal direction of flow of EOF in a fused-silica capillary is towards the cathode, which occurs as a result of the negative charge on the capillary wall, caused by ionization of silanol groups. Thus, it becomes necessary to reverse the EOF, if the desired co-EOF separation is to be achieved. Such EOF reversal can be achieved by dynamic modification of the inner wall of the fused-silica capillary by adsorption of cationic compounds or by permanent (covalent bonding) of cationic groups to the inner wall of the fused-silica capillary to provide an overall-positive charge on the wall. Dynamic modification is used most commonly. It can be achieved by allowing suitable cationic surfactants (such as C_{12} to C_{16} alkyltrimethylammonium salts) to be adsorbed, either by adding these to the background electrolyte (BGE) or by flushing the capillary with them between the runs.

When the separation voltage is applied, the anions migrate towards the anode at the detection end in the order of their effective electrophoretic mobilities. The electrophoretic mobilities of inorganic anions, shown schematically in Fig. 13.11, cover a wide range from *ca.* 30×10^{-9} $m^2V^{-1}sec^{-1}$ to $> 100 \times 10^{-9}$ $m^2V^{-1}sec^{-1}$. As can be seen in Fig. 13.11, most inorganic anions have a unique value of electrophoretic mobility and can therefore be separated. However, there are examples where electrophoretic mobilities of ions are

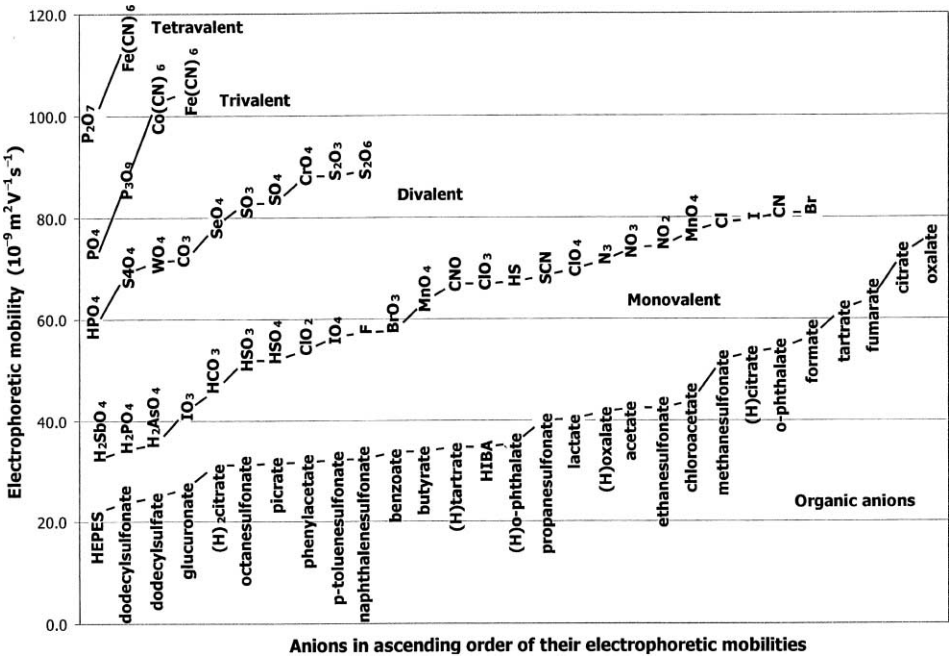


Fig. 13.11. Electrophoretic mobilities of inorganic and some organic anions in ascending order of their mobilities. (H) = protonated form, $(H)_2$ = diprotonated form, charges omitted for simplicity. Electrophoretic mobilities were calculated from tabulated ionic conductances or taken from published values. (Reproduced from Ref. 266 with permission.)

similar, and some manipulation of separation selectivity is needed in order to achieve a separation. The most straightforward approaches for manipulating selectivity are to alter the solvation of the analyte anions by adding organic solvents or to vary the pH of the BGE so as to alter the charge on particular analytes. For the latter approach to be successful, the BGE must be well buffered to avoid pH variations within the migrating sample zone.

However, perhaps the most powerful method for the control of separation selectivity for inorganic anions is to introduce ion-exchange interactions into the separation. This can be achieved by adding an anion exchange stationary phase to the capillary (to make a CEC system) or by using an anion-exchange pseudo-stationary phase in the BGE. The latter approach is termed *electrokinetic capillary chromatography* (EKC) (Chap. 7). It is conceptually identical to CEC and differs from it only in that a pseudo-stationary phase, rather than a true stationary phase, is used. Studies undertaken by both CEC and EKC suggest that the latter is most convenient because the pseudo-stationary phase can be simply added to the BGE. A typical pseudo-stationary phase used for this purpose is poly(diallyldimethylammonium)chloride (PDDAC), which is a water-soluble cationic polymer exhibiting strong anion-exchange interactions with inorganic anions. PDDAC has been used extensively for selectivity manipulation, and the resultant EKC system can be modeled mathematically to a high degree of accuracy, thereby enabling rapid optimization of separations [267,268].

13.3.2.2 Detection

While some inorganic anions show appreciable UV absorbance in the wavelength range 200–220 nm, most do not. For this reason, the most widely used detection method in electroseparations of inorganic anions is indirect photometric detection. This has the prime advantage of being universal in its applicability. An absorbing co-ion (commonly referred to as the probe ion) is added to the BGE, and the detector monitors a suitable absorbing wavelength of the probe. Migrating bands of analytes displace the probe from the BGE, and indirect detection is possible due to the resulting decrease in absorbance. There are three key factors which must be considered in maximizing the detection sensitivity in indirect photometric detection [269]: first, the electromigrational dispersion of the analytes should be minimized. Second, the transfer ratio, which is defined as the number of moles of the probe that are displaced by one mole of analyte, should be maximized. Third, the presence of co-ions (*i.e.*, ions having the same charge sign as the probe) in the BGE should be avoided, since these co-ions can compete with the probe for displacement by analyte anions. The first of these factors affects the width of the migrating sample band, while the other two factors affect the magnitude of the detection signal. Maximum detection sensitivity results when the mobility of the probe matches closely that of the analytes. A schematic listing of the electrophoretic mobilities of some inorganic anions and some commonly used anionic probes is given in Fig. 13.12.

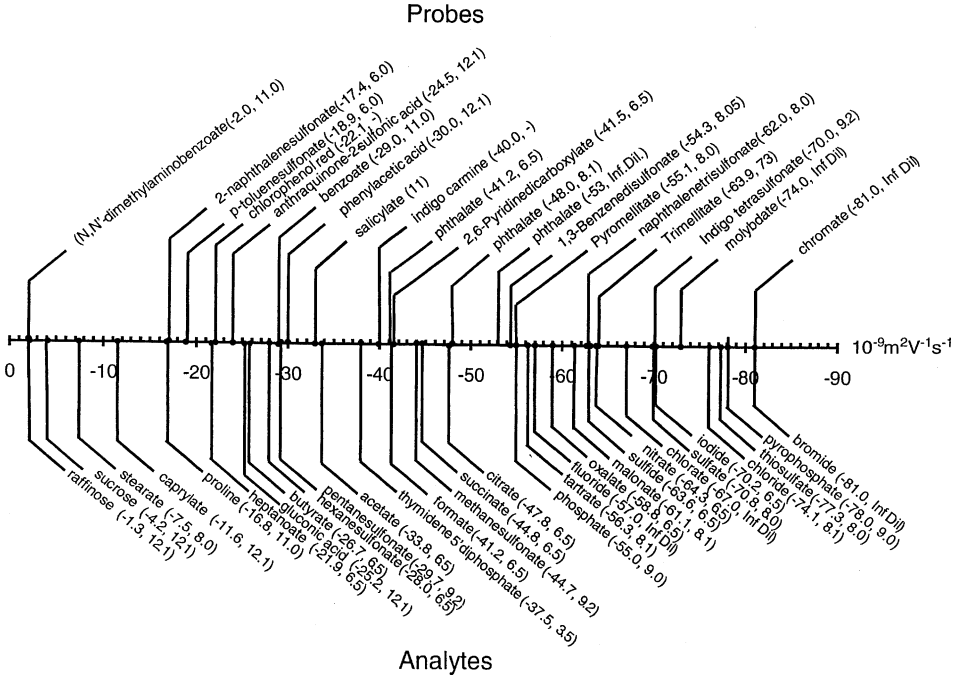


Fig. 13.12. Matching of electrophoretic mobilities of some inorganic anionic analytes and UV-absorbing probes. Given in brackets are mobility and, optionally, a pH. (Data taken from Ref. 273; reproduced from Ref. 270 with permission.)

Typical detection limits for indirect detection are in the low-μM region, although sub-μM detection limits have been achieved by using highly absorbing dyes as probes. Finally, it is noteworthy that special precautions are needed when buffering BGEs for use with indirect detection, since buffering must be achieved without the introduction of co-ions. This can be accomplished either by using a buffering counter-ion (*i.e.* one having a charge sign opposite to the probe) or by using buffering species of extremely low mobility, such as buffering ampholytes employed at a pH close to their pI. Strategies for successful buffering have been reviewed by Macka *et al.* [270,271].

Electrochemical detection by conductometric, amperometric, and potentiometric methods can be employed in CZE of inorganic anions. The first one is the most successful. The conductivity detector can either be mounted at the end of the capillary (provided suitable precautions are taken to isolate the detector from the electric field used to effect the separation) or it may take the form of a contactless detector, which can be positioned anywhere along the length of the capillary. In the latter case, the conductivity electrodes sit outside the capillary and operate through capacitive coupling of an applied high-frequency field with the interior of the capillary, containing the sample to be detected [272].

13.3.2.3 Applications

The exceptional separating power of CZE for inorganic anions is illustrated in Fig. 13.13, which shows the separation achieved for a co-EOF set-up, with chromate ion as the indirect detection probe. The most common sample type is some kind of water (drinking water, mineral water, river water, ground water, well water etc.), and other common samples are fruit juices and beverages of all kinds. The fact that CE is not useful for high-ionic-strength samples is reflected in the infrequent application of CE to samples such as seawater, unless considerable dilution or modification of the sample is undertaken. A further general limitation is that electroseparation methods for inorganic anions are less sensitive than IC. This limitation is due to the short detection light path across the capillary. Therefore, high sensitivity can be achieved only with indirect detection and very strongly absorbing probes or by employing on-line sample enrichment methods, such as stacking techniques or sample pre-concentration. The latter approaches have been reviewed recently [273]. Table 13.5 lists some applications of CZE, CEC, and EKC to the determination of inorganic anions. Several reviews on CE of anions are available [274–278].

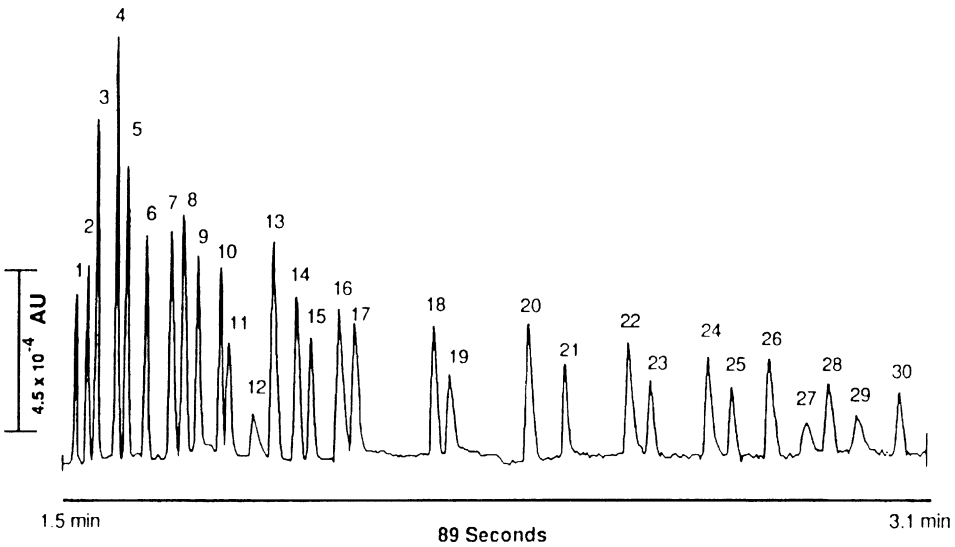


Fig. 13.13. Separation of 30 anions in a chromate BGE. Conditions: capillary, fused silica, 75 μm ID, length 0.600 m, 0.527 m to detector; BGE, 5 m*M* chromate, 0.5 m*M* tetradecyltrimethylammonium bromide (pH 8.0); separation voltage, 30 kV; detection, indirect at 254 nm; injection, electrokinetic at 1 kV for 15 sec; sample, 0.3–1.7 ppm of each anion. Peak identification: 1 = thiosulfate, 2 = bromide, 3 = chloride, 4 = sulfate, 5 = nitrite, 6 = nitrate, 7 = molybdate, 8 = azide, 9 = tungstate, 10 = monofluorophosphate, 11 = chlorate, 12 = citrate, 13 = fluoride, 14 = formate, 15 = phosphate, 16 = phosphite, 17 = chlorite, 18 = galactarate, 19 = carbonate, 20 = acetate, 21 = ethanesulfonate, 22 = propionate, 23 = propanesulfonate, 24 = butyrate, 25 = butanesulfonate, 26 = valerate, 27 = benzoate, 28 = L-glutamate, 29 = pentanesulfonate, 30 = D-gluconate. (Reproduced from Ref. 279 with permission.)

TABLE 13.5

EXAMPLES OF CAPILLARY-ELECTROPHORETIC SEPARATIONS OF ANIONS

Analyte	Capillary, voltage	Electrolyte	Detection	Sample	Ref.
$S_2O_3^{2-}$, NO_2^-, NO_3^-, S^{2-}, MoO_4^{2-}	FS 60 cm × 75 μm, 20 kV	10 m*M* Na_2SO_4, 0.5 m*M* CIA-Pak OFM-OH (pH 11.4)	Direct UV at 229 nm	Waste water	[280]
NO_3^-, SCN^-, I^-	FS 52 cm × 75 μm, 10 kV	100 m*M* NaCl, 2.0 m*M* CTACl	Direct UV at 214 nm	Subterranean water	[281]
$S_2O_3^{2-}$, $S_4O_6^{2-}$, SO_3^{2-}, S^{2-}	FS 60 cm × 75 μm, 20 kV	1.5 m*M* pyromellitate, 10 m*M* Tris, 0.5 m*M* EDTA (pH 8.0)	Indirect UV at 214 nm	Clay water	[282]
Br^-, BrO_3^-, I^-, IO_3^-, NO_2^-, NO_3^-, SeO_3^{2-}	FS 50 cm × 75 μm, 20 kV	Phosphate buffer (pH 2.9)	Direct UV at 200 nm	River-water	[283]
Br^-, Cl^-, SO_4^{2-}, NO_2^-, NO_3^-, F^-, PO_4^{3-}, CO_3^{2-}	FS 60 cm × 75 μm, 20 kV	5 m*M* Na_2CrO_4, 0.5 m*M* CIA-Pak OFM Anion BT (pH 8)	Indirect UV at 254 nm	Soil extract	[284]
$S_2O_3^{2-}$, Cl^-, SO_4^{2-}, NO_3^-, S^{2-}, SO_3^{2-}, F^-, PO_4^{3-}, CO_3^{2-}, OH^-	FS 45 cm × 50 μm, 20 kV	6 m*M* Na_2CrO_4, 3 m*M* boric acid, 32 μ*M* CTABr (pH 8)	Indirect UV at 372 nm	Juice, milk	[285]

TABLE 13.5 (continued)

Analyte	Capillary, voltage	Electrolyte	Detection	Sample	Ref.
Br^-, NO_2^-, NO_3^-	FS 50 cm × 75 μm, 6 kV	$NaPO_4^{2-}/Na_2PO_4^-$, 1.4% NaCl, 0.1% PEG 8000	Direct UV at 214 nm	Serum, urine	[286]
$S_2O_3^{2-}$, Br^-, Cl^-, SO_4^{2-}, NO_2^-, NO_3^-, F^-, PO_4^{3-}	FS 60 cm × 50 μm, 20 kV	2.25 m*M* pyromellitate, 6.5 m*M* NaOH, 0.75 m*M* HMAOH, 1.6 m*M* TEA (pH 7.7)	Indirect UV at 250 nm	Serum, urine	[287]
Cl^-, SO_4^{2-}, F^-, PO_4^{3-}, CO_3^{2-}, organic acids	FS 52 cm × 75 μm, 20 kV	5.5–7.5 m*M* Na_2CrO_4, 3 − 5 m*M* TTABr, 1–3 m*M* DTABr (pH 9)	Indirect UV at 254 nm	Bayer liquor	[288]
$S_2O_3^{2-}$, Cl^-, SO_4^{2-}, CO_3^{2-}, organic acids	FS 60 cm × 75 μm, 20 kV	5 m*M* Na_2CrO_4, OFM Anion-BT (pH 10)	Indirect UV at 254 nm	Kraft Black Liquor	[289]
Cl^-, SO_4^{2-}	FS 52 cm × 75 μm, 20 kV	10 m*M* $LiNO_3$, 1 m*M* NaSCN, 0.8% (v/v) 1-butanol (pH 6)	Direct UV at 214 nm	Dissolved concrete	[290]

13.3.3 Inorganic cations

13.3.3.1 Separation strategy

As in the case of inorganic anions, inorganic cations are usually separated with co-EOF. In this case, the positive electrode is placed at the injection side and, when a bare fused-silica capillary is used, the EOF is towards the detection side (cathode). Under these conditions, the migration order is such that the analyte with the highest positive electrophoretic mobility migrates fastest and the analyte having the lowest positive mobility migrates slowest. Fig. 13.14 provides a schematic illustration of the electrophoretic mobilities of common inorganic cations. It can be seen that many inorganic cations exhibit very similar mobilities, *e.g.*, the whole group of rare-earth metals and numerous transition-metal ions. Additional sources of separation selectivity are therefore needed in order to separate these species.

The desired separation selectivity is usually achieved through the addition of an auxiliary ligand to the BGE in order to complex partially the analyte cations. Provided the degree of complexation is different for each analyte cation, the effective charge and, hence, the effective mobility will be unique, and separation will be possible. Auxiliary ligands are usually weakly complexing (*e.g.*, HIBA or 18-crown-6) and normally form

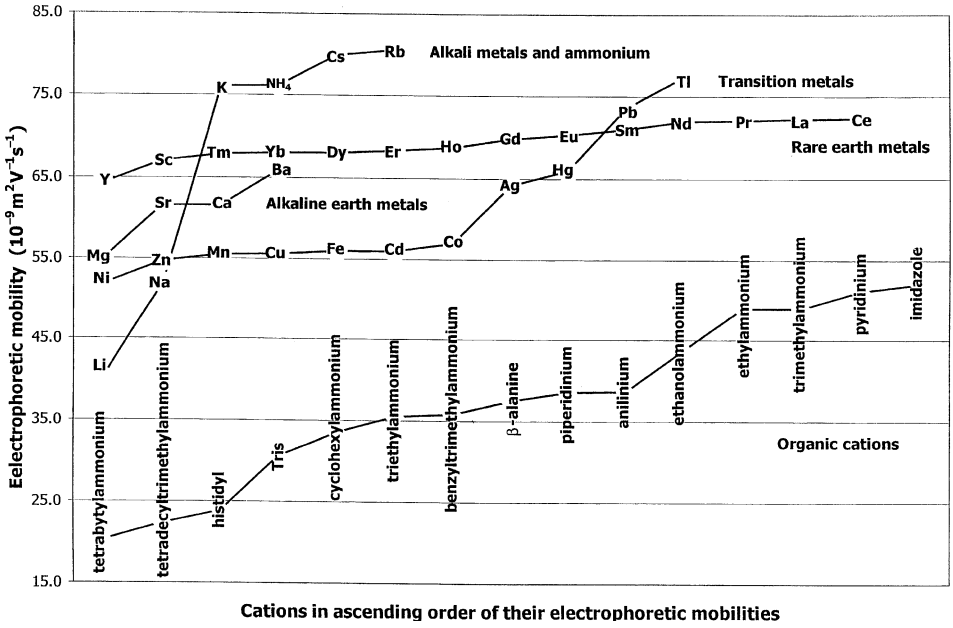


Fig. 13.14. Electrophoretic mobilities of metal ions and some organic cations in ascending order of their mobilities. Ammonium was included with the alkali metals because of its frequent importance in analysis of metal ions. Legend: charges of metal cations left out for simplicity, transition metal ions divalent apart from monovalent Ag and Tl; organic cations all bearing a charge of +1. (Reproduced from Ref. 266 with permission.)

part of the BGE into which samples containing free metal ions are injected, *i.e.*, *on-capillary complexation* is utilized. A substantial part of the metal remains in the uncomplexed form, and therefore indirect absorption detection with addition of a cationic absorbing probe to the BGE is normally applied. Rapid complexation kinetics are desirable. For a metal ion forming several complexes with the auxiliary ligand, which are in equilibrium with rapid kinetics of interchange between the forms, the effective mobility of the analyte is given by the weighted average of the mobilities of each of the forms. For a metal ion, M, migrating in a BGE containing a ligand, L, forming complexes $ML, ML_2, \ldots ML_i$, the effective mobility of the metal, $\bar{\mu}_M$, can then be expressed as

$$\overline{\mu}_M = \sum_{i=0}^{n} \mu_i \alpha_i = \frac{\sum_{i=0}^{n} \mu_i [ML_i]}{\sum_{i=0}^{n} [ML_i]} = \frac{\sum_{i=0}^{n} \mu_i \beta_i [L]^i}{\sum_{i=0}^{n} \beta_i [L]^i} \tag{13.3}$$

where μ_i is mobility and α_i is the fraction of metal ion existing in form i, $[ML_i]$ is the concentration of the complex ML_i, $[L]$ is the concentration of the form of ligand forming the complex, and β is the overall stability constant (here, $ML_0 = M$, $\beta_0 = 1$).

As an alternative approach, strongly complexing ligands which form anionic complexes can be used for *pre-capillary complexation* reactions, followed by separation as anions, using the same approaches as those discussed earlier. Ligands, such as EDTA or PAR, have been utilized for this purpose and are usually added to the sample. Since most of the metal ion is complexed under all BGE conditions, the complex formation/dissociation cannot be used to govern the separation selectivity as in the case of weakly complexing ligands. The separation selectivity therefore depends strongly on the nature of the ligand used. It should be noted that metal ions are prone to become adsorbed on the surface of silica capillaries through interaction with silanol groups. Such adsorption can lead to changes in the EOF and poor peak shapes for some metal ions.

13.3.3.2 Detection

Indirect photometric detection is again the most commonly used detection method in the separation of metal ions, being employed in about 85% of publications. The underlying principles for indirect photometric detection of inorganic cations are the same as for inorganic anions, and Fig. 13.15 illustrates the electrophoretic mobilities of some metal cations as analytes and some common cationic probes.

Direct detection of complexed cations can also be employed, and in this case, the auxiliary ligand, which in the earlier discussion was shown to play a crucial role in the separation of metal ions, also governs the detectability of these species. Metallochromic ligands form highly absorbing (*i.e.*, having a molar absorptivity, ε, of *ca.* 10^5 L mol^{-1} cm^{-1}), colored complexes with metal ions, and when these complexes are stable and the separation results in well-shaped peaks, very good detection sensitivity can be obtained. For example, determination of transition metals in the form of

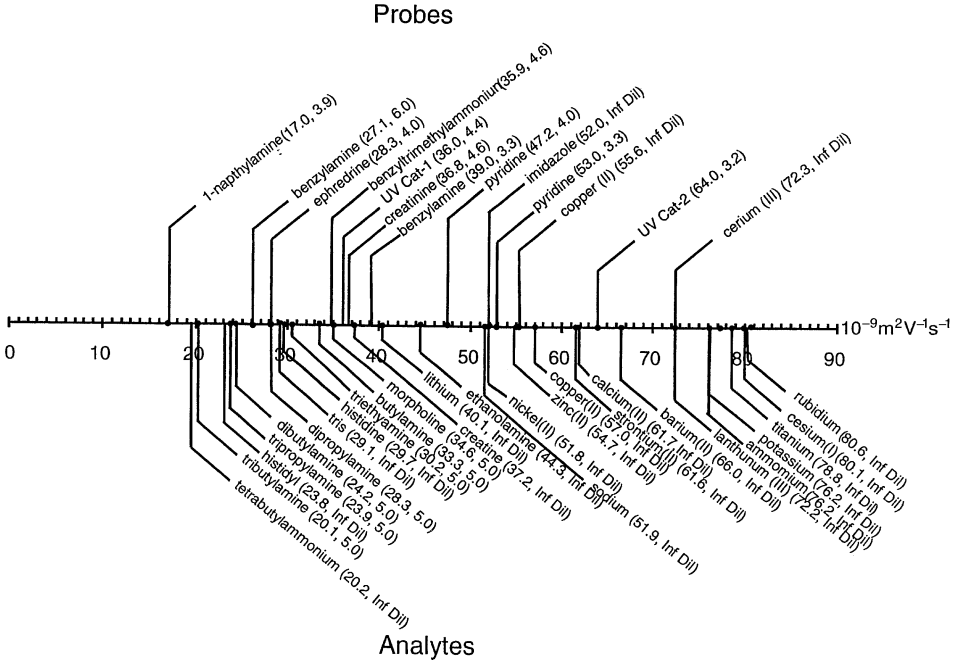


Fig. 13.15. Matching of electrophoretic mobilities of some metal ion analytes and probes. Given in brackets are mobility and, optionally, a pH. Data taken from Ref. 273. (Reproduced from Ref. 270 with permission.)

complexes with PAR achieved concentration limits of detction (LODs) in the order of 10^{-7} mol/L or absolute LODs at fmol levels. Even lower LODs (80 *nM* or 0.5 fmol for Zn) were obtained for porphinate complexes (ε *ca.* $7{\cdot}10^5$ L mol^{-1}cm^{-1}), but the detection method is less universal, because different metal ions exhibit different absorption maxima and absorptivities.

End-capillary conductometric detection has been used for a range of cations with weakly conductive BGEs. Success was similar to that in anion analysis (detection limits are in the low-μ*M* range). CZE coupled with electrospray mass spectrometry (CZE/MS) or inductively coupled-plasma mass spectrometry (CZE/ICPMS) can be used since the advent of special nebulizers, such as the direct-injection nebulizer, which introduces 100% of the sample into the plasma and does not cause any detectable peak-broadening. A successful interface for CZE/ICPMS should satisfy three main requirements: first, the interface must provide an electrical connection and a stable electrical current for reproducible electrophoretic separations. Second, the interface must be able to adapt the electro-osmotic flow inside the CZE capillary to the flow-rate of the nebulizer. Third, the interface should prevent pressure-induced flow inside the CZE capillary resulting from a pressure difference at the end of the capillary and caused by the nebulizer. Despite the disadvantages of high operating costs and equipment complexity, CZE/ICPMS has been applied successfully, often with limits of detection in the sub-nM range.

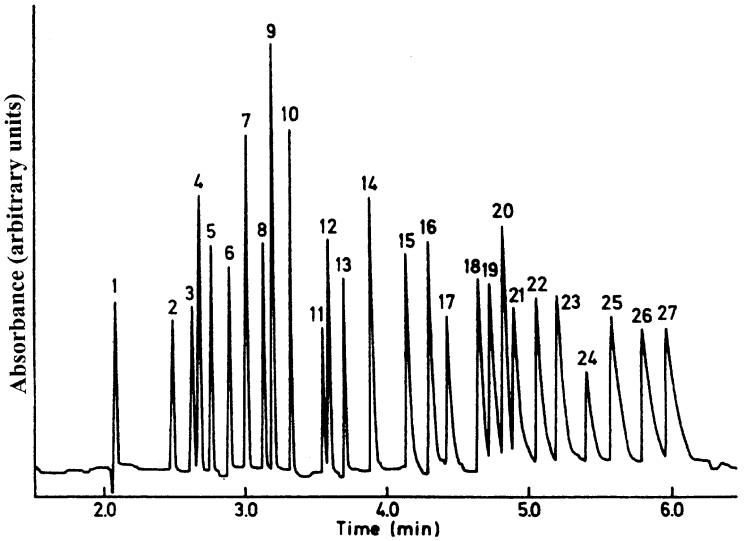


Fig. 13.16. Separation of 27 alkali, alkaline-earth, transition and rare-earth metal ions in a single run with lactate as auxiliary ligand. Conditions: capillary, fused silica, 75 μm ID, length 0.600 m, 0.527 m to detector; BGE, 15 m*M* lactic acid, 8 m*M* 4-methylbenzylamine, 5% methanol (pH 4.25); separation voltage, 30 kV; detection, indirect at 214 nm; injection, hydrostatic at 100 mm for 30 sec; sample, 1–5 ppm of each metal. Peak identification: 1 = K^+, 2 = Ba^{2+}, 3 = Sr^{2+}, 4 = Na^+, 5 = Ca^{2+}, 6 = Mg^{2+}, 7 = Mn^{2+}, 8 = Cd^{2+}, 9 = Li^+, 10 = Co^{2+}, 11 = Pb^{2+}, 12 = Ni^{2+}, 13 = Zn^{2+}, 14 = La^{3+}, 15 = Ce^{3+}, 16 = Pr^{3+}, 17 = Nd^{3+}, 18 = Sm^{3+}, 19 = Gd^{3+}, 20 = Cu^{2+}, 21 = Tb^{3+}, 22 = Dy^{3+}, 23 = Ho^{3+}, 24 = Er^{3+}, 25 = Tm^{3+}, 26 = Yb^{3+}, 27 = Lu^{3+}. (Reproduced from Ref. 291 with permission.)

13.3.3.3 Applications

Up to 30 metal ions can be separated in a single run, in a BGE containing a weakly complexing auxiliary ligand and a suitable indirect photometric detection probe. Fig. 13.16 illustrates such a separation and, as for anions, demonstrates the excellent separation capability of CZE when applied to inorganic cations. Typical precision for migration times, peak areas, and peak heights show a relative standard deviation ranging from 3 to 10%. As with anions, many of the real samples analyzed have been various water samples, fruit juices, beverages, and food. Typical applications are included in Table 13.6.

13.3.4 Simultaneous electroseparations of anions and cations

While it is normal for anions and cations to be separated in individual runs, simultaneous separations are possible using a range of strategies. One approach is to inject the sample into both ends of the capillary and to locate the detector approximately in the middle of the capillary. When indirect photometric detection is to be used, both an anionic and a cationic UV-absorbing probe need to be present. One way to achieve this without the introduction of unwanted co-ions, which would interfere with the separation, is to mix the free acid and free

TABLE 13.6

EXAMPLES OF CAPILLARY-ELECTROPHORETIC SEPARATIONS OF CATIONS

Analyte	Capillary, voltage	Electrolyte	Detection	Sample	Ref.
Cs^+, NH_4^+, Na^+, Li^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Ni^{2+}, Cd^{2+}, Al^{3+}, Ce^{3+}	FS 57 cm × 50 μm, 30 kV	4 m*M* 4-methylaminophenolsulfate, 20% acetone	Indirect UV at 220 nm	Seawater	[292]
Li^+, K^+, Ca^{2+}, Cr^{3+}, Zn^{2+}, Al^{3+}, Cu^{2+}	FS 50 cm × 50 μm, 15 kV	5.2 m*M* ephedrine, 4.7 mM hydroxyisobutyric acid (pH 2.8)	Indirect UV at 204 nm	Standards	[293]
K^+, Ba^{2+}, Na^+, Li^+	FS 50 cm × 75 μm, 7.5 kV	10 *mM p*-anisic acid, Tris (pH 8.24)	Indirect UV at 270 nm	Standards	[294]
Na^+, K^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}	FS 52 cm × 75 μm, 20 kV	5 m*M* UV Cat-1, 6.5 m*M* hydroxy-isobutyric acid, 6.2 m*M* 18-crown-6, 25% MeOH	Indirect UV at 185	Seawater	[295]
Co^{2+}, Ni^{2+}, Zn^{2+}, Hg^{2+}	FS 58 cm × 75 μm, 20 kV	50 m*M* acetate, 2 m*M* CTAB, 0.1 m*M* 5-Br-PADAP (pH 6.0)	Direct UV/Vis	Hair	[296]
Pb^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Fe^{2+}	FS 50 cm × 50 μm, 12 kV	7.5 m*M* salicylic acid, 0.1 m*M* EDTA, 0.02 m*M* CTAB (pH 4.01)	Direct UV at 200 nm	Standards	[297]
NH_4^+, K^+, Ca^{2+}, Na^+, Mg^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+}, Li^+	FS 50 cm × 75 μm, 20 kV	10 m*M* imidazole, 5 m*M* lactate, 0.5 m*M* 18-crown-6 (pH 4.5)	Indirect UV at 254 nm	Standards	[298]
Mn^{2+}, Zn^{2+}, NH_4^+	FS 60 cm × 75 μm, 20 kV	5 m*M* imidazole, 6.5 m*M* hydroxyisobutyric acid, 0.55 m*M* 18-crown-6, 20% MeOH (pH 4.5)	Indirect UV at 214 nm	Oyster tissue	[299]
U(VI), lanthanides	FS 52 cm × 75 μm, 30 kV	25 m*M* Arsenazo III, 15 m*M* citrate, 20 m*M* Tris (pH 4.3)	Vis at 654 nm	Standards	[300]
Mn^{2+}, Ni^{2+}, Co^{2+}, La^{3+}	FS 50 cm × 50 μm, 10 kV	20 m*M* CH_3COONH_4 (pH 7.0)	ICP-MS	Standards	[301]

FS = fused-silica, CTAB = cetyltrimethylammonium bromide, 5-Br-PADAP = 2-(5-bromo-2-pyridylazo)-5-(diethylamino)phenol, UV Cat-1 is a proprietary indirect detection probe produced by Waters Corporation.

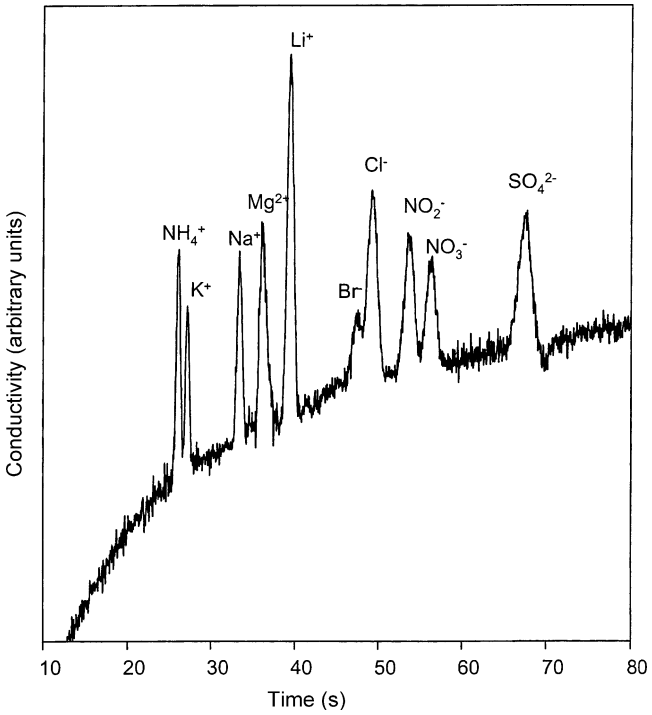


Fig. 13.17. High-speed simultaneous separation of inorganic anions and cations by injection into both ends of the capillary and detection with a contactless conductivity detector, placed in the middle of the capillary. The BGE was 50 m*M* MES/His, 0.001% sodium polyanethol sulfonate at pH 6. A separation voltage of + 25 kV was used. Sample: 5 ppm of each ion. (Reproduced from Ref. 302 with permission.)

base forms of the two probes. Contactless conductivity detection offers a simpler detection approach and is especially useful for simultaneous separation of anions and cations, because the position of the detector on the capillary can be manipulated easily. Thus, the detector can be placed at the point where the separation of the anions and cations, moving in opposite directions is maximized: *i.e.*, the apparent separation selectivity is optimized. Fig. 13.17 shows an example of such a separation.

13.3.5 Co-ordination compounds and organometallics

13.3.5.1 Co-ordination compounds

As in the case of liquid-chromatographic methods, a large amount of research has been conducted into the separation of co-ordination compounds by electroseparation methods. Typically, these separations are performed using micellar electrokinetic capillary chromatography (MEKC), in which neutral analytes partition into the interior of a micelle (typically sodium dodecylsulfate [SDS]). Separation results from the fact that differences in hydrophobicity between analytes will result in each analyte having a different partition coefficient with the micellar pseudo-stationary phase. This mechanism is therefore very

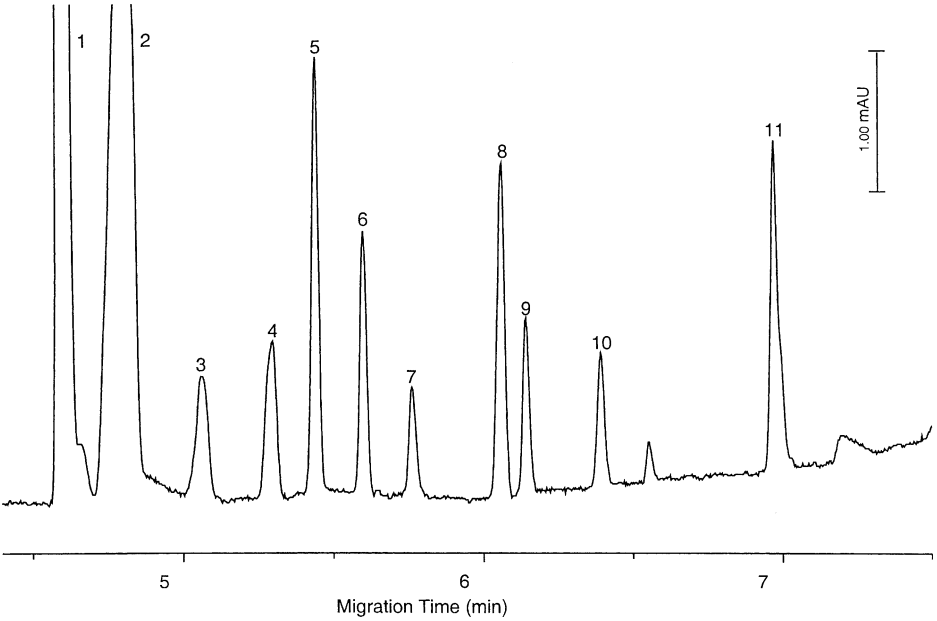


Fig. 13.18. Separation of HEDTC complexes, using a 4-morpholinepropanesulfonic acid(MOPS)/Tris BGE (60 m*M* MOPS, 30 m*M* Tris, 10 m*M* SDS, 100 μ*M* HEDTC [pH 7.2]). Peaks: 1 = EOF, 2 = oxidation product, 3 = Cd(II), 4 = Pb(II), 5 = Pt(II), 6 = Co(II), Ni(II), 8 = Bi(III), 9 = Cr(III), 10 = Cu(II), 11 = Hg(II). Concentration of each metal, 10 μ*M*. (Reproduced from Ref. 303 with permission.)

similar to that of reversed-phase HPLC, and thus it is not surprising that many of the ligands used in HPLC have also been applied to MEKC. This is especially true of dithiocarbamates. Fig. 13.18 shows a separation of some complexes of metal ions with *bis*(2-hydroxyethyl)dithiocarbamate (HEDTC). HEDTC has the advantage of forming water-soluble complexes which still retain sufficient hydrophobicity to permit their separation by MEKC. Further examples of electroseparations of metal complexes are included in Table 13.7.

13.3.5.2 Organometallic compounds

Electroseparation methods have been applied widely to the separation of organometallic compounds of tin, lead, mercury, arsenic, and selenium. A recent comprehensive review covering literature on this topic published since 1992 is available [316]. Aqueous BGEs are used; acetate, phosphate and borate are the most commonly employed buffers, and SDS is commonly added to enable neutral analytes to be separated by MEKC. Some analytes which are not normally soluble in aqueous solution were separated by adding organic solvents to the BGE in order to improve their solubility in the separation system. Complexing additives are sometimes used both for pre-capillary complexation of analytes prior to injection as well as in the form of BGE additives during the separation.

TABLE 13.7

EXAMPLES OF CAPILLARY-ELECTROPHORETIC SEPARATIONS OF ORGANOMETALLIC SPECIES AND CO-ORDINATION COMPOUNDS

Analyte	Capillary, volts	Electrolyte	Detection	Sample	Ref.
Se(IV), Se(VI), GSSeSG, SeM, SeC and SeCM	FS, 150 cm × 50 μm, 18 kV	10 m*M* Na_2CO_3 (adjusted to pH 11.5 with KOH)	ICP-MS	Human milk, blood, serum	[304]
As(III), As(V), DMA, MMA, DPA, PAA, and PABA	FS, 70 cm × 75 μm, 25 kV	15 m*M* phosphate (pH 6.5), containing SDS	UV, 200 nm	Urine	[305]
As(III), As(V), DMA, MMA, AsB and AsC	FS, 150 cm × 50 μm, 18 kV	20 m*M* Na_2HPO_4/NaH_2PO_4 (pH 5.6)/acetonitrile (1:1)	ICP-MS	Urine and sewage sludge	[306]
Methyl-Hg and inorganic Hg	FS, 75 cm × 75 μm, 21 kV	25 m*M* $CaCl_2$ (pH 7)	ICP-MS	Reference materials	[307]
Methyl-,ethyl-, phenyl-Hg	FS 50 cm × 75 μm, 18 kV	0.1 *M* sodium borate (pH 8.35)	UV, 200 nm	Marine sample	[308]
Triorganotin species	FS 60 cm × 75 μm, 300 V/cm	5 m*M* acetate, 3 m*M* 4-aminopyridine (pH 4.5)	Indirect UV	Sediment	[309]
TELC, DPS, TMLC, DPLC, Pb(II), Se(IV), PMA	FS 65 cm × 50 μm, 20 kV	40 m*M* SDS, 5 m*M* EDTA in phosphate/borate buffer (pH 7)	UV, 200 nm	Not applicable	[310]
Se(VI), Se(IV), As(V), Te(IV), MMA, SeC, SeM, Te(VI), As(III), Sb(V), DMA	FS 60 cm × 75 μm, − 10 kV	5 m*M* Na_2CrO_4, 0.5 m*M* TTAOH (pH 11.2)	UV, 254 nm	Soil	[311]
DEDTC complexes of TMLC, TELC, TMT, and TBT	FS 55 or 60 cm × 75 μm, 20 kV.	0.05 *M* SDS in 15 m*M* borate-30 m*M* phosphate buffer (pH 7.65)	UV, 200 nm.	Standards	[312]

(*Continued on next page*)

TABLE 13.7 (continued)

Analyte	Capillary, volts	Electrolyte	Detection	Sample	Ref.
TMLC, TELC, diphenyl selenide and phenylselenyl chloride	FS 44 cm × 50 μm, 15 kV	50 m*M* SDS, 5 m*M* β-CD in 25 m*M* phosphate/borate buffer (pH 6.0)	UV, 210 nm	Water	[313]
Cyanide complexes of Fe(II), Pd(II), Co(II), Pt(II), Fe(III), Cr(III), Au(I), Ag(I)	FS 44 cm × 50 μm, −20 kV	Phosphate-triethanolamine buffer (pH 8.), containing 0.8 m*M* hexamethonium bromide	UV, 214 nm	Standards	[314]
Bis(carboxymethyl)dithio-carbamate complexes of Cd(II), Ni(II), Pb(II), Hg(II), Cu(II)	FS 42 cm × 75 μm, 12 kV	20 m*M* sodium tetraborate (pH 9.1)	UV, 254 nm	Standards	[315]
2,6 pyridinedicarboxylic acid complexes of Cu(II), Ni(II), Co(II), Fe(II), Fe(III)	FS 45 cm × 50 μm, −30 kV	20 m*M* pyridinedicarboxylic acid, 0.5 m*M* CTAOH (pH 3.8)	UV, 230 nm	Plating bath	[304]

AsB = arsenobetaine, AsC = arsenocholine, CTA = cetyltrimethylammonium, DEDTC = diethyldithiocarbamate, DMA = dimethylarsinic acid, DPA = diphenylarsonic acid, DPLC = diphenyllead chloride, FS = fused-silica, GSSeSG = selenium-carrying glutathione, MMA = monomethylarsonic acid, PAA = phenylarsonate, PABA = *p*-aminobenzenearsonate, PMA = phenylmercuric acetate, SDS = sodium dodecylsulfate, SeC = selenocystine, SeCM = selenocystamine, SeM = selenomethionine, TBT = tributyltin, TELC = tetraethyllead chloride, TMLC = tetramethyllead chloride, TMT = trimethyltin, TTA = tetradecyltrimethylammonium.

On-capillary direct photometric detection in the UV region is the most commonly used detection method, but it suffers from poor sensitivity because of the short light-path lengths. This loss of sensitivity can be only partly compensated for by the very high efficiencies of CE separations. ICP-MS coupled with CZE has increasingly been applied as an extremely sensitive, element-specific, multi-element detector. Since ICP-MS was first coupled with CE, its applications in speciation have increased sharply. In all cases of speciation with CE/ICP-MS, an electrolyte liquid sheath-flow has been applied as a make-up solution to complete the electrical circuit. To eliminate the pressure-induced flow inside the capillary, the make-up solution also has the function of controlling the nebulizer suction. The flow-rate of the make-up solution is usually optimized, either by using a reciprocating pump or by self-aspiration with the nebulizer.

Because the organometallic species commonly determined by CZE are of great environmental significance, their determination in environmental samples at very low concentration is frequently required. The sensitivity limitations of CZE with spectrophotometric detection often limit the application of the technique in these cases, and for this reason, there has been a great deal of attention paid to the enhancement of detection sensitivity. Approaches to the achievement of this goal have included off-line methods (such as pre-concentration of tin species by solid-phase extraction [317,318] and a wide range of on-line sample-stacking methods [316]. These stacking methods all involve focusing, or zone compression, of analytes from a relatively large sample injection volume, prior to the separation step. Fig. 13.19 shows a typical example of the use of CZE for the separation of organometallic species (in this case, arsenic species), and further examples are included in Table 13.7.

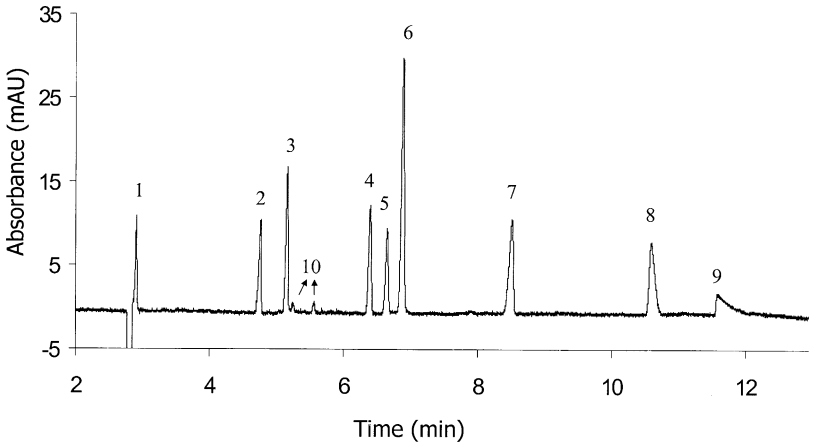


Fig. 13.19. Electropherogram of arsenic compounds separated in the counter-EOF mode. Conditions: Capillary, fused silica, 75 μm × 48.5 cm (effective length 40.0 cm); separation voltage, +18 kV; electrolyte, 20 mmol/L $NaHCO_3$–Na_2CO_3 buffer (pH 10.0); injection, 2 sec at 50 mbar, 4 mg/L As for aryl arsenic species, 20 mg/L As for others. Peaks: 1 = phenylarsenic oxide, 2 = dimethylarsinic acid, 3 = As(III), 4 = *p*-aminophenylarsonic acid, 5 = 4-nitrophenylarsonic acid, 6 = phenylarsonic acid, 7 = monomethylarsonic acid, 8 = Roxarsone, 9 = As(V), 10 = impurity in *p*-aminophenylarsonic acid. (Reproduced from Ref. 316 with permission.)

13.3.6 Comparison of electroseparation and liquid chromatography

The preceding discussion has shown that liquid-chromatographic and electroseparation methods can both be used for the separation of inorganic species. It is therefore pertinent to make a comparison of the two approaches and to point out the particular merits and drawbacks of each. It should be stressed that the following comments relate specifically to the applications of these techniques to inorganic analysis and are not intended to extend to the entire range of applications for which LC and CE can be used. In the case of separations of inorganic cations and anions, a direct comparison can be made between suppressed IC (with conductivity detection) and co-EOF CE (with indirect photometric detection), since these two approaches represent the most widely used methods in liquid chromatography and electroseparations, respectively. The following conclusions have been drawn [319]:

(a) IC has been developed much more extensively than CE, and there are many more published IC applications and IC regulatory methods than is the case for CE.
(b) CE offers higher separation efficiencies than IC (250,000 plates for a CE capillary *vs.* 4,000 plates for an IC column), but the efficiency is not maintained for all peaks in an electropherogram due to electromigration dispersion effects. This means that the peak capacity of CE over a practical time interval exceeds that of IC by only a relatively small factor (peak capacity of 90 for CE *vs.* 30 for IC).
(c) The detection limits attainable in suppressed IC are much lower than those for CE with indirect photometric detection for normal injection volumes (*ca.* 10 ppb for IC *vs.* 200 ppb for CE). Detection limits can be further lowered in IC by using pre-concentration methods and in CE by using electromigration injection or electrostacking.
(d) The precision of IC (typically 1% RSD) is generally superior to that of CE (typically 3–5% RSD).
(e) Under standard conditions, IC and CE exhibit different separation selectivities, as revealed by the elution or migration order of common analytes. In the case of IC, anions are eluted in the following order of retention times: $F^- < Cl^- < NO_2^- < Br^- < NO_3^- < PO_4^{3-} < SO_4^{2-} < I^-$. However, the migration times of these species in co-EOF CE are $Br^- < Cl^- < SO_4^{2-} < NO_2^- < I^- < NO_3^- < F^- < PO_4^{3-}$. A similar pattern emerges for inorganic cations for which the IC retention times follow the order $Li^+ < Na^+ < NH_4^+ < K^+ < Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$, while migration times for co-EOF CE are $NH_4^+ < K^+ < Ba^{2+} < Sr^{2+} < Ca^{2+} < Na^+ < Mg^{2+} < Li^+$. These sequences show that the selectivities of each of the two techniques are complementary, and this can be used to advantage in solving analytical problems. Thus, separation of fluoride from a difficult matrix is likely to be problematic in IC because of the early elution of this species, but it should be feasible by using co-EOF CE. The reverse situation would be expected to apply for bromide. Similarly, one would expect co-EOF CE to be best for analytes like lithium and sodium in complex matrices, but IC to be better for analytes like potassium.

The above factors suggest that IC and CE should be viewed as complementary rather than competitive techniques. When applied to other types of inorganic analytes

(co-ordination compounds and organometallic species), it is fair to say that CE offers generally superior separation efficiency, but it is hampered by relatively poor detection limits. Combination with sensitive spectroscopic detection methods, such as ICP-MS offers a solution to this drawback, but such a coupling is still at an experimental stage for CE. On the other hand, the combination with LC methods is well developed, and numerous commercial interfaces are available. For this reason, LC will probably remain the method of choice for practical applications until CE interfaces become more readily accessible.

REFERENCES

1 P.R. Haddad and E. Patsalides, in E. Heftmann (Ed.), *Chromatography*, 5th Edn. Elsevier, Amsterdam, 1992, pp. B1–B74.
2 Y.S. Drugov, *J. Anal. Chem.*, 53 (1998) 606.
3 C.M. Wai and S.F. Wang, *J. Biochem. Biophys. Methods*, 43 (2000) 273.
4 J. Hrouzek and J. Krupcik, *Chemicke Listy*, 87 (1993) 463.
5 J.M. Cano-Pavon, A.G. De Torres, F. Sanchez-Rojas and P. Canada-Rudner, *Int. J. Environ. Anal. Chem.*, 75 (1999) 93.
6 A.M. Carro and M.C. Mejuto, *J. Chromatogr. A*, 882 (2000) 283.
7 I.R. Pereiro and A.C. Diaz, *Anal. Bioanal. Chem.*, 372 (2002) 74.
8 V.G. Berezkin, *Russ. Chem. Bull.*, 48 (1999) 1807.
9 P.C. Uden, *J. Chromatogr.*, 703 (1995) 393.
10 J. Szpunar and R.S. Lobinski, *Fresenius J. Anal. Chem.*, 363 (1999) 550.
11 K. Fytianos, *J. AOAC Int.*, 84 (2001) 1763.
12 R.S. Lobinski, I.R. Pereiro, H. Chassaigne, A. Wasik and J. Szpunar, *J. Anal. Atomic Spectrom.*, 13 (1998) 859.
13 D.M. Templeton, F. Ariese, R. Cornelis, L.G. Danielsson, H. Muntau, H.P. Van Leeuwen and R. Lobinski, *Pure Appl. Chem.*, 72 (2000) 1453.
14 A. Sanz-Medel, *Spectrochim. Acta B*, 53 (1998) 197.
15 R. Lobinski, *Spectrochim. Acta B*, 53 (1998) 177.
16 L.E. Ebdon, S. Hill and R.W. Ward, *Analyst*, 111 (1986) 1113.
17 R. Lobinski, V. Sidelnikov, Y. Patrushev, I. Rodriguez and A. Wasik, *Trends Anal. Chem.*, 18 (1999) 449.
18 K. Sutton, R.M.C. Sutton and J.A. Caruso, *J. Chromatogr. A*, 789 (1997) 85.
19 L.A. Ellis and D.J. Roberts, *J. Chromatogr. A*, 774 (1997) 3.
20 H. Tao, *Bunseki Kagaku*, 46 (1997) 239.
21 S. Pedersenbjergaard and T. Greibrokk, *J. High Resolut. Chromatogr.*, 19 (1996) 597.
22 H. Emteborg, H.W. Sinemus, B. Radziuk, D.C. Baxter and W. Frech, *Spectrochim. Acta B*, 51 (1996) 829.
23 M.B.D. Guntinas, M. Ceulemans, C. Witte, R. Lobinski and F.C. Adams, *Mikrochim. Acta*, 120 (1995) 73.
24 M. Heisterkamp and F.C. Adams, *Fresenius J. Anal. Chem.*, 362 (1998) 489.
25 C. Gerbersmann, M. Heisterkamp, F.C. Adams and J.A.C. Broekaert, *Anal. Chim. Acta*, 350 (1997) 273.
26 Y. Liu, V. Lopezavila, M. Alcaraz, W.F. Beckert and E.M. Heithmar, *J. Chromatogr. Sci.*, 31 (1993) 310.

27 I.R. Pereiro, V.O. Schmitt and R. Lobinski, *Anal. Chem.*, 69 (1997) 4799.
28 I.R. Pereiro, A. Wasik and R. Lobinski, *J. Anal. Atomic Spectrom.*, 13 (1998) 743.
29 M. Ceulemans, R. Lobinski, W.M.R. Dirkx and F.C. Adams, *Fresenius J. Anal. Chem.*, 347 (1993) 256.
30 M. Ceulemans and F.C. Adams, *J. Anal. Atomic Spectrom.*, 11 (1996) 201.
31 J. Szpunarlobinska, M. Ceulemans, W. Dirkx, C. Witte, R. Lobinski and F.C. Adams, *Mikrochim. Acta*, 113 (1994) 287.
32 N.G. Orellana-Velado, R. Pereiro and A. Sanz-Medel, *J. Anal. Atomic Spectrom.*, 13 (1998) 905.
33 P. Read, H. Beere, L. Ebdon, M. Leizers, M. Hetheridge and S. Rowland, *Org. Geochem.*, 26 (1997) 11.
34 T. De Smaele, L. Moens, R. Dams and P. Sandra, *Fresenius J. Anal. Chem.*, 355 (1996) 778.
35 E.M. Krupp, R. Grumping, U.R.R. Furchtbar and A.V. Hirner, *Fresenius J. Anal. Chem.*, 354 (1996) 546.
36 M. Heisterkamp and F.C. Adams, *Fresenius J. Anal. Chem.*, 370 (2001) 597.
37 T. De Smaele, J. Vercauteren, L. Moens, R. Dams and P. Sandra, *Am. Lab.*, 33 (2001) 39.
38 S. Aguerre, G. Lespes, V. Desauziers and M. Potin-Gautier, *J. Anal. Atomic Spectrom.*, 16 (2001) 263.
39 U.M. Gruter, J. Kresimon and A.V. Hirner, *Fresenius J. Anal. Chem.*, 368 (2000) 67.
40 J. Feldmann, T. Riechmann and A.V. Hirner, *Fresenius J. Anal. Chem.*, 354 (1996) 620.
41 H. Frischenschlager, C. Mittermayr, M. Peck, E. Rosenberg and M. Grasserbauer, *J. Anal. Chem.*, 359 (1997) 21.
42 L. Ebdon, E.H. Evans, W.G. Pretorius and S.J. Rowland, *J. Anal. Atomic Spectrom.*, 9 (1994) 939.
43 G.B. Jiang, Q.F. Zhou and B. He, *Environ. Sci. Technol.*, 34 (2000) 2697.
44 B.V. Stolyarov, L.A. Kartsova and O.V. Filippova, *J. Anal. Chem.*, 52 (1997) 400.
45 G. Becker, A. Colmsjo and C. Ostman, *Anal. Chim. Acta*, 340 (1997) 181.
46 A.M. Gonzalez and P.C. Uden, *J. Chromatogr.*, 898 (2000) 201.
47 H.W. Jing and A. Amirav, *J. Chromatogr.*, 805 (1998) 177.
48 A. Marco, R. Compano, R. Rubio, I. Casals, L. Krotz, L. Ragaglia and G. Giazzi, *Analyst*, 126 (2001) 1820.
49 V.A. Krylov, S.G. Krasotskii, V.M. Malyshev and Y.M. Salganskii, *J. Anal. Chem.*, 56 (2001) 996.
50 G. Gryglewicz and S. Gryglewicz, *Fresenius J. Anal. Chem.*, 370 (2001) 60.
51 F. Zude, W. Fan, N. Trautmann, G. Herrmann and B. Eichler, *Radiochim. Acta*, 62 (1993) 61.
52 S. Taut, S. Hubener, B. Eichler, H.W. Gaggeler, M. Schadel and I. Zvara, *Radiochim. Acta*, 78 (1997) 33.
53 S. Taut, S. Hubener, B. Eichler, A. Turler, H.W. Gaggeler, S.N. Timokhin and I. Zvara, *J. Alloys Compounds*, 271 (1998) 316.
54 R. Eichler and M. Schadel, *J. Phys. Chem. B*, 106 (2002) 5413.
55 B. Eichler, H.P. Zimmermann and H.W. Gaggeler, *J. Phys. Chem.*, 104 (2000) 3126.
56 S. Monroe, *J. IEST*, 41 (1998) 21.
57 P.B. Hoke, J.J. McGrath and P.R. Loconto, *Am. Lab.*, 34 (2002) 13.
58 X.J. Zhou, P.A. Hines, K.C. White and M.W. Borer, *Anal. Chem.*, 70 (1998) 390.
59 R. Nussbaum, D. Lischke, H. Paxmann and B. Wolf, *Chromatographia*, 51 (2000) 119.
60 J. Jalbert, R. Gilbert and P. Tetreault, *Anal. Chem.*, 73 (2001) 3382.
61 I.N. Stan'kov, S.N. Tarasov and V.S. Polyakov, *J. Anal. Chem.*, 54 (1999) 473.
62 J. Maittala, S. Pennanen and J. Liesivuori, *Analyst*, 124 (1999) 665.
63 T. Ubuka, T. Abe, R. Kajikawa and K. Morino, *J. Chromatogr. B*, 757 (2001) 31.
64 J. Roels, H. Van Langenhove and W. Verstraete, *J. Chromatogr. A*, 952 (2002) 229.

65 K.N.T. Norman and K. Leonard, *J. Agric. Food Chem.*, 48 (2000) 4066.
66 R.E. Mauldin, D.A. Goldade, R.M. Engeman, M.J. Goodall, R.K. Craver and J.J. Johnston, *J. Agric. Food Chem.*, 44 (1996) 189.
67 A.G. Howard, *J. Anal. Atomic Spectrom.*, 12 (1997) 267.
68 A. Chatterjee, Y. Shibata, M. Yoneda, R. Banerjee, M. Uchida, H. Kon and M. Morita, *Anal. Chem.*, 73 (2001) 3181.
69 T. Tanaka, Y. Nakamura, A. Mizuike and A. Ono, *Anal. Sci.*, 12 (1996) 77.
70 T.S. Awad and K. Fujiwara, *Anal. Lett.*, 34 (2001) 2771.
71 J.L. Wang and C.H. Wu, *Anal. Chim. Acta*, 461 (2002) 85.
72 C.U. Galdiga and T. Greibrokk, *Chromatographia*, 46 (1997) 440.
73 J. Lasa, J. Rosiek and B. Drozdowicz, *J. Chromatogr. A*, 743 (1996) 301.
74 J.B. Laurens, J.P. de Coning and J.M. Swinley, *J. Chromatogr. A*, 911 (2001) 107.
75 M.K. Vollmer and R.F. Weiss, *Marine Chem.*, 78 (2002) 137.
76 M. Gartner, M. Boettger, B. Eichler, H.W. Gaggeler, M. Grantz, S. Hubener, D.T. Jost, D. Piguet, R. Dressler, A. Turler and A.B. Yakushev, *Radiochim. Acta*, 78 (1997) 59.
77 A.B. Yakushev, S.N. Timokhin, M.V. Vedeneev, H.G. Xu and I. Zvara, *J. Radioanal. Nucl. Chem.*, 205 (1996) 63.
78 R. Eichler, B. Eichler, H.W. Gaggeler, D.T. Jost, R. Dressler and A. Turler, *Radiochim. Acta*, 87 (1999) 151.
79 B. Eichler, *Radiochim. Acta*, 72 (1996) 19.
80 I.N. Stan'kov, V.V. Lysenko and S.N. Lanin, *J. Anal. Chem.*, 52 (1997) 580.
81 B. Cancho, F. Ventura and M.T. Galceran, *J. Chromatogr. A*, 897 (2000) 307.
82 E.V. Fedoseev, S.S. Travnikov, P.A. Korovaikov, A.V. Davydov and B.F. Myasoedov, *Radiochemistry*, 35 (1993) 540.
83 Y.G. Slizhov, V.G. Berezkin, M.A. Gavrilenko, T.T. Kuryaeva and I.M. Arkhipova, *Russ. J. Phys. Chem.*, 74 (2000) 632.
84 M.Y. Khuhawar, A.A. Memon and M.I. Bhanger, *J. Chromatogr. A*, 766 (1997) 159.
85 M.A. Arain, M.Y. Khuhawar and M.I. Bhanger, *Chromatographia*, 55 (2002) 349.
86 M.Y. Khuhawar, A.A. Memon and M.I. Bhanger, *J. Chem. Soc. Pakistan*, 22 (2000) 267.
87 M.Y. Khuhawar, A.A. Memon and M.I. Bhanger, *Chromatographia*, 49 (1999) 249.
88 J. Hrouzek, J. Krupcik and I. Skacani, *Collection of Czechoslovak Chem. Commun.*, 61 (1996) 1609.
89 S.K. Aggarwal, M. Kinter, J. Nicholson and D.A. Herold, *Anal. Chem.*, 66 (1994) 1316.
90 Y.D. Zeng and P.C. Uden, *J. High Resolut. Chromatogr.*, 17 (1994) 223.
91 I. Rykowska, S. Smyka, W. Urbaniak and W. Wasiak, *J. Chromatogr. A*, 844 (1999) 239.
92 M.Y. Khuhawar, A.A. Memon and M.I. Bhanger, *J. Chem. Soc. Pakistan*, 18 (1996) 276.
93 C.Y. Liu, S.H. Yang, M.H. Chau and C.C. Shiue, *J. Chromatogr. A*, 933 (2001) 117.
94 W.J. Kowalski, *Chem. Anal. (Warsaw)*, 42 (1997) 315.
95 R. Wawrzyniak and W. Wasiak, *Chromatographia*, 49 (1999) 147.
96 R. Wawrzyniak and W. Wasiak, *Chromatographia*, 49 (1999) 273.
97 V. Schurig, *J. Chromatogr. A*, 906 (2001) 275.
98 W.J. Kowalski, *Chem. Anal. (Warsaw)*, 40 (1995) 715.
99 M. Schleimer and V. Schurig, *J. Chromatogr. A*, 638 (1993) 85.
100 W.J. Kowalski, *Acta Chromatogr.*, 2 (1993) 984.
101 W.J. Kowalski, *Chem. Anal. (Warsaw)*, 43 (1998) 69.
102 K. Robards and E. Patsalides, *J. Chromatogr. A*, 844 (1999) 181.
103 K.J. Mulligan, *J. Microcolumn Sep.*, 7 (1995) 567.
104 D. Tsikas, R.H. Boger, S.M. Bodeboger, F.M. Gutzki and J.C. Frolich, *J. Chromatogr. B*, 661 (1994) 185.

105 S. Kage, K. Kudo and N. Ikeda, *J. Chromatogr. B*, 742 (2000) 363.
106 M. Guidotti, *J. AOAC Int.*, 83 (2000) 1082.
107 A. Elaseer and G. Nickless, *J. Chromatogr. A*, 664 (1994) 77.
108 A. Sarafrazyazdi and W.I. Stephen, *J. Chem. Soc. Pakistan*, 15 (1993) 168.
109 M.A. Arain, M.Y. Khuhawar and M.I. Bhanger, *J. Sep. Sci.*, 25 (2002) 462.
110 T. Stijve, H. Diserens, J.M. Oberson and A.A.R. Demeijer, *Dtsch. Lebensmittel-Rundschau*, 94 (1998) 112.
111 H.S. Shin, Y.S. Ohshin, J.H. Kim and J.K. Ryu, *J. Chromatogr. A*, 732 (1996) 327.
112 S. Mishra, V. Singh, A. Jain and K.K. Verma, *Analyst*, 125 (2000) 459.
113 K. Himata, T. Kuwahara, S. Ando and H. Maruoka, *Food Addit. Contam.*, 11 (1994) 559.
114 M.L. Magnuson, *Anal. Chim. Acta*, 377 (1998) 53.
115 R. Reuther, L. Jaeger and B. Allard, *Anal. Chim. Acta*, 394 (1999) 259.
116 N. Demuth and K.G. Heumann, *Anal. Chem.*, 73 (2001) 4020.
117 J. Kresimon, U.M. Gruter and A.V. Hirner, *Fresenius J. Anal. Chem.*, 371 (2001) 586.
118 T. De Smaele, L. Moens, R. Dams, P. Sandra, J. Vandereycken and J. Vandyck, *J. Chromatogr. A*, 793 (1998) 99.
119 I. Rodriguez, S. Mounicou, R. Lobinski, V. Sidelnikov, Y. Patrushev and M. Yamanaka, *Anal. Chem.*, 71 (1999) 4534.
120 I.R. Pereiro, A. Wasik and R. Lobinski, *J. Chromatogr. A*, 795 (1998) 359.
121 D.C. Baxter and W. Frech, *Analyst*, 118 (1993) 495.
122 S. Mothes and R. Wennrich, *Mikrochim. Acta*, 135 (2000) 91.
123 M.M. Bayon, M.G. Camblor, J.I.G. Alonso and A. Sanz-Medel, *J. Anal. Atomic Spectrom.*, 14 (1999) 1317.
124 C.M. Barshick, S.A. Barshick, E.B. Walsh, M.A. Vance and P.F. Britt, *Anal. Chem.*, 71 (1999) 483.
125 A.V. Hirner, U.M. Gruter and J. Kresimon, *Fresenius J. Anal. Chem.*, 368 (2000) 263.
126 R. Pongratz and K.G. Heumann, *Chemosphere*, 39 (1999) 89.
127 J.R. Encinar, I.L. Granadillo, J.I.G. Alonso and A. Sanz-Medel, *J. Anal. Atomic Spectrom.*, 16 (2001) 475.
128 J.R. Baena, M. Gallego, M. Valcarcel, J. Leenaers and F.C. Adams, *Anal. Chem.*, 73 (2001) 3927.
129 B. Pons, A. Carrera and C. Nerin, *J. Chromatogr. B*, 716 (1998) 139.
130 B. Salih, *Spectrochim. Acta B*, 55 (2000) 1117.
131 M. Crnoja, C. Haberhauer-Troyer, E. Rosenberg and N. Grasserbauer, *J. Anal. Atomic Spectrom.*, 16 (2001) 1160.
132 E.M. Krupp, C. Pecheyran, H. Pinaly, M. Motelica-Heino, D. Koller, S.M.M. Young, I.B. Brenner and O.F.X. Donard, *Spectrochim. Acta B*, 56 (2001) 1233.
133 E.M. Krupp, C. Pecheyran, S. Meffan-Main and O.F.X. Donard, *Fresenius J. Anal. Chem.*, 370 (2001) 573.
134 J. Feldmann and A.V. Hirner, *Int. J. Environ. Anal. Chem.*, 60 (1995) 339.
135 C.M. Barshick, S.A. Barshick, P.F. Britt, D.A. Lake, M.A. Vance and E.B. Walsh, *Int. J. Mass Spectrom.*, 178 (1998) 31.
136 B. Salih, R. Say, A. Denizli, O. Genc and E. Piskin, *Anal. Chim. Acta*, 371 (1998) 177.
137 Y. Cai, S. Monsalud, R. Jaffe and R.D. Jones, *J. Chromatogr. A*, 876 (2000) 147.
138 A. Dediego, C.M. Tseng, T. Stoichev, D. Amouroux and O.F.X. Donard, *J. Anal. Atomic Spectrom.*, 13 (1998) 623.
139 R.G. Fernandez, M.M. Bayon, J.I.G. Alonso and A. Sanz-Medel, *J. Mass Spectrom.*, 35 (2000) 639.
140 H. Emteborg, J. Snell, J. Qian and W. Frech, *Chemosphere*, 39 (1999) 1137.

141 B. Rosenkranz, J. Bettmer, W. Buscher, C. Breer and K. Cammann, *Appl. Organomet. Chem.*, 11 (1997) 721.
142 M.R. Brunetto, J.R. Luna, A. Zambrano, M. Gallignani, M. Burguera, J.L. Burguera and Y.P. de Pena, *Analyst*, 124 (1999) 1493.
143 B. He and G.B. Jiang, *Fresenius J. Anal. Chem.*, 365 (1999) 615.
144 H. Emteborg, E. Bjorklund, F. Odman, L. Karlsson, L. Mathiasson, W. Frech and D.C. Baxter, *Analyst*, 121 (1996) 19.
145 K. Mizuishi, M. Takeuchi and T. Hobo, *Chromatographia*, 44 (1997) 386.
146 M.S. Jimenez and R.E. Sturgeon, *J. Anal. Atomic Spectrom.*, 12 (1997) 597.
147 M.K. Donais, P.C. Uden, M.M. Schantz and S.A. Wise, *Anal. Chem.*, 68 (1996) 3859.
148 E. Rubi, R.A. Lorenzo, M.C. Casais and R. Cela, *Appl. Organomet. Chem.*, 8 (1994) 677.
149 Y. Cai, R. Jaffe, A. Alli and R.D. Jones, *Anal. Chim. Acta*, 334 (1996) 251.
150 Q. Tu, J. Qian and W. Frech, *J. Anal. Atomic Spectrom.*, 15 (2000) 1583.
151 L. Lambertsson, E. Lundberg, M. Nilsson and W. Frech, *J. Anal. Atomic Spectrom.*, 16 (2001) 1296.
152 C. Dietz, Y. Madrid, C. Camara and P. Quevauviller, *Anal. Chem.*, 72 (2000) 4178.
153 E. Cernichiari, T.Y. Toribara, L. Liang, D.O. Marsh, M.W. Berlin, G.J. Myers, C. Cox, C.F. Shamlaye, O. Choisy, P. Davidson and T.W. Clarkson, *Neurotoxicology*, 16 (1995) 613.
154 W. Frech, J.P. Snell and R.E. Sturgeon, *J. Anal. Atomic Spectrom.*, 13 (1998) 1347.
155 C.M. Tseng, D. Amouroux, G. Abril, E. Tessier, H. Etcheber and O.F.X. Donard, *Environ. Sci. Technol.*, 35 (2001) 2627.
156 R. Wagemann, E. Trebacz, R. Hunt and G. Boila, *Environ. Toxicol. Chem.*, 16 (1997) 1859.
157 Y.L. Feng and H. Narasaki, *Anal. BioAnal. Chem.*, 372 (2002) 382.
158 J.R. Encinar, M.I.M. Villar, V.G. Santamaria, J.I.G. Alonso and A. Sanz-Medel, *Anal. Chem.*, 73 (2001) 3174.
159 G.B. Jiang and Q.F. Zhou, *J. Chromatogr. A*, 886 (2000) 197.
160 J.R. Encinar, P.R. Gonzalez, J.I.G. Alonso and A. Sanz-Medel, *Anal. Chem.*, 74 (2002) 270.
161 G.B. Jiang, Q.F. Zhou, J.Y. Liu and D.J. Wu, *Environ. Pollut.*, 115 (2001) 81.
162 F. Pannier, A. Astruc and M. Astruc, *Anal. Chim. Acta*, 327 (1996) 287.
163 A.M. Caricchia, S. Chiavarini, C. Cremisini, R. Morabito and C. Ubaldi, *Int. J. Environ. Anal. Chem.*, 53 (1993) 37.
164 W.A.C. Anderson, S.A. Thorpe, L.M. Owen, S.E. Anderson, H.M. Crews and S.L. Reynolds, *Food Addit. Contam.*, 15 (1998) 288.
165 J. Feldmann, I. Koch and W.R. Cullen, *Analyst*, 123 (1998) 815.
166 M. Kumaresan and P. Riyazuddin, *Curr. Sci.*, 80 (2001) 837.
167 L.M. Smith, P.J. Craig and R.O. Jenkins, *Chemosphere*, 47 (2002) 401.
168 G.B. Jiang and F.C. Adams, *Anal. Chim. Acta*, 337 (1997) 83.
169 N. Molenat, A. Astruc, M. Holeman, G. Maury and R. Pinel, *Analusis*, 27 (1999) 795.
170 D.R. Killelea and J.H. Aldstadt, *J. Chromatogr. A*, 918 (2001) 169.
171 B. Bouyssiere, F. Baco, L. Savary, H. Garraud, D.L. Gallup and R. Lobinski, *J. Anal. Atomic Spectrom.*, 16 (2001) 1329.
172 C.A. Pohl, J.R. Stillian and P.E. Jackson, *J. Chromatogr. A*, 789 (1997) 29.
173 P.E. Jackson and C.A. Pohl, *Trends Anal. Chem.*, 16 (1997) 393.
174 Dionex IonPac AS11 Product Brochure, (2000) p. 23
175 W.W. Buchberger and P.R. Haddad, *J. Chromatogr. A*, 789 (1997) 67.
176 N. Furuta and T. Shinofuji, *Fresenius J. Anal. Chem.*, 355 (1996) 457.
177 L. Charles, D. Pepin and B. Casetta, *Anal. Chem.*, 68 (1996) 2554.
178 P.R. Haddad, S. Laksana and R.G. Simons, *J. Chromatogr.*, 640 (1993) 135.
179 Y. Okamoto, N. Sakamoto, M. Yamamoto and T. Kumamaru, *J. Chromatogr.*, 539 (1991) 221.

180 R.M. Montgomery, R. Saari-Nordhaus, L.M. Nair and J.M. Anderson, *J. Chromatogr. A*, 804 (1998) 55.
181 M. Andrew, I.M.V. Burholt, N.J. Kernoghan, T.P. Lynch, R. Mackison, D. Mealor, J.A. Price and P. Schofield, *J. Chromatogr.*, 640 (1993) 111.
182 P.R. Haddad, P. Doble and M. Macka, *J. Chromatogr. A*, 856 (1999) 145.
183 R.P. Singh, N.M. Abbas and S.A. Smesko, *J. Chromatogr.*, 733 (1996) 73.
184 E.T. Urbansky, *Crit. Rev. Anal. Chem.*, 30 (2000) 311.
185 E.T. Urbansky, *J. Environ. Monit.*, 2 (2000) 285.
186 D.E.C. Cole and J. Evrovski, *J. Chromatogr. A*, 789 (1997) 221.
187 R.P. Singh, S.A. Smesko and N.M. Abbas, *J. Chromatogr. A*, 774 (1997) 21.
188 U. Scheller, D. Siedenberg, U. Hubner, M. Siebold, G. Krezmer and K. Schugerl, *Anal. Chim. Acta*, 286 (1994) 179.
189 P.L. Buldini, S. Cavalli and A. Trifiro, *J. Chromatogr. A*, 789 (1997) 529.
190 E.O. Otu, J.J. Byerley and C.W. Robinson, *Int. J. Environ. Anal. Chem.*, 63 (1996) 81.
191 J. O'Reilly, G. Dicinoski, M. Shaw and P.R. Haddad, *Anal. Chim. Acta*, 432 (2001) 169.
192 B. Lopez-Ruiz, *J. Chromatogr. A*, 881 (2000) 607.
193 D.J. Pinkham and M.P. Knapp, *J. Liq. Chromatogr. Relat. Technol.*, 23 (2000) 1099.
194 R.P. Holmes and M. Kennedy, *Kidney Int.*, 57 (2000) 1662.
195 Y.W. Ko, T.J. Gremm, G. Abbt-Braun, F.H. Frimmel and P.C. Chiang, *Fresenius J. Anal. Chem.*, 366 (2000) 244.
196 Y. Sekiguchi, A. Matsunaga, A. Yamamoto and Y. Inoue, *J. Chromatogr. A*, 881 (2000) 639.
197 P.L. Buldini, A. Mevoli and A. Quirini, *J. Chromatogr. A*, 882 (2000) 321.
198 K. Ito, *J. Chromatogr.*, 764 (1997) 346.
199 J.M. Doyle, M.L. Miller, B.R. McCord, D.A. McCollam and G.W. Mushrush, *Anal. Chem.*, 72 (2000) 2302.
200 A.B. Kovacs, J. Prokisch, B. Kovacs, A.J. Palencsar, Z. Gyori and J. Loch, *Commun. Soil Sci. Plant Anal.*, 31 (2000) 1941.
201 V. Rizzo, L. Montalbetti, A.L. Rozza, W. Bolzani, C. Porta, G. Balduzzi, E. Scoglio and R. Moratti, *J. Chromatogr. A*, 798 (1998) 103.
202 A.A. Almeida, X. Jun and J.L.F.C. Lima, *Mikrochim. Acta*, 127 (1997) 55.
203 Z.L. Chen and M.A. Adams, *Anal. Chim. Acta*, 386 (1999) 249.
204 Dionex Application Note 25.
205 M. Lourdes Morales, A. Gustavo Gonzalez and A.M. Troncoso, *J. Chromatogr. A*, 822 (1998) 45.
206 B.K. Glod, G.A. Czapski and P.R. Haddad, *Trends Anal. Chem.*, 19 (2000) 492.
207 J.W. O'Reilly, M.J. Shaw, G.W. Dicinoski, A.C. Grosse, Y. Miura and P.R. Haddad, *Analyst*, 127 (2002) 906.
208 K. Tanaka and P.R. Haddad, *Trends Anal. Chem.*, 15 (1996) 266.
209 K. Tanaka, K. Ohta, P.R. Haddad and J.S. Fritz, *J. Chromatogr. A*, 804 (1998) 179.
210 K. Tanaka, K. Ohta, P.R. Haddad, J.S. Fritz, K.-P. Lee, K. Hasebe, A. Ieuji and A. Miyanaga, *J. Chromatogr. A*, 850 (1999) 311.
211 K. Tanaka, K. Ohta, P.R. Haddad, J.S. Fritz, A. Miyanaga, W. Hu and H. Hasebe, *J. Chromatogr. A*, 884 (2000) 167.
212 M.Y. Ding, K. Tanaka, W. Hu, K. Hasebe and P.R. Haddad, *Analyst*, 126 (2001) 567.
213 K. Tanaka, K. Ohta, P.R. Haddad, J.S. Fritz, A. Miyanaga, W. Hu, H. Hasebe, K.-P. Lee and C. Sarzanini, *J. Chromatogr. A*, 920 (2001) 239.
214 M.Y. Ding, K. Tanaka, W. Hu, P.R. Haddad and A. Miyanaga, *J. Liq. Chromatogr. Relat. Technol.*, 24 (2001) 3105.
215 T. Williams and N.W. Barnett, *Anal. Chim. Acta*, 259 (1992) 19.
216 C. Sarzanini, M. Aceto, G. Sacchero and E. Mentasti, *Ann. Chim. (Rome)*, 84 (1994) 71.

217 J. Carnevale and P.E. Jackson, *J. Chromatogr. A*, 671 (1994) 115.
218 M.D. Prat, R. Compano, M. Granados and E. Miralles, *J. Chromatogr. A*, 746 (1996) 239.
219 C.A. Lucy and L. Ye, *J. Chromatogr. A*, 671 (1994) 121.
220 C.A. Lucy and L. Ye, *Anal. Chem.*, 67 (1995) 79.
221 B. Yan, S.W. Lewis, P.J. Worsfold, J.S. Lancaster and A. Gachanja, *Anal. Chim. Acta*, 250 (1991) 145.
222 M.J. Tomlinson, J. Wang and J.A. Caruso, *J. Anal. Atom. Spectrom.*, 9 (1994) 957.
223 F. Gaucheron and Y. le Graet, *J. Chromatogr. A*, 893 (2000) 133.
224 J. Kerth, C. Rattmann and D. Jensen, *GIT Labor. Fachz.*, 44 (2000) 1324.
225 Y. Huang, S.F. Mou and J.M. Riviello, *J. Chromatogr. A*, 868 (2000) 209.
226 P. Bruno, P. Caselli, G. de Gennaro, P. Ielpo and A. Traini, *J. Chromatogr. A*, 888 (2000) 145.
227 Y. Yokoyama, S. Horikoshi, T. Takahashi and H. Sato, *J. Chromatogr. A*, 886 (2000) 297.
228 X.J. Ding, S.F. Mou, K.N. Liu, A. Siriraks and J.M. Riviello, *Anal. Chim. Acta*, 407 (2000) 319.
229 Y.T. Xie, L.L. Bao and W.Z. Wei, *Curr. Microbiol.*, 40 (2000) 101.
230 O.N. Obrezkov, V.A. Tolkacheva, G.I. Zaikanova, V.A. Yamnikov, O.V. Krokhin, S.R. Zhukov and O.A. Shpigun, *Ind. Lab.*, 66 (2000) 18.
231 A.W. Al-Shawi and R. Dahl, *Anal. Chim. Acta*, 333 (1996) 23.
232 C. Sarzanini, M.C. Bruzzoniti, O. Abollino and E. Mentasti, *J. Chromatogr. A*, 847 (1999) 233.
233 K. Ndungu, N.K. Djane and L. Mathiasson, *J. Chromatogr. A*, 826 (1998) 103.
234 R.T. Gettar, E.A. Gautier, R.E. Servant and D.A. Batistoni, *J. Chromatogr. A*, 855 (1999) 111.
235 P.A. Fagan and P.R. Haddad, *J. Chromatogr.*, 550 (1991) 559.
236 E.A. Gautier, R.T. Gettar and R.E. Servant, *Anal. Chim. Acta*, 283 (1993) 350.
237 D.J. Barkley, L.A. Bennett, J.R. Charbonneau and L.A. Pokrajac, *J. Chromatogr.*, 606 (1992) 195.
238 M. Kumar and P.K. Srivastava, *Analyst*, 118 (1993) 193.
239 P. Jones, O.J. Challenger, S.J. Hill and N.W. Barnett, *Analyst*, 117 (1992) 1447.
240 P. Jones, O.J. Challenger, S.J. Hill and N.W. Barnett, *Anal. Proc.*, 29 (1992) 91.
241 O.J. Challenger, S.J. Hill and P. Jones, *J. Chromatogr.*, 639 (1993) 197.
242 B. Paull, M. Foulkes and P. Jones, *Anal. Proc.*, 31 (1994) 209.
243 P. Jones, M. Foulkes and B. Paull, *J. Chromatogr. A*, 673 (1994) 173.
244 B. Paull and P. Jones, *Chromatographia*, 42 (1996) 528.
245 B. Paull, P. Nesterenko, M. Nurdin and P.R. Haddad, *Anal. Commun.*, 35 (1998) 17.
246 B. Paull, M. Clow and P.R. Haddad, *J. Chromatogr. A*, 804 (1998) 95.
247 B. Paull and P.R. Haddad, *Trends Anal. Chem.*, 18 (1999) 107.
248 P. Jones and P.N. Nesterenko, *J. Chromatogr. A*, 789 (1997) 413.
249 C. Sarzanini and E. Mentasti, *J. Chromatogr. A*, 789 (1997) 301.
250 A.R. Timberbaev and G.K. Bonn, *J. Chromatogr.*, 640 (1993) 195.
251 C. Sarzanini, *J. Chromatogr. A*, 850 (1999) 213.
252 P. Wang and K.K. Lee, *J. Chromatogr. A*, 789 (1997) 437.
253 T.P. Rao and V.M. Biju, *Crit. Rev. Anal. Chem.*, 30 (2000) 179.
254 M.J. Marques, A. Salvador, A. Morales-Rubio and M. de la Guardia, *Fresenius J. Anal. Chem.*, 367 (2000) 601.
255 M. Betti, *J. Chromatogr. A*, 789 (1997) 369.
256 M. Pilar da Silva, J.R. Procopio and L. Hernandez, *J. Chromatogr.*, 761 (1997) 139.
257 J.T. van Elteren and Z. Slejkovec, *J. Chromatogr. A*, 789 (1997) 339.
258 J. Mattusch and R. Wennrich, *Anal. Chem.*, 70 (1998) 3649.
259 N. Ulrich, *Anal. Chim. Acta*, 359 (1998) 245.

260 M. Johansson, G. Bordin and A.R. Rodriguez, *Analyst*, 125 (2000) 273.
261 B. Gammelgaard, K.D. Jessen, F.H. Kristensen and O. Jons, *Anal. Chim. Acta*, 404 (2000) 47.
262 F. Hao, B. Paull and P.R. Haddad, *J. Chromatogr.*, 749 (1996) 103–113.
263 S.M. Bird, H. Ge, P.C. Uden, J.F. Tyson, E. Block and E. Denoyer, *J. Chromatogr. A*, 789 (1997) 349.
264 C. Leal, M. Granados, R. Compano and M.D. Prat, *J. Chromatogr. A*, 809 (1998) 39.
265 N. Vachirapatama, M. Macka, B. Paull, C. Munker and P.R. Haddad, *J. Chromatogr. A*, 850 (1999) 257.
266 M. Macka and P.R. Haddad, *Inorganic ions – capillary electrophoresis*, in I. Wilson (Ed.), *Encyclopedia of Separation Science*, Academic Press, London, 2000, p. 3128.
267 J.S. Fritz, M.C. Breadmore, E.F. Hilder and P.R. Haddad, *J. Chromatogr. A*, 942 (2002) 11.
268 M.C. Breadmore, P.R. Haddad and J.S. Fritz, *J. Chromatogr. A*, 920 (2001) 31.
269 P. Doble and P.R. Haddad, *J. Chromatogr. A*, 834 (1999) 189.
270 M. Macka, C. Johns, P. Doble and P.R. Haddad, *LC-GC*, 19 (2001) 38.
271 M. Macka, C. Johns, P. Doble and P.R. Haddad, *LC-GC*, 19 (2001) 178.
272 A.J. Zemann, E. Schnell, D. Volgger and G.K. Bonn, *Anal. Chem.*, 70 (1998) 563.
273 M.C. Breadmore and P.R. Haddad, *Electrophoresis*, 22 (2001) 2464.
274 D. Kaniansky, M. Masar, J. Marak and R. Bodor, *J. Chromatogr. A*, 834 (1999) 133.
275 T. Wang and S.F.Y. Li, *J. Chromatogr. A*, 834 (1999) 233.
276 F.S. Stover, *J. Chromatogr. A*, 834 (1999) 243.
277 J. Sadecka and J. Polonsky, *J. Chromatogr. A*, 834 (1999) 401.
278 C.A. Lucy, *J. Chromatogr. A*, 850 (1999) 319.
279 W.R. Jones and P. Jandik, *J. Chromatogr.*, 546 (1991) 445.
280 J. Font, J. Gutierrez, J. Lalueza and X. Perez, *J. Chromatogr. A*, 740 (1996) 125.
281 L. Song, Q. Ou, W. Yu, L. Fang and Y. Jin, *J. Chromatogr. A*, 715 (1995) 376.
282 S. Motellier, K. Gurdale and H. Pitsch, *J. Chromatogr. A*, 770 (1997) 311.
283 M.B. Ambran, M.D. Lakkis, F. Lagarde, M.J.F. Leroy, J.F. Lopez-Sanchez and G. Rauret, *Fresenius J. Anal. Chem.*, 345 (1993) 420.
284 P.E. Jackson and P.R. Haddad, *J. Chromatogr.*, 640 (1993) 481.
285 P. Kuban and B. Karlberg, *Anal. Chem.*, 69 (1997) 1169.
286 M.A. Friedberg, M.E. Hinsdale and Z.K. Shihabi, *J. Chromatogr. A*, 781 (1997) 491.
287 M.M. Rhemrev-Boom, *J. Chromatogr. A*, 680 (1994) 675.
288 P.R. Haddad, A.H. Harakuwe and W. Buchberger, *J. Chromatogr. A*, 706 (1995) 571.
289 J. Romano, P. Jandik, W.R. Jones and P.E. Jackson, *J. Chromatogr.*, 546 (1991) 411.
290 A.H. Harakuwe, P.R. Haddad and R. Thomas, *J. Chromatogr. A*, 793 (1998) 187.
291 Y.C. Shi and J.S. Fritz, *J. Chromatogr.*, 640 (1993) 473.
292 J. Boden, T. Ehmann, T. Groh, I. Haumann and K. Bächmann, *Fresenius J. Anal. Chem.*, 348 (1994) 572.
293 W.R. Barger, R.L. Mowery and J.R. Wyatt, *J. Chromatogr. A*, 680 (1994) 659.
294 J. Collet and P. Gareil, *J. Chromatogr. A*, 716 (1995) 115.
295 A. Tangen, W. Lund and R.B. Frederiksen, *J. Chromatogr. A*, 767 (1997) 311.
296 S. McClean, E. O'Kane, D.J.M. Coulter, S. McLean and W.F. Smyth, *Electrophoresis*, 19 (1998) 11.
297 S. Conradi, C. Vogt, H. Wittrich, G. Knobloch and G. Werner, *J. Chromatogr. A*, 745 (1996) 103.
298 C. Francois, Ph. Morin and M. Dreux, *J. Chromatogr. A*, 717 (1995) 393.
299 Q. Yang, C. Hartmann, J. Smeyers-Verbeke and D.L. Massart, *J. Chromatogr. A*, 717 (1995) 415.
300 M. Macka, P. Nesterenko, P. Andersson and P.R. Haddad, *J. Chromatogr. A*, 803 (1998) 279.

301 V. Majidi and N.J. Miller-Ihli, *Analyst*, 123 (1998) 803.
302 V. Unterholzner, M. Macka, P.R. Haddad and A. Zemann, *Analyst*, 127 (2002) 715.
303 E.F. Hilder, M. Macka and P.R. Haddad, *Analyst*, 123 (1998) 2865.
304 B. Michalke and P. Schramel, *J. Chromatogr. A*, 807 (1998) 71.
305 H. Greschonig, M.G. Schmid and G. Gübitz, *Fresenius J. Anal. Chem.*, 362 (1998) 218.
306 B. Michalke and P. Schramel, *Electrophoresis*, 19 (1998) 2220.
307 A. Tangen, W. Lund, B. Josefsson and H. Borg, *J. Chromatogr. A*, 826 (1998) 87.
308 I. Medina, E. Rubi, M.C. Mejuto and R. Cela, *Talanta*, 40 (1993) 1631.
309 K.S. Whang and C.W. Whang, *Electrophoresis*, 18 (1997) 241.
310 W. Liu and H.K. Lee, *Electrophoresis*, 20 (1999) 2475.
311 C. Casiot, M.C. Barciela-Alonso, J. Boisson, O.F.X. Donard and M. Potin, *Analyst*, 123 (1998) 2887.
312 K. Li, S.F.Y. Li and H.K. Lee, *J. Liq. Chromatogr.*, 18 (1995) 1325.
313 C.L. Ng, H.K. Lee and S.F.Y. Li, *J. Chromatogr. A*, 652 (1993) 547.
314 W. Buchberger and P.R. Haddad, *J. Chromatogr. A*, 687 (1994) 342.
315 E.B. Walker, J.C. Walker, S.E. Zaugg and R. Davidson, *J. Chromatogr. A*, 745 (1996) 111.
316 B. Sun, M. Macka and P.R. Haddad, *Electrophoresis*, 23 (2002) 2821.
317 E. Pobozy, B. Glod, S. Marczak and M. Trojanowicz, *J. Chromatogr. A*, 718 (1995) 329.
318 B. Glod, E. Pobozy, S. Marczak and M. Trojanowicz, *Acta Chromatogr.*, 6 (1996) 39.
319 P.R. Haddad, *J. Chromatogr. A*, 770 (1997) 281.

This page is intentionally left blank

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 14

Chromatography of amino acids and peptides

MASSIMO CASTAGNOLA, SILVIO LIPPA, CECILIA ZUPPI and IRENE MASSANA

CONTENTS

14.1 INTRODUCTION

The first automated chromatographic separation of amino acids (AAs), by Spackman *et al.* [1], who utilized anion-exchange chromatography and ninhydrin post-column detection, is still remarkable for the reliability of the separation, the chemical performance, the automation and, above all, for the enormous quantity of information obtained. Several commercial automated instruments, based on this principle, are still on the market. The most exciting recent developments in the AA and peptide field are in the use of capillary techniques, namely capillary zone electrophoresis (CE or CZE), micellar electrokinetic capillary chromatography (MEKC) and capillary electrochromatography (CEC). Other chapters in Part A of this book are devoted to detailed descriptions of these techniques; in this chapter only to the applications to AA and peptide analysis will be discussed.

In the fifth edition of this book, Mant *et al.* [2] elegantly described the classical chromatographic separations of AAs and peptides. The three- and one-letter symbols used for the 21 "natural" amino acids and the nomenclature for peptides can be found there. It should be pointed out that selenocysteine (U and Sec symbols), is now known to be the 21st "natural" amino acid, occurring in several proteins. Its mRNA codon is UGA, usually serving as a stop codon. However, in the presence of a specific downstream sequence, forming a loop (termed SECIS element), and a specific translational elongation factor, the UGA codon is recognized for selenocysteine incorporation into protein sequences by a unique tRNA [3,4].

14.2 AMINO ACIDS

14.2.1 General analytical approaches

The approach to AA analysis depends on the origin of the specimen. Specimens may be divided into three principal classes:

(a) mixtures of amino acids derived from peptide hydrolysis,
(b) amino acids derived from peptide sequencing, and
(c) amino acids present in some biological fluid.

The first class is related to the classical protein analysis technique. When the analysis is intended for protein identification, a sample as homogeneous as possible is needed. Conversely, the analysis can be also utilized to determine protein and peptide purity.

This technique, recently reviewed by Fountoulakis and Lahm [5], requires two steps, hydrolysis of the protein and chromatographic identification of the products. Classical hydrolysis is preferentially performed on purified proteins, in an inert atmosphere, by azeotropic HCl at a temperature of 100°C, in either liquid or vapor state. Hydrolysis time can be reduced either by working at high temperature in the vapor state or by using microwave radiation energy [5]. The pH of the hydrolyzate is adjusted with an appropriate buffer, and AA analysis may be performed by pre-column derivatization with well-known reagents (Table 14.1), such as phenylisothiocyanate, to produce phenylthiocarbamyl amino acids (PTC-AAs), dansyl chloride, dabsyl chloride, 9-fluorenylmethyl chloroformate, or *o*-phthaldialdehyde [2]. Chromatographic separation is almost always performed on a stationary phase of octadecylsilica, using either water/acetonitrile or water/methanol gradients as mobile phases. Earlier, columns having a diameter of 4.6 mm (normal-bore) and a length of 25 cm, packed with 5-μm particles, were typically used. More recently, medium (2-mm) and micro (1-mm) columns with particles of 3 μm have come into use. Monitoring can be performed either by absorbance in the visible/UV range or by fluorescence, depending on the derivatization reagents. Even though post-column derivatization with ninhydrin provides reliable data, this method is falling into disuse due to its low sensitivity, excessive analysis time, and limited flexibility of the dedicated automated instruments.

In AA analysis for sequence determination, the most common choice is the automated phenylthiohydantoin amino acid (PTH-AA) separation. Alternatively, dimethylaminoazobenzene isothiocyanate (DABITC) sequencing can be performed [6]. Few laboratories are still engaged in the tedious subtractive manual Edman sequencing. Even though triphenylgermanyl isothiocyanate (TPG-ITC) and tribenzylsilyl isothiocyanate (TBS-ITC), more than trimethylsilyl isothiocyanate, are promising reagents for chemical C-terminal sequencing of peptides and proteins [7,8], this approach is still far from reliable and affordable [9]. Today, the automated N-terminal sequencing with classical Edman degradation is the technique of choice. PTH-AAs are usually separated on medium-bore octadecylsilica with complex gradients.

The major task in AA analysis is the determination of free amino acids in biological fluids. In this case, the specimen may contain many unknown substances that can affect the reliability of the analysis. Derivatization reagents can generate either artifacts or overlapping. For this reason, matrix effects must be always considered [10]. Furthermore, the analyte of interest may have a concentration very different from other analytes present in the specimen, causing serious interference problems. For correct analyte quantification the use of an internal standard is often advisable. It is relevant to point out that no reference method exists and technological developments are still in a state of flux.

14.2.2 Methods of separation

14.2.2.1 Conventional methods

Conventional amino acid chromatography is currently performed mainly on octadecylsilica by RP-HPLC with water/organic solvent gradients. Optimization studies

TABLE 14.1

SELECTED REAGENTS FOR PRE-COLUMN DERIVATIZATION OF AMINO ACIDS

Reagent	Abbrev.	Detect.	$\lambda_{abs(ex.)}$	λ_{em}	Separation	Refs.
o-Phthaldialdehyde	OPA	UV–Fl	340/260	455	RP, MEKC	[13,52]
Phenylisothiocyanate	PTH/PTC	UV	254		RP, MEKC, CEC	[11,53–57]
4,4-*N*,*N*-Dimethylaminoazobenzene-4′-isothiocyanate	DABTH/DABITC	Vis	463		RP	[6]
5-Dimethylaminonaphtalene-1-sulfonyl chloride	DNS	UV–Fl	360/254	570	RP, MEKC	[58–60]
4-Dimethylaminoazobenzene-4′-sulfonyl chloride	Dabsyl	Vis	420		RP	[61]
9-Fluorenylmethyl chloroformate	FMOC	Fl	265	315, 630	RP, MEKC	[62–64]
Naphthalene-2,3-dicarboxaldehyde	NDA	Fl	462	490	RP, MEKC, CEC	[42,43,47,65,66]
Fluorescamine	–	UV–Fl	280/390	475	RP, CE	[67,68]
Fluorescein isothiocyanate	FTH/FITC	Fl	490	519	RP, MEKC	[69–71]
3-(*p*-Carboxybenzoyl)quinoline-2-carboxaldehyde	CBQ	Fl	466	544	MEKC	[72]
3-(4-Carboxybenzoyl)-2-quinolinecarboxyaldehyde	CBQCA	Fl	450	550	MEKC	[73]
3-(4-Tetrazolbenzoyl)-2-quinolinecarboxyaldehyde	TBQCA	Vis-Fl	488/465	550	MEKC	[74]
1-Methoxycarbonylindolizine-3,5-dicarbaldehyde	IDA	Vis-Fl	416/280	482	RP, CE, MEKC	[75,76]
2-(9-Anthryl)ethyl chloroformate	AEOC	UV–Fl	366/256	402	RP, MEKC	[77,78]
6-Aminoquinolyl-*N*-hydroxy-succinimidyl carbamate	AQC	Fl	250	400	RP, MEKC	[79,80]
Carbazole-*N*-(2-methyl)acetyl chloride	CMA-Cl	Fl	335	360	RP	[81]
Acridone-*N*-acetyl chloride	ARC-Cl	Fl	404	430	RP	[82]
Carbazole-9-acetyl chloride	CRA-Cl	Fl	335	360	RP	[82,83]
Carbazole-9-propionyl chloride	CRP-Cl	Fl	340	365	RP	[82]
Tetramethylrhodamine isothiocyanate	TRITC	Fl	540	567	MEKC	[84]
2,4-Dinitrofluorobenzene	DNP	UV	254		CE	[85]
9-Isothiocyanatoacridine	–	UV	280		RP	[86]
Benzylisothiocyanate	BZITC	UV	246/238		RP	[87]

with respect to temperature, eluent flow-rate and composition, and stationary phases allowed the resolution of 27 PTH-AAs [11]. Isocratic separations are simple [12], but they may be plagued by short column life and unsatisfactory selectivity. Ion-exchange chromatography (IEC) is still used, but pre-column derivatization by OPA is preferable to post-column ninhydrin detection, because it is faster and more sensitive [13]. RP-HPLC analysis of urinary AAs was improved by a pre-column of strong cation-exchange resin [14], while a pellicular anion-exchange column was found particularly suitable for arginine determination in biological fluids [15]. Thin-layer chromatography (TLC) is usually replaced by HPLC methods, as quantification by TLC is unreliable [16]. Gas chromatography (GC) of derivatized amino acids is characterized by very good performance. It is frequently coupled with mass spectrometry (MS) for the detection of particular AA derivatives [17]. The most interesting developments in AA analysis are occurring in electro-driven capillary methods.

14.2.2.2 Electro-driven capillary methods

The techniques of capillary electrophoresis are largely described in Chaps. 7 and 9. However, in connection with AA and peptide analysis, it is worth remembering that separations performed by free-zone electrophoresis depend on a frictional factor. This factor, according to the Stokes–Einstein equation, is proportional to the ratio between analyte charge and hydrodynamic radius. Peptides are characterized by distinctly different charge and hydrodynamic radius values. Thus, CE is a very good choice for selective peptide separations (Chap. 15). In contrast, charge and Stokes radius values of many amino acids are very similar, and CE in free solution is not so applicable to selective amino acid separations. However, taking advantage of electro-osmosis, other capillary techniques, such as MEKC and CEC, can be exploited. These methods should be considered to be electro-driven chromatographic techniques, rather than straight-forward electrophoretic methods (Chaps. 7 and 9). The electroosmotic flow (EOF) offers a very high separating power. It is strongly dependent on the pH of the electrophoretic medium, acting on the dissociation of silanol groups of the silica capillaries. Strong EOF and ζ potential are also generated in capillaries made of plastic material, such as polyfluorocarbon, polyethylene, and polyvinyl chloride [18], even though the molecular events responsible for the potential are not completely understood. The EOF values can be high [19–21]; *e.g.*, in a silica capillary with 53 μm ID, at pH 6.0, the EOF reaches a value of about 200 nL/min, and the capillary solution is renewed in few minutes [22]. Various factors influence the EOF values. Apart from its dependence on the pH value, EOF increases as the capillary diameter and ionic strength decrease. Moreover, it is related to the hydrated radius of metal ions present in the solution [23,24], and it can be reversed at low pH by the use of appropriate amines [25]. Thus, EOF values are very sensitive to matrix effects, capillary uniformity, adhesion of various ions to the capillary wall, temperature, and the presence of organic solvents or zwitter-ions. All these parameters have conspired against quantification of EOF values by simple equations. Righetti *et al.* [26], having measured EOF values under identical experimental conditions, but using

different electrophoretic apparatus, concluded that different radial electrical fields were responsible for the observed differences.

An important technique that takes advantage of electro-osmosis is MEKC. This technique, first reported by Terabe *et al.* [27], utilizes a detergent, normally sodium dodecylsulfate (SDS) above its critical micellar concentration (CMC), in the electrophoretic solution. Negatively charged micelles migrate towards the positive electrode at a velocity v_{ep}. At pH values above 5, in uncoated silica capillaries, the velocity due to EOF, v_{eo}, driving towards the negative electrode is larger than v_{ep}. Thus, the net micellar velocity, v_{mc}, resulting from the vectorial sum of v_{eo} and v_{ep}, is directed toward the cathode. The migration time of each analyte depends on its partition coefficient between the aqueous and micellar phases. Analytes present only in the aqueous phase migrate according to v_{eo} (at time t_o). Those totally incorporated into the micellar phase (*e.g.*, Sudan III) migrate according to v_{mc} (at time t_{mc}). Migration times of electrically neutral compounds are thus confined in a detection window between t_{mc} and t_o. The micellar phase acts as a pseudo-stationary chromatographic phase. In analogy with classical chromatography, equations were derived describing MEKC resolution as a function of capacity factors [28]. The chromatographic selectivity of MEKC may be notably modulated by the use of different separation conditions. Important factors that must be considered in the selection of a pseudo-stationary phase are the length of the hydrophobic chain and the structure of the hydrophilic group. The chain-length is important in order to have a CMC in agreement with the electrophoretic experimental requirements, since surfactants with short alkyl chain have a higher CMC and conductivity. The hydrophilic group affects principally the micelle selectivity. SDS usually represents the best choice, but good alternatives are also offered by cationic surfactants, such as cetyltrimethylammonium bromide and chloride (CTAB, CTAC) and dodecyltrimethylammonium bromide (DTAB), nonionic surfactants, such as Tween or octylglucoside, zwitter-ionic and chiral surfactants, such as cholate or digitonin. The possible use of mixed micelles offers practically infinite separation options. In addition, organic solvents, such as methanol and acetonitrile, are often used in order to increase analyte solubility, to stabilize micellar phases and, to a minor extent, to reduce EOF. Selection of different buffers (normally borate/phosphate) and use of further additives, such as cyclodextrin or chaotropic agents, permits a choice of favorable conditions for particular analytical problems. With this wealth of possibilities, the contemporaneous optimization of all separation parameters may be difficult. In this context, various optimization strategies were developed [29]. However, due to the complexity and to the number of available schemes, the analyst's experience always plays the most relevant role in the choice of optimal conditions.

MEKC, more than classical CZE, is widely used for AA separations, and it is characterized by high sensitivity, selectivity, and resolution [30–32]. AA separations are usually performed by MEKC with pre-column derivatization (Sec. 14.2.4.1). MEKC also forms the basis for indirect AA detection (Sec. 14.2.4.4) and for many chiral separations (Secs. 14.2.5.1 and 14.2.5.2). SDS, by far the preferred detergent, is commonly used in aqueous/organic buffers in basic medium to ensure fast EOF and short analysis time.

Due to problems with pumps that can deliver a constant flow-rate of a few microliters, necessary in capillary HPLC, capillary electrochromatography (CEC) represents a

powerful emerging alternative to micro-LC (Chap. 7). CEC is based on the application of high electric fields between the ends of a capillary, containing a chromatographic stationary phase. The mobile phase in the capillary is driven by electro-osmosis. The stationary phase can be either covalently bonded to the capillary wall (open-tubular CEC) or packed in the capillary (packed CEC). The flat profile of the electro-osmotic flow promises higher efficiencies than capillary HPLC. A good packing procedure is essential for a successful separation. In this respect, good results were obtained by preparing narrow silica frits with a thermal wire stripper [33]. The EOF in packed CEC is usually lower than that measured in CE under similar conditions [34,35]. This behavior can derive partly from random alignment of the flow channels in the packed phase and partly from surface modifications, leading to a decrease of free silanol surface density.

Due to its relatively recent development, only few AA separations by CEC have been reported. Ru *et al.* [36] reported a good separation of 18 AA derivatives by pressurized CEC on an octadecylsilica phase. Qi *et al.*[37] described a CEC separation of PTH-AAs on a capillary column packed with a 3-μm C_{18} stationary phase, interfaced with an ultraviolet/laser-based thermo-optical absorbance detector. A separation efficiency of 216,000 plates/m and an absolute sensitivity at the attomole level were obtained. Extensive utilization of CEC in AA analysis is likely, even though several technical improvements are still necessary. In this respect, the ability to perform reliable gradient elution is very attractive. Yan *et al.*[38] showed that a dynamic gradient can be generated by merging two electro-osmotic flows, regulated by computer-controlled voltage. These flows were elegantly delivered by two fused-silica arms, attached to a T-connector, where they were mixed and driven into a capillary column, electrokinetically packed with reversed-phase material. This technique is well suited for generating not only solvent gradients, but also for delivering other types of gradients, such as pH and ionic-strength gradients. Further improvements of CEC have resulted from the use of monolithic supports [39–41], which are continuous unitary porous structures, prepared by *in situ* polymerization or consolidation inside the column. If necessary, the surface can be functionalized and converted to a sorbent with the desired chromatographic binding properties. Good separations of naphthalene-2,3-dicarboxaldehyde-labeled AAs on UV-initiated acrylate-based porous polymer monoliths, followed by absorbance or laser-induced fluorescence detection, have recently been described [42,43].

14.2.3 Sensitivity

Amino acid analyses performed about 40 years ago were characterized by an absolute limit of detection (LOD) in the range of a few nanomoles. Therefore, in order to exploit the whole structure of a complex peptide about one micromole of the analyte was necessary, corresponding to about 6×10^{17} molecules. This enormous number of molecules has generated serious problem for the structure determination of protein and peptides, which are present in tissues at very low concentration. Just as an example, to obtain the few milligrams of TSH-releasing factor necessary for structure determination, the researchers of the Guillemin and Burgus group had to process tons of sheep brain [44]. As a consequence, one of the most relevant objectives for real progress in AA analysis is the improvement in the

LOD. The chromatographic parameters that affect the LOD most are the following:

(a) resolution and number of theoretical plates,
(b) detector response, and
(c) the volume of sample submitted for analysis.

Continuous evolution of separation methods have worked to improve all these factors materially. The presently available commercial instruments can perform analyses with a LOD of a few femtomoles (10^{-15} moles; *ca.* 6×10^8 molecules). The number of molecules necessary for an analysis, even though reduced more than nine orders of magnitude, is still impressive. Success in building ever smaller analytical instruments, combined with the ability to perform detection by laser-induced fluorescence (LIF), has greatly improved the sensitivity. The combined use of either CE or CEC with LIF detection has dropped the LOD to the level of attomoles [45,46]. Taking advantage of this level of sensitivity, several one-cell analyses have been reported. On-column derivatization by naphtalene-2,3-dicarboxaldehyde of the content of a single pheochromocytoma cell and CE/LIF detection have made it possible to quantify five amino acids and dopamine [47] (Fig. 14.1). The aspartic acid concentration accounted for *ca.* 100 amol/cell (about 60 million molecules per cell). Exceptional analyses now allow the detection of few molecules. Davies and Hounsell [46] described the detection of as little as 0.1 zeptomoles in a miniaturized separation systems with a CE/laser-induced fluorescence instrument. Since a zeptomole (10^{-21} moles) accounts for about 600 molecules, these analyses were performed on a signal derived from 60 molecules of the same analyte! At these LOD levels, it seems more correct to define

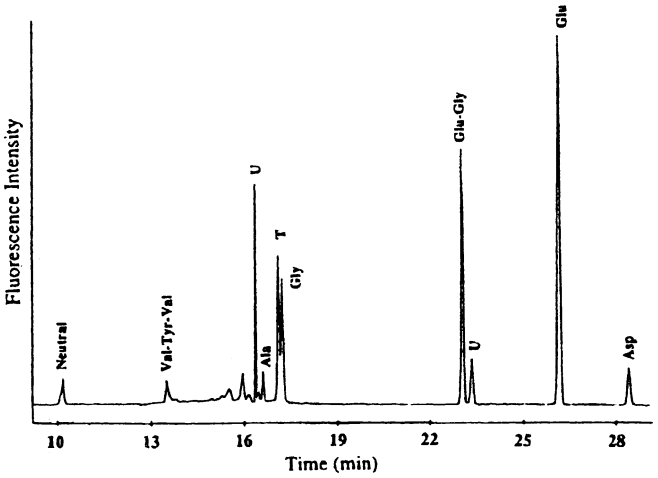


Fig. 14.1. Electropherogram of the NDA-derivatized contents of a single rat pheochromocytoma cell. CE separation carried out using a 17-μm ID fused-silica capillary (length 100 cm; 85 cm at the detection window). Separation performed in 100 m*M* borate buffer (pH 9.5) using a 30 kV separation potential. LIF detection, 442 nm beam from a He–Cd laser. Val-Tyr-Val and γ-Glu-Gly are internal standards. Unidentified peaks are labeled U, and taurine is T. (Reprinted from Ref. 47 with permission. Copyright 1995 American Chemical Society.)

sensitivity in terms of molecules than in term of fractions of a mole. Moreover, the new term, yoctomole (10^{-24} moles), is not plausible, because it is not compatible with Avogadro's number [48,49].

14.2.4 Derivatizing reagents

It is well known that only a few amino acids contain groups that show a strong absorbance in the working wavelength range of the usual UV detectors, but derivatization with suitable chromo- and fluorophores permits AA detection. Derivatization also provides properties that may enhance the separation. It can be carried out pre-, post- and on-column; the choice is principally linked to the requirements of the application and to the available instrumentation [50]. Post-column derivatization was preferred in the past, due to the difficulties of achieving satisfactory separation of derivatized AAs before the advent of HPLC. However, the improved resolution and selectivity of HPLC made it possible to separate analytes with minor structural differences. Now that optimal separations of almost all amino acid derivatives can be achieved, pre-column derivatization is becoming the preferred option. One disadvantage of post-column derivatization is the requirement of additional pumps to dispense the derivatizating reagents. Moreover, the post-column reactor can lead to peak-broadening and to a decrease in sensitivity. On the other hand, disadvantages of pre-column derivatization include possible interference by the excess reagent and the formation of artifacts. A comparison between automated pre- and post-column AA analysis, based on the classical OPA derivatization, showed that pre-column derivatization provides better separation, higher sensitivity, and shorter analysis time [51].

14.2.4.1 Pre-column derivatization

A list of selected pre-column derivatization reagents is presented in Table 14.1. OPA is probably the most popular pre-column derivatization reagent. OPA derivatization with various SH-group-containing additives has been recently reviewed [88]. The easy coupling of different isothiocyanates with the amine function at slightly basic pH values generates various thiocarbamyl derivatives. Various efforts devoted to substitutes for the common phenylisothiocyanate reagent are in progress; *e.g.*, derivatization with fluoresceinisothiocyanate [69–71] and benzylisothiocyanate [87] (Table 14.1). Other, similar reagents are butylisothiocyanate [89] and 4-(5′,6′-dimethoxybenzothiazolyl)phenylisothiocyanate [90]. All the reagents shown in Table 14.1 react with the amine function of the AAs, but several reagents for the carboxylic function, such as 5-bromomethylfluorescein [91] and 5-maleimidefluorescein [92], have also been described. These derivatives were separated by MEKC, coupled to LIF detection. Derivatives of the carboxylic function are commonly prepared for capillary GC in order to increase volatility. The derivatization with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide by a single-step reaction allowed separation of the *N*(*O*)-(*tert*-butyldimethylsilyl) derivatives of 22 protein AAs by GC on a HP-1 capillary column [93]. Derivatization

with isobutyl chloroformate produced *N*(*O*,*S*)-iso-butyloxycarbonyl AAs, which, after solid-phase extraction, were converted to stable (*tert*-butyldimethylsilyl) derivatives. Their analysis in wine by dual-capillary-column GC and by GC/MS was performed with the aid of pattern recognition [94]. Kataoka *et al.* [95] analyzed the *N*(*O*,*S*)-isobutylcarbonylmethyl ester derivatives by GC on a DB-17 capillary column with a hydrogen-flame-ionization detector. Under these conditions, the 21 protein AAs and 33 nonprotein AAs were quantitatively resolved within 25 min. These authors recently published an interesting review on the GC analysis of protein and nonprotein AAs in biological samples [96]. MEKC separation, coupled with LIF detection of the fluoresceinethiocarbamate derivatives of 28 biogenic amines and amino acids was used to assess wine ageing [97]. In this case, derivatization of the amine function in biological samples allowed the detection of many other compounds. Almost all the pre-column derivatives are detected either by UV or by fluorescence, often under LIF conditions. However, derivatization with 6-aminoquinolyl-*N*-hydroxysuccinimidylcarbamate to obtain 6-aminoquinolylurea derivatives can be exploited for electrochemical detection after RP-HPLC separation. The amperometric response is linear in the range from 5 to 2500 pmol, and the separation is highly reliable [98]. Thermo-optical absorbance detection, based on a 248-nm krypton fluoride excimer laser, coupled with CEC separation on a 3-μm C_{18} stationary phase allowed analysis of PTH-AAs with a detection limit of about 100 attomoles, appreciably higher than that obtainable by MEKC under similar conditions [99].

14.2.4.2 Post-column derivatization

As explained in Sec. 14.2.4, post-column derivatization of AAs is now used less than pre-column derivatization, and the number of references is noticeably smaller. The classical post-column ninhydrin detection was recently reviewed [100]. The problems connected with protein interference in plasma samples were solved by the use a hydroxyapatite cartridge for pre-column deproteinization [101]. Separation time was approximately 2 h, and the method was superior to 5-sulfosalicylic acid deproteinization. Proper design of sheath-flow reactors allowed a noteworthy performance of OPA post-column derivatization for several AAs, following CE separation [102,103]. A LOD of about 100 amol of glycine and a separation efficiency of 200,000 theoretical plates were achieved. Otherwise, automated post-column derivatization with 1,2-naphtoquinone-4-sulfonate, following ion-pair RP-HPLC, provided satisfactory results [104]. A specific application of post-column derivatization is the automated detection of methylated arginines in plasma samples [105].

14.2.4.3 On-column derivatization

Although attractive, on-column derivatization has until now not attained widespread acceptance. The main reason is probably the need for a reaction to be completed during the separation. This requirement is in contrast with the actual trend to reduce the separation

time. Latorre *et al.* [106] have discussed the attractive aspects of this procedure. On-capillary derivatization with 1,2-naphtoquinone-4-sulfonate during CE separations allowed the UV detection of several AAs. Sample and reagent solutions were separately injected under pressure into the capillary prior to application of the running voltage. Derivatization occurred after application of the electric field, by mixing the sample and reagent inside the capillary. Different configurations (tandem, sandwich) were evaluated. On-line AA derivatizations with IDA [76] and OPA [107] (Table 14.1) during CE separation have been described. It was found that the sensitivity is greater for OPA than for IDA. Moreover, CE separation is enhanced by adding β-cyclodextrin to the running buffer. On-column OPA derivatization during CE separation was studied by Taga *et al.* [108]. Variations of peak intensity, attributed to different overlapping periods and therefore to different reaction times, were observed. The technique, affected by various factors, including sample/reagent introduction times and applied voltage, showed a lower reproducibility than pre-column derivatization. However, Ewing *et al.* [47,109] demonstrated that this procedure can be utilized to quantify AAs within a single cell.

14.2.4.4 Detection of nonderivatized amino acids

The most attractive option in amino acid analysis is represented by procedures for quantitative and sensitive analysis without derivatization. Fluorimetric detection, taking advantage of the inherent fluorescence of aromatic AAs, allowed the determination of Phe and Tyr in human plasma [110]. The excitation and emission wavelengths were 215 and 283 nm, respectively, and the LOD was in the μmol/L range, providing a quick and simple procedure for monitoring patients with phenylketonuria. Klampfl *et al.* [111] showed that direct UV detection (at 185 nm) can be utilized during MEKC separation for the detection of nonderivatized AAs in beverage samples. Nonderivatized AAs can be detected by other detectors, such as the chemiluminescent nitrogen detector (CLND), evaporative light-scattering detector (ELSD), nuclear magnetic resonance (NMR) spectrometer, conductivity detectors, refractive-index (RI) detectors, and mass spectrometer in either single or tandem mode. The different capabilities of these detectors have been discussed recently [112]. ELSD and CLND require volatile mobile phases. Underivatized taurine, hypotaurine, hydroxy-Pro, N, S, G, Q, C, D, E, T, and A were simultaneously determined with comparable sensitivity by both ELSD and CLND detectors after HPLC separations. However, the ELSD detector showed a nonlinear response. Although controversial, because of their very high cost, the MS and NMR detectors offer important structural information, which facilitates analyte identification. Refractive-index and conductivity detectors are usually not compatible with gradient elution, and conductivity detectors require low-conductivity eluents. Ivanov *et al.* [113] recently proposed the coupling of a refractometric detector with MEKC, performed in borate/SDS running buffers. For this technique, the LOD is typically in the order of 100 fmol. Amperometric detection also offers promising properties. A nickel-based composite electrode, prepared by anodic electro-deposition on a gold electrode, has been successfully applied to the determination of free

AAs [114]. Amperometric detections, based on the immobilization of amino acid oxidase on the surface of a $CuPtCl_6$-modified electrode, gave a satisfactory performance [115].

However, the most interesting option for the detection of nonderivatized amino acids is the indirect detection mode, available through the characteristics of CE and MEKC. Indirect detection depends on the presence of an absorbing compound in the running buffer. AAs can be detected as negative peaks, due to the displacement of the absorbing background electrolyte (BGE). Several studies have dealt with the theoretical aspects related to indirect CE detection [116–119]. These studies indicated that the BGE should have a mobility similar to that of the amino acids and that the sensitivity is inversely proportional to the electrolyte concentration. The majority of indirect detection applications in CE and related techniques have been devoted to sugar analyses, which present serious difficulties for either direct detection or various derivatizations (Chap. 18). Some amino acid separations by CE and MEKC have utilized indirect detection [120–129]. Separations were usually performed in basic media, and some of the BGE compounds were salicylate [116], *p*-aminosalicylate and 4-(*N*,*N*,-dimethylamino)benzoic acid [121,122], benzoate [125], quinine sulfate [120], 2,6-pyridinedicarboxylic acid [126, 127], iron(II)-1,10-phenanthroline [129], and adenosine monophosphate [124]. This technique allowed the detection of not only protein AAs, but also of phospho-AAs [124]. Optimal separations were observed for acetylated derivatives [129], but this strategy misses the advantages inherent in the indirect detection mode. Soga and Imaizumi [127] showed that the choice of proper conditions, such as high pH and reversed electro-osmosis, provided outstanding separations and allowed the analysis of 206 analytes, including the physiological AAs, inorganic and organic anions, carbohydrates and phosphorylated saccharides, nucleotides, aromatic acids, oxyhalides, and complexes (Fig. 14.2). These studies clearly indicate that indirect detection plays a relevant role in biological analysis. Indirect fluorescence detection has been utilized for single-cell analysis [130,131]. Indirect amperometric [132,133] and conductimetric [134] detection of non-derivatized AAs have been described. The use of these detectors allowed AAs to be analyzed not only by CE [132], but also by isocratic HPLC [133,134]. The detection of underivatized AAs utilizing the quenched phosphorescence of biacetyl as a constituent of the buffer in CE separations should be also included among the indirect detection modes [135].

14.2.5 Chiral separations

It is widely accepted that the D(*R*)-enantiomers of amino acids have biological properties different from those of the L(*S*)-enantiomers [136–154]. Due to the great interest in the biochemistry of D-amino acids, the number of papers dealing with their analysis has considerably increased in the past decade, and some recent reviews have covered this subject [155,156]. Wan and Blomberg [157] have presented an outstanding review on the chiral separation of amino acids and peptides by CE, and many references to chiral AA separations can be found in more general reviews, devoted to CE [30,31, 158]. General information on enantioselectivity in electro-driven chiral capillary separations can be found in the reviews of Fanali [159], Terabe *et al.* [160], and Chankvetadze and Blaschke [161]. Here, we must restrict ourselves to a discussion of a

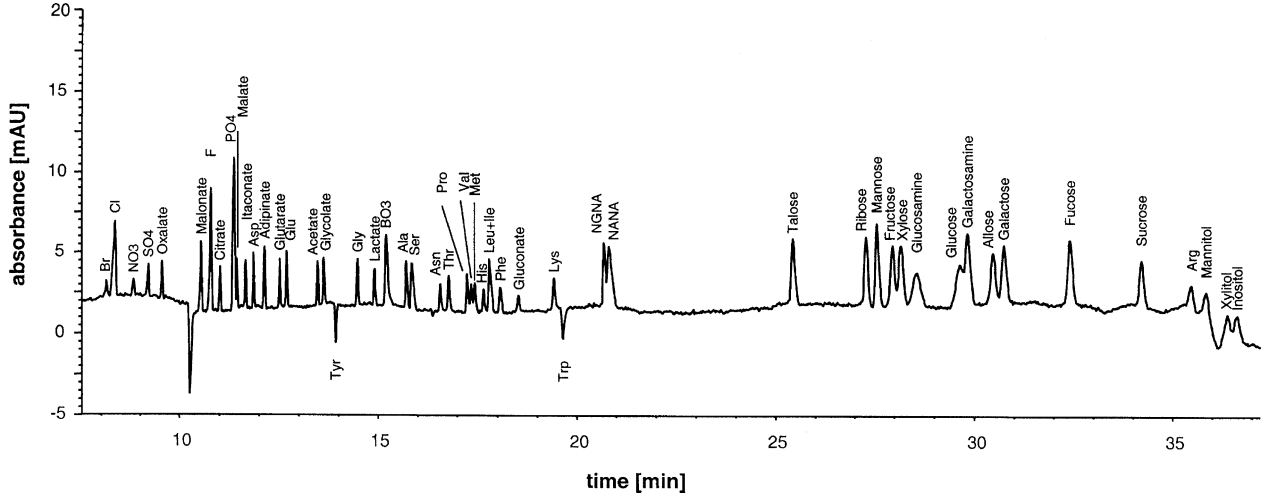


Fig. 14.2. Separation of inorganic and organic anion, amino acid, and carbohydrate standard mixture by CE with indirect UV detection. Conditions: capillary, fused silica (50 μm ID), 112.5 cm (104 cm at the detection window); electrolyte, 20 m*M* 2,6-pyridinedicarboxylic acid (background electrolyte), 0.5 m*M* cetyltrimethylammonium hydroxide (pH 12.1); voltage, −30 kV; injection, 6 sec at 50 mbar; temperature, 15°C; signal, 350 nm; reference, 230 nm; concentrations, chloride, 88 mg/L; phosphate, 80 mg/L; Arg and carbohydrates, 200 mg/L each; others, 40 mg/L each. (Reprinted from Ref. 127 with permission.)

few recent developments. Chiral separations can be divided in two main areas: direct (or enantiomeric) separations and indirect (or diastereomeric) separations. The first deals with differential interactions of the two enantiomers with a chiral ligand or surface, whereas the second requires the reaction of the two enantiomers with a chiral reactant, with subsequent separation of the two diastereoisomers formed under achiral conditions.

14.2.5.1 Direct chiral separations

14.2.5.1.1 Conventional chromatographic chiral separations

Chromatographic chiral stationary phases (CSPs) can be divided into several classes according to different nomenclatures. Wainer's nomenclature [162] proposed five classes of CPSs, based on the mechanism of interaction with the solute. They are:

(a) attractive interactions (*e.g.,* hydrogen bonds, π/π interactions);
(b) mixed attractive interactions and inclusion complexes (*e.g.*, cellulose derivatives);
(c) inclusion complexes (*e.g.*, cyclodextrin, crown ethers, helical polymers);
(d) diastereomeric metal complexes {also introduced by Davankov [163] as chiral ligand-exchange chromatography (CLEC)};
(e) hydrophobic and polar interactions (*e.g.*, proteins).

Since this scheme was proposed, other CPSs have come into use, and in some cases it is not clear to which of the above classes they belong. Therefore, a second classification, according to the chemical type, divides CPSs in: 1) brush type; 2) cellulose; 3) cyclodextrin (CD); 4) crown ethers; 5) macrocyclic antibiotics; 6) proteins; and 7) ligands. Not all these CPSs are suitable for AA separations. The best choices are the macrocyclic antibiotics and the ligand-exchange CPSs. However, crown ethers, cyclodextrin and proteins are also sometimes used profitably. Concerning macrocyclic antibiotics, ristocetin-A CPS allowed the enantiomeric resolution of 28 natural and unnatural amino acid derivatives [164]. Teicoplanin CSP was used for the enatiomeric separations of native amino acids and their *N-tert*-butyloxycarbonyl (t-Boc) derivatives [165]. Native AAs were better resolved than the blocked AAs, and the presence of triethylamine in the eluent improved the separation of t-Boc derivatives. Teicoplanin CPS also effected the enantiomeric resolution of 15 underivatized proteinogenic and nonproteinogenic AAs under isocratic conditions in less than 25 min [166]. Binding of a single enantiomer of a crown ether (diphenyl-substituted 1,1′-binaphthyl) to a 5-μm silica support allowed the enantiomeric resolution of various amino acids, with the exception of Pro [167]. The enantiomeric separation of cyclic β-substituted α-quaternary α-amino acids was achieved by ligand exchange on a Cu(II)/D-penicillamine CSP [168]. The elution order of the enantiomers of some AAs was reversed by changing the acetonitrile content of the eluent. This phenomenon was explained by the formation of two different Cu(II) complexes of the tridentate ligand penicillamine. Ligand-exchange chromatography was also performed on porous-graphitic carbon columns, coated with different *N*-substituted L-proline selectors [169]. The best separation of 36 AA enantiomers was obtained by naphthylmethyl-L-proline-coated columns. The mechanism of the enantiomeric separation of unmodified amino acid racemates by CLEC with Cu(II) complexes of tetradentate diaminodiamido-type ligands has been investigated [170]. The most important factor responsible for chiral discrimination seems to be the affinity of the

diastereomeric ternary complexes for the stationary phase (Fig. 14.3). For the chiral separation of DNS-amino acids a column with immobilized human serum albumin was investigated. The influence of tetrabutylammonium (TBA) as a hydrophobic, charged additive suggested that the coulombic interactions between a DNS-derivative and the binding cavity are of crucial importance in the association process [171]. The enantiomeric resolution of 18 PTH-amino acids was achieved with two types of CSP, bonded with β-cyclodextrin [172]. BF_3 was used as conversion reagent instead of anhydrous TFA, and HCl/methanol instead of aqueous TFA, in order to suppress racemization during Edman degradation. GC of several amino acid derivatives, prepared with different chloroformates, was performed on a Chirasil-L-Val capillary column and indicated that the best compromise between short retention times, high-yield responses, and good resolution is obtained by using *N*(*O*,*S*)-ethoxycarbonylheptafluorobutyl ester derivatives [173]. The use of impregnated TLC for the separation of amino acid enantiomers has been reviewed [174].

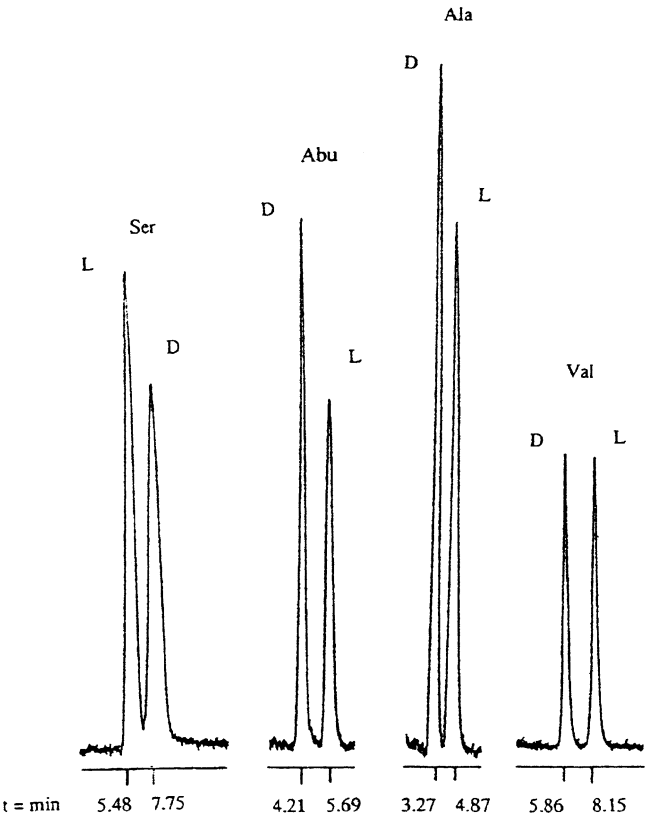


Fig. 14.3. Enantiomer separation of D,L-amino acids by ligand-exchange HPLC with a copper complex of (*S*,*S*)-*N*,*N*′-*bis*-(methylphenylalanyl)ethanediamine (Me_2PheNN-2). Conditions: eluent, 0.5 m*M* Me_2PheNN-2 and 0.5 m*M* copper(II) acetate in water (pH 6.0); column, Spherisorb ODS2 (3 μm, 15 × 0.46 cm); flow-rate, 1.0 mL/min; fluorescence detection of OPA derivatives. (Reprinted from Ref. 170 with permission.)

14.2.5.1.2 Capillary electrochromatography and micro-high-performance liquid chromatography

Several studies on the chiral amino acid discrimination by CEC (and micro-HPLC) have been reported. These techniques are obviously attractive for improving resolution while simultaneously decreasing the sample size, thus saving eluent and costly CSP. A β-cyclodextrin CSP was used to resolve DNS-amino acids by CEC, albeit with a separation efficiency lower than that obtained in CE separation [175]. Hydroxypropyl-β-CDs were either dynamically coated on a diol-silica stationary phase [176] or bonded to silica having a sulfonated sublayer [177]. The first option [176] allowed the separation of several DNS-amino acids at relatively low EOF, whereas the sulfonated sublayer [177] provided comparable efficiencies at relatively strong EOF. Teicoplanin and poly-tergulide as macrocyclic-antibiotic-bonded CSPs were investigated [178,179]. The results showed an efficiency similar to that obtained by conventional chiral HPLC, whereas quinine-derived chiral selectors showed high enantioselectivity in CEC of FMOC-amino acids [180,181]. Some CEC enantioseparations with the ligand-exchange strategy were studied. The chiral selectors included L-prolinamide [182], *N*-(2-hydroxy-3-allyloxypropyl)-L-4-hydroxyproline [183,184], and L-phenylalaninamide [185]. Interestingly, these monolithic silica columns were used for DNS-amino acids [182, 185], and continuous beds for CEC separations of nonderivatized AAs [183,184]. The monolithic matrix was prepared by sol/gel procedures, whereas continuous beds were prepared by a one-step co-polymerization procedure, allowing the formation of a covalent bond with the capillary wall. L-phenylalaninamide was also used for the preparation of molecularly imprinted polymers for CEC separations of amino acid enantiomers [186]. Several CE chiral AA separations with gels, modified by chiral selectors, can also be included in this class. Gels incorporating, as selectors, either β-CDs [187–189] or BSA [190,191] gave satisfactory enantiomer separations of several AA derivatives. However, the short lifespan of gels and the difficulties connected with filling the capillary are serious obstacles to effective use in analytical applications. All these approaches should be considered as challenges for the development of new CSPs, suitable for fast CEC enantiomer separations, than rather as solutions to analytical problems.

14.2.5.1.3 Capillary electrophoresis and micellar electrokinetic capillary chromatography

Capillary electro-driven separations performed in free solution, particularly MEKC, are characterized by low reagent consumption, high efficiency, and short analysis time. Therefore, application of these techniques to chiral separations is particularly attractive, since only minimal amounts of expensive chiral selectors are needed. Moreover, the facile replacement of the separation buffer allows testing of many different conditions and consequently rapid development and optimization of methods [30,31,157,158]. Table 14.2 summarizes selected separations obtained by using host/guest compounds (cyclodextrin and crown ethers), chiral surfactants, and polymers as selectors. The most widely used separation procedures take advantage of the good enantiomeric selectivity of CDs. Often, in order to increase solubility, derivatives of CD are employed, and organic solvents are added to the separation buffers. For instance, the presence of 2-propanol in the

TABLE 14.2

SELECTED CE AND MEKC ENANTIOMERIC AMINO ACID SEPARATIONS BY HOST/GUEST, CHIRAL SURFACTANT AND CHIRAL POLYMER SEPARATION MODES

Chiral selector	AA der.	Observations	Refs.
Host/guest: cyclodextrins	PTH	*o*-Trimethyl-β-CD, CE	[192]
	Underivatized	Various CDs, indirect detection, CE	[193]
	DTDP (triazine)	β-CD, 2-propanol, borate, MEKC	[194]
	DNS or FTH	CD mixed mode, MEKC and LIF	[195]
	AEOC	β-, γ-CD, CE and MEKC	[196]
	DNS	β-CD, methanol, CE	[197]
	AQC	Various CDs, methanol, CE	[198]
	DNP	Various CDs, CE	[199]
	Aromatic	α-CD, CE	[200]
Host/guest: crown ethers	Underivatized	(+)-18-crown-6-tetracarboxylic acid	[201,202]
Chiral surfactants (MEKC)	DNS	Na deoxycholate and Na taurocholate	[203]
	PTH	Digitonin	[204]
	NDA	Na taurodeoxycholate, β-CD	[205]
	PTH	*N*-dodecanoyl-L-serine, -L-glutamate	[206,207]
	AQC	(*R*)-*N*-dodecoxycarbonylvaline	[208]
	DNS	(*S*)-*N*-octoxycarbonylleucine, β-CD	[209]
	DNS	*n*-Octyl-β-D-maltopyranoside	[210]
	DNS	Cyclohexyl-pentyl-β-D-maltoside	[211]
Chiral polymers	DNB	Poly(Na *N*-undecylenyl-L-valinate)	[212]
	DNS	Dextran polymers	[213]
	DNS	Dextrin	[214]
	Trp esters	β-CD polymers	[215]
	Various derivs.	Polymeric dipeptide	[216,217]
	DNB	Dimeric quinines	[218]

buffer is requisite for chiral separations with 3-(4,6-dichloro-1,3,5-triazinylamino)-7-dimethylamino-2-methylphenazine (DTDP), a new triazine spectroscopic reagent [194]. Many separations are performed with mixtures, including CDs in suitable chiral or nonchiral micellar phases. A paper of El Rassi [219] describes the most recent developments in the use of chiral glycosidic surfactants, and Haddadian *et al.* [220] reviewed chiral separations with dipeptide polymers. The use of different polymers showed good resolution of various dinitrobenzoyl (DNB) AA derivatives [212,218]. Table 14.3 cites selected AA chiral separations achieved with CE and MEKC by making use of macrocyclic antibiotics and the ligand-exchange strategy. The use of vancomycin as a chiral selector in CE was amply discussed [238]. The resolving power of macrocyclic antibiotics is usually due to enantiomer discrimination rather than high affinities. Application of ligand-exchange complexation could be limited, since it requires two polar groups at a proper distance from each other. However, for their promising characteristics

TABLE 14.3

SELECTED CE AND MEKC ENANTIOMERIC AMINO ACID SEPARATIONS BY MACROCYCLIC ANTIBIOTICS AND LIGAND-EXCHANGE SEPARATION MODES

Chiral selector	AA der.	Observations	Ref.
Macrocyclic antibiotics	Underiv. Asp, Glu	Vancomycin, teicoplanin, indirect detn., CE	[221]
	Various deriv.	Vancomycin	[222]
	Various deriv.	Ristocetin A	[223]
	FMOC	Vancomycin, organic modifiers	[224]
	FMOC	Vancomycin, hexadimetrine bromide	[225]
	DNS	Rifamycin SV, indirect det.	[226]
	FMOC	Teicoplanin, 40% acetonitrile	[227]
	DBA	Avoparcin, organic modifiers	[228]
	DNS	A35512B antibiotic	[229]
Ligand-exchange	Underivatized	*N*-(2-hydroxyoctyl)-L-4-hydroxyproline/Cu(II)	[230]
	Underivatized	*N*-alkyl-L-4-hydroxyprolines/Cu(II)	[231]
	DNS	L-histidine/Cu(II)	[232]
	DNS	L-(+)-tartrate/Co(III)	[233]
	Underivatized	L-4-hydroxyproline/Cu(II), urea, SDS	[234]
	DNS	*N*,*N*,-didecyl-alanine/Cu(II), SDS	[235]
	DNS	Cu(II)/aspartame complex	[236]
	DNS	L-arginine/Cu(II)	[237]

both strategies need further investigations. Chiral separations by CE and MEKC offer the opportunity to modify many parameters simultaneously, *e.g.*, the concentration of the chiral selector, buffer additives and detergents, organic solvents and acidity of the buffer, temperature, and voltage. The optimization of these separation parameters is certainly a complex task, which often requires the application of chemometric experimental designs. Since this subject is not essential for the purpose of this chapter, interested readers are referred to excellent recent reviews [29,157,239].

14.2.5.2 Indirect chiral separations

Indirect chiral discrimination is based on racemate derivatization with an optically pure reagent. Subsequent separation of diastereoisomers can be usually achieved with achiral stationary phases. Table 14.4 lists selected derivatizating reagents for indirect AA chiral separations. Marfey's reagent always represents a good choice. This derivatization was

TABLE 14.4

SELECTED CHIRAL REAGENTS FOR PRE-COLUMN DERIVATIZATION OF AMINO ACIDS

Reagent	Abbrev.	Detect.	$\lambda_{abs(ex.)}$	λ_{em}	Separation	Refs.
1-(9-Anthryl)-2-propyl chloroformate	APOC	UV–Fl	256/351	412	MEKC	[240]
1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent)	FDAA	UV	340		RP, MEKC	[241–246]
1-(9-Fluorenyl)-ethyl chloroformate	FLEC	Fl	248/265	310/330	MEKC	[247–249]
N-α-9-Fluorenylmethyloxycarbonyl-amino acid-*N*-carboxyanhydrides	FMOC-AA-NCAs	UV	254		RP	[250]
(1*R*,2*R*)-*N*-[(2-isothiocyanato)cyclo-hexyl]-6-methoxy-4-quinolinylamide	CDITC	Fl	333	430	RP, CEC	[251]
1-(1-Naphtyl)-ethyl isothiocyanate	SNEIT	UV	210		CE	[252]
α-Methylbenzyl isothiocyanate	SAMBI	UV	254		CE	[252]
R(−)-4-(3-isothiocyanatopyrrolidin-1-yl)-7-(*N*,*N*-dimethylaminosulfonyl)-2,1,3-benzoxadiazole	DBD-PyNCS	Fl	460	550	RP, MEKC	[253–256]
Diacetyl-L-tartaric anhydride	DAT	UV	233		MEKC	[257]
Dibenzoyl-L-tartaric anhydride	DBT	UV	233		MEKC	[258]
1,3-Diacetoxy-1-(4-nitrophenyl)-2-propyl isothiocyanate	DANI	UV	245		RP	[259]
OPA + *N*-*tert*-butylcarbonyl-L-cysteine	OPA + BocC	UV/Fl	230	340	RP, MEKC	[260,261]
OPA + *N*-acetyl-L-cysteine	OPA − NAC	UV/Fl	230	340	RP	[262]
OPA + 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranose	OPA + TATG	UV/Fl	340	415	MEKC	[263]
2,3,4,6-Tetra-*O*-acetyl-1-thio-β-D-glucopyranosyl isothiocyanate	GITC	UV	210		RP, MEKC	[264,265]

used in quantifying the racemization of amino acids anchored to solid supports for peptide synthesis [266]. Derivatization with OPA and chiral cysteine derivatives has been applied in the determination of enantiomeric amino acids in fossil samples [267]. Indirect derivatization with 1-fluoro-2,4-dinitro-5-L-alanine was used in the determination of the chirality of amino acid residues during subtractive Edman peptide degradation [268]. High optical purity is very important for the reagents in indirect chiral separations, especially when the determination of minimal enantiomeric impurity is the analytical purpose. In any case, when the optical purity of the reagent is known, suitable corrections can be applied to calculate the effective enantiomeric impurity [269]. A further limitation of this technique concerns the requirement of close chiral centers for satisfactory diastereoisomer separations.

14.2.6 Particular amino acid groups

Several chromatographic methods have been recently developed for the separation of specific amino acid groups relevant to biology. The following sections describe some of these specific methods, briefly outlining their potential applications.

14.2.6.1 Aromatic amino acids and derivatives

Taking advantage of the particular characteristics of aromatic AAs, specific chromatographic separations that satisfy medical requirements for fast and reliable determinations have been carried out. Measurement of the plasma Phe/Tyr ratio is of relevance to the early assessment of phenylketonuria, an inborn metabolic error with a decreased conversion of Phe to Tyr. Tyrosinemia can be detected in a disease involving mainly liver, kidney and peripheral nerves [270]. Moreover, an increase of aromatic amino acids (particularly Phe) in the blood has been observed in children with *falciparum* malaria [271]. As previously reported [110], the Phe/Tyr ratio can be measured by RP-HPLC, taking advantage of the inherent fluorescence of the two AAs, with excitation and emission wavelengths of 215 and 283 nm, respectively, and by using *N*-methyl-Phe as internal standard. MEKC separations with LIF detection in plasma microdialyzates also showed a satisfactory performance [272]. Recently, molecularly imprinted polymers for open-tubular and filled CEC permitted the fast separation of DNS-Phe [273], although this method was not tested in biological fluids. The absorption characteristics of Phe and Tyr can be used for their direct UV detection after either RP-HPLC [274–278] or CE [279] separations. Alternatively, specific Phe analysis in plasma and urine, either by electrochemical [280] or by photolysis/electrochemical [281] detection can be performed. Low-capacity cation-exchange chromatography on hexadecylsulfonate-coated silica allowed the direct UV detection of Tyr, Phe, His, 1- and 3-methyl-His, anserine, carnosine, and homocarnosine in urine samples [282,283]. Modification of a tunable UV/vis detector for the simultaneous absorbance and fluorescence detection in CE permitted the monitoring of underivatized fluorescent compounds in MEKC [284]. More general approaches utilized CBQCA derivatization with LIF detection during CE separations of 23

amino acids [285] (Fig. 14.4) and OPA derivatization, followed by isocratic RP-HPLC with fluorimetric detection of amino acids, both in plasma [286]. Quantification of tryptophan and its derivatives may be relevant for assessing mental disorders, aromatic L-amino acid decarboxylase deficiency [287] and chronic renal failure [288]. Molnár-Perl [289] has reviewed the analysis of Trp and related compounds by chromatography and CE. Trp, 5-hydroxytryptamine and related indoles, like Tyr and Phe, can be detected in plasma by their inherent fluorescence, in either RP-HPLC [290] or MEKC, with LIF detection [291]. Trp and its kynurenine derivatives can also be determined in plasma by using RP-HPLC with UV, fluorescence, and electrochemical detection [288].

The diffusion-limited reaction of nitric oxide and superoxide anion generates peroxynitrite, a potent, unstable oxidant that readily reacts with tyrosine to produce 3-nitrotyrosine [292]. To a lesser extent, peroxynitrite also reacts with tryptophan, producing

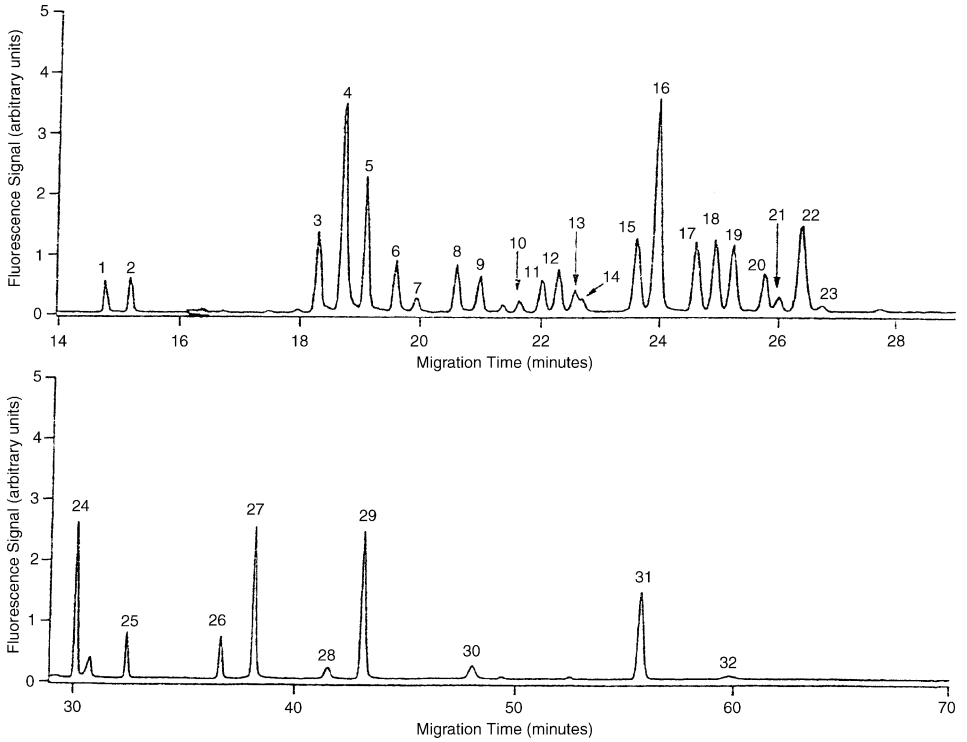


Fig. 14.4. Capillary electrophoresis/laser-induced fluorescence of 29 CBQCA-Aas. All Aas are 100 μ*M* except Ans, β-Ala, Car (30 μ*M*), Asn, Gly, Tau (60 μ*M*), and Glu, Arg, Asp (200 μ*M*). Peak identification: (1 and 2) Lys; (3) 1-Me-His; (4) Cit; (5) Gln; (6) Ans; (7) Asn; (8) 3-Me-His; (9) Tyr; (10) Orn; (11) β-Ala; (12) Car; (13) $(Hcy)_2$; (14) Ser; (15) Gly; (16) Ala; (17) D-Abu; (18) Tau; (19) L-Abu; (20) Val; (21) $(Hcy)_2$; (22) Met; (23) Thr; (24) N-Val; (25) Ile; (26) Phe); (27) Leu; (28) Aad; (29) N-Leu; (30) Glu; (31) Arg; (32) Asp. Ce parameters: BGE: 160 m*M* borate, 130 m*M* SDS, 7.5 m*M* γ-CD and 20 m*M* NaCl (pH 9.5); capillary, 67 cm length (60 cm to the detection window), 50 μm ID; separation voltage, 30 kV. (Reprinted from Ref. 285 with permission.)

formylkynurenine and different oxindols [293]. Some procedures have recently been developed for 3-nitro-Tyr analysis. They include RP-HPLC with tandem electrochemical and UV detection [294,295] and GC/MS assays [296]. Analytical methods utilized for the determination of 3-nitro-Tyr have been reviewed recently [297]. Similarly, 3-chloro-Tyr, a marker of the toxicity of endogenous and exogenous hypochlorous acid [298], can be specifically determined, either by RP-HPLC with electrochemical detection [299] or by GC with electron-capture negative-ion chemical ionization (EC-NICI) MS [300], which seems more sensitive than HPLC/MS and allows the artifact-free quantification of 3-halogen-Tyr derivatives in plasma [300].

14.2.6.2 Phospho-amino acids

The fast identification of protein phosphorylation sites by the coupling of 2-D PAGE and MS is important in proteome analysis [301–306]. Classical phospho-amino acid analysis can be based on the molybdate assay [307], but the low detection limit of this staining procedure calls for more sensitive analytical methods [308]. They include [γ-^{32}P]-phosphate radiolabeling and MS analysis and fluorescent labeling [309]. Chromatographic methods for the separations of [γ-^{32}P]-phosphate radiolabeled amino acids include TLC, performed on cellulose and silica plates [310,311]. Taking advantage of the strong acidic properties of phosphoamino acids, anion-exchange chromatography, coupled to pre-column derivatization, is the preferred method of analysis [308]. Nevertheless, CE and MEKC methods can provide specific phospho-amino acid determinations. In CE, adenosine monophosphate is added as BGE for indirect detection section [124]. Another strategy for the localization of nonradioactive phosphorylated AAs in proteins is based on CE separation of dabsyl derivatives [312]. The determination of phosphoamino acids in urine hydrolyzate was carried out either by GC with flame-photometric detection [313] or by GC/MS [314]. For both techniques, the sample was partially purified by anion-exchange chromatography and the phospho-amino acids were converted to their *N-iso*-butoxycarbonyl trimethylester derivatives. A CE separation involving selective fluorophore labeling and LIF detection allowed the specific detection of phosphoserine at the amol level [315]. Phosphoserine was derivatized with 1,2-ethanedithiol and then subjected to an iodoacetate reaction, yielding a highly fluorescent adduct. Dual HPLC separation on the dabsyl AA derivatives permitted the simultaneous determination of phospho-Tyr, phospho-Thr, phospho-Ser, and dityrosine [316]. Another chromatographic method for the specific detection of phospho-histidines has recently been reported [317], but a reliable, widely applicable and fast method for phospho-amino acid analysis is awaiting development.

14.2.6.3 Sulfur-containing amino acids and their NO-derivatives

The most important sulfur-containing amino acids are Cys, homocysteine (with their oxidation products, cystine and homocystine), Met, and taurine. Cys and Met are determined by the general chromatographic and MEKC procedures described in the previous sections. Specific Cys determination in biological fluid is achieved by selective derivatization of the sulfhydryl group [50,318–320], and procedures have

been designed for cysteine and disulfide analysis [321,322]. The Ellman reagent (5,5′-dithio-*bis*(2-nitrobenzoic acid)) can be used for post-column derivatization in the presence of cationic micelles in order to increase the sensitivity at 412 nm [323]. Moreover, 4,4′-dithiodipyridine (DTDP) was recently proposed as a more quantitative reagent for the detection of sulfhydryls in proteins [324]. The simultaneous determination of cysteine, glutathione, and homocysteine in blood was achieved by narrow-bore isocratic RP-HPLC following derivatization by monobromobimane (MBBr) [325]. Pre-column derivatization by means of MBBr permitted the determination of urinary *S*-phenylmercapturic and *S*-benzylmercapturic acids, markers of benzene and toluene exposure, by RP-HPLC, coupled to fluorimetric detection ($\lambda_{exc} = 375$ nm; $\lambda_{em} = 480$ nm) [326]. MBBr derivatization was also utilized for the determination of glutathione in a single human erythrocyte [327]. The same result can be obtained by using CE with electrochemical detection [328]. Specific determination of urinary cysteine was carried out by using UV labeling with 2-chloro-1-methylquinolinium tetrafluoroborate [329]. Total and free cysteine can be quantified by derivatization before and after sample reduction with tri-*n*-butylphosphine. The specific oxidation properties of thiols are utilized for the specific analysis of cysteine and glutathione by HPLC, coupled to electrochemical detection [330–332].

Homocysteine is connected with the folate pool and plays a central role in methylation pathways [333]. An increase of homocysteine levels in the plasma is considered a risk factor for coronary diseases. Therefore, many specific chromatographic methods for the analysis of homocysteine have recently been developed [334]. They are based on RP-HPLC and pre-column derivatization with MBBr [325,334], 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonate (SBD-F) [334], 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) [335], or 2-chloro-1-methylquinolinium tetrafluoroborate [336]. CE after derivatization by 6-iodoacetamidofluorescein (IAF) and LIF detection permitted homocysteine determination in minute amounts [337]. However, even though HPLC with electrochemical detection, HPLC/MS, GC, CE, MEKC, and immunoassay methods are available, the between-method and between-laboratory variability of homocysteine analysis is still unsatisfactory [334].

Several links exist between altered taurine metabolism and the development of diabetes sequelae [338]. Moreover, taurine possesses trophic properties in neural tissue, which makes it a likely essential micronutrient for the development and regeneration of the central nervous system and of the retina [339]. Many methods have been developed for the determination of taurine, hypotaurine, and their precursors in plasma [340]. The chemical properties of taurine and hypotaurine permit all derivatization reactions involving the amino group to be utilized. Consequently, taurine and hypotaurine can be determined together with many other amino acids occurring in plasma by the methods cited in the previous sections of this chapter. For instance, HPLC [341] and CE [342] determinations in plasma made use of pre-column fluorescamine derivatizations, while a specific urine analysis by HPLC was based on pre-column dabsyl derivatization [343]. Another topic relevant to thiol-containing compounds concerns their oxidative reaction with nitric oxide. This event seems to be critically involved in the modulation of intracellular and intercellular signal transduction [344]. Thus, *e.g.*, intra-erythrocytic *S*-nitrosoglutathione seems to be implicated in the storage, transport, and metabolism of NO. Naturally,

methods for the quantification of *S*-nitroso compounds, despite their instability and low concentrations, are valuable. RP-HPLC [345] and CE [346] methods, based on direct detection of *S*-nitrosoglutathione were reported, but they show low sensitivity, at the limit for determination of *S*-nitrosoglutathione in biological samples.

14.2.6.4 Seleno-amino acids

Selenium, in the form of selenocysteine occurs at the active site of a wide range of proteins, *e.g.*, glutathione peroxidase enzymes and sperm capsule selenoprotein [347,348]. Approximately 50% of the Se in plasma is in selenoprotein P, which contains up to ten selenocysteine residues in its chain and probably is the protein for Se transport and storage [349], whereas the role of selenoprotein W, a protein found in muscle, heart, and brain, is still unknown [350]. Dietary Se seems to decrease the incidence of genetic damage and cancer risk in adults [351]. GC with inductively coupled plasma mass spectrometry (ICP-MS), following solid-phase micro-extraction, allowed the selective determination of selenocysteine, selenomethionine, and selenoethionine [352]. A pre-column derivatization with *iso*butylchloroformate was an essential step in these determinations. ICP-MS, coupled with CE [353] can take the place of pre-column derivatization. The resolution of CE, combined with specificity of ICP-MS detection of the element, promises to become a powerful tool for selenium speciation. Free Se(IV) and Se(VI), selenoglutathione, selenomethionine, selenocysteine, and selenocystamine can be detected with high sensitivity in extremely small amounts of sample. Because of its high cost, ICP-MS is not always available. More accessible options are the determination of selenocysteine and selenomethionine as OPA-derivatives by RP-HPLC [354] and the direct determination by anion-exchange chromatography with electrochemical detection [355]. The enantiomeric resolution of selenoamino acid derivatives was achieved by MEKC, performed with SDS and a mixture of β-CD and taurodeoxycholic acid as a chiral pseudo-stationary phase [356]. Satisfactory enantioresolution of selenomethionine in urinary samples was also obtained after derivatization with ethylchloroformate and GC/ICP-MS on a Chirasil-L-Val capillary column [357]. Mendez *et al.* [358] compared different chiral separation methods for the enantioresolution of selenomethionine, such as HPLC on either a β-CD column or on a Chirobiotic T column, MEKC with CD as chiral selector, and GC on a Chirasil-L-Val capillary column, coupled with an ICP-MS detector. Whereas for HPLC and MEKC separations SeMet was derivatized with naphthalene-2,3-dicarboxaldehyde (NDA), for GC separation SeMet was converted to its *N*-trifluoroacetyl-*O*-alkyl ester. Best results were obtained by utilizing the HPLC separation on a Chirobiotic T column.

14.3 PEPTIDES

14.3.1 Conventional methods

Common bonded alkylsilica RP packings give adequate performance for peptide and protein purification and separation [359]. The most common method for peptide analysis nowadays utilizes a HPLC procedure with the following characteristics: a column of

25 cm length and 4.1 mm ID, packed with a 5-μm, wide-pore (usually 300 Å) octadecyl- or octyl-bonded stationary phase, eluted with a water/acetonitrile gradient and 0.1 to 0.2% trifluoroacetic acid (TFA) as ion-pairing and solubilizing agent. This can be considered as the reference method for peptide analysis. It features high resolution and selectivity, the eluent is characterized by transparency, and peptide recovery is simple. However, this technique often fails in the separation of either small polar peptides or large apolar peptides. Moreover, it shows poor selectivity in the separation of several post-translationally modified peptides, such as glycosylated, deamidated, oxidized, and phosphorylated peptides. Therefore, there are studies in progress, both for a better understanding of reversed-phase ion-pairing mechanisms and for the development of new stationary phases. The molecular dynamics of solute interaction with the reversed-phase stationary phase under specific chromatographic conditions are still not well understood. In order to predict peptide retention times in RP-HPLC separations, many authors have proposed several set of empirical retention coefficients, linked to amino acid composition, which have been discussed in the previous edition of this book [2]. These semi-empirical approaches are suitable for the optimization of peptide separations. However, the peptide chain-length, as well as the presence of several domains, may produce appreciable deviations of experimental retention times from the theoretical values calculated with these coefficients. In addition, when peptides are protonated, particularly at C- and N-terminal groups, or assume particular conformations, other, nonpredictable contributions to the observed retention times may enter [360,361]. These predictions must therefore be considered as suggesting the peptide elution order, and they mandate peptide identification in unknown samples.

14.3.2 New stationary phases

Recently, Buchmaiser [362] reviewed new synthetic strategies for the preparation of HPLC packings for peptide chromatography with special reference to pellicular stationary phases [363]. They consist of fluid-impervious microspheres, coated with an appropriate retentive layer of stationary phase. The enhancement of speed and efficiency of the separation is due to the pellicular configuration. In fact, intraparticular mass transfer resistance is confined to the thin outer shell, and therefore it is drastically reduced. A paradigm of this separation medium is the Hi-Tach C_{18} column, which features a thin, porous octadecylsilica layer, successfully employed for rapid and high-resolution separation of peptides and proteins [364]. These pellicular columns frequently operate at high temperatures in order to reduce mobile-phase viscosity. Good performance, coupled with very short analysis times was obtained with 2-μm porous microspherical reversed-phase silica gel, having a 12-nm mean pore diameter [365]. With a 50 mm × 4.6 mm column, a flow-rate of 1.5 mL/min, and a 2-min linear gradient, 13 m*M* perchloric acid/acetonitrile separated several peptides, including bombesin, somatostatin, oxytocin, Leu-enkephalin, and α- and γ-endorphin, within 1 min. The properties of superficially porous silica microsphere column packings (called "Poroshell") were extensively investigated [366]. They consist of ultra-pure "biofriendly" silica microspheres, composed of solid cores and a thin outer shell. The excellent kinetic properties of these chromatographic supports are judged to be particularly suitable for the

separation of different natural polymers, such as peptides, in a fraction of the time usually required for conventional separations. Although ion-exchange chromatography is devoted to protein rather than peptide separation, several interesting recent studies with the so-called "tentacle-type" LiChrospher-1000 SO_3 were performed [367]. These studies showed that peptides and proteins are adsorbed on "tentacular" ligand systems through a multilayer dissolution mechanism: the peptide interacts with a diffuse or extended Donnan double layer in the ion-exchange environment, resulting in a multi-site binding process. Several silica coatings were tested for peptide separations. Polyvinylpyrrolidone-coated silica was synthetized by the interaction of a copolymer of vinylmethyldiethoxysilane and vinylpyrrolidone with LiChrospher Si 300 and Si 500 silicas [368]. This coating permitted satisfactory peptide separations according to a hydrophobic mechanism. A temperature-responsive polymer, poly-(*N*-isopropylacrylamide), was grafted to aminopropylsilica by an activated ester/amine coupling. Since the surface properties of this stationary phase depended on external temperature, isocratic elution of three peptides was achieved with an aqueous mobile phase alone by changing the column temperature [369]. Hydrophilic-interaction chromatography on amine bonded-phase silica columns provides peptide separations that are usually complementary to those obtained by RP-HPLC [370].

Silica-based packing materials have good mechanical strength, but are unstable at high pH. Several studies were directed toward the development of phases suitable for use in alkaline medium. With the polymer-based POROS resin, which is stable at high pH, separation of the phosphorylated and nonphosphorylated forms of phosrestin I was accomplished by taking advantage of the different charges of the peptide at high pH [371]. In the field of alkali-stable phases, zirconia-based materials received special attention, although column cost can be a serious bar to extensive application. For instance, Wirth *et al.* [372,373] have modified porous zirconia particles with reversed-phase stationary phases, carbohydrate, concanavalin A, Cibacron Blue F3GA, and imidoacetic/Cu(II) complexes. Polyphosphates with various chain-lengths were linked to zirconia in order to prepare stationary phases for the separation of nucleic acids and proteins [374]. Yu and El Rassi [375] succeeded in synthesizing microspherical octadecyl- and octylzirconia particles that were highly stable at extremely high pH and applicable to peptide separations. Phosphate-modified polybutadiene-coated zirconia has both reversed-phase and cation-exchange characteristics at low pH and gave excellent resolution of standard peptides [376]. An alkali-stable strong-anion-exchange material was synthesized by the deposition of particles of polyethylenimine on porous zirconia, followed by cross-linking and quaternarization [377]. The retention coefficients of selected homopeptides showed that ion exchange was the primary mechanism of retention. Additionally, microspherical carbon packings may offer further approaches to less-expensive stationary phases. Gradients of acetonitrile/water with trifluoroacetic acid as ion-pairing agent were successfully employed in the separation of many peptides on microspherical porous carbon beads (Carbonex) with an average particle size of 3.5 μm [378]. Comparison with the results obtained using octadecylsilica columns indicated that peptides with aromatic residues are more strongly adsorbed on the carbon column and suggested an interaction based on the graphitic nature of the carbon surface. The retention behavior of 14 peptides on a porous graphitized carbon column showed that hydrophobicity and steric and

electronic parameters affect peptide retention similarly, confirming that separation is based on mixed mechanisms [379]. Recently, peptide separations on a polyethylene-coated alumina column packing was investigated [380]. In this case, steric parameters seemed to exert the highest influence on the mechanisms of peptide separation. Pesek and Matyska [381] have reviewed the use of modified aluminas as chromatographic media for HPLC separation. A number of successful applications were described for various modified silica packings in order to illustrate their potential usefulness and to compare alumina-modified phases with the more conventional silica-based materials.

Extensive studies have been performed for the development of immobilized metal-chelate phases for metal-chelate-interaction chromatography. The geometry of metal chelates as well as the nature of the incorporated metal ion strongly affect peptide retention as a function of its amino acid content and consequently affect the separation specificity. The immobilization of histidine residues on a stationary phase allowed the separation of peptides in the presence of various divalent metal ions, following both classical affinity procedures and hydrophobic-interaction chromatography [382]. Alternatively, Cu(II) can be immobilized on the stationary phase. The most studied metal-chelate phases are based on iminodiacetic/Cu^{2+} complexes on a silica support. They are used for the separation of His-containing peptides [383], and several columns are commercially available. Recently, a phage-displayed examer random peptide library was used to select for peptides with affinity for immobilized Cu(II). Peptides with two His residues showed a higher affinity than those containing a higher number [384]. This finding was explained by invoking the differences between the pH-dependent Cu(II) affinity of the different peptides.

14.3.3 Chromatographic conditions

Eluents and ion-pair reagents have been adequately described [2], acetonitrile being the most commonly used organic phase and TFA the ion-pairing reagent of choice. However, pentafluoropropionic, heptafluorobutyric, perfluoropentanoic, perfluorohexanoic, and perfluoroheptanoic acids were investigated as an alternative [385]. Increasing the *n*-alkyl chain-length can help to resolve sample components that are otherwise eluted in the void volume of TFA-based RP-HPLC separations on octadecyl-silica.

Direct peptide detection is usually performed with good sensitivity in the 210- to 220-nm range, taking advantage of the peptide-bond absorbance. Nonetheless, this detection wavelength range does not allow correct quantification, because the absorbance depends on the lateral chains, sequence, and conformation. However, extinction coefficients at a wavelength of 280 nm can be accurately calculated from the sequence, being mainly related to Trp, Tyr, and Cys-Cys content. Therefore, the concentration of a peptide containing these residues can be calculated at this wavelength from the peak area relative to an internal standard, even at a lower sensitivity [386]. The fact that Trp and Tyr have absorption maxima at different positions can be utilized in the diode-array detection mode by first- or second-derivative UV spectroscopy, in the 240- to 320-nm range, to discriminate between peptides having different ratios of these aromatic amino acids [387–389]. The observation that Tyr-containing peptides show a hypsochromic shift of the absorbance maximum upon Tyr phosphorylation allowed the mapping of Tyr phosphorylation sites in several tryptic digests by second-order derivative spectra [390].

Detection of either low-absorbing or low-concentrated peptides can be improved by pre-column (or post-column) derivatization with almost any of the reagents suitable for amino acids. Examples are fluorescamine [391], *N*-hdroxysuccinimidyl-α-(9-acridine) acetate [392], and 9-fluorenylmethyl chloroformate (FMOC) [393]. FMOC was successfully used for fluorescence detection of the human β-endorphin 28–31 fragment and analogs in cerebrospinal fluid. Specific strategies can require particular procedures. For example, hydrolysis in deuterated acid, derivatization with Marfey's reagent, and analysis by HPLC/ESI-MS allowed the chiral purity of a peptide to be established [394]. The classical RP-HPLC technique for peptide separation is now the reference method for proteolytic digest mapping. Starting from purified proteins, completely automated mapping, including the protection of Cys residues after alkylation with iodoacetamide or vinylpyridine, can be performed in less than 2 h [395]. Therefore, HPLC peptide mapping (often by coupling with MS) is the method of choice for the identification of the sites of post-translational modifications of proteins [396].

14.3.4 Monolithic columns and imprinted stationary phases

Monolithic columns are made of either acrylate or methacrylate derivatives, obtained by polymerization of a monomer and a cross-linker by an initiator in the presence of at least one, generally two porogenic solvents [362]. By *in situ* polymerization of either divinylbenzene with styrene or ethylene methacrylate with butyl methacrylate, Chirica and Remcho [397] prepared monolithic columns with templated porosity. A convective flow-through pore can be achieved and, due to significantly enhanced mass transfer, these sorbents can allow efficient separation of natural polymers. Cast as continuous homogeneous phases, monoliths increase chromatographic productivity by at least one order of magnitude, compared to particle-based chromatography. Short columns seem to be the best option for the purification of large molecules [398]. A commercial monolithic C_{18}-bonded silica rod column (Merck Chromolith) was used for the separations of different microcystins and nodularin-R. Resolution, selectivity, efficiency, and peak asymmetry equaled or exceeded the performance of particle-based columns [399]. Chromolith columns cut total analysis time to *ca.* 4 min with a flow-rate of 4 mL/min. Under electro-driven conditions, acrylate-based porous monoliths, containing quaternary amine moieties have been used to achieve high EOF at low pH values, and monoliths with sulfonic acid groups were used for high-pH separations [42]. Native peptides, PTH-AAs, NDA-derivatized amino acids and peptides were separated with high efficiency. A monolith column, prepared by co-polymerization of lauryl methacrylate and ethylene dimethacrylate to form a C_{12} hydrophobic stationary phase, showed good efficiency in the separation of some basic and acidic model peptides, although the separation was characterized by a low EOF value [400]. Coupling of capillary monolithic columns with ESI-MS yielded efficiencies comparable, or even superior to those observed with conventional microcolumns [401]. Macroporous poly(styrene/divinylbenzene) monoliths, prepared by *in situ* polymerization in PEEK, fused-silica, or stainless-steel tubing produced an efficient separation of protein digests and standard peptides and their characterization by ESI-MS [402]. A surface octadecylation was not necessary for the separation, since both modified and unmodified columns provided comparable results.

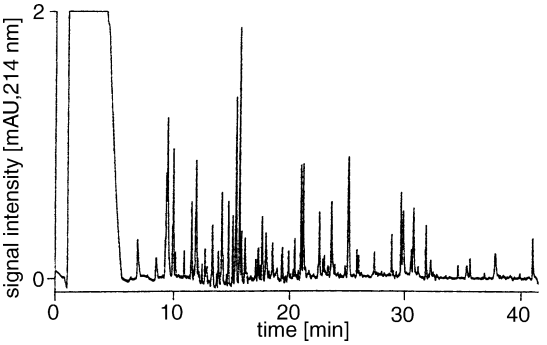


Fig. 14.5. High-resolution capillary RP-HPLC of the tryptic digest of human transferrin in a monolithic column. Column, monolithic PS/DVB, 60 × 0.2 mm ID. Mobile phases: (A) 2.0% acetonitrile, 0.050% TFA in water; (B) 80% acetonitrile, 0.050% TFA in water; linear gradient, 0–40% B in 30 min, 40–100% B in 10 min; flow-rate: 1.7 μL/min; temperature, 50°C; detection, 214 nm; sample, tryptic digest of 1.0 pmol of human transferrin. (Reprinted from Ref. 403 with permission. Copyright 2001 American Chemical Society.)

Using similar monoliths, the mass fingerprinting of tryptic digests of bovine catalase, human transferrin (Fig. 14.5) and membrane proteins related to the photosystem II antenna complex, was achieved by ESI-MS coupling [403].

By methods similar to those employed for monoliths, molecularly imprinted stationary phases can be prepared [362,404]. Imprinted phases are of special use for direct chiral AA separations (Sec. 14.2.5.1.2). Some peptide separations were also described [404–406]. A co-polymer of methacrylic acid and trimethylolpropane trimethacrylate, imprinted with *Z*-L-Ala-L-Ala-O-Me allowed the resolution of the racemate of the dipeptide at a high load capacity [404]. Taking advantage of the epitope approach, *i.e.*, the use of a short peptide that represents only part of a larger peptide as template, a stationary phase capable of recognizing oxytocin and oxytocin-related peptides [405,406] was prepared. The ability to use an imprinted phase for on-line sample pre-concentration in capillary electrophoresis has recently been reported [407].

14.3.5 Capillary electrochromatography

Some excellent reviews are devoted to the description of the principles of CEC, stationary phases, and applications [408–410] (Chap. 7), others deal specifically with peptide separations [411,412]. Hearn *et al.* [412,413] described the analysis of peptides by different capillary techniques, placing emphasis on their orthogonality. CEC separations of bradikynin as a test solute in diol- and octadecyl-modified, etched, fused-silica capillaries showed that the surface area of the inner wall was sufficiently increased to induce solute/bonded-phase interactions and reversed-phase behavior of the C_{18}-modified capillaries [414,415]. Similar conclusions were drawn for the separation of a tryptic digest of transferrin [416], for the separation of basic standard peptides on a C_{12} porous-layer open-tubular column [417], and for standard synthetic peptides on open C_{18} and cholesterol-modified etched capillaries under isocratic conditions [418,419].

A porphyrin-modified capillary was prepared for the separation of structurally closely related peptides, containing aromatic amino acids [420]. The separation was characterized by good selectivity, further demonstrating a solute/bonded-phase interactions. Peptide separation by CEC in filled capillaries was usually achieved with strong-cation-exchange stationary phases [421–423], either in microsphere or monolithic configuration, in order to take advantage of high EOF. All of these studies have led to the conclusion that efficient peptide separations by CEC are possible and that the separation is governed by a dual mechanism that involves the complex interplay between selective chromatographic retention and differential electrophoretic migration. Efficient separation of peptides by CEC on a mixed reversed-phase and cation-exchange monolithic stationary phase was achieved [424]. A mixed-mode mechanism of separation was at work, including hydrophobic and electrostatic interactions as well as electrophoretic migration at the low pH value of the mobile phase. Studies on the effect of increasing the temperature to 50–60°C on peptide separations demonstrated that EOF and column efficiency increased whereas retention coefficients usually decreased [425,426]. Usually, Van't Hoff plots are not linear, suggesting a molecular transition of the stationary phase related to the temperature increase. A HPLC apparatus connected by a T-split to a high-voltage power supply was used for peptide mapping by gradient CEC and nano-HPLC on a C_{18} capillary column. The resolution of a tryptic digest of cytochrome c, obtained by CEC was higher than that obtained by nano-HPLC [427]. The coupling of CEC with MS can be utilized with good success for the detection of peptides and their mixtures [428,429]. CEC/MS coupling, at its present state of development, may be considered one of the most sensible systems for peptide analysis [430].

Based on phase transition with temperature change, very interesting studies on the use of lyotropic pluronic liquid crystals as stationary phases have been performed [431–435]. Pluronic F127 (BASF) solutions are liquid at 5°C or less, and they can be introduced into the capillary at this temperature. A self-supporting gel-like liquid crystalline phase is formed as the temperature is raised over 20°C. This liquid crystalline phase consists of spherical micelles with diameters of 17–18 nm that pack with local cubic symmetry [431,432]. Separations of peptide mixtures, such as CNBr fragments of collagen, at low pH values revealed that the mechanism is mainly driven by the charge/mass ratio with little sieving effect [433,434] (Fig. 14.6). The addition of SDS as BGE modifier at low pH allowed selective separations of positively charged polypeptides, such as poly-Lys, provided that their relative molecular mass was sufficiently low (<3500 Da) [435].

14.3.6 Micellar electrokinetic capillary chromatography

Since the CE separation of peptides is extensively discussed in the Chap. 15 and Chap. 16 is devoted to chromatographic techniques suitable for peptide and protein analysis, this section will treat only micellar electrokinetic capillary chromatography of peptides. Some reviews on its general use [436–440] and on some more specific applications to peptide separations have been published [441,442]. The influence of SDS concentration, percentage of organic modifier, applied temperature, and ionic strength of the buffer have been investigated for various enkephalin-related peptides with a multivariate

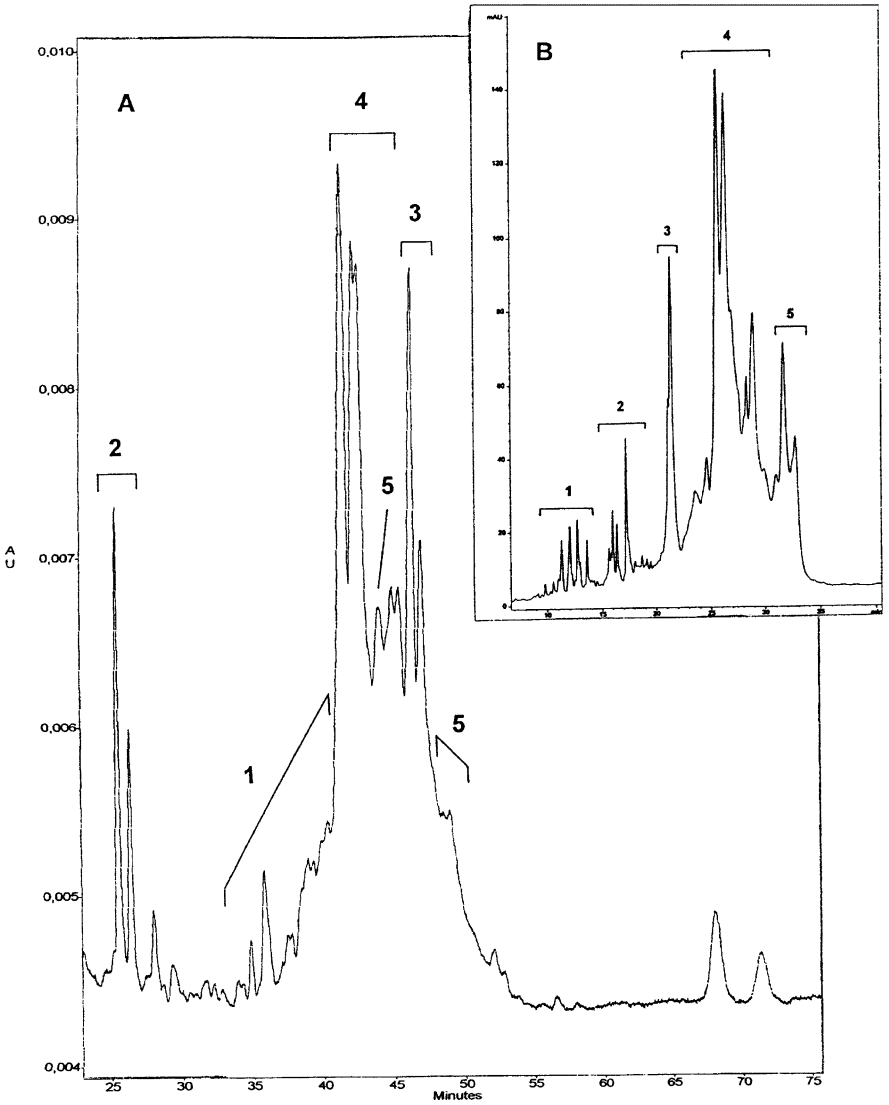


Fig. 14.6. Separation of rat-tail-tendon collagen-CNBr peptides by (A) CE in the Pluronic co-polymer media and by (B) HPLC. CE conditions: bare fused-silica capillary, 47 cm (40 cm to the detector), 75 μm ID; applied voltage, 15 kV; temperature, 20°C; BGE 10 m*M* Tris, 75 m*M* phosphate buffer (pH 2.5), containing 7.5% Pluronic F127. HPLC conditions: column, Zorbax 300SB-C_4, narrow-bore, 150 × 2.1 mm, 5 μm; mobile phase: (A) 0.1% heptafluorobutyric acid (HFBA) in water; (B) 50% acetonitrile, 0.1% HBFA in water; linear gradient, 0–100% B in 40 min; flow-rate, 0.25 mL/min; temperature, 60°C; detection, 214 nm. Peptide identification: (1) α_2(I)CB_2, α_1(I)CB_2, α_1(I)CB_5; (2) α_1(I)CB_4, α_1(III)CB_3, α_1(III)CB_6, α_1(I)CB_3, α_1(III)CB_4; (3) α_1(I)CB_6; (4) α_1(III)CB_5, α_1(I)CB_7, α_1(I)CB_8, α_2(I)CB_4 and incomplete cleavage products; (5) α_2(I)$CB_{3,5}$, $[\alpha_1$(III)$CB_9]_3$. (Reprinted from Ref. 433 with permission.)

approach [443]. In the presence of SDS, at least three mechanisms operate on selectivity: hydrophobic and ionic interactions and electrophoretic mobility. Since a pseudo-stationary phase may be easily changed, many options for the modulation of selectivity are available. Among the different materials, anionic and non-ionic, zwitter-ionic, micro-emulsion, macro-cyclic and macro-molecular phases, micelle polymers, polymeric surfactants, vesicles, resorcarenes, dendrimers, and polymer ions can be used successfully as pseudo-stationary phases [440]. Even though the large number of possible phases may generate confusion about the most suitable choice for a selected application, MEKC can be said to be particularly useful for the separation of peptides having similar structures and charge but different polarity, since it is based on selectivity principles different from those of RP-HPLC.

Numerous examples have been reported, and a complete listing is impossible. Large peptides, such as motilins and insulins, were purified in SDS or CTAB micelles [444]. The number of separations of closely related peptides is continuously increasing. For instance, eleven angiotensins II analogs were separated, using Tween 20 as non-ionic surfactants in acidic media [445]. Moreover, a mixture of structurally related dynorphin analogs was resolved, employing anionic, cationic, and zwitter-ionic surfactants [446]. Oxytocin and vasopressin and their analogs can be analyzed without derivatization in SDS [447], gonadorelin and its analogs in CTAB and other cationic surfactants [448], and galanines in SDS/borate buffers [449]. Glutathione can be determined either without derivatization [450,451] or by on-column derivatization with 2,2′-dipyridyldisulfide [452]. MEKC can be utilized for comparative analysis of protease maps. It was applied to the analysis of tryptic maps of human hemoglobin [453], where the peak pattern was appreciably different from that observed in CE and HPLC separations. MEKC was also employed as a tool for the detection of protease activity [454]. By utilizing high-ionic-strength SDS/borate buffer, a high-resolution separation of different glycoforms of recombinant human γ-interferon was achieved [455]. MEKC selectivity permitted the complete resolution of imidodipeptide mixtures in urine samples of prolidase-deficient patients [456]. Pre-concentration on an Immobilon membrane, followed by derivatization with 3-(2-furoyl)-quinoline-2-carboxaldehyde, and MEKC separation permitted insulin detection at very low concentration [457]. As for modifications of MEKC separation parameters, external electric fields can be utilized for EOF control in uncoated and coated capillaries for peptide separations [458], even though this option was tested more under CE than under MEKC conditions. High separation temperatures (about 110°C) produced an appreciable increase in the resolution of different cyclosporins without causing sample degradation or solvent boiling [459]. Acidic conditions (pH below 3) and SDS (at least 50 mmol/L) are of great potential in peptide separations by MEKC [460]. Under these conditions,

(a) the surfactant exerts a washing effect upon the capillary wall, thus preventing peptide binding;
(b) the surfactant interacts with peptides that bear considerable negative charge and can be separated on this basis;
(c) the procedure can be applied to peptides insoluble in neutral and alkaline media.

Owing to the low pH, EOF is negligible, and the separation of negative SDS/peptide aggregates is obtained with reversed polarity (Fig. 14.7). Although not applied to peptides,

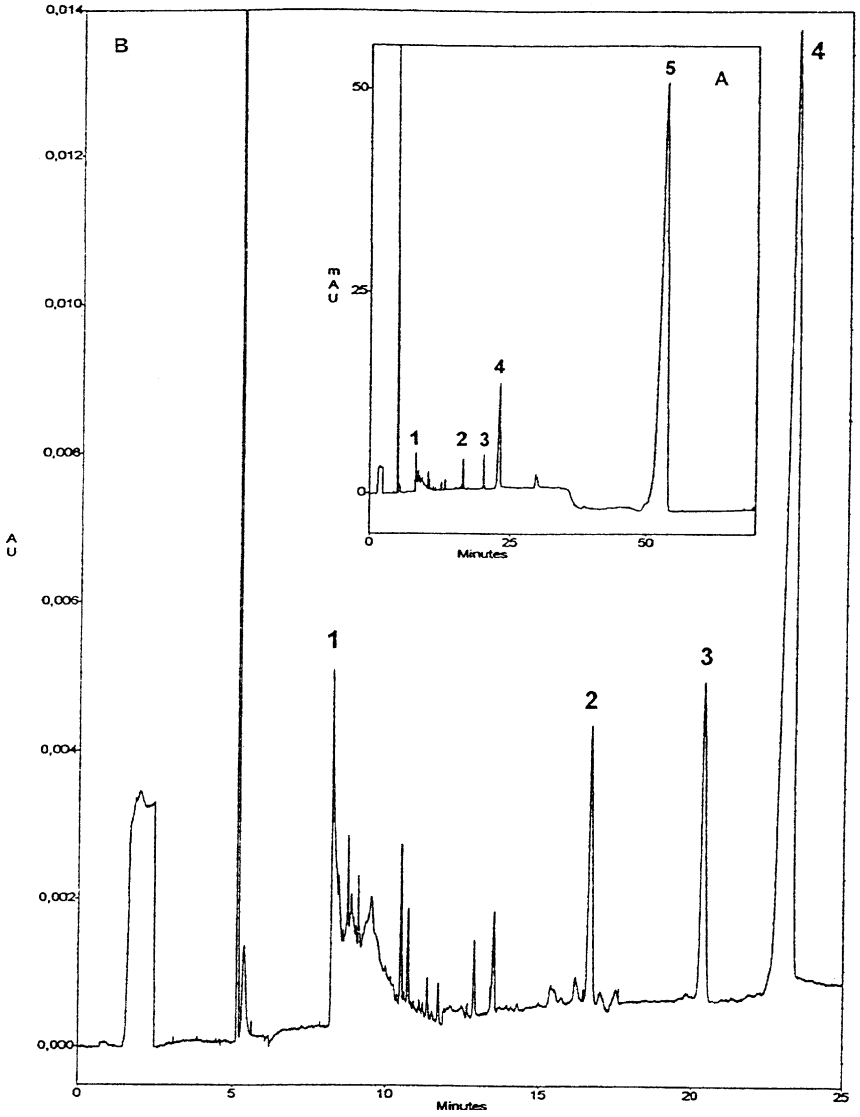


Fig. 14.7. Separation of CNBr fragments of egg-shell proteins by CE in acidic buffers, containing a high concentration of surfactant (SDS). Separation voltage, − 15 kV (reversed polarity mode). CE conditions: bare fused-silica capillary, 57 cm (50 cm to the detection window) 75 μm ID; BGE, 50 m*M* phosphate buffer (pH 2.5, adjusted with HCl) containing 50 m*M* SDS. Inset (A): whole separation indicating the presence of a large fraction, associating poorly with the surfactant. Main figure (B): enlarged front section of the electropherogram, showing the peaks of protein fragments associating easily with the surfactant, emerging within the first 25 min of running time. (Reprinted from Ref. 460 with permission.)

a further increase in buffer acidity (pH below 1), and a polyethylenimine-coated capillary gave very fast MEKC separations (< 2 min) [461].

14.4 MICROCHIP TECHNOLOGY

This topic is adequately covered in Chap. 11 and a number of reviews [462–465]. As for the application of microfluidics to peptides, LIF is a good choice for rapid clinical diagnosis [466]. Some amino acid and peptide separations were investigated on electro-driven micro-chips [462–466]. Several options previously described in different sections of this chapter, such as monolith technology and indirect detection, can be of particular interest in the microchip assembly. For example, poly(dimethylsiloxane) monolithic chips, modified by Ce(IV)-catalyzed polymerization, showed reproducible separations for over a month of synthetic peptide mixtures, such as a tryptic digest of bovine serum albumin [467]. Photopatterned rigid polymeric monoliths can be utilized for RP-electrochromatography of NDA-derivatized amino acids and peptides with LIF detection [468]. Indirect fluorescence detection permits the analysis of some free amino acid with enough sensitivity for application to body fluids [469].

ACKNOWLEDGMENTS

We acknowledge the kind assistance of Mrs. Roberta Monti in checking references.

REFERENCES

1 D.H. Spackman, W.H. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
2 C.T. Mant, N.E. Zhou and R.S. Hodges, in E. Heftmann (Ed.), *Chromatography, 5th edition. Fundamentals and Applications of Chromatography and Related Differential Migration Methods. Part B: Applications*, Elsevier, Amsterdam, 1992, p. B75.
3 R.M. Tujebajeva, P.R. Copeland, X.M. Xu, B.A. Carlson, J.W. Harney, D.M. Driscoll, D.L. Hatfield and M.J. Berry, *EMBO Rep.*, 1 (2000) 158.
4 K.V. Korotkov, S.V. Novoselov, D.L. Hatfield and V.N. Gladyshev, *Mol. Cell. Biol.*, 22 (2002) 1402.
5 M. Fountoulakis and H.-W. Lahm, *J. Chromatogr. A*, 826 (1998) 109.
6 M. Salva and F.X. Aviles, *Anal. Biochem.*, 180 (1989) 374.
7 J. Li and S. Liang, *Anal. Biochem.*, 302 (2002) 108.
8 Y. Liu and S. Liang, *J. Protein Chem.*, 20 (2001) 535.
9 F. Casagranda and J.F. Wilshire, *Methods Mol. Biol.*, 64 (1997) 243.
10 F. Lai and T. Sheehan, *Biotechniques*, 14 (1993) 642.
11 A. Vasanits and I. Molnár-Perl, *J. Chromatogr. A*, 832 (1999) 109.
12 Y.V. Tcherkas, L.A. Kartsova and I.N. Krasnova, *J. Chromatogr. A*, 913 (2001) 303.
13 D. Fekkes, A. Voskuilen-Kooyman, R. Jankie and J. Huijmans, *J. Chromatogr. B*, 744 (2000) 183.
14 K. Hara, Y. Hijikata, E. Hiraoka, N. Ogawa and H. Takahashi, *Ann. Clin. Biochem.*, 36 (1999) 202.

15 P. Jandik, J. Cheng, D. Jensen, S. Manz and N. Avdalovic, *Anal. Biochem.*, 287 (2000) 38.
16 R. Bhushan and J. Martens, *Biomed. Chromatogr.*, 15 (2001) 155.
17 A. Namera, M. Yashiki, M. Nishida and T. Kojima, *J. Chromatogr. B*, 776 (2002) 49.
18 W. Schützner and E. Kenndler, *Anal. Chem.*, 64 (1992) 1991.
19 T.T. Lee, R. Dadoo and R.N. Zare, *Anal. Chem.*, 66 (1994) 2694.
20 B.J. Wanders, T.A.A.M. van der Goor and F.M. Everaerts, *J. Chromatogr.*, 652 (1993) 291.
21 V. Rohlícek, Z. Deyl and I. Miksik, *J. Chromatogr. A*, 662 (1994) 369.
22 R.M. McCormick, *Anal. Chem.*, 60 (1988) 2322.
23 B. Gas, M. Stedry and E. Kenndler, *J. Chromatogr. A*, 709 (1995) 63.
24 J.E. Dickens, J. Gorse, J.A. Everhart and M. Ryan, *J. Chromatogr. B*, 657 (1994) 401.
25 D. Corradini, A. Rhomberg and C. Corradini, *J. Chromatogr. A*, 661 (1994) 305.
26 M.S. Bello, L. Capelli and P.G. Righetti, *J. Chromatogr. A*, 684 (1994) 311.
27 S. Terabe, K. Otsuka, K. Ichikawa, A. Tsuchiya and T. Ando, *Anal. Chem.*, 56 (1984) 111.
28 S. Terabe, K. Otsuka and T. Ando, *Anal. Chem.*, 57 (1985) 834.
29 H. Corstjen, H.A.H. Billiet, J. Frank and K.Ch.A.M. Luyben, *J. Chromatogr. A*, 715 (1995) 1.
30 J.T. Smith, *Electrophoresis*, 18 (1997) 2377.
31 C. Prata, P. Bonnafous, N. Fraysse, M. Treilhou, V. Poinsot and F. Couderc, *Electrophoresis*, 22 (2001) 4129.
32 S. Oguri, *J. Chromatogr. B*, 747 (2000) 1.
33 J.J. Pesek and M.T. Matyska, *J. Chromatogr. A*, 736 (1996) 255.
34 F. Lelièvre, C. Yan, R.N. Zare and P. Gareil, *J. Chromatogr. A*, 723 (1996) 145.
35 H. Yamamoto, J. Baumann and F. Erni, *J. Chromatogr.*, 593 (1992) 313.
36 Q.H. Ru, J. Yao, G.A. Luo, Y.X. Zhang and C. Yan, *J. Chromatogr. A*, 894 (2000) 337.
37 M. Qi, X.F. Li, C. Stathakis and N.J. Dovichi, *J. Chromatogr. A*, 853 (1999) 131.
38 C. Yan, R. Dadoo, R.N. Zare, D.J. Rakestraw and D.S. Anex, *Anal. Chem.*, 68 (1996) 2726.
39 S. Xie, R.W. Allington, J.M. Frechet and F. Svec, *Adv. Biochem. Eng. Biotechnol.*, 76 (2002) 87.
40 A. Strancar, A. Podgornik, M. Barut and R. Necina, *Adv. Biochem. Eng. Biotechnol.*, 76 (2002) 49.
41 H. Zou, X. Huang, M. Ye and Q. Luo, *J. Chromatogr. A*, 954 (2002) 5.
42 R. Shediac, S.M. Ngola, D.J. Throckmorton, D.S. Anex, T.J. Shepodd and A.K. Singh, *J. Chromatogr. A*, 925 (2001) 251.
43 D.J. Throckmorton, T.J. Shepodd and A.K. Singh, *Anal. Chem.*, 74 (2002) 784.
44 R. Burgus, T.F. Dunn, D. Desiderio, D.N. Ward, W. Vale and R. Guillemin, *Nature*, 226 (1970) 321.
45 M. Ummadi and B.C. Weimer, *J. Chromatogr. A*, 964 (2002) 243.
46 M.J. Davies and E.F. Hounsell, *Biomed. Chromatogr.*, 10 (1996) 285.
47 S.D. Gilman and A.G. Ewing, *Anal. Chem.*, 67 (1995) 58.
48 M. Castagnola, *Trends Biochem. Sci.*, 23 (1998) 283.
49 M. Castagnola, *Trends Biochem. Sci.*, 24 (1999) 129.
50 H.A. Bardelmeijer, J.C. Waterval, H. Lingeman, R. van't Hof, A. Bult and W.J. Underberg, *Electrophoresis*, 18 (1997) 2214.
51 J. Chow, J.B. Orenberg and K.D. Nugent, *J. Chromatogr.*, 386 (1987) 243.
52 M. Albin, R. Weinberger, E. Sapp and S. Moring, *Anal. Chem.*, 63 (1991) 417.
53 S. Terabe, Y. Ishihama, H. Nishi, T. Fukuyama and K. Otsuka, *J. Chromatogr.*, 545 (1991) 359.
54 M. Castagnola, D.V. Rossetti, L. Cassiano, R. Rabino, G. Nocca and B. Giardina, *J. Chromatogr.*, 638 (1993) 327.
55 E.L. Little and J.P. Foley, *J. Microcol. Sep.*, 4 (1992) 145.
56 N.J. Kim, J.H. Kim and K.-J. Lee, *Electrophoresis*, 16 (1995) 510.
57 M. Castagnola, L. Cassiano, R. De Cristofaro, R. Landolfi, D.V. Rossetti and G.B. Marini Bettolo, *J. Chromatogr.*, 440 (1988) 231.

58 N.B. Levina and I.V. Nazimov, *J. Chromatogr.*, 286 (1984) 207.
59 E. Skocir, J. Vindevogel and P. Sandra, *Chromatographia*, 39 (1994) 7.
60 N. Matsubara and S. Terabe, *J. Chromatogr. A*, 680 (1994) 311.
61 D. Drnevich and T.C. Vary, *J. Chromatogr.*, 613 (1993) 137.
62 R.A. Bank, E.J. Jansen, B. Beekman and J.M. te Koppele, *Anal. Biochem.*, 240 (1996) 167.
63 K.C. Chan, G.M. Janini, G.M. Muschik and H.J. Issaq, *J. Chromatogr.*, 653 (1993) 93.
64 H. Wan and L.G. Blomberg, *J. Chromatogr. Sci.*, 34 (1996) 540.
65 A.J. Shah, V. de Biasi, S.G. Taylor, C. Roberts, P. Hemmati, R. Munton, A. West, C. Routledge and P. Camilleri, *J. Chromatogr. B*, 735 (1999) 133.
66 T. Ueda, F. Kitamura, R. Mitchell, T. Metcalf, T. Kuwana and A. Nakamoto, *Anal. Chem.*, 63 (1991) 2979.
67 K.J. Hunter and A.H. Fairlamb, *Methods Mol. Biol.*, 79 (1998) 125.
68 W. Zhan, T. Wang and S.F. Li, *Electrophoresis*, 21 (2000) 3593.
69 K. Muramoto, K. Nokihara, A. Ueda and H. Kamiya, *Biosci. Biotechnol. Biochem.*, 58 (1994) 300.
70 Y. Qu, Y. Li, E. Vandenbussche, F. Vandesande and L. Arckens, *Brain Res. Brain Res. Protoc.*, 7 (2001) 45.
71 S.P.D. Lalljie and P. Sandra, *Chromatographia*, 40 (1995) 513.
72 E.A. Arriaga, Y. Zhang and N.J. Dovichi, *Anal. Chim. Acta*, 299 (1995) 319.
73 J. Liu, Y. Hsieh, D. Wiesler and M. Novotny, *Anal. Chem.*, 63 (1991) 408.
74 P. Camilleri, D. Dhanak, M. Druges and G.N. Okafo, *Anal. Proc.*, 31 (1994) 99.
75 S. Oguri, C. Uchida, M. Mishina, Y. Miki and K. Kakehi, *J. Chromatogr. A*, 724 (1996) 169.
76 S. Oguri, T. Fujiyoshi and Y. Miki, *Analist*, 121 (1996) 1683.
77 J. Björklund, S. Einarsson, A. Engström, A. Grzegorczyk, H.-D. Becker and B. Josefsson, *J. Chromatogr. A*, 798 (1998) 1.
78 A. Engström, P.E. Andersson, B. Josefsson and W.D. Pfeffer, *Anal. Chem.*, 67 (1995) 3018.
79 H. Liu, M.C. Sañuda-Peña, J.D. Harvey-White, S. Kalra and S.A. Cohen, *J. Chromatogr. A*, 828 (1998) 383.
80 S.A. Cohen and D.P. Michaud, *Anal. Biochem.*, 211 (1993) 279.
81 J. You, W. Lao, Q. Ou and X. Sun, *J. Chromatogr. A*, 848 (1999) 117.
82 X. Fan, J. You, J. Kang, Q. Ou and Q. Zhu, *Anal. Chim. Acta*, 367 (1998) 81.
83 J. You, H. Sun, W. Lao and Q. Ou, *Anal. Chim. Acta*, 382 (1999) 51.
84 J.-Y. Zhao, D.-Y. Chen and N.J. Dovichi, *J. Chromatogr.*, 608 (1992) 117.
85 D.J. Pietrzyk, S. Chen and B. Chanthawat, *J. Chromatogr. A*, 775 (1997) 327.
86 P. Oravec and D. Podhradsky, *J. Biochem. Biophys. Methods*, 30 (1995) 145.
87 W. Kang-Lyung and A. Young-Kyoo, *J. Chromatogr. A*, 740 (1996) 41.
88 I. Molnár-Perl, *J. Chromatogr. A*, 913 (2001) 283.
89 K.-L. Woo, Q.-C. Hwang and H.-S. Kim, *J. Chromatogr. A*, 740 (1996) 31.
90 T. Iwata, H. Mitoma and M. Yamaguchi, *Anal. Chim. Acta*, 416 (2000) 69.
91 P.S. Mukherjee, K.H. DeSilva and H.T. Karnes, *Pharm. Res.*, 12 (1995) 930.
92 C. Chassaing, J. Gonin, C.S. Wilcox and I.W. Wainer, *J. Chromatogr. B*, 735 (1999) 219.
93 L.W. Kang and S.L. Dong, *J. Chromatogr. B*, 665 (1995) 15.
94 K.R. Kim, J.H. Kim, E. Cheong and C. Jeong, *J. Chromatogr. A*, 722 (1996) 303.
95 H. Kataoka, S. Matsumura, H. Koizumi and M. Makita, *J. Chromatogr. A*, 758 (1997) 167.
96 H. Kataoka, S. Matsumura, S. Yamamoto and M. Makita, *Methods Mol. Biol.*, 159 (2000) 101.
97 G. Nouadje, N. Siméon, F. Dedieu, M. Nertz, P. Puig and F. Couderc, *J. Chromatogr. A*, 765 (1997) 337.
98 G.-D. Li, I.S. Krull and S.A. Cohen, *J. Chromatogr. A*, 724 (1996) 147.
99 M. Qi, X.-F. Li, C. Stathakis and N.J. Dovichi, *J. Chromatogr. A*, 853 (1999) 131.

100 F.D. Macchi, F.J. Shen, R.G. Keck and R.J. Harris, *Methods Mol. Biol.*, 159 (2000) 9.
101 H. Iwase, S. Ozawa, M. Ikuta and I. Ono, *J. Chromatogr. B*, 663 (1995) 15.
102 P.G. Coble and A.T. Timperman, *J. Chromatogr. A*, 829 (1998) 309.
103 K.E. Oldenburg, X. Xi and J.V. Sweedler, *Analyst*, 122 (1997) 1581.
104 J. Saurina and S. Hernández-Cassou, *J. Chromatogr. A*, 676 (1994) 311.
105 Y. Dobashi, T. Santa, K. Nakagomi and K. Imai, *Analyst*, 127 (2002) 54.
106 R.M. Latorre, S. Hernández-Cassou and J. Saurina, *J. Chromatogr. A*, 934 (2001) 105.
107 S. Oguri, K. Yokoi and Y. Motohase, *J. Chromatogr. A*, 787 (1997) 253.
108 A. Taga, M. Sugimura and S. Honda, *J. Chromatogr. A*, 802 (1998) 243.
109 P. Beyer Hietpas and A.G. Ewing, *J. Liq. Chromatogr.*, 18 (1995) 58.
110 K.R. Allen, T.J. Degg, P.A. Rushworth, M. Smith and M.J. Henderson, *Ann. Clin. Biochem.*, 36 (1999) 207.
111 C.W. Klampfl, W. Buchberger, M. Turner and J.S. Fritz, *J. Chromatogr. A*, 804 (1998) 349.
112 K. Petritis, C. Elfakir and M. Dreux, *J. Chromatogr. A*, 961 (2002) 9.
113 A.R. Ivanov, I.V. Nazimov, A.P. Lobazov and G.B. Popkovich, *J. Chromatogr. A*, 894 (2000) 253.
114 I.G. Casella, M. Gatta and T.R.I. Cataldi, *J. Chromatogr. A*, 878 (2000) 57.
115 J. Pei and X. Li, *Fresenius J. Anal. Chem.*, 367 (2000) 707.
116 G.J.M. Bruin, A.C. van Asten, X. Xu and H. Poppe, *J. Chromatogr.*, 608 (1992) 97.
117 M. Macka, P.R. Haddad, P. Gebauer and P. Bocek, *Electrophoresis*, 18 (1997) 1998.
118 B. Lu and D. Westerlund, *Electrophoresis*, 19 (1998) 1683.
119 B. Gas, P. Coufal, M. Jaros, J. Muzikar and I. Jelinek, *J. Chromatogr. A*, 905 (2001) 269.
120 Y. Ma, R. Zhang and C.L. Cooper, *J. Chromatogr.*, 608 (1992) 93.
121 Y.H. Lee and T.I. Lin, *J. Chromatogr. A*, 680 (1994) 287.
122 Y.H. Lee and T.I. Lin, *J. Chromatogr. A*, 716 (1995) 335.
123 H. Gao, J. Carlson, A.M. Stalcup and W.R. Heineman, *J. Chromatogr. Sci.*, 36 (1998) 146.
124 M.W. Crowder, A. Numan, F. Haddadian, M.A. Weitzel and N.D. Danielson, *Anal. Chim. Acta*, 384 (1999) 127.
125 Z.L. Chen, C.R. Warren and M.A. Adams, *Chromatographia*, 51 (2000) 180.
126 T. Soga and G.A. Ross, *J. Chromatogr. A*, 837 (1999) 231.
127 T. Soga and M. Imaizumi, *Electrophoresis*, 22 (2001) 3418.
128 G. Zunic, Z. Jelic-Ivanovic, M. Colic and S. Spasic, *J. Chromatogr. B*, 772 (2002) 19.
129 D. Yuan and D.J. Pietrzyk, *J. Chromatogr.*, 557 (1991) 315.
130 W.G. Kuhr and E.S. Yeung, *Anal. Chem.*, 60 (1988) 1832.
131 B.L. Hogan and E.S. Yeung, *Anal. Chem.*, 64 (1992) 2841.
132 T.M. Olefirowicz and A.G. Ewing, *J. Chromatogr.*, 499 (1990) 713.
133 K. Sato, J.Y. Jin, T. Takeuchi, T. Miwa, Y. Takekoshi, S. Kanno and S. Kawase, *Analyst*, 125 (2000) 1041.
134 O.-W. Lau and C.-S. Mok, *Anal. Chim. Acta*, 300 (1995) 183.
135 J. Kuijt, U.A. Brinkman and C. Gooijer, *Electrophoresis*, 21 (2000) 1305.
136 H. Kleinkauf and H. von Dohren, *Eur. J. Biochem.*, 28 (1990) 192.
137 G. Kreil, *Annu. Rev. Biochem.*, 66 (1997) 337.
138 D. Soyez, J.Y. Toullec, C. Ollivaux and G. Geraud, *J. Biol. Chem.*, 275 (2000) 37870.
139 K. Imai, T. Fukushima, T. Santa, H. Homma, Y. Huang, K. Sakai and M. Kato, *Enantiomer*, 2 (1997) 143.
140 H. Wang, H. Wolosker, J.F. Morris, J. Pevsner, S.H. Snyder and D.J. Selkoe, *Neuroscience*, 109 (2002) 1.
141 A. Hashimoto and T. Oka, *Prog. Neurobiol.*, 52 (1997) 325.
142 S.H. Snyder and C.D. Ferris, *Am. J. Psychiatry*, 157 (2000) 1738.

143 S.H. Snyder and P.M. Kim, *Neurochem. Res.*, 25 (2000) 553.
144 K. Hamase, T. Inoue, A. Morikawa, R. Konno and K. Zaitsu, *Anal. Biochem.*, 15 (2001) 253.
145 G.H. Fisher, *EXS*, 85 (1998) 109.
146 D. Ingrosso and A.F. Perna, *EXS*, 85 (1998) 119.
147 H. Bruckner, R. Wittner, M. Hausch and H. Godel, *Fresenius Z. Anal. Chem.*, 333 (1989) 775.
148 K.M. Williams and G.G. Smith, *Orig. Life*, 8 (1977) 91.
149 G. Lubec, M. Weninger and S.R. Anderson, *FASEB J.*, 8 (1994) 1166.
150 T. De Torres, J.E. Ortiz, M.J. Garcia, J.F. Llamas, L. Canoira, M.A. De La Morena and R. Julia, *Chirality*, 13 (2001) 517.
151 L.D. Hutt, D.P. Glavin, J.L. Bada and R.A. Mathies, *Anal. Chem.*, 71 (1999) 4000.
152 H.K. Lee, *Curr. Pharm. Des.*, 8 (2002) 795.
153 E.M. Taylor, D.A. Otero, W.A. Banks and J.S. O'Brien, *J. Pharmacol. Exp. Ther.*, 295 (2000) 190.
154 M. Friedman, *J. Agric. Food Chem.*, 47 (1999) 3457.
155 K. Imai, T. Fukushima, T. Santa, H. Homma, K. Hamase, K. Sakai and M. Kato, *Biomed. Chromatogr.*, 10 (1996) 303.
156 T. Iida, T. Santa, A. Toriba and K. Imai, *Biomed. Chromatogr.*, 15 (2001) 319.
157 H. Wan and L.G. Blomberg, *J. Chromatogr. A*, 875 (2000) 43.
158 H.J. Issaq and K.C. Chan, *Electrophoresis*, 16 (1995) 467.
159 S. Fanali, *J. Chromatogr. A*, 792 (1997) 227.
160 S. Terabe, K. Otsuka and J. Nishi, *J. Chromatogr. A*, 666 (1994) 295.
161 B. Chankvetadze and G. Blaschke, *J. Chromatogr. A*, 906 (2001) 309.
162 I.W. Wainer, *Trends Anal. Chem.*, 6 (1987) 125.
163 V.A. Davankov, *Adv. Chromatogr.*, 18 (1980) 139.
164 A. Peter, G. Torok, D.W. Armstrong, G. Toth and D. Tourwe, *J. Chromatogr. A*, 904 (2000) 1.
165 E. Tesarova, Z. Bosáková and V. Pacáková, *J. Chromatogr. A*, 838 (1999) 121.
166 K. Petritis, A. Valleix, C. Elfakir and M. Dreux, *J. Chromatogr. A*, 913 (2001) 331.
167 M.H. Hyun, S.C. Han, B.H. Lipshutz, Y.J. Shin and C.J. Welch, *J. Chromatogr. A*, 910 (2001) 359.
168 M. Schlauch, F.J. Volk, K.P. Fondekar, J. Wede and A.W. Frahm, *J. Chromatogr. A*, 897 (2000) 145.
169 Q.-H. Wan, P.N. Shaw, M.C. Davies and D.A. Barrett, *J. Chromatogr. A*, 786 (1997) 249.
170 G. Galaverna, R. Corradini, F. Dalla Valle, G. Folesani, A. Dossena and R. Marchelli, *J. Chromatogr. A*, 922 (2001) 151.
171 E. Peyrin and Y.C. Guiliaume, *Talanta*, 49 (1999) 415.
172 T. Iida, H. Matsunaga, T. Santa, T. Fukushima, H. Homma and K. Imai, *J. Chromatogr. A*, 813 (1998) 267.
173 S. Casal, M.B. Oliveira and M.A. Ferreira, *J. Chromatogr. A*, 866 (2000) 221.
174 R. Bhushan and J. Martens, *Biomed. Chromatogr.*, 15 (2001) 155.
175 S. Li and D.K. Llyod, *J. Chromatogr. A*, 666 (1994) 321.
176 M. Zhang and Z. El Rassi, *Electrophoresis*, 21 (2000) 3126.
177 M. Zhang and Z. El Rassi, *Electrophoresis*, 21 (2000) 3135.
178 A.S. Carter-Finch and N.W. Smith, *J. Chromatogr. A*, 848 (1999) 375.
179 M. Sinibaldi, M. Vinci, F. Federici and M. Flieger, *Biomed. Chromatogr.*, 11 (1997) 307.
180 M. Lämmerhofer and W. Lindner, *J. Chromatogr. A*, 829 (1998) 115.
181 M. Lämmerhofer and W. Lindner, *J. Chromatogr. A*, 839 (1999) 167.
182 Z. Chen and T. Hobo, *Electrophoresis*, 22 (2001) 3339.
183 M.G. Schmid, N. Grobuschek, O. Lecnik, G. Gubitz, A. Vegvari and S. Hjerten, *Electrophoresis*, 22 (2001) 2616.

184 M.G. Schmid, N. Grobuschek, C. Tuscher, G. Gubitz, A. Vegvari, E. Machtejavas, A. Maruska and S. Hjerten, *Electrophoresis*, 21 (2000) 3141.
185 Z. Chen and T. Hobo, *Anal. Chem.*, 73 (2001) 3348.
186 J.M. Lin, T. Nakagama, K. Uchiyama and T. Hobo, *J. Pharm. Biomed. Anal.*, 15 (1997) 1351.
187 I.D. Cruzado and G.Y. Vigh, *J. Chromatogr.*, 608 (1992) 421.
188 J.M. Lin, T. Nakagama and T. Hobo, *Chromatographia*, 42 (1996) 559.
189 T. Kaneta, H. Shiba and T. Imasaka, *J. Chromatogr. A*, 805 (1998) 295.
190 S. Birnbaum and S. Nilsson, *Anal. Chem.*, 64 (1992) 2872.
191 G.E. Barker, P. Russo and R.A. Hartwick, *Anal. Chem.*, 64 (1992) 3024.
192 Y. Kurosu, K. Murayama, N. Shindo, Y. Shisa, Y. Satou, M. Senda and N. Ishioka, *J. Chromatogr. A*, 802 (1998) 129.
193 Y.-H. Lee and T.-I. Lin, *J. Chromatogr. A*, 716 (1995) 335.
194 H.-M. Ma, Z.-H. Wang and M.-H. Su, *J. Chromatogr. A*, 955 (2002) 125.
195 J. Liu, T.T. Dabrah, J.A. Matson, S.E. Klohr, K.J. Volk, E.H. Kerns and M.S. Lee, *J. Pharm. Biomed. Anal.*, 16 (1997) 207.
196 H. Wan, A. Engström and L.G. Blomberg, *J. Chromatogr. A*, 731 (1996) 283.
197 T.J. Ward, M. Nichols, L. Sturdivant and C.C. King, *Amino Acids*, 8 (1995) 337.
198 W. Lindner, B. Böhs and V. Seidel, *J. Chromatogr. A*, 697 (1995) 549.
199 N. Egashira, O. Mutoh, Y. Kurauchi and K. Ohga, *Anal. Sci.*, 12 (1996) 503.
200 P. Dzygiel, P. Wieczorek and J.Å. Jönsson, *J. Chromatogr. A*, 793 (1998) 414.
201 R. Kuhn, F. Stöcklin and F. Erni, *Chromatographia*, 33 (1992) 32.
202 R. Kuhn, F. Erni, T. Bereuter and J. Häusler, *Anal. Chem.*, 64 (1992) 2815.
203 S. Terabe, M. Shibata and Y. Miyashita, *J. Chromatogr.*, 480 (1989) 403.
204 K. Otsuka and S. Terabe, *J. Chromatogr.*, 515 (1990) 221.
205 C. Brown, P. Cutler, C. Eckers, W. Neville, G. Okafo and P. Camilleri, *Anal. Commun.*, 33 (1996) 433.
206 K. Otsuka, H. Kawakami, W. Tamaki and S. Terabe, *J. Chromatogr. A*, 716 (1995) 319.
207 K. Otsuka, H. Karuhaka, M. Higashimori and S. Terabe, *J. Chromatogr. A*, 680 (1994) 317.
208 M.E. Swartz, J.R. Mazzeo, E.R. Grover and P.R. Brown, *Anal. Biochem.*, 231 (1995) 65.
209 W. Ding and J.S. Fritz, *J. Chromatogr. A*, 831 (1999) 311.
210 Y. Mechref and Z. El Rassi, *Electrophoresis*, 18 (1997) 912.
211 Y. Mechref and Z. El Rassi, *J. Chromatogr. A*, 724 (1996) 285.
212 A. Dobashi, M. Hamada, Y. Dobashi and J. Yamaguchi, *Anal. Chem.*, 67 (1995) 3011.
213 P. Sun, G.E. Barker, G.J. Mariano and R.A. Hartwick, *Electrophoresis*, 15 (1994) 793.
214 C. Quang and M.G. Khaledi, *J. High Resolut. Chromatogr.*, 17 (1994) 609.
215 B.A. Ingelse, F.M. Everaerts, C. Desiderio and S. Fanali, *J. Chromatogr. A*, 709 (1995) 89.
216 F. Haddadian, E.J. Billiot, S.A. Shamsi and I.M. Warner, *J. Chromatogr. A*, 858 (1999) 219.
217 H.H. Yarabe, S.A. Shamsi and I.M. Warner, *Anal. Chem.*, 71 (1999) 3992.
218 V. Piette, W. Lindner and J. Crommen, *J. Chromatogr. A*, 948 (2002) 295.
219 Z. El Rassi, *J. Chromatogr. A*, 875 (2000) 207.
220 F. Haddadian, S.A. Shamsi and I.M. Warner, *Electrophoresis*, 20 (1999) 3011.
221 P. Bednar, Z. Aturki, Z. Stransky and S. Fanali, *Electrophoresis*, 22 (2001) 2129.
222 D.W. Armstrong, K.L. Rundlett and J.R. Chen, *Chirality*, 6 (1994) 496.
223 D.W. Armstrong, M.P. Gasper and K.L. Rundlett, *J. Chromatogr. A*, 689 (1995) 285.
224 H. Wan and L.G. Blomberg, *J. Microcol. Sep.*, 8 (1996) 339.
225 J.-W. Kang, Y.-T. Yang, J.-M. You and Q.-Y. Ou, *J. Chromatogr. A*, 825 (1998) 81.
226 T.J. Ward, C. Dann, III and A. Blaylock, *J. Chromatogr. A*, 715 (1995) 337.
227 H. Wan and L.G. Blomberg, *Electrophoresis*, 18 (1997) 943.

228 K.H. Ekborg-Ott, G.A. Zientara, J.M. Schneiderheinze, K. Gahm and D.W. Armstrong, *Electrophoresis*, 20 (1999) 2438.
229 D.S. Risley, L. Trelli-Seifert and Q.J. McKenzie, *Electrophoresis*, 20 (1999) 2749.
230 A. Vegvari, M.G. Schmid, F. Kilar and G. Gübitz, *Electrophoresis*, 19 (1998) 2109.
231 M.D. Schmid, R. Rinaldi, D. Dreveny and G. Gübitz, *J. Chromatogr. A*, 846 (1999) 157.
232 E. Gassmann, J.E. Kuo and R.N. Zare, *Science*, 230 (1985) 813.
233 S. Fanali, L. Ossicini, F. Foret and P. Bocek, *J. Microcol. Sep.*, 1 (1989) 190.
234 M.G. Schmid and G. Gübitz, *Enantiomer*, 1 (1996) 23.
235 N.G. Sundin, T.M. Dowling, N. Grinberg and G. Bicker, *J. Microcol. Sep.*, 8 (1996) 323.
236 P. Gozel, E. Gassmann, H. Michelsen and R.N. Zare, *Anal. Chem.*, 59 (1987) 44.
237 Z. Yuan, L. Yang and S.-S. Zhang, *Electrophoresis*, 20 (1999) 1842.
238 R. Vespalec, H.A. Billiet, J. Frank and P. Bocek, *Electrophoresis*, 17 (1996) 1214.
239 K.D. Altria, B.J. Clark, S.D. Filbey, M.A. Kelly and D.R. Rudd, *Electrophoresis*, 16 (1995) 2143.
240 G. Thorsén, A. Engström and B. Josefsson, *J. Chromatogr. A*, 786 (1997) 347.
241 D.B. Goodnough, M.P. Lutz and P.L. Wood, *J. Chromatogr. B*, 672 (1995) 290.
242 S. Kochhar, B. Mouratou and P. Christen, *Methods Mol. Biol.*, 159 (2000) 49.
243 A. Peter, E. Olajos, R. Casimir, D. Tourwe, Q.B. Broxterman, B. Kaptein and D.W. Armstrog, *J. Chromatogr. A*, 871 (2000) 105.
244 S. Kochhar and P. Christen, *Anal. Biochem.*, 178 (1989) 17.
245 G. Szokan, G. Mezo and F. Hudecz, *J. Chromatogr.*, 444 (1988) 115.
246 A.D. Tran, T. Blanc and E.J. Leopold, *J. Chromatogr.*, 516 (1990) 241.
247 K.C. Chan, G.M. Muschik and H.J. Issaq, *Electrophoresis*, 16 (1995) 504.
248 H. Wan and L.G. Blomberg, *J. Chromatogr. A*, 758 (1997) 303.
249 H. Wan, S. Schmidt, L. Carlsson and L.G. Blomberg, *Electrophoresis*, 20 (1999) 2705.
250 M. Pugniere, H. Mattras, B. Castro and A. Previero, *J. Chromatogr. A*, 767 (1997) 69.
251 O.P. Keidernigg and W. Lindner, *J. Chromatogr. A*, 795 (1998) 251.
252 R. Bonfichi, C. Dallanoce, S. Lociuro and A. Spada, *J. Chromatogr. A*, 707 (1995) 355.
253 D. Jin, K. Nagakura, S. Murofushi, T. Miyahara and T. Toyo'oka, *J. Chromatogr. A*, 822 (1998) 215.
254 D. Jin, T. Miyahara, T. Oe and T. Toyo'oka, *Anal. Biochem.*, 269 (1999) 124.
255 T. Toyo'oka, D. Jin, N. Tomoi, T. Oe and H. Hiranuma, *Biomed. Chromatogr.*, 15 (2001) 56.
256 Y.M. Liu, M. Schneider, C.M. Sticha, T. Toyo'oka and J.V. Sweedler, *J. Chromatogr. A*, 800 (1998) 345.
257 W. Schützner, S. Fanali, A. Rizzi and E. Kenndler, *J. Chromatogr.*, 639 (1993) 375.
258 W. Schützner, S. Fanali, A. Rizzi and E. Kenndler, *J. Chromatogr. A*, 719 (1996) 411.
259 M. Peter, A. Peter and F. Fulop, *J. Chromatogr. A*, 871 (2000) 115.
260 A. Hashimoto, T. Nishikawa, T. Oka, K. Takahashi and T. Hayashi, *J. Chromatogr.*, 582 (1992) 41.
261 L. Kang and R.H. Buck, *Amino Acids*, 2 (1992) 103.
262 M. Zhao and J.L. Bada, *J. Chromatogr. A*, 690 (1995) 55.
263 A. Tivesten and S. Folestad, *J. Chromatogr. A*, 708 (1995) 323.
264 A. Peter, L. Lazar, F. Fulop and D.W. Armstrom, *J. Chromatogr. A*, 926 (2001) 229.
265 H. Nishi, T. Fukyama and M. Matuso, *J. Microcol. Sep.*, 2 (1990) 234.
266 J.G. Adamson, T. Hoang, A. Crivici and G.A. Lajoie, *Anal. Biochem.*, 202 (1992) 210.
267 H.P. Fitznar, J.M. Lobbes and G. Kattner, *J. Chromatogr. A*, 832 (1999) 123.
268 A. Scaloni, M. Simmaco and F. Bossa, *Anal. Biochem.*, 197 (1991) 305.
269 A. Engström, H. Wan, P. Andersson and B. Josefsson, *J. Chromatogr. A*, 715 (1995) 151.
270 P.A. Russo, G.A. Mitchell and R.M. Tanguay, *Pediatr. Dev. Pathol.*, 4 (2001) 212.

271 C.O. Enwonwu, B.M. Afolabi, L.A. Salako, E.O. Idigbe, H. al-Hassan and R.A. Rabiu, *QJM*, 92 (1999) 495.
272 X. Paez, P. Rada and L. Hernandez, *J. Chromatogr. B*, 739 (2000) 247.
273 Z.J. Tan and V.T. Remcho, *Electrophoresis*, 19 (1998) 2055.
274 J.L. Rudy, J.C. Rutledge and S.L. Lewis, *Clin. Chem.*, 33 (1987) 1152.
275 N.D. Atherton, *Clin. Chem.*, 35 (1989) 975.
276 Y. Qu, J.B. Miller, R.H. Slocum and E. Shapira, *Clin. Chim. Acta*, 203 (1991) 191.
277 Y. Yokoyama, H. Sato, M. Tsuchiya and H. Kakinuma, *J. Chromatogr.*, 566 (1991) 19.
278 Y. Yokoyama, M. Tsuchiya, H. Sato and H. Kakinuma, *J. Chromatogr.*, 583 (1992) 1.
279 F. Tagliaro, S. Moretto, R. Valentini, G. Gambaro, C. Antonioli, M. Moffa and L. Tato, *Electrophoresis*, 15 (1994) 94.
280 L. Dou and I.S. Krull, *J. Pharm. Biomed. Anal.*, 8 (1990) 493.
281 V.F. Ruban, *J. Chromatogr.*, 563 (1991) 134.
282 Y. Yokoyama, S. Horikoshi, T. Takahashi and H. Sato, *J. Chromatogr. A*, 886 (2000) 297.
283 T. Page, *J. Autism Dev. Disord.*, 30 (2000) 463.
284 J. Caslavska, E. Gassmann and W. Thormann, *J. Chromatogr. A*, 709 (1995) 147.
285 O. Boulat, D.G. McLaren, E.A. Arriaga and D.D. Chen, *J. Chromatogr. B*, 754 (2001) 217.
286 D.W. Vollmer, D.C. Jinks and R. Guthrie, *Anal. Biochem.*, 189 (1990) 115.
287 K. Hyland and P.T. Clayton, *Clin. Chem.*, 38 (1992) 2405.
288 D. Pawlak, A. Tankievicz, P. Mysliwiek and W. Buczo, *Nephron*, 90 (2002) 328.
289 I. Molnar-Perl, *Adv. Exp. Med. Biol.*, 467 (1999) 801.
290 N. Aymard, P. Honore and I. Carbuccia, *Prog. Neuropsychopharmacol. Biol. Psychiatry*, 18 (1994) 77.
291 K.C. Chan, G.M. Muschik and H.J. Issaq, *J. Chromatogr. A*, 718 (1995) 203.
292 J.S. Beckman, *FASEB J.*, 15 (2001) 2355.
293 Y. Kato, S. Kawakishi, T. Aoki, K. Itakura and T. Osawa, *Biochem. Biophys. Res. Commun.*, 234 (1997) 82.
294 M.K. Shigenaga, H.H. Lee, B.C. Blount, S. Christen, E.T. Shigeno, H. Yip and B.N. Ames, *Proc. Natl Acad. Sci. USA*, 94 (1997) 3211.
295 M.K. Shigenaga, *Methods Enzymol.*, 301 (1999) 27.
296 H. Jiang and M. Balazy, *Nitric Oxide*, 2 (1998) 350.
297 C. Herce-Pagliai, S. Kotecha and D.E. Shuker, *Nitric Oxide*, 2 (1998) 324.
298 S. Fu, H. Wang, M. Davies and R. Dean, *J. Biol. Chem.*, 275 (2000) 10851.
299 J.P. Crow, *Methods Enzymol.*, 301 (1999) 151.
300 J.P. Gaut, J. Byun, H.D. Tran and J.W. Heinecke, *Anal. Biochem.*, 300 (2002) 252.
301 A. Sickmann and H.E. Meyer, *Proteomics*, 1 (2001) 200.
302 P. Blume-Jensen and T. Hunter, *Methods Mol. Biol.*, 124 (2001) 49.
303 P.J. Kennelly and M. Potts, *J. Bacteriol.*, 178 (1996) 4759.
304 M. Kuba, H. Ohmori and A. Kumon, *Eur. J. Biochem.*, 208 (1992) 747.
305 M. Meins, P. Jeno, D. Muller, W.J. Richter, J.P. Rosenbusch and B. Erni, *J. Biol. Chem.*, 268 (1993) 11604.
306 Y. Shibagaki, H. Gotoh, M. Kato and K. Mizumoto, *J. Biochem. Tokio*, 118 (1995) 1303.
307 F. Hatzack and S.K. Rasmussen, *J. Chromatogr. B*, 736 (1999) 221.
308 J.X. Yan, N.H. Packer, A.A. Gooley and K.L. Williams, *J. Chromatogr. A*, 808 (1998) 23.
309 P. Wang and R.W. Giese, *Anal. Biochem.*, 230 (1995) 329.
310 P. van der Geer and T. Hunter, *Electrophoresis*, 15 (1994) 544.
311 P.A. de Witte, J.F. Cuveele, W.J. Merleved and P.M. Agostinis, *J. Pharm. Biomed. Anal.*, 14 (1996) 1063.

312 H.E. Meyer, B. Eisermann, M. Heber, E. Hoffmann-Posorske, H. Korte, C. Weigt, A. Wegner, T. Hutton, A. Donella-Deana and J.W. Perich, *FASEB J.*, 7 (1993) 776.
313 H. Kataoka, K. Nakai, Y. Katagiri and M. Makita, *Biomed. Chromatogr.*, 7 (1993) 184.
314 H. Kataoka, K. Nakai and M. Makita, *J. Chromatogr.*, 615 (1993) 136.
315 P. Fadden and T.A. Haystead, *Anal. Biochem.*, 225 (1995) 81.
316 D.A. Malencik, Z.Z. Zhao and S.R. Anderson, *Anal. Biochem.*, 184 (1990) 353.
317 P.G. Besant, L. Byrne, G. Thomas and P.V. Attwood, *Anal. Biochem.*, 258 (1998) 372.
318 I.S. Krull, Z. Deyl and H. Lingeman, *J. Chromatogr. B*, 659 (1994) 1.
319 H.A. Bardelmeijer, H. Lingeman, C. de Ruiter and W.J.M. Underberg, *J. Chromatogr. A*, 807 (1998) 3.
320 J.C.M. Waterval, H. Lingeman, A. Bult and W.J.M. Underberg, *Electrophoresis*, 21 (2000) 4029.
321 A. Aitken, *Methods Mol. Biol.*, 32 (1994) 351.
322 K. Shimada and K. Mitamura, *J. Chromatogr. B*, 659 (1994) 227.
323 M.J. Nozal, J.L. Bernal, L. Toribio, P. Marinero, O. Moral, L. Manzanas and E. Rodriguez, *J. Chromatogr. A*, 778 (1997) 347.
324 C.K. Riener, G. Kada and H.J. Gruber, *Anal. Bioanal. Chem.*, 373 (2002) 266.
325 A.R. Ivanov, I.V. Nazimov, L. Baratova, A.P. Lobazov and G.B. Popkovich, *J. Chromatogr. A*, 913 (2001) 315.
326 M. Buratti, G. Brembilla, S. Fustinoni, O. Pellegrino, S. Pulvirenti and A. Colombi, *J. Chromatogr. B*, 751 (2001) 305.
327 B.L. Yogan and E.S. Leung, *Anal. Chem.*, 64 (1992) 2841.
328 W. Jin, W. Li and Q. Xu, *Electrophoresis*, 21 (2000) 774.
329 E. Bald, R. Glowacki and J. Drzewoski, *J. Chromatogr. A*, 913 (2001) 319.
330 Y. Hiraku, M. Murata and S. Kawanishi, *Biochim. Biophys. Acta*, 1570 (2002) 47.
331 F. Xu, L. Wang, M. Gao, L. Jin and J. Jin, *Anal. Bioanal. Chem.*, 372 (2002) 791.
332 I.G. Casella, M. Contursi and E. Desimoni, *Analyst*, 127 (2002) 647.
333 L.J. Langman and D.E. Cole, *Crit. Rev. Clin. Lab. Sci.*, 36 (1999) 365.
334 J.B. Ubbink, *Semin. Thromb. Hemost.*, 26 (2000) 233.
335 I.J. Kim, S.J. Park and H.J. Kim, *J. Chromatogr. A*, 877 (2000) 217.
336 G. Chwatko and E. Bald, *J. Chromatogr. A*, 949 (2002) 141.
337 E. Causse, C. Issac, P. Malatray, C. Bayle, P. Valdiguie, L. Salvayre and F. Couderc, *J. Chromatogr. A*, 895 (2000) 173.
338 S.H. Hansen, *Diabetes Metab. Res. Rev.*, 17 (2001) 330.
339 L. Lima, F. Obregon, S. Cubillos, F. Fazzino and I. Jaimes, *Nutr. Neurosci.*, 4 (2001) 439.
340 Z.K. Shihabi, H.O. Goodman and R.P. Holmes, *Adv. Exp. Med. Biol.*, 315 (1992) 423.
341 G.P. McMahon, R. O'Kennedy and M.T. Kelly, *J. Pharm. Biomed. Anal.*, 14 (1996) 1287.
342 M.T. Kelly, H. Fabre and D. Perrett, *Electrophoresis*, 21 (2000) 699.
343 S. Futani, T. Ubuka and T. Abe, *J. Chromatogr. B*, 660 (1994) 164.
344 T. Akaike, *Free Radic. Res.*, 33 (2000) 461.
345 D. Tsikas, K. Denker and J.C. Frolich, *J. Chromatogr. A*, 915 (2001) 107.
346 I. Messana, D.V. Rossetti, F. Misiti, F. Vincenzoni, E. Tellone, B. Giardina and M. Castagnola, *Electrophoresis*, 21 (2000) 1606.
347 K.M. Brown and J.R. Arthur, *Public Health Nutr.*, 4 (2001) 593.
348 D. Behne and A. Kyriakopoulos, *Annu. Rev. Nutr.*, 21 (2001) 453.
349 M.P. Moschos, *Cell Mol. Life Sci.*, 57 (2000) 1836.
350 P.D. Whanger, *Cell Mol. Life Sci.*, 57 (2000) 1846.
351 K. El-Bayoumy, *Mut. Res.*, 475 (2001) 123.
352 A.P. Vonderheide, M. Montes-Bayon and J.A. Caruso, *Analyst*, 127 (2002) 49.

353 B. Michalke and P. Schramel, *Electrophoresis*, 19 (1998) 270.
354 C. Hammel, A. Kyriakopoulos, U. Rosick and D. Behne, *Analyst*, 122 (1997) 1359.
355 S. Cavalli and N. Cardellicchio, *J. Chromatogr. A*, 706 (1995) 429.
356 S.P. Mendez, E.B. Gonzalez and A. Sanz-Medel, *Biomed. Chromatogr.*, 14 (2000) 8.
357 C. Devos, K. Sandra and P. Sandra, *J. Pharm. Biomed. Anal.*, 27 (2002) 507.
358 S.P. Mendez, E.B. Gonzalez and A. Sanz-Medel, *Biomed. Chromatogr.*, 15 (2001) 181.
359 J.G. Dorsey, W.T. Cooper, J.F. Wheeler, H.G. Barth and J.P. Fooley, *Anal. Chem.*, 66 (1994) 500R.
360 C.T. Mant, T.W.L. Burke, J.A. Black and R.S. Hodges, *J. Chromatogr.*, 458 (1988) 193.
361 T.J. Sereda, C.T. Mant, F.D. Sönnichsen and R.S. Hodges, *J. Chromatogr. A*, 676 (1994) 139.
362 M.R. Buchmeiser, *J. Chromatogr. A*, 918 (2001) 233.
363 D. Corradini, K. Kalghatgi and C. Horváth, *J. Chromatogr. A*, 728 (1996) 225.
364 K. Kalghatgi and C. Horváth, *J. Chromatogr.*, 443 (1988) 343.
365 H. Moriyama, M. Anegayama and Y. Kato, *J. Chromatogr. A*, 729 (1996) 81.
366 J.J. Kirkland, F.A. Truszkowski, C.H. Dilks, Jr. and G.S. Engel, *J. Chromatogr. A*, 890 (2000) 3.
367 F. Fang, M.I. Aguilar and M.T.W. Hearn, *J. Chromatogr. A*, 729 (1996) 49.
368 A. Kurganov, Y. Puchkova, V. Davankov and F. Eisenbeiss, *J. Chromatogr. A*, 663 (1994) 163.
369 H. Kanazawa, K. Yamamoto, Y. Kashiwase, Y. Matsushima, N. Takai, A. Kikuchi, Y. Sakurai and T. Okano, *J. Pharm. Biomed. Anal.*, 15 (1997) 1545.
370 A.R. Oyler, B.L. Armstrong, J.Y. Cha, M.X. Zhou, Q. Yang, R.I. Robinson, R. Dunphy and D.J. Burinsky, *J. Chromatogr. A*, 724 (1996) 378.
371 H. Matsumoto, E.S. Kahn and N. Komori, *Anal. Biochem.*, 251 (1997) 116.
372 H.J. Wirth, K.O. Eriksson, P. Holt, M. Aguilar and M.T.W. Hearn, *J. Chromatogr.*, 646 (1993) 129.
373 H.J. Wirth and M.T.W. Hearn, *J. Chromatogr.*, 646 (1993) 142.
374 B. Lorenz, S. Marmé, W.E.G. Müller, K. Unger and H.C. Schröder, *Anal. Biochem.*, 216 (1994) 118.
375 J. Yu and Z. El Rassi, *J. Chromatogr.*, 631 (1993) 91.
376 L. Sun and P.W. Carr, *Anal. Chem.*, 67 (1995) 2517.
377 C. McNeff and P.W. Carr, *Anal. Chem.*, 67 (1995) 3886.
378 S. Yamaki, T. Isobe, T. Okuyama and T. Shinoda, *J. Chromatogr. A*, 729 (1996) 143.
379 V. Nèmeth-Kiss, E. Forgács and T. Cserháti, *J. Chromatogr. A*, 776 (1997) 147.
380 E. Forgács and T. Cserháti, *J. Chromatogr. A*, 948 (2002) 69.
381 J.J. Pesek and M.T. Matyska, *J. Chromatogr. A*, 952 (2002) 1.
382 S. Hu and D.D. Do, *J. Chromatogr.*, 646 (1993) 31.
383 R.J. Todd, R.D. Johnson and F.H. Arnold, *J. Chromatogr.*, 662 (1994) 13.
384 A.V. Patwardhan, G.N. Goud, R.R. Koepsel and M.M. Ataai, *J. Chromatogr. A*, 787 (1997) 91.
385 J.D. Pearson and M.C. McCroskey, *J. Chromatogr. A*, 746 (1996) 277.
386 F. Moffatt, P. Senkans and D. Ricketts, *J. Chromatogr. A*, 891 (2000) 235.
387 J. Bertini, C. Mannucci, R. Noferini, A. Perico and P. Rovero, *J. Pharm. Sci.*, 82 (1993) 179.
388 L. Miclo, E. Perrin, A. Driou, M. Mellet and G. Linden, *Int. J. Pept. Protein Res.*, 46 (1995) 186.
389 C.W. Turk, *Methods Mol. Biol.*, 64 (1997) 109.
390 C.W. Turck, *Pept. Res.*, 5 (1992) 156.
391 V.K. Boppana, C. Miller-Stein, J.F. Politowski and G.R. Rhodes, *J. Chromatogr.*, 548 (1991) 319.
392 J. You, X.J. Fan, Q. Zhu and Y. Su, *Anal. Chim. Acta*, 367 (1998) 69.

393 J.R. Lewis, J.S. Morley and R.F. Venn, *J. Chromatogr.*, 615 (1993) 37.
394 D.R. Goodlett, P.A. Abuaf, P.A. Savage, K.A. Kowalski, T.K. Mukherjee, J.W. Tolan, N. Corkum, G. Goldstein and J.B. Crowther, *J. Chromatogr. A*, 707 (1995) 233.
395 T. Nadler, C. Blackburn, J. Mark, N. Gordon, F.E. Regnier and G. Vella, *J. Chromatogr. A*, 743 (1996) 91.
396 K.R. Williams and K.L. Stone, *Methods Mol. Biol.*, 40 (1995) 157.
397 G.S. Chirica and V.T. Remcho, *J. Chromatogr. A*, 924 (2001) 223.
398 A. Strancar, A. Podgornik, M. Barut and R. Necina, *Adv. Biochem. Eng. Biotechnol.*, 76 (2002) 49.
399 L. Spoof and J. Meriluoto, *J. Chromatogr. A*, 947 (2002) 237.
400 R. Wu, H. Zou, M. Ye, Z. Lei and J. Ni, *Anal. Chem.*, 73 (2001) 4918.
401 R.E. Moore, L. Licklider, D. Shumann and T.D. Lee, *Anal. Chem.*, 70 (1998) 4879.
402 X. Huang, S. Zhang, G.A. Schultz and J. Henion, *Anal. Chem.*, 74 (2002) 2336.
403 A. Premstaller, H. Oberacher, W. Walcher, A.M. Timperio, L. Zolla, J.P. Chervet, N. Cavusoglu, A. van Dorsselaer and C.G. Huber, *Anal. Chem.*, 73 (2001) 2390.
404 M. Kempe and K. Mosbach, *J. Chromatogr. A*, 691 (1995) 317.
405 A. Rachkov and N. Minoura, *J. Chromatogr. A*, 889 (2000) 111.
406 A. Rachkov and N. Minoura, *Biochim. Biophys. Acta*, 1544 (2001) 255.
407 T. Stroink, E. Paarlberg, J.C. Waterval, A. Bult and W.J. Underberg, *Electrophoresis*, 22 (2001) 2375.
408 A. Dermaux and P. Sandra, *Electrophoresis*, 20 (1999) 3027.
409 M. Pursch and L.C. Sander, *J. Chromatogr. A*, 887 (2000) 313.
410 C.Y. Liu, *Electrophoresis*, 22 (2001) 612.
411 I.S. Krull, A. Sebag and R. Stevenson, *J. Chromatogr. A*, 887 (2000) 137.
412 M.T. Hearn, *Biologicals*, 29 (2001) 159.
413 K. Walhagen, K.K. Unger and M.T. Hearn, *J. Chromatogr. A*, 887 (2000) 165.
414 J.J. Pesek and M.T. Matyska, *J. Chromatogr. A*, 736 (1996) 255.
415 J.J. Pesek, M.T. Matyska and L. Mauskar, *J. Chromatogr. A*, 763 (1997) 307.
416 J.J. Pesek, M.T. Matyska, S. Swdberg and S. Udivar, *Electrophoresis*, 20 (1999) 2343.
417 X. Huang, J. Zhang and C. Horvath, *J. Chromatogr. A*, 858 (1999) 91.
418 M.T. Matyska, J.J. Pesek, R.I. Boysen and M.T. Hearn, *Anal. Chem.*, 73 (2001) 5116.
419 M.T. Matyska, J.J. Pesek, R.I. Boysen and M.T. Hearn, *Electrophoresis*, 22 (2001) 2620.
420 J. Charvátová, V. Král and Z. Deyl, *J. Chromatogr. B*, 770 (2002) 155.
421 M. Ye, H. Zou, Z. Liu and J. Ni, *J. Chromatogr. A*, 869 (2000) 385.
422 S. Zhang, X. Huang, J. Zhang and C. Horvath, *J. Chromatogr. A*, 887 (2000) 465.
423 S. Zhang, J. Zhang and C. Horvath, *J. Chromatogr. A*, 965 (2002) 83.
424 R. Wu, H. Zou, H. Fu, W. Jin and M. Ye, *Electrophoresis*, 23 (2002) 1239.
425 S. Zhang, J. Zhang and C. Horvath, *J. Chromatogr. A*, 914 (2001) 189.
426 K. Walhagen, K.K. Unger and M.T. Hearn, *J. Chromatogr. A*, 893 (2000) 401.
427 B. Behnke and J.W. Metzger, *Electrophoresis*, 20 (1999) 80.
428 G. Choudhary, A. Apffel, H. Yin and W. Hancock, *J. Chromatogr. A*, 887 (2000) 85.
429 A. von Brocke, G. Nicholson and E. Bayer, *Electrophoresis*, 22 (2001) 1251.
430 M. Gucek, M. Gaspari, K. Walhagen, R.J. Vreeken, E.R. Verheij and J. van der Greef, *Rapid Commun. Mass Spectrom.*, 14 (2000) 1448.
431 R.L. Rill, B.R. Locke, Y. Liu and D.H. Van Winkle, *Proc. Natl Acad. Sci. USA*, 95 (1998) 1534.
432 R.L. Rill, Y. Liu, D.H. Van Winkle and B.R. Locke, *J. Chromatogr. A*, 817 (1998) 287.
433 I. Miksik and Z. Deyl, *J. Chromatogr. B*, 739 (2000) 109.
434 I. Miksik, Z. Deyl and V. Kasicka, *J. Chromatogr. B*, 741 (2000) 37.

435 I. Miksik, A. Eckhardt, E. Forgacs, T. Cserhati and Z. Deyl, *Electrophoresis*, 23 (2002) 1882.
436 M.G. Khaledi, *J. Chromatogr. A*, 780 (1997) 3.
437 H.J. Issaq, *Electrophoresis*, 21 (2000) 1921.
438 J.P. Quirino and S. Terabe, *J. Chromatogr. A*, 856 (1999) 465.
439 K. Otsuka and S. Terabe, *Mol. Biotechnol.*, 9 (1998) 253.
440 P.G. Muijselaar, K. Otsuka and S. Terabe, *J. Chromatogr. A*, 780 (1997) 41.
441 N. Matsubara and S. Terabe, in P.G. Righetti (Ed.), *Capillary Electrophoresis in Analytical Biotechnology*, CRC Press, New York, 1996, p. 155.
442 V. Kasicka, *Electrophoresis*, 22 (2001) 4139.
443 M. Thorsteinsdóttir, C. Ringbom, D. Westerlund, G. Andersson and P. Kaufmann, *J. Chromatogr. A*, 831 (1999) 293.
444 T. Yashima, A. Tsuchiya, O. Morita and S. Terabe, *Anal. Chem.*, 64 (1992) 2981.
445 N. Matsubara, K. Koezuka and S. Terabe, *Electrophoresis*, 16 (1995) 580.
446 A. Furtos-Matei, R. Day, S.A. St-Pierre, L.G. St-Pierre and K.C. Waldron, *Electrophoresis*, 21 (2000) 715.
447 N. Sutcliffe and P.H. Corran, *J. Chromatogr.*, 636 (1993) 95.
448 P.H. Corran and N. Sutcliffe, *J. Chromatogr.*, 636 (1993) 87.
449 T. Janaky, E. Szabo, L. Balaspiri, B. Adi and B. Penke, *J. Chromatogr. B*, 676 (1996) 7.
450 M. Castagnola, D. Di Pierro, R. Scatena, B. Tavazzi, G. Nocca, D.V. Rossetti and B. Giardina, *Biomed. Chromatogr.*, 7 (1993) 220.
451 H. Havel, K. Pritts and T. Wielgos, *J. Chromatogr. A*, 853 (1999) 215.
452 Z. Glatz and H. Maslanova, *J. Chromatogr. A*, 895 (2000) 179.
453 M. Castagnola, I. Messana, L. Cassiano, R. Rabino, D.V. Rossetti and B. Giardina, *Electrophoresis*, 16 (1995) 1492.
454 S. Viglio, G. Zanaboni, M. Luisetti, G. Cetta, M. Guglielminetti and P. Iadarola, *Electrophoresis*, 19 (1998) 2083.
455 D.C. James, R.B. Freedman, M. Hoare and N. Jenkins, *Anal. Biochem.*, 222 (1994) 315.
456 R. Grimm, G. Zanaboni, S. Viglio, K. Dyne, M. Valli, G. Cetta and P. Iadarola, *J. Chromatogr. A*, 768 (1997) 57.
457 D.M. Pinto, E.A. Arriaga, S. Sia, Z. Li and N.J. Dovichi, *Electrophoresis*, 16 (1995) 534.
458 V. Kasicka, Z. Prusik, P. Sazelova, M. Chiari, I. Miksik and Z. Deyl, *J. Chromatogr. B*, 741 (2000) 43.
459 N.M. Djordjevic, F. Fitzpatrick and F. Houdiere, *Electrophoresis*, 22 (2001) 2398.
460 I. Miksik and Z. Deyl, *J. Chromatogr. A*, 852 (1999) 325.
461 M.A. Rodriguez-Delgado, F.J. Garcia-Montelongo and A. Cifuentes, *Anal. Chem.*, 74 (2002) 257.
462 C.S. Effenhauser, G.J. Bruin and A. Paulus, *Electrophoresis*, 18 (1997) 2203.
463 F.E. Regnier, B. He, S. Lin and J. Busse, *Trends Biotechnol.*, 17 (1999) 101.
464 V. Dolnik, S. Liu and S. Jovanovich, *Electrophoresis*, 21 (2000) 41.
465 N.A. Lacher, K.E. Garrison, R.S. Martin and S.M. Lunte, *Electrophoresis*, 22 (2001) 2526.
466 J. Ferrance and J.P. Landers, *Luminescence*, 16 (2001) 79.
467 B.E. Slentz, N.A. Penner and F.E. Regnier, *J. Chromatogr. A*, 948 (2002) 225.
468 D.J. Throckmorton, T.J. Shepodd and A.K. Singh, *Anal. Chem.*, 74 (2002) 784.
469 N.J. Munro, Z. Huang, D.N. Finegold and J.P. Landers, *Anal. Chem.*, 72 (2000) 2765.

This page is intentionally left blank

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 15

Electrophoresis of proteins and peptides

PIER GIORGIO RIGHETTI, MAHMOUD HAMDAN, FRANCESCA ANTONUCCI, BARBARA VERZOLA and ALESSANDRA BOSSI

CONTENTS

15.1 INTRODUCTION

This chapter cannot possibly deal with all known forms of electrophoresis of proteins and peptides, since a number of them are only of historical value and are scarcely used in modern laboratories. This applies, *e.g.*, to cellulose acetate electrophoresis [1], kept still barely alive in clinical chemistry analyses; to agar/agarose electrophoresis [2],

today certainly much in vogue but essentially only in DNA analysis; to starch gel electrophoresis [3]; to the Ornstein–Davis [4,5] disc electrophoresis, mostly abandoned (except for the concept of buffer discontinuities); and to isotachophoresis [6], a declining star after its brief apparition in the Seventies. For a survey of the vast array of electrokinetic separation methods, the reader is referred to the relevant chapter in the 5th edition of this book [7].

Having amply stated what one will not possibly find in this survey, we ought to say what readers might be able to find in these pages. They will, we hope, locate a survey of the surviving techniques, still widely adopted in modern separation science. Those will be divided into two categories: gel-based and free-solution methodologies. In the first group, we will deal with: (a) conventional isoelectric focusing (IEF) in soluble carrier ampholyte buffers; (b) IEF in immobilized pH gradients (IPG); (c) sodium dodecylsulfate gel slab electrophoresis (SDS-PAGE) and (d) two-dimensional (2-D) maps, as engendered by an orthogonal combination of IEF and IPG (charge/size separation). In the second group we will discuss just capillary zone electrophoresis (CZE), the only useful approach today for performing fast separations of proteins/peptides in free solution (although one could easily perform size separations in capillaries filled with appropriate solutions of sieving polymers). The last part (free-solution CZE) will end with a discussion on models for predicting the migration of peptides and will offer a glimpse of recent studies aimed at assessing folding/unfolding/misfolding processes which proteins undergo during denaturation/renaturation cycles.

15.2 GEL ELECTROPHORESIS

15.2.1 Isoelectric focusing in soluble amphoteric buffers

The 1960s saw three major events in electrokinetic methodologies, which concurred in shaping modern technologies:

(a) The description of disc electrophoresis by Ornstein [4] and Davis [5], a method which dramatically increases resolution by introducing in the matrix and buffers a series of discontinuities that sharpen the bands and form incredibly thin starting zones;
(b) The discovery of sodium dodecyl sulfate electrophoresis [8], which separates detergent-saturated proteins in a polyacrylamide gel, mostly according to their mass, rather than in accordance with combined mass and charge effects, as is expected in conventional electrophoresis;
(c) The invention of isoelectric focusing [9–11], which separates proteins essentially on the basis of their net charge (at a given value, the isoelectric point).

A major breakthrough indeed was the discovery of conventional IEF in carrier ampholytes (CA, soluble, amphoteric buffers) reported by Svensson-Rilbe in a series of now classical articles [9–11]. At just about the same time, Meselson *et al.* [12] described isopycnic centrifugation (IPC), a related, high-resolution technique–in fact, another member of a family Kolin [13] called isoperichoric focusing. Unlike conventional chromatographic and electrophoretic techniques available up to then, which could not

prevent peak decay during the transport process, IEF and IPC had built-in mechanisms opposing entropic forces that try to dissipate the zone. As the analyte reaches an environment (the *perichoron*, in Greek) in which its physico-chemical parameters are equal (*iso*) with those of the surroundings, it is focused, or condensed, in an ultra-thin zone, kept stable and sharp by two opposing force fields: diffusion (tending to dissipate the zone) and external fields [voltage gradients (in IEF) or centrifugal fields (in IPC)] forcing the "escaping" analyte back into its "focusing" zone [14].

IEF is an electrophoretic technique for fractionating amphoteric compounds according to their pIs along a continuous pH gradient [15,16]. Contrary to zone electrophoresis, where the constant (buffered) pH of the separation medium establishes a constant charge density at the surface of the molecule and causes it to migrate with constant mobility (in the absence of molecular sieving), the surface charge of an amphoteric compound in IEF keeps changing, and decreasing, according to its titration curve, as it moves along a pH gradient until it reaches its equilibrium position, *i.e.* the region where the pH matches its pI. There, its mobility equals zero and the molecule comes to a stop.

The gradient is created, and maintained, by the passage of an electric current through a solution of amphoteric compounds which have closely spaced pIs, encompassing a given pH range. The electrophoretic transport causes these carrier ampholytes (CA) to stack according to their pIs, so that a pH gradient, increasing from anode to cathode, is established. At the beginning of the run, the medium has a uniform pH, which equals the average pI of the CAs. Thus, most ampholytes have a net charge and a net mobility. The most acidic CA moves toward the anode, where it concentrates in a zone where the pH equals its pI, while the more basic CAs are driven toward the cathode. A less acidic ampholyte migrates adjacent and just cathodal to the previous one and so on, until all the components of the system reach a steady state. After this stacking process is completed, some CAs still enter zones of higher, or lower, pH by diffusion where they are no longer in isoelectric equilibrium. But as soon as they enter these zones, the CAs become charged, and the applied voltage forces them back to their equilibrium position. This pendulum movement, diffusion *vs.* electrophoresis, is the primary cause of the residual current observed under isoelectric steady-state conditions. Finally, as time progresses, the sample protein molecules also reach their isoelectric point.

In chemical terms, CAs are oligo-amino, oligocarboxylic acids, available from different suppliers under different trade names. There are three basic synthetic approaches: Vesterberg's approach, which involves reacting different oligo-amines (tetra-, penta- and hexa-amines) with acrylic acid [17]; the Söderberg *et al.* synthetic process [18], which involves the co-polymerization of amines, amino acids and dipeptides with epichlorohydrin, and the Pogacar–Grubhofer approach, which utilizes ethyleneimine and propylenediamine for subsequent reaction with propanesultone, sodium vinyl sulfonate and sodium chloromethyl phosphonate [19,20]. Accordingly, there are 3 types of products on the market: the Amersham-Pharmacia Biotech Ampholines (formerly from LKB-Produkter AB) and Bio-Rad Biolytes, which belong to the first class; the Amersham-Pharmacia Biotech Pharmalytes, which should be listed in the second class; and the Novex-Servalyt Ampholytes and Genomic Solutions (pH 3–10) Ampholytes, which should be classified into the third category. The wide-range synthetic mixtures (pH 3–10) contain hundreds, possibly thousands, of different amphoteric chemicals having pIs evenly distributed

along the pH scale. Since they are obtained by different synthetic approaches, CAs from different manufacturers are bound to have somewhat different pIs. Thus, if higher resolution is needed, particularly for two-dimensional maps of complex samples, we suggest using blends of the different commercially available CAs. A useful blend is 50% Pharmalyte, 30% Ampholine and 20% Biolyte (by volume). CAs from any source should have an average molecular mass of about 750 (size interval 600–900, the higher molecular mass referring to the more acidic CA species) [21]. Thus, CAs should be readily separable (unless they are hydrophobically complexed to proteins) from macromolecules by size-exclusion chromatography. The hallmark of a "carrier ampholyte" is the absolute value of $pI - pK_{prox}$ (or $\frac{1}{2}\Delta pK$): the smaller this value, the higher are the conductivity and buffering capacity (at $pH = pI$) of the amphotere. A $\Delta pK = \log 4$ (*i.e.* $pI - pK = 0.3$) would provide an incredibly great molar buffering power (β) at pI 2.0 (unfortunately, such compounds do not exist in nature). A $\Delta pK = \log 16$ (*i.e.* $pI - pK = 0.6$) offers a β value of 1.35 at $pH = pI$ [16].

It would be impossible to cover here adequately the vast literature on IEF. Suffice it to quote a book that gives a broad overview of the field and thoroughly covers the methodology and a number of applications [22]. We will, nevertheless, end this section with an illuminating experiment, displayed in Fig. 15.1. It shows the analysis of active fragments of the human growth hormone (hGH), synthesized by the solid-phase method of Merrifield [23]. Early on, it was thought that IEF of peptides would not be feasible, first of all because they have a higher diffusion coefficient than proteins, and secondly because they would not be precipitated and fixed in the gel matrix by the common protein stains, like alcoholic solutions of Coomassie Blue. Fig. 15.1 dispelled both doubts and proved that IEF of peptides was not only feasible, but would also produce very sharp bands [24]. The problem of fixation was solved by adopting a leuco-stain, a micellar suspension of Coomassie Brilliant Blue G-250 (G stands for its greenish hue, which cannot be appreciated in monomeric solutions, but is clearly visible in the micellar state) in TCA: as the focused gel is bathed directly in this solution, the peptides adsorb the dye from the micelle and are fixed by the dye molecules, which probably act as cross-links over the different peptide chains, thus forming a macromolecular aggregate, which is trapped in the gel matrix fibers. This experiment was very important for peptide chemists, since it helped redirecting their synthetic strategies. As is evident from Fig. 15.1, when medium-length peptides were produced *via* the Merrifield approach, the amount of failed and truncated sequences was in large excess over the desired product, to the point that the latter could not be recognized any longer. Although this had been theoretically predicted, it had not been experimentally verified up to that time, due to lack of high-resolution techniques. As the news spread, *in vitro* synthesis of peptides took an important turn: it was done only in short sequences (5–6 amino acids at most), which were ultimately joined by splicing.

15.2.2 Isoelectric focusing in immobilized pH gradients

Some major problems associated with CA-IEF were already recognized in the early Seventies, but no remedies could be found. Some problems are quite severe; for example, the low ionic strength of the IEF milieu often induces near-isoelectric precipitation and smearing of proteins, even in analytical runs at low protein loads. The problem of uneven

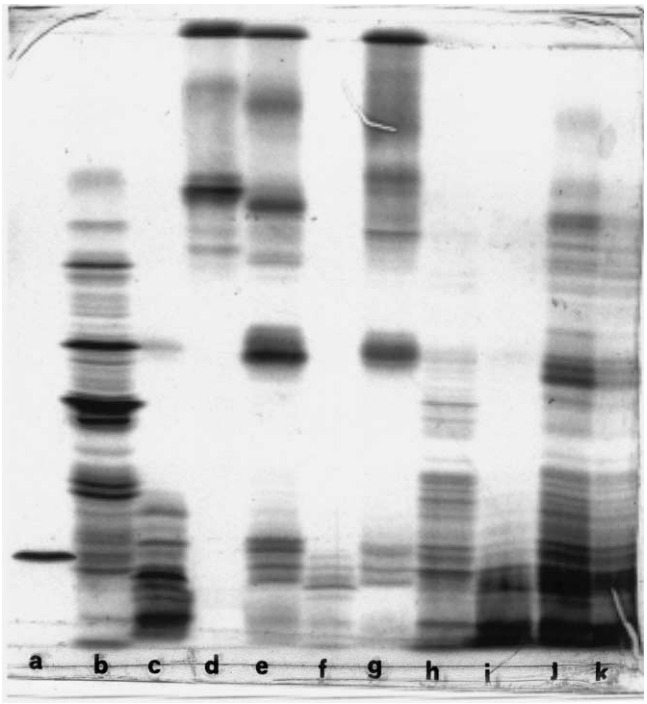


Fig. 15.1. Isoelectric focusing of peptides in the range of 8–54 amino acids. The gel slab was 0.7 mm thick and contained 7% acrylamide, 2% Ampholine (pH 3.5–10) and 8 *M* urea. Ten to fifteen μL of sample (10 mg/mL) were applied in filter paper strips at the anode after 1-h prefocusing. Total running time, 4 h at 10 W (1000 V at equilibrium). The gel was then dipped in a colloidal dispersion of Coomassie Brilliant Blue G-250 in 12% TCA. The samples were the following synthetic fragments of the human growth hormone (hGH): a = hGH 31–44; b = hGH 15–36; c = hGH 111–134; d = hGH 1–24; e = hGH 166–191; f = hGH 25–51; g = hGH 157–191; h = hGH 1–36; i = hGH 96–134; j = hGH 115–156; and k = hGH 103–156. Shorter fragments (octa-, nona- and decapeptides) were neither fixed in the gel nor stained. (Reprinted from Ref. 24 with permission.)

conductivity (and buffering capacity as well!) is magnified in poor ampholyte mixtures, like the Poly Sep 47 (a mixture of 47 amphoteric and non-amphoteric buffers, claimed to be superior to CAs) [25]. Due to their poor composition, huge conductivity gaps form along the migration path, against which proteins of different pIs are stacked, with disastrous results [26]. The cathodic drift is also a major unsolved problem of CA-IEF, resulting in extensive loss of proteins at the gel cathodic extremity upon prolonged runs. For all these reasons, Bjellqvist *et al.* [27] in 1982 launched the technique of immobilized pH gradients (IPGs). IPGs represent perhaps the ultimate development in all focusing techniques, a revolution in the field, in fact. Due to the possibility of engineering the pH gradient at whim, from the narrowest (which, for practical purposes, has been set at 0.1 pH units over a 10-cm distance) to the widest possible gradient (pH 2.5–12), IPGs permit the highest possible resolving power, on the one hand, and the widest possible collection of

spots (in 2-D maps), on the other hand. The chemistry is precise and amply developed; so are all algorithms for implementing any possible width and shape of the pH gradient. Due to their unique performance, IPGs now represent the best first dimension for 2-D maps and are increasingly adopted for this purpose.

IPGs are based on the principle that the pH gradient, which exists prior to the IEF run itself, is co-polymerized, and thus insolubilized, within the fibers of a polyacrylamide matrix. This is achieved by using, as buffers, a set of six non-amphoteric weak acids and bases, having the general chemical composition, $CH_2{=}CH{-}CO{-}NH{-}R$, where R denotes either two different weak carboxyl groups, with pKs of 3.6 and 4.6, or four tertiary amino groups, with pKs of 6.2, 7.0, 8.5, and 9.3 (available under the trade name Immobiline from Pharmacia-Upjohn). Their synthesis has been described by Chiari *et al.* [28,29]. A more extensive set, comprising 10 chemicals (a pK 3.1 acidic buffer, a pK 10.3 basic buffer and two strong titrants, a pK 1 acid and a pK $>$ 12 quaternary base) is available as "pI select" from Fluka (see Tables 15.1 and 15.2 for their formulas) [30]. All of the above chemicals have been reported by Righetti *et al.* [31]. The synthesis of the pK 3.1 buffer was utilized for the separation of isoforms of a very acidic protein (pepsin). The pK 10.3 species was first adopted by Sinha and Righetti [32] for creating alkaline gradients for the separation of elastase isoforms. Over the years, Righetti *et al.* have reported the synthesis of a number of other buffering ions, produced with the aim of closing some gaps between the available Immobilines, especially in the pH 7.0 to 8.5 interval. These are: a pK 6.6, 2-thiomorpholinoethylacrylamide; and a pK 7.4, 3-thiomorpholinopropylacrylamide [33]; a pK 6.85, 1-acryloyl-4-methylpiperazine [34]; an alternative pK 7.0, 2-(4-imidazolyl)ethylamine-2-acrylamide [35]; and a pK 8.05, *N*,*N*-*bis*(2-hydroxyethyl) *N*′-acryloyl-1,3-diaminopropane [36]. Additional species have recently been described by Bellini and Manchester [37].

Since it would be impossible to cover properly the vast literature and applications in the field of IPGs, we are forced to refer the readers to a comprehensive book on this topic [38]. Nevertheless, some examples will be given here, just to show the versatility and unique resolving power of IPGs. One of them regards the engineering of the pH gradients. Although originally most IPG formulations for extended pH intervals had been given only in terms of rigorously linear pH gradients, this may not be the optimal solution in some cases. The pH slope might need to be altered in pH regions that are overcrowded with proteins. This is particularly important in the general case involving the separation of proteins in a complex mixture, such as cell lysates, and is thus imperative when performing two-dimensional (2-D) maps. Given the relative abundance of various species (70% of all proteins having acidic pI values) [39], it is clear that an optimally resolving pH gradient should have a gentler slope in the acidic portion and a steeper profile in the alkaline region. Such a course has been calculated by assigning to each 0.5 pH unit interval in the pH 3.5–10 region a slope inversely proportional to the relative abundance of proteins in that interval. This has generated the ideal curve (dotted line in Fig. 15.2) [40]. Although non-linear gradients of just about any shape are much in vogue in chromatography, it should be emphasized that the gradients displayed here are somewhat unique in that they are created and optimized simply with the aid of a two-vessel gradient mixer, by manipulating the composition (in Immobilines) of the two limiting solutions. In contrast, in chromatography this is achieved by drawing solutions of different composition

TABLE 15.1

ACIDIC ACRYLAMIDO BUFFERS

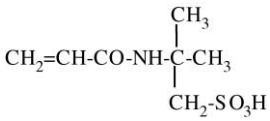
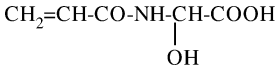
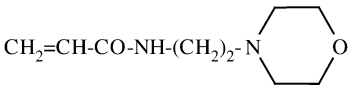
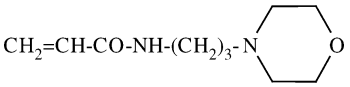
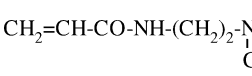
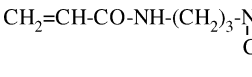
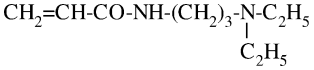
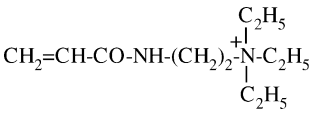
pK	Formula	Name	M_r
1.2		2-acrylamido-2-methylpropane-sulfonic acid	207
3.1		2-acrylamido-glycolic acid	145
3.6	CH_2=CH–CO–NH–CH_2–COOH	*N*-acryloylglycine	129
4.6	CH_2=CH–CO–NH–$(CH_2)_3$–COOH	4-acrylamido-butyric acid	157

TABLE 15.2

BASIC ACRYLAMIDO BUFFERS

pK	Formula	Name	M_r
6.2		2-morpholino-ethylacrylamide	184
7.0		3-morpholino-propylacrylamide	199
8.5		*N*,*N*-dimethyl-aminoethyl-acrylamide	142
9.3		*N*,*N*-dimethyl-aminopropyl-acrylamide	156
10.3		*N*,*N*-diethyl-aminopropyl-acrylamide	184
>12		*N*,*N*,*N*-triethyl-aminoethyl-acrylamide	198

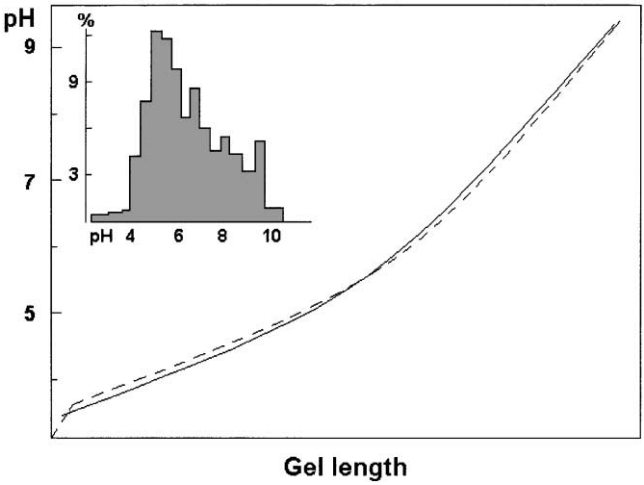


Fig. 15.2. Non-linear pH 4–10 gradient: "ideal" (solid line) and actual (– – –) courses. The shape of the "ideal" profile was computed from data on statistical distribution of protein pIs. The relevant histogram is redrawn in the figure inset. (Reprinted from Ref. 40 with permission.)

from several vessels and/or altering their pumping speed. The second example shows the exquisite resolving power of IPGs. Resolution, in IEF and IPGs, is expressed in ΔpI, defined as the difference in surface charge, in pI units, between two barely resolved protein species. One can push the ΔpI, in IPGs, to the limit of 0.001 pH unit, the corresponding limit in CA-IEF being only 0.01 pH unit. Cossu and Righetti [41] have investigated the possibility of resolving neutral mutants, which carry a point mutation, involving amino acids with non-ionizable side chains, and are, in fact, described as "electrophoretically silent", because they cannot be distinguished by conventional electrophoretic techniques. Fetal hemoglobin (Hb F), which is an envelope of two components, called Aγ and Gγ, carrying a Gly → Ala mutation at γ-136, could be resolved into two main bands by using a very shallow pH gradient, spanning 0.1 pH unit (over a standard 10-cm migration length) [41] (Fig. 15.3). The resolution was close to the practical limit of $\Delta pI = 0.001$.

15.2.3 Sodium dodecylsulfate, polyacrylamide gel electrophoresis

SDS-PAGE is perhaps the most popular and direct method for assessing, in a fast and reproducible manner, the molecular mass (*Mr*) of denatured polypeptide chains and the purity of a protein preparation [8]. Several reviews on SDS-PAGE are available [42–46]. In small or large gel slab formats, it is the standard second dimension of 2-D maps. Typically, in disc electrophoresis, proteins migrate according to both surface charge and mass, so that discriminating the two contributions is not an easy task (although this can be done by a number of mathematical treatments, such as the Ferguson plots) [47]. A possible approach to *Mr* measurements of proteins would be to cancel out differences in molecular charge by chemical means, so that migration would then occur solely according to size. SDS, an amphipatic molecule, is known to form complexes with both, nonpolar side-chains

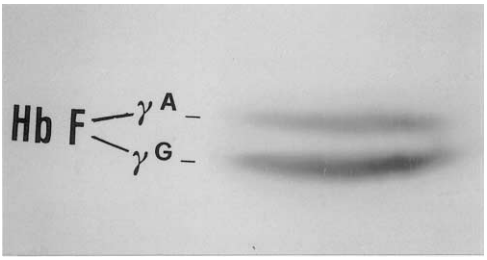


Fig. 15.3. Focusing of umbilical cord lysates from an individual, heterozygous for fetal hemoglobin (HbF) Sardinia (only the HbF bands are shown, since the two other major components of cord blood, *i.e.* HbA and HbF_{ac}, are lost in very shallow pH gradients). In an IPG gel, spanning 0.1 pH unit, over a standard 10-cm gel length, two bands are resolved, identified as the Gly *vs.* Ala gene products. The resolved Aγ/Gγ bands are in a 20:80 ratio, as theoretically predicted from gene expression. Their identity was established by eluting the two zones and fingerprinting the γ-chains. (Reprinted from Ref. 41 with permission.)

and charged groups of amino acid residues in polypeptides of all possible sizes and shapes, without rupturing polypeptide bonds. Surprisingly large amounts of SDS can be bound to proteins, estimated by a number of authors at *ca.* 1.4 g SDS per g protein [48–51]. This means that the number of SDS molecules is of the order of half the number of amino acid residues in the polypeptide chain. This amount of highly charged surfactant molecules is sufficient to overwhelm effectively the intrinsic charges on the polymer chain, so that the net charge per unit mass becomes approximately constant. Electrophoretic migration is then proportional to the effective molecular radius, *i.e.* to the molecular mass of the polypeptide chain [8]. Although it is an oversimplification to which a number of exceptions are to be found, this relationship does, in fact, hold true for a very large number of proteins [52,53], and the method has become one of the most widely used means of measuring of protein molecular mass. The unique properties of SDS are due to its long, flexible alkyl tail, which is capable of hydrophobic interactions with all combinations of amino acids, leading to massive unfolding of proteins. The action of SDS is also due to its ionic head, which can break ionic interactions between proteins and drive an important electrostatic repulsion between SDS/protein complexes. This prevents reassociation of such complexes, even at the very high concentrations encountered in gel electrophoresis. Another important property of SDS is due to the fact that the ionic head is a strong electrolyte, so that it is fully ionized in the pH 2–12 interval, where most of the biochemical separations take place. According to Reynolds and Tanford [50], a concentration of SDS above 0.5 m*M* is sufficient for binding 1.4 g SDS per g of protein by a primarily hydrophobic mechanism, provided the disulfide bridges are reduced and the polymer chain is thus in an extended conformation. Under the influence of SDS, proteins assume the shape of rod-like particles, the length of which varies uniquely with the molecular mass of the protein moiety, occupying 0.074 nm per amino acid residue. Certain proteins, such as papain, pepsin, and glucose oxidase [54] and two different classes of proteins, the glycoproteins [55] and the histones [56], show an "anomalous" behavior towards SDS. Either they bind a relatively low amount of detergent, or SDS cannot compensate for their intrinsic charges, as in the

case of histones. But these examples are not sufficient to invalidate the *Mr* estimation by SDS [57,58]. As a matter of fact, SDS-PAGE has become by far the most popular method for *Mr* assessments of denatured polypeptide chains.

Among the various models proposed for protein/SDS complexes, the one suggested by Lundahl *et al.* [59] has become the most convincing. It pictures the polypeptide chain as helically coiled around an SDS micelle, attached by hydrogen bonds between sulfate group oxygens and peptide bond nitrogens. Over the years, Lundahl's group has refined this picture, which has now become the "protein-decorated micelle model" [60–64]. Based on small-angle neutron-scattering data, this model proposes that adjacent, protein-decorated, spherical micelles are formed, rather than cylindrical structures, as previously suggested by the same group [59]. They have studied the formation of SDS/protein complexes at SDS levels in the proximity of its critical micellar concentration (1.8 m*M* SDS in 100 m*M* buffer) and propose the following series of events in complex formation. First, the dodecyl chains of the detergent penetrate the surface of the protein and come into contact with the hydrophobic interior of the protein or of its domains. As a consequence, polypeptide segments from the interior of the protein become displaced toward the surface of the complex, since they are less hydrophobic than the dodecyl chain. Many SDS molecules become locked in the inserted position by ion-pair formation and hydrogen bonding. Additional SDS molecules then become included in the complex until a spherical SDS micelle is completed, around which the polypeptide is wound. Any length of polypeptide that, for steric reasons, cannot be accommodated in direct contact with the micelles forms the core for growth of another protein-covered micelle. This process is repeated until the whole polypeptide is coiled around adjacent SDS micelles, which are linked with short polypeptide segments. For a protein of average size (50,000 Da) it is believed that this complex could be composed of three protein-decorated SDS micelles, not necessarily of equal size. For instance, in the case of the enzyme *N*-5′-phosphoribosylanthranilate isomerase/indole-3-glycerol-phosphate synthase (*Mr* = 49,484 Da) the SDS/protein complex contains the dodecyl hydrocarbon moieties in three globular cores, the central one being the largest. Each core is surrounded by a hydrophilic shell, formed by the hydrophilic and amphiphilic stretches of the polypeptide chain, and by the sulfate groups of the detergent, whereas, presumably, most or all nonpolar side-groups of the polypeptide chain penetrate partly or completely into the hydrophobic micelle cores. The model has received support from recent data by Samsò *et al.* [65] and by Westerhuis *et al.* [66].

As in detergent-free systems, multiphasic buffers in SDS-PAGE give much sharper bands and better resolution, and are therefore almost universally used nowadays. The theory of zone stacking and of the other processes involved in SDS-containing gels has been comprehensively discussed by Wyckoff *et al.* [67]. It is important to recall here that the nature of stacking is somewhat altered in the presence of SDS, since SDS-coated proteins have a constant charge-to-mass ratio. As a consequence, they will migrate with the same mobility and thus will automatically stack. Moreover, as the net charge of SDS-protein micelles does not vary in the pH 7–10 interval, mobility is not affected within this pH range. Although two methods have been described for disc-SDS-PAGE [68,69], the one finally adopted in every laboratory today is that of Laemmli [69]. In this method, the upper (cathodic) buffer reservoir contains Tris–glycinate at pH 8.3; the stacking gel buffer

is Tris–HCl (pH 6.8), and the running gel buffer is Tris–HCl (pH 8.8). This forms a classic isotachophoretic train, in which the Cl^- acts as leading ion, the glycinate (as it is titrated near its pI value upon entering the stacking gel) as trailing ion, and all protein species as intermediate-mobility poly-ions, stacked in between, until they enter the separation gel, where sorting proceeds on the basis of the mass of the various polypeptide coils. Another important aspect of SDS-PAGE, which also substantially helps in augmenting resolution, is the use of acrylamide concentration gradients. There seems to be little doubt that a gradient gel can resolve a complex mixture better than a gel of single concentration, and for this it is not necessary that all components have migrated as far as their pore limits. Part of this high resolution results from the fact that, throughout the run, the leading edge of any particular band is moving through more concentrated gel than the trailing edge and, hence, it encounters greater resistance, resulting in a band-sharpening effect [70]. There are two further practical advantages of this method. Firstly, since after the initial "sorting-out" process a relatively stable band pattern is formed, it is not necessary to control the electrophoretic conditions so precisely as in other electrokinetic procedures, at least for qualitative work. Secondly, once bands have migrated well into the gel and have approached their pore limits, diffusion is greatly reduced, so that the gels can be kept unfixed for long periods of time with little loss of resolution [71]. Gels can be prepared with any shape of concentration gradient to suit the particular requirements of the separation. However, the bulk of published work refers to the use of linear or simple, concave gradients; in fact, in proteome analysis, most users now prefer simple, linear gradients, a typical recipe being in the 7.5–20%T interval [72].

Here too, it would be impractical to give a list of references on the use of SDS-PAGE: it is the gel electrophoretic technique most popular in every laboratory. We will simply refer the reader to Refs. 42–46 and to Andrews [73] for the application of SDS-PAGE in peptide mapping.

15.2.4 Two-dimensional maps

For the last 25 years, two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) has been the technique of choice for analyzing the protein composition of a given cell type and for monitoring changes in gene activity through quantitative and qualitative analysis of the thousands of proteins that orchestrate various cellular functions. Proteins are usually the functional molecules and, therefore, the most likely components to reflect qualitative (expression of new proteins, post-translational modifications) and quantitative (up and down regulation, co-ordinated expression) differences in gene expression [74]. Just as an example of the incredible growth in the field, in the journal *Electrophoresis* alone an avalanche of special issues has appeared, over the years, collecting several hundreds of articles that deal with 2-D PAGE and its application in the many areas of biological research [75–98]. A number of new journals, dedicated entirely to proteome analysis, have appeared: *Proteomics* (Wiley-VCH), *Journal of Proteome Research* (American Chemical Society), and *Molecular and Cellular Proteomics* (American Society for Biochemistry and Molecular Biology). Among the recent books dedicated to this topic, we quote those of Wilkins *et al.* [99], Kellner *et al.* [100], Rabilloud [101], Righetti *et al.* [102] and James [103]; and among book chapters, Westermeier [104] and Hanash [105], to name just a few.

It was the elegant work of O'Farrell in 1975 [106] that really demonstrated the full capabilities of this approach and set in motion the entire work on 2-D maps (but *cf.* Klose [107] and Scheele [108]). He was able to resolve and detect about 1100 different proteins from lysed *E. coli* cells on a single 2-D map and suggested that the maximum resolving capability may have been as high as 5000 different proteins. Apart from his meticulous attention to detail, major reasons for the advance in resolution obtained by O'Farrell, compared to earlier workers, included the use of samples labeled with ^{14}C or ^{35}S to high specific activity, and the use of thin (0.8-mm) gel slabs for the second dimension, which could then be dried easily for autoradiography. With this detection method he was able to detect protein zones corresponding to one part in 10^7 of the sample (usually 1–20 μg was applied initially, since higher loads caused zone spreading, although up to 100 μg could be loaded). Coomassie blue, in comparison, was about 3 orders of magnitude less sensitive and could reveal only about 400 spots. For the first dimension, O'Farrell adopted gel rods, 13 cm in length and 2.5 mm in diameter. The idea was to run samples fully denatured, in what became known as the "O'Farrell lysis buffer" (9 *M* urea, 2% Nonidet P-40, 2% β-mercaptoethanol, and 2% carrier ampholytes, in any desired pH interval). For the second SDS-PAGE dimension, O'Farrell [106] used the discontinuous buffer system of Laemmli [69] and, for improved resolution, a concave exponential gradient of polyacrylamide gel (usually in the intervals 9–15 or 10–14%T, although wide-porosity gradients, *e.g.*, 5–22.5%T, were also suggested). Thus, O'Farrell carefully selected all the best conditions available at the time; it is no wonder that his system was adopted, as such, in the avalanche of reports that soon followed. He went as far as to recognize that some protein losses could occur during the equilibration of the IEF gel prior to running of the SDS-PAGE. Depending on the identity of the protein and the duration of equilibration, losses were estimated to vary from 5 up to 25%. Even for that, O'Farrell proposed a remedy: he reported that, in such cases, the equilibration step could be omitted and, to minimize streaking in the second dimension, due to only partial SDS saturation, one could increase the depth of the stacking gel in the SDS-PAGE run from 2.5 up to 5 cm. The increased length of the stacking gel, coupled to lower initial voltage gradients and to higher amount of SDS in the cathodic reservoir, allowed for proper saturation of the protein species with the SDS moiety. It is no surprise that, with such a thorough methodological development, hardly any modifications to this technique were needed.

Although the power of 2-D electrophoresis as a biochemical separation technique has been well recognized since its introduction, its application has become particularly significant in the past few years, as a result of a number of developments, outlined below:

(a) The 2-D technique has been substantially improved to generate 2-D maps that are superior in terms of resolution and reproducibility. This improved methodology utilizes a unique first dimension, which replaces the CA-generated pH gradients with immobilized pH gradients and replaces the tube gels with gel strips, supported by a plastic film backing [27].

(b) Methods for the rapid analysis of proteins have been improved to the point that single spots eluted or transferred from single 2-D gels can be rapidly identified. Mass spectroscopic techniques have been developed that allow analysis of very small quantities of proteins and peptides and also permit a proper assessment of their possible *in vivo* or *in vitro* modifications [109–113]. Chemical

microsequencing and amino acid analysis can be performed on increasingly smaller samples [114]. Immunochemical identification is now possible with a wide assortment of available antibodies.

(c) More powerful, less expensive computers and software are now available, allowing routine computerized evaluations of the highly complex 2-D patterns.

(d) Data for entire genomes (or substantial fractions thereof) are now available for a number of organisms, permitting rapid identification of the genes encoding a protein separated by 2-D electrophoresis.

(e) The World Wide Web (WWW) provides simple, direct access to spot pattern databases [115–120] for the comparison of electrophoretic results and to genome sequence databases for assignment of sequence information.

We now briefly outline what is perhaps the most popular approach to 2-D map analysis today. The 1st dimension is preferably performed in individual IPG strips, laid side by side on a cooling platform, with the sample often absorbed in the strips during rehydration. At the end of the IEF step, the strips must be interfaced with the 2nd dimension, almost exclusively performed by mass discrimination *via* saturation with the anionic surfactant SDS. After this equilibration step, the strip is embedded in the top of a SDS-PAGE slab, where the second run is carried out perpendicular to the first migration. The 2-D map displayed at the end of these steps is the stained SDS-PAGE slab, where polypeptides are seen, after staining, as (ideally) round spots, each characterized by an individual set of pI/*Mr* coordinates. As mentioned earlier, for decades, the most popular lysis solution has been the O'Farrell cocktail (9 *M* urea, 2% Nonidet P-40, 2% β-mercaptoethanol, and 2% carrier ampholytes, in any desired pH interval). Although it is still much in vogue, over the years new, even more powerful, solubilizing mixtures have been devised. Great efforts were dedicated to such developments, especially in view of the fact that many authors noted that hydrophobic proteins were largely absent from 2-D maps. For instance, Wilkins *et al.* [121] reported that, quite strikingly, in three different micro-organisms (*Escherichia coli*, *Bacillus subtilis*, and *Saccharomyces cerevisiae*), all proteins above a given hydrophobicity value were completely missing, independent of the mode of IEF (soluble CAs or IPG). This suggested that the initial sample solubilization was the primary cause for the loss of such hydrophobic proteins. The progress made in solubilizing cocktails can be summarized as follows (see also the reviews by Molloy [122] and by Rabilloud and Chevallet [123]):

(a) *Chaotropes*. Although urea (up to 9.5 *M*) has for decades been the only chaotrope used in IEF, recently, thiourea has been found to further improve solubilization, especially that of membrane proteins [124–127]. The inclusion of thiourea is recommended for use with IPGs, which are prone to adsorptive losses of hydrophobic and isoelectrically neutral proteins. Typically, thiourea is added at concentrations of 2 *M* in conjunction with 5–7 *M* urea. The high concentration of urea is essential for solvating thiourea, which is poorly water-soluble (only *ca.* 1 *M* in plain water) [128]. Among all substituted ureas (alkyl ureas, both symmetric and asymmetric) Rabilloud *et al.* [127] found thiourea to be still the best additive.

(b) *Surfactants*. These compounds are always included in solubilizing cocktails to act synergistically with chaotropes. Surfactants are important in preventing hydrophobic interactions due to exposure of hydrophobic domains of proteins induced by

chaotropes [128]. Both the hydrophobic tails and the polar head groups of detergents play an important role in protein solubilization [129]. The surfactant tail binds to hydrophobic residues, allowing dispersal of these domains in an aqueous medium, while the polar head groups of detergents can disrupt ionic and hydrogen bonds, aiding in dispersion. Detergents typically used in the past include Nonidet P-40 and Triton X-100, in concentrations ranging from 0.5 to 4%. More and more, zwitterionic surfactants, such as CHAPS, are replacing those neutral detergents [130,131], often in combination with small amounts (0.5%) of Triton X-100. Small amount of CAs ($<1\%$) are also added, since they appear to reduce protein/matrix hydrophobic interactions and to overcome detrimental effects of salt boundaries [132]. Linear sulfobetaines are now emerging as perhaps the most powerful surfactants, especially those with at least a 12-carbon tail (SB 3–12). They were, in fact, demonstrated earlier to be potent solubilizers of hydrophobic proteins (*e.g.*, plasma membranes), but they had the serious drawback of being precipitated out of solution due to low urea compatibility (maximally 4 *M* urea for SB 3–12) [133]. This drawback of low solubility of many non-ionic or zwitter-ionic surfactants with long, linear hydrophobic tails in urea solution seems to be a general problem [134,135] which has prompted the synthesis of more soluble variants [136]. The inclusion of an amido group along the hydrophobic tail greatly improves urea tolerance (up to 8.5 *M*) and ameliorates separation of some proteins of the erythrocyte membranes [137]. Recently, Chevallet *et al.* [138] introduced ASB 14 (amidosulfobetaine), containing a 14-C linear alkyl tail, as a most powerful reagent, in combination with urea and thiourea, for solubilizing integral membrane proteins of both *E. coli* [139] and *Arabidopsis thaliana* [140,141].

(c) *Reducing agents*. Thiol agents are typically used to break intramolecular and intermolecular disulfide bridges. Cyclic reducing agents, such as dithiothreitol (DTT) or dithioerythritol (DTE) are the most common reagents admixed to solubilizing cocktails. These chemicals are used in large excess (*e.g.*, 20 to 40 m*M*) so as to shift the equilibrium toward oxidation of the reducing agent with concomitant reduction of the protein disulfides. Because this is an equilibrium reaction, loss of the reducing agent through migration of proteins away from the sample application zone can permit reoxidation of free Cys to disulfides in proteins. This would result not only in horizontal streaking, but also, possibly, in the formation of spurious extra bands, due to scrambled –S–S– bridges and their cross-linking different polypeptide chains. Even if the sample is directly re-swollen in the dried IPG strip, as is customary today, the excess DTT or DTE will not remain in the gel at a constant concentration, since, due to their weakly acidic character, both compounds will migrate above pH 7 and be depleted from the alkaline gel region. Thus, this will deteriorate focusing of alkaline proteins and be one of the many factors responsible for poor focusing in the alkaline region. The situation would be aggravated when β-mercaptoethanol is used, since the latter has an even lower pK value and is thus more depleted in the alkaline region and forms a concentration gradient towards pH 7, with a distribution in the gel according to its degree of ionization at any given pH value along the IEF strip [142]. The most recent solution to all of the above problems appears to be the use of phosphines as alternative reducing agents. Phosphines,

which were already described in 1977 by Ruegg and Rüdinger, [143] operate in a stoichiometric reaction, thus allowing the use of low concentrations (barely 2 m*M*). The use of tributyl phosphine (TBP) was recently proposed by Herbert *et al.* [144], who reported much improved protein solubility for both ovary cell lysates and intractable, highly disulfide-cross-linked wool keratins. TBP thus offers two main advantages: it can be used at much lower levels than other thiolic reagents (at least one order of magnitude lower concentration) and, additionally, it can be uniformly distributed in the IPG gel strip (when re-hydrated with the entire sample solution), since, being uncharged, it will not migrate in the electric field. Of course, the best solution remains reducing and alkylating proteins prior to any electrophoretic step, as recently proposed [145–147].

15.2.5 Conclusion No. 1: *Quo vadis*, proteome?

The proteome picture has changed dramatically in the course of years. Up to the early 1970s, chemists could separate only a handful of proteins by various electrophoretic approaches. The major break-through was surely the O'Farrell 2-D map [106], which showed that, *e.g.*, even a simple microorganism, such as *E. coli*, contains at least 1100 polypeptide chains. Later on, working with human tissues, Klose and Zeindl [148] showed that eukaryotes displayed at least 10,000 spots in a 2-D map. The second major event in proteome analysis came with the demonstration, in the late Eighties, that proteins and peptides could be ionized and analyzed by mass spectrometry. This paved the way to protein recognition on a large scale. The third major event in proteomics was the explosion of informatics, the building of genome and proteome databases, and the development of new, powerful algorithms allowing normalization and comparison of various 2-D maps for spotting events of up- and down-regulation. As in astronomy, we can now look at the starry sky, represented by a 2-D map with a few thousand protein spots, and count each one of them, give them three-dimensional coordinates, such as pI, *Mr* and spot volume, and perhaps assign a biological function to them. The repertoire of a human proteome may be as complex as:

(a) $> 10^4$ polypeptides per cell;
(b) 10^6 polypeptides per individual at any moment;
(c) 10^7 polypeptides per individual during the entire life span;
(d) 10^8 polypeptides per species.

15.3 FREE ZONE ELECTROPHORESIS

15.3.1 Acidic, isoelectric buffers in capillary zone electrophoresis of proteins/peptides

For analyzing proteins/peptides by CZE, the capillary wall must be conditioned, since these macromolecules can be adsorbed (even irreversibly), due to multi-point attachment to ionized silanols. Various ways of modifying the silica surface and a number of additives to the background electrolyte have been discussed in Chap. 9. Here we will discuss only what had been left out of Chap. 9, namely, the use of acidic, isoelectric buffers. In order to

facilitate peptide/protein analysis, a number of authors have advocated the use of strongly acidic buffers, based on phosphate, titrated to *ca.* pH 2.0–2.3. This buffer seems to have some unique advantages: it completely protonates the surface silanols, leaving the silica wall essentially uncharged; in addition, it impedes accidental adsorption of peptides/proteins to any residual, charged group on the capillary surface, due to the fact that the phosphate ion itself is strongly adsorbed on the silica surface [149]. It also has the drawback that its very high conductivity negates the use of high voltage gradients, which permit fast separations and thus high resolution due to minimal peak-spreading, which is induced by diffusion. Attempting to preserve the advantages of such acidic buffers while eliminating their negative aspects, Righetti and Nembri [150] introduced isoelectric, acidic buffers in peptide separations. Four such buffers were described: Asp (pH = pI = 2.75) [150], iminodiacetic acid (IDA) (pH = pI = 2.23) [151], cysteic acid (pH = pI = 1.85) [152], and Glu (pH = pI = 3.32) [153]. Although some unique and very fast peptide separations were obtained in such buffers, it was discovered, later on, that larger peptides and proteins would still stick to the silica wall, even under such drastic acidic conditions [154]. After a method for quantifying this adsorption was devised [155–157], it was found that such acidic buffers, when supplemented with small amounts (1–3 m*M*) of oligoamines [notably spermine and TEPA (tetraethylenepentamine)] worked exceedingly well in the separation of macro-analytes. The newest dynamic coating is a simple diamine [158], acting in a peculiar way: when confronted with a silica surface, at pH values of pH 8.8 to 9.0, it will interact with the wall by two different mechanisms, hydrogen bonding and ionic interaction, *via* its tertiary and quaternary nitrogens, respectively. These transient interactions are soon followed by a permanent link, namely a covalent bond spontaneously formed with neighboring ionized silanols, *via* its terminal iodine atom in the butyl chain (Fig. 15.4). Several additional compounds in the same family have been described, showing even more pronounced reactivity with the wall [159]; their performance in protein separations was found to be unique [160]. This series of improvements in performance should allow protein/peptide separations to be easily performed in any laboratory without requiring the skills and laborious permanent-coating method used up to now.

15.3.2 Capillary zone electrophoretic analysis of peptides and tryptic fragments of proteins

CE of peptides, either natural or derived from proteins by digestion with proteases, is becoming an important area of biochemical research, due to its peculiar advantage of CE to require only truly minute sample amounts (microliters at the injection port, but just nanoliters of loaded sample volume). Nevertheless, its main disadvantage is the fact that the detection window monitors a miniaturized channel, so that the sensitivity is rather low. This is, in part, offset by the fact that fused silica is compatible with UV detection at as low as 200–210 nm, *i.e.* the region of absorbance of the peptide bond. This makes the sensitivity of detection at least one order of magnitude greater than monitoring of Trp in Tyr in proteins at 280 nm. At *ca.* 200 nm, micromolar concentrations of peptides can usually be detected. However, when exploiting laser-induced fluorescence (LIF) detection, the sensitivity can be greater by several orders of magnitude, reaching, in principle, the absolute limit of detection

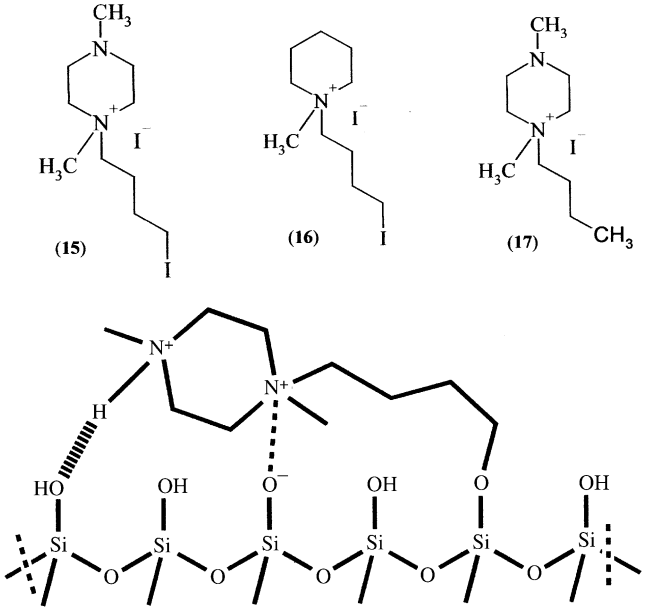


Fig. 15.4. A: Formulas of some derivatives of the "scorpion" family (*N*-iodobutyl,*N*-methyl-*N*′-methylpiperazinium iodide). B: proposed mechanism of interaction with the wall of Compound No. 15: covalent bonding *via* reaction of the iodine in the butyl tail with a silanol; salt bridge with the quaternary nitrogen and hydrogen bond with the tertiary nitrogen. (Reprinted from Ref. 162, with permission.)

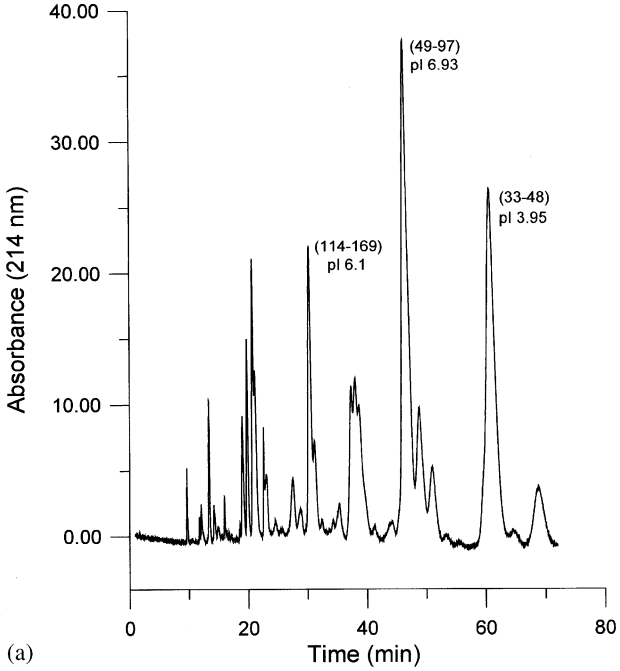
of a single molecule [161]. Unfortunately, native fluorescence can only be utilized for detection of peptides containing Trp and Tyr, *e.g.*, by adopting a frequency-doubled Ar laser operating at 257 nm and Kr laser pumping at 284 nm [162]. Alternatively, peptides can be derivatized before or after electrophoresis with a number of fluorescent dyes [163,164], *e.g.*, fluorescein isothiocyanate, fluorescamine, the near-infrared fluorescent dye NN382 [165], and the fluorogenic dye 5-furoylquinoline-3-carboxyaldehyde [166]. Mass spectrometry, in addition, represents perhaps the ultimate in peptide detection, since it couples excellent sensitivity (low femtomoles) with the ability to identify peptides by sequencing in the post-source decay mode by MALDI-TOF instrumentation [167,168].

There are plenty of examples of separations of peptides by CE, a few of which will be given here. CE in buffers at pH 9.35 and 2.70 has been used for the determination of the dipeptide sweetener aspartame (α-L-Asp-L-Phe-O-Me) and of its potential degradation products, such as Phe and Phe-O-Me [169]. CE was instrumental in assessing the purity of the synthetic nonapeptide hormone bradykinin [170], for the analysis of cyclic peptide growth promoters [171], of thymosins [172], of lipopeptides [173] and of ginseng polypeptides [174]. Three opioid peptides: β-endorphin, Met-enkephalin, Leu-enkephalin; and four other neuropeptides (Substance P, somatostatin, vasopressin, and oxytocin) were determined by CE in brain tissue homogenates and plasma with detection limits at the sub-picomole level [175]. The *in vivo* release of multiple neuropeptides, luteinizing hormone-releasing hormone (LHRH), β-endorphin and neuropeptide Y in perfusate

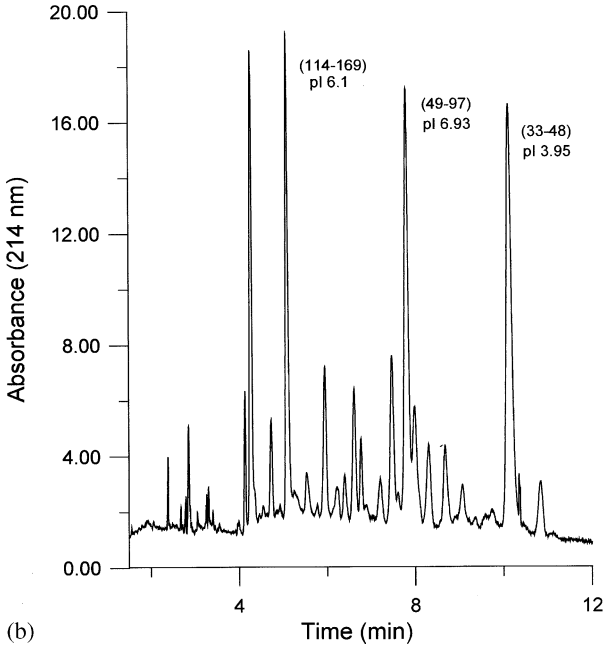
samples from the brain hypothalamic median eminence was determined by CE with argon-ion LIF detection [176]. By exploiting the same principle of LIF detection, D-penicillamine-2,5-enkephalin in serum was analyzed by CE with a sensitivity of *ca.* 1 attomole [177]. Additional examples of peptide determination in biological matrices are the analyses of insulin-like growth factors in cell-conditioned human osteoblast medium [178], of phytochelatins and their desglycyl peptides (class II metallothioneins) and precursors, of glutathione and γ-glutamylcysteine in the extracts from root cultures [179], the determination of insulin in pancreatic tissue homogenates [180], the identification of antigen peptides in melanoma tumor cell extracts [181], the analysis of polypeptides in cerebrospinal fluid [182], in urine, and in aqueous humor [183]. CE has also been used for monitoring dynamic changes in peptide preparations, *i.e.*, their chemical and enzymatic modifications and reactions, cleavage, degradation, etc. As an example, modifications and/or alterations of amino acid residues, such as oxidation, reduction, deamidation, hydrolysis, arginine conversion, and β-elimination have been surveyed and summarized in Refs. 184 and 185. CE was also used for stability research on goserelin, an analog of LHRH [186], for the separation of asparagine-containing hexapeptides and their deamidation products [187], for monitoring the enzymatic hydrolysis of glycine-containing peptides by a protease from *Pseudomonas aeruginosa* [188], for assessing the glycosylation of the peptide hormones delargin and desmopressin [189] as well as the glycosylation of Thr- and Ser-rich peptides [190]. Peptide map analysis of complex mixtures could be facilitated by using high-surface area capillaries, etched with hydrogen difluoride. Thus, *e.g.*, Pesek *et al.* [191] have demonstrated that, in the analysis of tryptic digests of transferrin, only 26 peaks could be resolved in standard capillaries, compared to 36 in etched ones.

We will now give some examples of peptide separations, as obtained by the novel methodology described above, exploiting isoelectric buffers. Righetti and Nembri [150] proposed the use of isoelectric aspartic acid as a background electrolyte, operating at pH = pI = 2.77 (at 25°C and 50 m*M* concentration). These authors could produce peptide maps of casein in only 10–12 min, as opposed to >70 min in standard phosphate buffer (pH 2.0) at voltage gradients as high as 800 V/cm, with much increased resolution. Adsorption of some larger peptides on the wall was completely eliminated by adding 0.5% hydroxyethylcellulose (HEC) and 5% trifluoroethanol (TFE) to the isoelectric Asp buffer. Fig. 15.5 shows the results of standard CZE of a tryptic digest of β-casein (Fig. 15.5A) and CZE in the presence of isoelectric Asp (Fig. 15.5B). However, there are some intrinsic

Fig. 15.5. Capillary zone electrophoresis of tryptic digests of β-casein. Conditions (upper panel): capillary 37 cm long, 100 μm ID, bathed in 80 m*M* phosphate buffer (pH 2.0). Sample application by pressure (0.5 psi for 3 sec). Separations were performed at 110 V/cm (85 μA) and detection was at 214 nm. The three major peaks are: 1 = pI 6.1, Fragment β-CN (114–169); 2 = pI 6.93, Fragment β-CN (49–97); and 3 = pI 3.95, Fragment β-CN (33–48). Note that the total running time is >70 min. B: CZE of tryptic digests of β-casein in a 37-cm long capillary, 100 μm ID, bathed in 50 m*M* isoelectric aspartic acid (pH approximating the pI value of 2.77), containing 0.5% HEC (M_n: 27,000 Da) and 5% trifluoroethanol; running conditions, 600 V/cm (58 μA). (Reprinted from Ref. 154 with permission.)


40.00
30.00
20.00
10.00
0.00
Absorbance (214 nm)
(49-97)
pI 6.93
(33-48)
pI 3.95
(114-169)
pI 6.1
0
20
40
60
80
Time (min)

(a)


20.00
16.00
12.00
8.00
4.00
0.00
Absorbance (214 nm)
(114-169)
pI 6.1
(49-97)
pI 6.93
(33-48)
pI 3.95
4
8
12
Time (min)

(b)

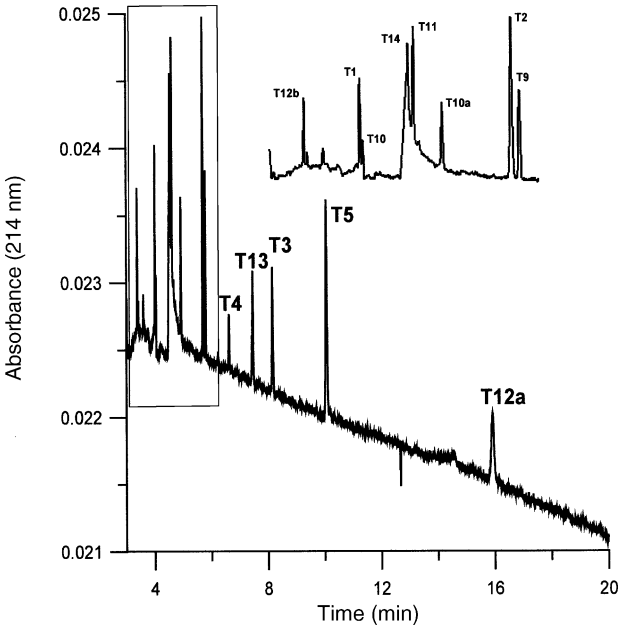


Fig. 15.6. Capillary zone electrophoretic separation of a peptide digest of β-globin chains. Running buffer, 30 m*M* Asp (pH 3.0), containing 0.5% HEC, 10% TFE and 50 m*M* NDSB-195. Sample injected for 15 sec by hydrostatic pressure. Note in the inset (an enlargement of the boxed area on the left side) the splitting of peaks T2 and T9. (Reprinted from Ref. 196 with permission.)

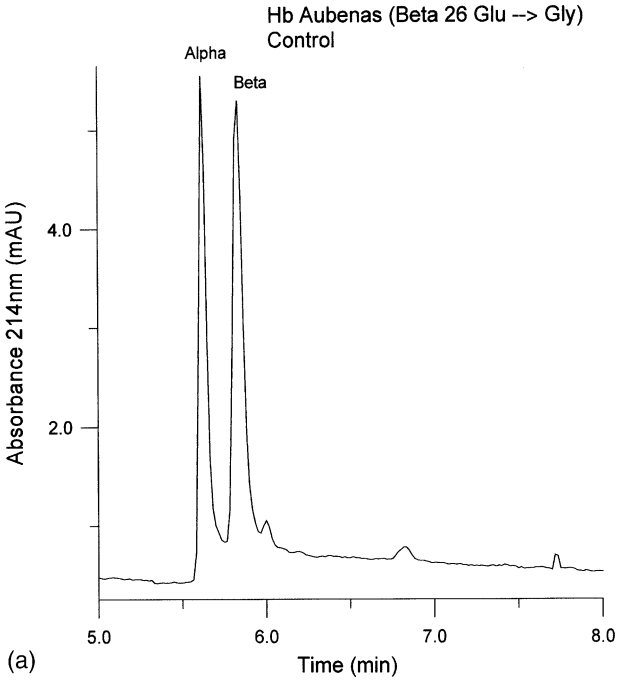
problems with the use of isoelectric buffers. A case in point is exemplified by Fig. 15.6, which represents the CZE separation of tryptic digests of β-globin chains [192]. At the pH prevailing in isoelectric Asp (pH 2.77) two peaks (labeled T2 and T9) are eluted in a single zone. By simulating the theoretical pH/mobility curves of the 13 peptides in the pH 2–4 interval, it was determined that at this pH value there was a cross-over point in the titration curves of these two peptides, which could only be eliminated by working at slightly different pH values, such as pH 3.0 or 3.1. However, if one were to work at this pH with a conventional, non-amphoteric buffer, one would automatically lose the benefit of adopting high voltages and thus considerably shorten the separation time while gaining in resolving power. It should be noted that, as shown by Bossi and Righetti [151] in the case of IDA, the pI of 2.77 is only a theoretical value, reached at a high enough concentration of Asp (in this case 50 m*M*). By progressive dilution of the buffering ion, one can span a small pH interval, up to 0.4 pH units. Thus, it is possible, while still working under isoelectric conditions, to modulate the pH of the background electrolyte simply by varying its concentration. In a buffer composed of 30 m*M* Asp, which still ensures adequate buffering power, the final pH of the background electrolyte is 3.0. By adopting this last buffer (Fig. 15.6), it was possible to separate all thirteen β-globin peptides. By enlarging the 3–6 min separation window (Fig. 15.6, inset) one can appreciate that the T2 and T9 β-peptides, which had migrated as a single zone, are now base-line resolved. However, as the pH was increased to 3.0, there was

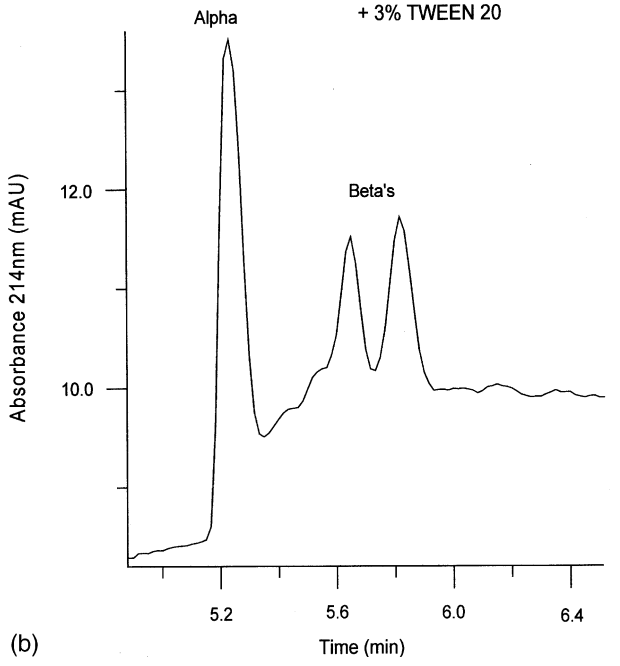
a higher risk of peptide adsorption on the naked silica wall, and addition of a zwitter-ion (non-detergent sulfobetaine, NDSB-195) was necessary in order to quench such interaction. Nowadays, oligoamines, such as spermine and TEPA, would be used instead [193]. Additional references on CZE separations of peptides can be found in Kašièka [194,195] and Righetti *et al.* [196].

15.3.3 Capillary zone electrophoretic analysis of proteins

Here, again, we can only present some limited examples and refer the readers to a number of extensive reviews [197–209]. Such examples will be limited mostly to proteins of medical and biotechnological interest. McNerney *et al.* [210] reported the analysis of recombinant human growth hormone (rhGH) in *E. coli* by the use of phosphate-deactivated capillaries. They were able to resolve a number of variants, such as des-Phe (des = desamido), des-Phe-Pro, mono-deamidated, and di-deamidated hGH forms. Recombinant $NADP^+$-dependent formate dehydrogenase, expressed in *E. coli*, was analyzed by CE in sieving liquid polymers [211]. By the same technique, a protective antigen protein, a major component of human anthrax vaccine, also expressed in *E. coli*, was purified and analyzed [212]. Roddy *et al.* [213] used CE at low pH to screen preparations of acidic fibroblast growth factor, a protein which tends to aggregate and precipitate. Synaptobrevin thioredoxin protein, a substrate for cleavage by botulin toxin B, was expressed in *E. coli* and analyzed by CE by Asermely *et al.* [214], while Kundu *et al.* [215] reported CE analysis of GB virus-C (also known as hepatitis G virus) protein, which presents the problem of forming inclusion bodies. CE has been used extensively for monitoring glycosylation patterns of rDNA proteins as a function of fermentation conditions. Thus, *e.g.*, when interferon-ω was obtained from fermentations in the presence of fetal calf serum, the glycosylation pattern observed by CE was different from the profile of a fermentation in a serum-free medium, as reported by Kopp *et al.* [216]. Goldman *et al.* [217] analyzed interferon-γ, after purifying it by immunoaffinity, using an uncoated silica capillary and SD/borate buffer in the micellar mode. Recombinant erythropoietin (rEPO) is one of the most important biotechnological therapeutics. Since rEPO formulations contain human serum albumin (HSA), it was important to achieve good resolution by CE, which was obtained by Bietlot *et al.* [218] in the presence of 1 m*M* $NiCl_2$ and an amine-coated capillary. For the same analysis, Nieto *et al.* [219] adopted a C_{18} derivatized capillary and 30 m*M* phosphate buffer at pH 7.0, whereas Zhou *et al.* [220] reported CE separations in uncoated capillaries in acetate buffer (pH 4.0). Tryptic peptides of rEPO were also analyzed by Boss *et al.* [221], using a combination of techniques, such as HPLC, CE, and MS. In addition to zone electrophoretic methods, Cifuentes *et al.* [222] have reported the analysis of rEPO by cIEF: all the isoforms were resolved in the pH 2.5–5.0 interval. Isoelectric focusing techniques were also used for studying the heterogeneity of tissue plasminogen activator, as reported by Thorne *et al.* [223] and by Moorhouse *et al.* [224].

Here, it is of interest to go into details of some separations, and some unique phenomena occurring in a fast analysis of human globin chains may be instructive. In 1979, Righetti *et al.* [225] proposed, as a fast and reliable method for detecting point mutations of human hemoglobin or in thalassemia, IEF of heme-free, denatured globin


Hb Aubenas (Beta 26 Glu --> Gly)
Control
Alpha
Beta
Absorbance 214nm (mAU)
4.0
2.0
5.0
6.0
7.0
8.0
Time (min)
(a)


Alpha
+ 3% TWEEN 20
Beta's
Absorbance 214nm (mAU)
12.0
10.0
5.2
5.6
6.0
6.4
Time (min)
(b)

chains, dissolved in 8 *M* urea plus a reducing agent (2-mercaptoethanol). It was observed that the addition of a neutral surfactant (Nonidet P-40) to the solubilization mixture brought about a unique phenomenon: it greatly improved the separation between β- and γ-(fetal) chains and, additionally, induced the splitting of the γ-zone into two peaks, identified as the products of two genes, coding for Ala (Aγ) or for Gly (Gγ) in position 136 of the fetal chains [226]. This surfactant effect on the resolution of the two genetic variants of fetal chains, Aγ and Gγ, could also be reproduced by zone electrophoresis on cellulose acetate strips. This method of CE in isoelectric buffers was applied to the fast analysis of such mutant globin chains. An interesting phenomenon can be observed in Fig. 15.7: in this particular case, a charged mutant was analyzed (Hb Aubenas, β26 Glu → Gly) [227]. One would have expected resolution of these two chains also in the control run (Fig. 15.7A), in the absence of surfactant. However, this was not the case, and splitting occurred only in the presence of 3% Tween 20 (Fig. 15.7B). In a way, this should have been expected: Given the operative pH of the isoelectric buffer used, the Glu residue should be extensively protonated, so that the charged amino acid substitution went undetected, but it could still be revealed in the presence of a surfactant, due to the large difference in hydrophobicity of these two residues.

15.3.4 Determination of absolute mobility and of its relation to the charge/mass ratio in peptides

The recent increased use of peptides in medical therapy is a result of their large range of activity and specificity, usually accompanied by low toxicity and rapid metabolization. An increasing activity in design and synthesis of new peptide-based drugs is predicted, as a result of combined advances in proteomic research and biotechnology. Thus, the possibility of predicting the mobility of peptides and their separation in CZE analysis, even prior to an actual electrophoretic run, is becoming increasingly important in pharmaceutical research.

Various models for predicting the effects of charge and Stokes radii on peptide mobilities have been proposed over the years, and we will attempt to present the different view points of a variety of research groups, which are often in conflict. The problem of determining absolute mobilities alone is not an easy one. In the early days, the technique of choice for this was isotachophoresis (ITP) [228–230]. However, since ITP experiments were quite cumbersome and difficult to interpret, CZE has now replaced ITP measurements. Some authors [231] went as far as to propose a double detector

Fig. 15.7. Capillary zone electrophoretic separation of hemoglobin Aubenas (β26 Glu → Gly). Conditions, 50 m*M* IDA buffer, containing 7 *M* urea and 0.5% HEC (apparent pH 3.2) in the absence (upper panel) or presence (lower panel) of 3% surfactant (Tween 20); capillary, uncoated, 50 μm ID, 375 μm OD, 30 cm long (22 cm to detector); run in a Water's Quanta 4000E, at 600 V/cm, *ca.* 20 μA, 15°C; sample injection at the anodic side for 0.4 sec at 5 psi; detection, 214 nm. Upper panel: control run; lower tracing: addition of 3% Tween 20. Note that, although this mutation involves a charged amino acid, it is not detected in the control run, but only when the surfactant is added. (Reprinted from Ref. 231 with permission.)

system for measuring mobilities in CZE, thus obtaining data comparable to those in the literature, with a relative standard deviation of *ca.* 1%. It should be remembered that, even when everything is held constant (ionic strength, pH, voltage gradient, solvent type, and dielectric constant), a most important parameter is the temperature: all ionic species show a similar dependence of their absolute mobilities on temperature (about 2% per °C).

There has been much debate on what relationship would give the best fit in correlating μ_{ep} (electrophoretic mobility) with Z_e/r (charge-to-mass ratio) for proteins and peptides. The earliest attempt is attributed to Offord [232], who has shown a linear relationship between paper-electrophoretic mobilities of a series of charged peptides and their molecular mass to the power of $-2/3$. In principle, according to Eqn. 15.1

$$\mu_{ep} = Z_e/(6\pi\eta r) \tag{15.1}$$

(where η is the solvent viscosity, Z_e is the electronic charge and r is the radius of the ion), there should be a straight, linear relationship between μ_{ep} and Z_e/r. However, Grossman *et al.* [233] proposed a semi-empirical model which related μ_{ep} of a range of positively charged peptides to their size, charge, and hydrophobicity. The effects of size and charge were determined independently and then combined to give the following relationship:

$$\mu_{ep} = [\mathrm{A}\log(Z+1)/n^{0.43}] + \mathrm{B} \tag{15.2}$$

where n is the number of amino acids in the peptide and A and B are constants related to the solvent system used. Compton and O'Grady [234,235] modified Eqn. 15.1 by applying the Debye–Hückel–Henry theory to account for ionic effects:

$$\mu_{ep} = Z_e\Phi(kr)/[6\pi\eta r(1+k)] \tag{15.3}$$

where $\Phi(kr)$ is Henry's function and k is the Debye screening parameter. By expressing the size dependence of μ_{ep} in terms of molecular mass (M) rather than radius (r), they obtained a general equation of the form:

$$\mu_{ep} = \mathrm{K}_1 Z/(\mathrm{K}_2 M^{1/3} + \mathrm{K}_3 M^{2/3}) \tag{15.4}$$

where K_1, K_2, and K_3 are three terms which include common physical constants, the solution ionic strength, and the frictional ratio (f/f_o). Further, they indicated that, after some simplifications and transformations of Eqn. 15.3, a relationship similar to Eqn. 15.2 is obtainable. On the other hand, if $K_2 \ll K_3$, Eqn. 15.4 becomes similar to the correlation observed by Rickard *et al.* [236], where

$$\mu_{ep} = Z/M^{2/3} \tag{15.5}$$

Thus, it would appear that the different approaches in Refs. 233 and 236 provide similar results, because both are particular cases, deriving from a more general theory elaborated by Compton and O'Grady [234,235]. There are at least two major discrepancies here to be reconciled: (a) is the relationship between μ_{ep} and Z/r linear or logarithmic? (b) which power function of molecular mass ($M^{1/3}$, $M^{2/3}$ or $M^{1/2}$) fits this relationship best? Answering the last question first, if we assume that the structure of the peptide in the capillary can be approximated by a sphere of constant density,

then the radius of the sphere would be proportional to $M^{1/3}$. Hence, if frictional drag is governed by Stokes' Law, then μ_{ep} would be related to the radius of the species, *i.e.*, to $M^{1/3}$. However, if frictional drag is related to the surface area (or cross-sectional area) of the molecule, then μ_{ep} would be proportional to $M^{2/3}$. On the other hand, studies on synthetic polymers have shown that the radius of gyration is proportional to the square root of the number of monomer units in the polymer, multiplied by the length of a single residue [237]. If frictional drag were proportional to the average radius of gyration, then μ_{ep} would be proportional to the square root of the number of residues, *i.e.*, to *ca.* $M^{1/2}$. In an extensive analysis of multiple phophoseryl-containing casein peptides, Adamson *et al.* [238] tried various possible fits and found the best correlation ($R^2 = 0.0993$) to be between μ_{ep} and $Z/M^{2/3}$. The other two possible correlations, with $Z/M^{1/3}$ and $Z/M^{1/2}$, gave poor fits. This would appear to settle at least the second question, but evidence to the contrary has recently been provided by Kálman *et al.* [239], this time not even with peptides (which should be easier to handle) but with native proteins (*Staphyloccus* nuclease mutants). The best linear fit these authors could obtain was μ_{ep} *vs.* $Z/M^{1/3}$, thus the question of what power of M should be accepted may still be debatable. As for the first question (linear or log relationship), the story is quite interesting. According to Castagnola *et al.* [240–244], the log relationship proposed by Grossman *et al.* [233] is intrinsically erroneous because it is based on calculations of net charge assuming fixed pK values of amino acids. Conversely, Castagnola *et al.* [241,242] performed microtitrations of the peptides, found experimentally accurate pK values, and used them for calculating Z. Under these conditions, the correct relationship was found to be a linear one, as predicted by Eqn. 15.1. These authors thus argued that the apparent good correlation between μ_{ep} and $log(Z + 1)$ was simply due to the fact that a log function is less sensitive to errors in pK values than the correct linear relationship between μ_{ep} and Z, where a good fit is only obtained by using very accurate pK values. This observation could also explain the results of Hilser *et al.* [245], who found a better correlation between μ_{ep} and $log(Z + 1)$ than between μ_{ep} and Z. In agreement with the arguments of Castagnola *et al.* [241, 242], Adamson *et al.* [238], when re-plotting their mobility data according to the dependence $log(Z + 1)$, found a much worse fit than with the linear relationship. Yet, it cannot be denied that other subtle parameters could play a role in peptide separation. An interesting example has been offered by Meyer *et al.* [246]: when working with the thioxo peptide Ala-Phe-Ψ[CS-N]-Pro-Phe-4-nitroanilide, peak splitting was observed at 25°C. Both peaks merged as the temperature was increased to 60°C, but reappeared when the heated sample was allowed to cool. This behavior was attributed to the electrophoretic separation of the *cis*/*trans* prolyl bond isomers of the thioxo peptide. These authors calculated that the mobility of the *cis* form is 1.027 times higher than that of the *trans* isomer. In fact, molecular modeling showed the *cis* conformer to be of more compact cylindrical shape than the *trans* isomer, behaving as a slightly more expanded, nearly spherical object. Computations revealed a 1.015-fold increase in the average volume of the *trans* isomer, hence the mobility of the *trans* form was slightly less than that of the *cis* form. More on these topics can be found in the recent literature [247–250].

15.3.5 Probing folding/unfolding/refolding/misfolding of proteins by capillary zone electrophoresis

It would not be fair to end this review without a glimpse at some of the most exciting recent developments: studying folding/unfolding transitions of proteins by CE. A number of reports have already appeared in this field [251–264], and two major reviews cover the progress in this area [265,266]. Recent data [262] suggest that CE can now be accepted as a tool for studying these transitions, which up to the present had to be monitored by a host of specialized techniques, such as circular dichroism and intrinsic fluorescence variations. Misfolded states of proteins seem to have important implications in some human diseases [267–269], including prion protein disease. Thus, a system which could not only detect their existence, but physically separate these forms, would be very valuable in trying to understand the mechanisms leading to misfolded and aggregated states of proteins. In general, folding/unfolding processes are rather fast phenomena; thus, when studying them by CZE, it is impossible to see the two species (the folded and unfolded forms) as separate peaks, since their interconversion rate is much faster than the electrophoretic separation time. One just sees a sharp peak when injecting the native form and another sharp peak when analyzing the fully denatured state. In the intermediate states, a peak of reduced height is seen that broadens at progressively higher denaturant concentration, and represents an envelope of the two species. An interesting case is the one in which the two forms are in slow equilibrium, such as the case of acyl phosphatase (AcP) [262]. AcP is a small (98 residues), mildly basic (pI 9.6), globular protein, exhibiting rather slow isomerization transitions, and it is therefore ideally suited for CZE studies. The unfolding process is typically observed over a period of several hours. Fig. 15.8 shows a standard run in 7 *M* urea. In order to facilitate CZE analysis, an acidic, isoelectric buffer (50 m*M* Glu, pH = pI = 3.2), was adopted, which contained 1 m*M* tetraethylene pentamine (final pH = 3.3), a potent quencher of protein interaction with the wall. The unfolded peak (UAcP) is already seen after 5 min of incubation, as a small peak, eluted after the Gly-His marker. At the end of a 100-min incubation period, the ratio of the areas of the folded/unfolded zones becomes *ca.* 9:1. Since the denaturing event of AcP is a unimolecular process (folded → unfolded) and thus follow first-order kinetics. Such experiments indicate that CZE can be used just as efficiently as spectroscopic techniques for monitoring such isomerization processes [262].

15.3.6 Conclusion No. 2: *Quo vadis*, capillary zone electrophoresis?

It is perhaps more difficult to predict where CZE is headed, than developments in the field of DNA analysis, surely a vast field of applications for CZE. There, developments have reached a plateau, at least in terms of the chemistry of sieving liquid polymers and in equipment miniaturization and automation. It was thanks to major developments in the protein field that massive and parallel DNA sequencing became possible and substantially fulfilled the world-wide effort for completing the human genome sequencing project in the year 2000. Surely, one quite promising field of development is electrokinetic chromatography (Chap. 7), in which chromatography is performed in a capillary filled

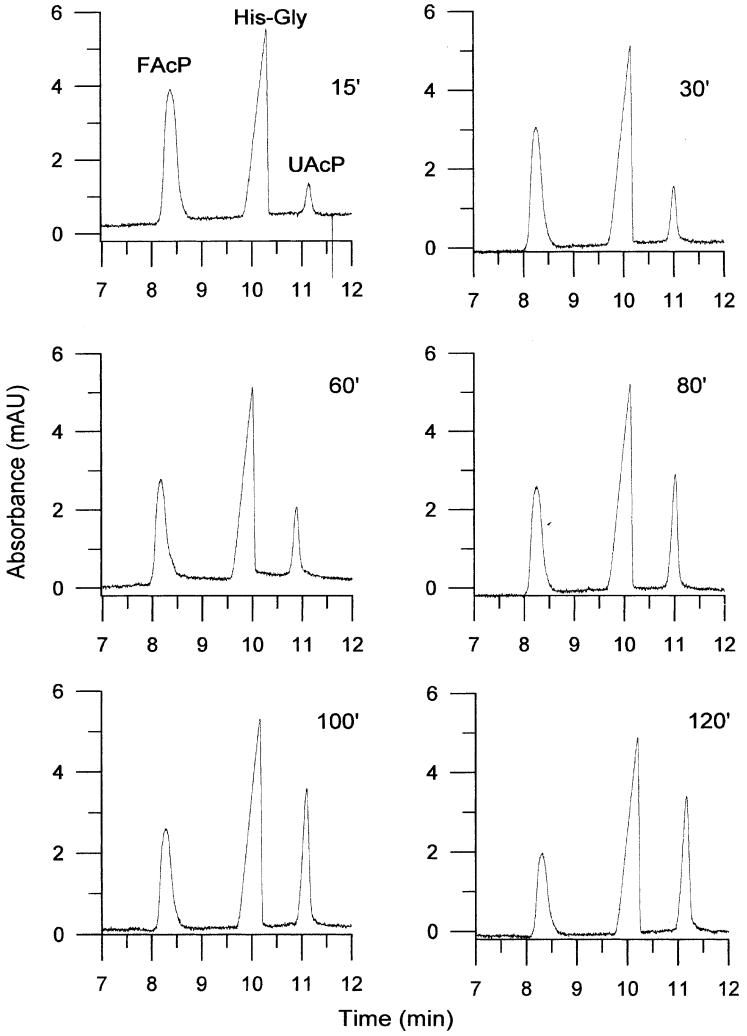


Fig. 15.8. Unfolding kinetics of acyl phosphatase in 7 *M* urea solutions. At the indicated times (from 5 to 100 min of incubation outside the capillary) the samples were injected in a capillary, containing 50 m*M* Glu, 7 *M* urea and 1 m*M* TEPA. Running conditions: 10 kV, 18 μA, 20°C; detection, 214 nm; sample, 0.5 mg/mL of AcP and 0.2 mg/mL of His-Gly. FacP = folded AcP; UacP = unfolded AcP. (Reprinted from Ref. 266 with permission.)

with a stationary phase of functionalized tiny beads. The electric field is just used to pump the liquid *via* electroendo-osmotic propulsion, a nice way to avoid parabolic fluid profiles! Perhaps one of the most attractive developments is the CAE instrument conceived by Mathies' group [270,271], and shown in Fig. 11.10: a compact disk, imprinted with 96 channels for massive parallel analyses.

ACKNOWLEDGMENTS

The work of our group, cited here, is supported by grants from ASI (Agenzia Spaziale Italiana, grant No. I/R/194/02), by the European Community, grant No. QLG2-CT-2001-01903, and by a GlaxoSmithKline grant No. GEC 556-2000 to PGR. A.B. is supported by MURST 2001 (prot. 2001033797_003) and by MURST 2003 "Ingegneria di Sistemi di Separazione ad Elevate Prestazioni, Sensori ed Arrays".

REFERENCES

1 J. Kohn, *Nature*, 180 (1957) 986.
2 P. Grabar and C.A. Williams, *Biochim. Biophys. Acta*, 10 (1953) 193.
3 O. Smithies, *Biochem. J.*, 61 (1955) 629.
4 L. Ornstein, *Ann. NY Acad. Sci.*, 121 (1964) 321.
5 B.J. Davis, *Ann. NY Acad. Sci.*, 121 (1964) 404.
6 P. Gebauer, V. Dolnik, M. Deml and P. Bocek, *Adv. Electr.*, 1 (1987) 281.
7 P.G. Righetti, in E. Heftmann (Ed.), *Chromatography*, 5th Edn., Elsevier, Amsterdam, 1992, pp. 481–517.
8 A.L. Shapiro, E. Vinuela and J.V. Maizel, *Biochem. Biophys. Res. Commun.*, 28 (1967) 815.
9 H. Svensson, *Acta Chem. Scand.*, 15 (1961) 325.
10 H. Svensson, *Acta Chem. Scand.*, 16 (1962) 456.
11 H. Svensson, *Arch. Biochem. Biophys.*, Suppl. 1 (1962) 132.
12 M. Meselson, F.W. Stahl and J. Vinogradov, *Proc. Natl Acad. Sci. USA*, 43 (1957) 581.
13 A. Kolin, in B.J. Radola and D. Graesslin (Eds.), *Electrofocusing and Isotachophoresis*, de Gruyter, Berlin, 1977, pp. 3–33.
14 J.C. Giddings, *Unified Separation Science*, Wiley, New York, 1991, pp. 86–109.
15 H. Rilbe, *Ann. NY Acad. Sci.*, 209 (1973) 11.
16 H. Rilbe, *pH and Buffer Theory. A New Approach*, Wiley, Chichester, 1996, pp. 1–192.
17 O. Vesterberg, *Ann. NY Acad. Sci.*, 209 (1973) 23.
18 L. Söderberg, D. Buckley, G. Hagström and J. Bergström, *Prot. Biol. Fluids*, 27 (1980) 687.
19 P. Pogacar and R. Jarecki, in R. Allen and H. Maurer (Eds.), *Electrophoresis and Isoelectric Focusing in Polyacrylamide Gels*, de Gruyter, Berlin, 1977, pp. 153–158.
20 N. Grubhofer and C. Borja, in B.J. Radola and D. Graesslin (Eds.), *Electrofocusing and Isotachophoresis*, de Gruyter, Berlin, 1974, pp. 111–120.
21 A. Bianchi-Bosisio, R.S. Snyder and P.G. Righetti, *J. Chromatogr.*, 209 (1981) 265.
22 P.G. Righetti, *Isoelectric Focusing: Theory, Methodology and Applications*, Elsevier, Amsterdam, 1983.
23 R.B. Merrifield, *Adv. Enzymol.*, 32 (1969) 221.
24 P.G. Righetti and F. Chillemi, *J. Chromatogr.*, 157 (1978) 243.
25 C.B. Cuono and G.A. Chapo, *Electrophoresis*, 3 (1982) 65.
26 P.G. Righetti, M. Fazio and C. Tonani, *J. Chromatogr.*, 440 (1988) 367.
27 B. Bjellqvist, K. Ek, P.G. Righetti, E. Gianazza, A. Görg, W. Postel and R. Westermeier, *J. Biochem. Biophys. Methods*, 6 (1982) 317.
28 M. Chiari, E. Casale, E. Santaniello and P.G. Righetti, *Theor. Appl. Electr.*, 1 (1989) 99.
29 M. Chiari, E. Casale, E. Santaniello and P.G. Righetti, *Theor. Appl. Electr.*, 1 (1989) 103.

30 P.G. Righetti, C. Gelfi and M. Chiari, in B.L. Karger and W.S. Hancock (Eds.), *Methods in Enzymology: High Resolution Separation and Analysis of Biological Macromolecules, Part A: Fundamentals*, Vol. 270, Academic Press, San Diego, 1996, pp. 235–255.
31 P.G. Righetti, M. Chiari, P.K. Sinha and E. Santaniello, *J. Biochem. Biophys. Methods*, 16 (1988) 185.
32 P.K. Sinha and P.G. Righetti, *J. Biochem. Biophys. Methods*, 15 (1987) 199.
33 M. Chiari, L. Pagani, P.G. Righetti, T. Jain, R. Shor and T. Rabilloud, *J. Biochem. Biophys. Methods*, 21 (1990) 165.
34 M. Chiari, C. Ettori, A. Manzocchi and P.G. Righetti, *J. Chromatogr.*, 548 (1991) 381.
35 M. Chiari, M. Giacomini, C. Micheletti and P.G. Righetti, *J. Chromatogr.*, 558 (1991) 285.
36 M. Chiari, P.G. Righetti, P. Ferraboschi, T. Jain and R. Shorr, *Electrophoresis*, 11 (1990) 617.
37 M.P. Bellini and K.L. Manchester, *Electrophoresis*, 19 (1998) 1590.
38 P.G. Righetti, *Immobilized pH Gradients: Theory and Methodology*, Elsevier, Amsterdam, 1990.
39 E. Gianazza and P.G. Righetti, *J. Chromatogr.*, 193 (1980) 1.
40 E. Gianazza, P. Giacon, B. Sahlin and P.G. Righetti, *Electrophoresis*, 6 (1985) 53.
41 G. Cossu and P.G. Righetti, *J. Chromatogr.*, 398 (1987) 211.
42 A.T. Andrews, *Electrophoresis: Theory, Techniques and Biochemical and Clinical Applications*, Clarendon Press, Oxford, 1986, pp. 118–141.
43 G.M. Rothe and W.D. Maurer, in M.J. Dunn (Ed.), *Gel Electrophoresis of Proteins*, Wright, Bristol, 1986, pp. 37–140.
44 G.M. Rothe, in A. Chrambach, M.J. Dunn and B.J. Radola (Eds.), *Advances in Electrophoresis*, VCH, Weinheim, 1991, pp. 252–358.
45 R. Westermeier, *Electrophoresis in Practice*, VCH, Weinheim, 1993, pp. 167–188.
46 Q. Shi and G. Jackowski, in B.D. Hames (Ed.), *Gel Electrophoresis of Proteins: A Practical Approach*, Oxford University Press, Oxford, 1998, pp. 13–34.
47 K.A. Ferguson, *Metabolism*, 13 (1964) 985.
48 R. Pitt-Rivers and F.S.A. Impiombato, *Biochem. J.*, 109 (1968) 825.
49 W.W. Fish, J.A. Reynolds and C. Tanford, *J. Biol. Chem.*, 245 (1970) 5166.
50 J.A. Reynolds and C. Tanford, *Proc. Natl Acad. Sci. USA*, 66 (1970) 1002.
51 J.A. Reynolds and C. Tanford, *J. Biol. Chem.*, 245 (1970) 5161.
52 K. Weber and M. Osborn, *J. Biol. Chem.*, 244 (1969) 4406.
53 A.K. Dunker and R.R. Rueckert, *J. Biol. Chem.*, 244 (1969) 5074.
54 C.A. Nelson, *J. Biol. Chem.*, 246 (1971) 3895.
55 J.P. Segrest, R.L. Jackson, E.P. Andrews and V.T. Marchesi, *Biochem. Biophys. Res. Commun.*, 44 (1971) 390–395.
56 S. Panyim and R. Chalkley, *Arch. Biochem. Biophys.*, 130 (1969) 337.
57 T.V. Waehneldt, *Bio-Systems*, 6 (1975) 176.
58 P. Lambin, *Anal. Biochem.*, 85 (1978) 114.
59 P. Lundahl, E. Greijer, M. Sandberg, S. Cardell and K.O. Eriksson, *Biochim. Biophys. Acta*, 873 (1986) 20.
60 E. Mascher and P. Lundahl, *J. Chromatogr.*, 476 (1989) 147.
61 M. Wallstén and P. Lundahl, *J. Chromatogr.*, 512 (1990) 3.
62 K. Ibel, R.P. May, K. Kirschner, H. Szadkowski, E. Mascher and P. Lundahl, *Eur. J. Biochem.*, 190 (1990) 311.
63 P. Lundahl, Y. Watanabe and T. Takagi, *J. Chromatogr.*, 604 (1992) 95.
64 K. Ibel, R.P. May, M. Sandberg, E. Mascher, E. Greijer and P. Lundahl, *Biophys. Chem.*, 53 (1994) 77.
65 M. Samsò, J.R. Daban, S. Hansen and G.R. Jones, *Eur. J. Biochem.*, 232 (1995) 818.
66 W.H. Westerhuis, J.N. Sturgis and R.A. Niederman, *Anal. Biochem.*, 284 (2000) 143.

67 M. Wyckoff, D. Rodbard and A. Chrambach, *Anal. Biochem.*, 78 (1977) 459.
68 D.M. Neville, *J. Biol. Chem.*, 246 (1971) 6328.
69 U.K. Laemmli, *Nature*, 227 (1970) 680–685.
70 J. Margolis and K.G. Kenrick, *Anal. Biochem.*, 25 (1968) 347.
71 J. Margolis, *Lab. Pract.*, 22 (1973) 107.
72 C. Piubelli, M. Galvani, M. Hamdan, E. Domenici and P.G. Righetti, *Electrophoresis*, 23 (2002) 298.
73 A.T. Andrews, in B.D. Hames (Ed.), *Gel Electrophoresis of Proteins: A Practical Approach*, 3rd Edn. Oxford University Press, Oxford, 1998, pp. 213–235.
74 J.E. Celis, M. Ostergaard, N.A. Jensen, I. Gromova, H.H. Rasmussen and P. Gromov, *FEBS Lett.*, 430 (1998) 64.
75 R.D. Appel, M.J. Dunn and D.F. Hochstrasser (Guest Eds.), Paper Symposium: Biomedicine and Bioinformatics, *Electrophoresis*, 18 (1997) 2703.
76 R.D. Appel, M.J. Dunn and D.F. Hochstrasser (Guest Eds.), Paper Symposium: Biomedicine and Bioinformatics, *Electrophoresis*, 20 (1999) 3481.
77 J.E. Celis (Guest Ed.), Paper Symposium: Protein Databases in Two Dimensional Electrophoresis, *Electrophoresis*, 10 (1989) 71.
78 J.E. Celis (Guest Ed.), Paper Symposium: Cell Biology, *Electrophoresis*, 11 (1990) 189.
79 J.E. Celis (Guest Ed.), Paper Symposium: Two Dimensional Gel Protein Databases, *Electrophoresis*, 11 (1990) 987.
80 J.E. Celis (Guest Ed.), Paper Symposium: Two Dimensional Gel Protein Databases, *Electrophoresis*, 12 (1991) 763.
81 J.E. Celis (Guest Ed.), Paper Symposium: Two Dimensional Gel Protein Databases, *Electrophoresis*, 13 (1992) 891.
82 J.E. Celis (Guest Ed.), Paper Symposium: Electrophoresis in Cancer Research, *Electrophoresis*, 15 (1994) 307.
83 J.E. Celis (Guest Ed.), Paper Symposium: Two Dimensional Gel Protein Databases, *Electrophoresis*, 15 (1994) 1347.
84 J.E. Celis (Guest Ed.), Paper Symposium: Two Dimensional Gel Protein Databases, *Electrophoresis*, 16 (1995) 2175.
85 J.E. Celis (Guest Ed.), Paper Symposium: Two Dimensional Gel Protein Databases, *Electrophoresis*, 17 (1996) 1653.
86 J.E. Celis (Guest Ed.), Genomics and Proteomics of Cancer, *Electrophoresis*, 20 (1999) 223.
87 M.J. Dunn (Guest Ed.), Paper Symposium: Biomedical Applications of Two-Dimensional Gel Electrophoresis, *Electrophoresis*, 12 (1991) 459.
88 M.J. Dunn (Guest Ed.), 2D Electrophoresis: from Protein Maps to Genomes, *Electrophoresis*, 16 (1995) 1077.
89 M.J. Dunn (Guest Ed.), From Protein Maps to Genomes, Proceedings of the Second Siena Two-Dimensional Electrophoresis Meeting, *Electrophoresis*, 18 (1997) 305.
90 M.J. Dunn (Guest Ed.), From Genome to Proteome: Proceedings of the Third Siena Two-Dimensional Electrophoresis Meeting, *Electrophoresis*, 20 (1999) 643.
91 M.J. Dunn (Guest Ed.), Proteomic Reviews, *Electrophoresis*, 21 (2000) 1037.
92 M.J. Dunn (Guest Ed.), From Genome to Proteome: Proceedings of the Fourth Siena Two-Dimensional Electrophoresis Meeting, *Electrophoresis*, 22 (2001) 1591.
93 C. Damerval and D. de Vienne (Guest Eds.), Paper Symposium: Two Dimensional Electrophoresis of Plant Proteins, *Electrophoresis*, 9 (1988) 679.
94 L.A. Huber (Guest Ed.), Paper Symposium: Proteomics of Cell Organelles, *Electrophoresis*, 21 (2000) 3329.

95 I. Humphery-Smith (Guest Ed.), Paper Symposium: Microbial Proteomes, *Electrophoresis*, 18 (1997) 1207.
96 F. Lottspeich (Guest Ed.), Paper Symposium: Electrophoresis and Amino Acid Sequencing, *Electrophoresis*, 17 (1996) 811.
97 B. Tümmler (Guest Ed.), Microbial Genomes: Biology and Technology, *Electrophoresis*, 19 (1998) 467.
98 K.L. Williams (Guest Ed.), Strategies in Proteome Research, *Electrophoresis*, 19 (1998) 1853.
99 M.R. Wilkins, K.L. Williams, R.D. Appel and D.F. Hochstrasser (Eds.), *Proteome Research: New Frontiers in Functional Genomics*, Springer, Berlin, 1997, pp. 1–243.
100 R. Kellner, F. Lottspeich and H.E. Meyer, *Microcharacterization of Proteins*, Wiley-VCH, Weinheim, 1999.
101 T. Rabilloud, *Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods*, Springer, Heidelberg, 2000, pp. 1–248.
102 P.G. Righetti, A. Stoyanov and M. Zhukov, *The Proteome Revisited: Theory and Practice of the Relevant Electrophoretic Steps*, Elsevier, Amsterdam, 2001, pp. 1–400.
103 P. James, *Proteome Research: Mass Spectrometry*, Springer, Berlin, 2001, pp. 1–243.
104 R. Westermeier, *Electrophoresis in Practice*, VCH, Weinheim, 1997, pp. 213–228.
105 S. Hanash, in B.D. Hames (Ed.), *Gel Electrophoresis of Proteins*, Oxford University Press, Oxford, 1998, pp. 189–212.
106 P.H. O'Farrell, *J. Biol. Chem.*, 250 (1975) 4007.
107 J. Klose, *Humangenetik*, 26 (1975) 231.
108 G.A. Scheele, *J. Biol. Chem.*, 250 (1975) 5375.
109 R. Aebersold and J. Leavitt, *Electrophoresis*, 11 (1990) 517.
110 R. Aebersold and J. Leavitt, *Electrophoresis*, 11 (1990) 517.
111 H.W. Lahm and H. Langen, *Electrophoresis*, 21 (2000) 2105.
112 M. Hamdan, M. Galvani, E. Bordini and P.G. Righetti, *Electrophoresis*, 22 (2001) 1633.
113 M. Hamdan, M. Galvani and P.G. Righetti, *Mass Spectrom. Rev.*, 20 (2001) 121.
114 F. Lottspeich, T. Houthaeve and R. Kellner, in R. Kellner, F. Lottspeich and H.E. Meyer (Eds.), *Microcharacterization of Proteins*, Wiley-VCH, Weinheim, 1999, pp. 141–158.
115 R.D. Appel, in M.R. Wilkins, K.L. Williams, R.D. Appel and D.F. Hochstrasser (Eds.), *Proteome Research: New Frontiers in Functional Genomics*, Springer, Berlin, 1997, pp. 149–175.
116 A. Bairoch, in M.R. Wilkins, K.L. Williams, R.D. Appel and D.F. Hochstrasser (Eds.), *Proteome Research: New Frontiers in Functional Genomics*, Springer, Berlin, 1997, pp. 93–148.
117 A. Bairoch and R. Apweiler, *Nucleic Acids Res.*, 28 (2000) 45.
118 R.C. Beavis and D. Fenyö, in W. Blackstock and M. Mann (Eds.), *Proteomics: A Trends Guide*, Elsevier, London, 2000, pp. 22–27.
119 C. Hoogland, J.C. Sanchez, L. Tonella, P.A. Binz, A. Bairoch, D.F. Hochstrasser and R.D. Appel, *Nucleic Acids Res.*, 28 (2000) 286.
120 M.C. Peitsch and N. Guex, in M.R. Wilkins, K.L. Williams, R.D. Appel and D.F. Hochstrasser (Eds.), *Proteome Research: New Frontiers in Functional Genomics*, Springer, Berlin, 1997, pp. 177–186.
121 M.R. Wilkins, E. Gasteiger, J.C. Sanchez, A. Bairoch and D.F. Hochstrasser, *Electrophoresis*, 19 (1998) 1501.
122 M.P. Molloy, *Anal. Biochem.*, 280 (2000) 1.
123 T. Rabilloud and M. Chevallet, in T. Rabilloud (Ed.), *Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods*, Springer, Heidelberg, 2000, pp. 9–29.

124 M.P. Molloy, B.R. Herbert, B.J. Walsh, M.I. Tyler, M. Traini, J.C. Sanchez, D.F. Hochstrasser, K.L. Williams and A.A. Gooley, *Electrophoresis*, 19 (1998) 837.
125 L. Musante, G. Candiano and G.M. Ghiggeri, *J. Chromatogr. A*, 705 (1997) 351.
126 C. Pasquali, I. Fialka and L.A. Huber, *Electrophoresis*, 18 (1997) 2574.
127 T. Rabilloud, C. Adessi, A. Giraudel and J. Lunardi, *Electrophoresis*, 18 (1997) 307.
128 J.A. Gordon and W.P. Jencks, *Biochemistry*, 2 (1963) 47.
129 M.N. Jones, *Int. J. Pharm.*, 177 (1999) 137.
130 D.F. Hochstrasser, M.G. Harrington, A.C. Hochstrasser, M.J. Miller and C.R. Merill, *Anal. Biochem.*, 173 (1988) 424.
131 P. Holloway and P. Arundel, *Anal. Biochem.*, 172 (1988) 8.
132 M.A. Rimpilainen and P.G. Righetti, *Electrophoresis*, 6 (1985) 419.
133 D. Satta, G. Schapira, P. Chafey, P.G. Righetti and J.P. Wahrmann, *J. Chromatogr.*, 299 (1984) 57.
134 K.E. Willard, C. Giometti, N.L. Anderson, T.E. O'Connor and N.G. Anderson, *Anal. Biochem.*, 100 (1979) 289.
135 M.J. Dunn and A.H.M. Burghes, *Electrophoresis*, 4 (1983) 97.
136 E. Gianazza, T. Rabilloud, L. Quaglia, P. Caccia, S. Astrua-Testori, L. Osio, G. Grazioli and P.G. Righetti, *Anal. Biochem.*, 165 (1987) 247.
137 T. Rabilloud, E. Gianazza, N. Cattò and P.G. Righetti, *Anal. Biochem.*, 185 (1990) 94.
138 M. Chevallet, V. Santoni, A. Poinas, D. Rouquie, A. Fuchs, S. Kieffer, M. Rossignol, J. Lunardi, J. Garin and T. Rabilloud, *Electrophoresis*, 19 (1998) 1901.
139 M.P. Molloy, B.R. Herbert, M.B. Slade, T. Rabilloud, A.S. Nouwens, K.L. Williams and A.A. Gooley, *Eur. J. Biochem.*, 267 (2000) 1.
140 V. Santoni, T. Rabilloud, P. Doumas, D. Rouquie, A. Fuchs, S. Kieffer, J. Garin and M. Rossignol, *Electrophoresis*, 20 (1999) 705.
141 V. Santoni, S. Kieffer, D. Desclaux, F. Masson and T. Rabilloud, *Electrophoresis*, 21 (2000) 3329.
142 P.G. Righetti, G. Tudor and E. Gianazza, *J. Biochem. Biophys. Methods*, 6 (1982) 219.
143 U.T. Ruegg and J. Rudinger, *Methods Enzymol.*, 47 (1977) 111.
144 B.R. Herbert, M.P. Molloy, B.A.A. Gooley, J. Walsh, W.G. Bryson and K.L. Williams, *Electrophoresis*, 19 (1998) 845.
145 B. Herbert, M. Galvani, M. Hamdan, E. Olivieri, J. McCarthy, S. Pedersen and P.G. Righetti, *Electrophoresis*, 22 (2001) 2046.
146 M. Galvani, M. Hamdan, B. Herbert and P.G. Righetti, *Electrophoresis*, 22 (2001) 2058.
147 M. Galvani, L. Rovatti, M. Hamdan, B. Herbert and P.G. Righetti, *Electrophoresis*, 22 (2001) 2066.
148 J. Klose and E. Zeindl, *Clin. Chem.*, 30 (1984) 2014.
149 S.E. Moring, in P.G. Righetti (Ed.), *Capillary Electrophoresis in Analytical Biotechnology*, CRC Press, Boca Raton, 1996, pp. 37–60.
150 P.G. Rigetti and F. Nembri, *J. Chromatogr. A*, 772 (1997) 203.
151 A. Bossi and P.G. Righetti, *Electrophoresis*, 18 (1997) 2012.
152 A. Bossi and P.G. Righetti, *J. Chromatogr. A*, 840 (1999) 117.
153 A. Bossi, E. Olivieri, L. Castelletti, C. Gelfi, M. Hamdan and P.G. Righetti, *J. Chromatogr. A*, 853 (1999) 71.
154 E. Olivieri, R. Sebastiano, A. Citterio, C. Gelfi and P.G. Righetti, *J. Chromatogr. A*, 894 (2000) 273.
155 B. Verzola, C. Gelfi and P.G. Righetti, *J. Chromatogr. A*, 868 (2000) 85.
156 B. Verzola, C. Gelfi and P.G. Righetti, *J. Chromatogr. A*, 874 (2000) 293.

157 L. Castelletti, B. Verzola, C. Gelfi, A. Stoyanov and P.G. Rigetti, *J. Chromatogr. A*, 894 (2000) 281.
158 R. Sebastiano, C. Gelfi, P.G. Righetti and A. Citterio, *J. Chromatogr. A*, 894 (2000) 53.
159 R. Sebastiano, C. Gelfi, P.G. Righetti and A. Citterio, *J. Chromatogr. A*, 924 (2001) 71.
160 C. Gelfi, A. Viganò, M. Ripamonti, P.G. Righetti, R. Sebastiano and A. Citterio, *Anal. Chem.*, 73 (2001) 3862.
161 M.A. Cousino, T.B. Jarbawi, H.B. Halsall and W.R. Heineman, *Anal. Chem.*, 69 (1997) A544.
162 A.T. Timperman, K.E. Oldenburg and J.V. Sweedler, *Anal. Chem.*, 67 (1995) 3421.
163 H.A. Bardelmeijer, J.C.M. Waterval, H. Lingeman, R. Van't Hof, A. Bult and W.J.M. Underberg, *Electrophoresis*, 18 (1997) 2214.
164 H.A. Bardelmeijer, H. Lingeman, C. Deruiter and W.J.M. Underberg, *J. Chromatogr. A*, 807 (1998) 3.
165 M.J. Baars and G. Patonay, *Anal. Chem.*, 71 (1999) 667.
166 D.M. Pinto, E.A. Arriaga, D. Craig, G. Angelova, N. Sherma, H. Ahmadzadeh, N.J. Dovichi and C.A. Boulet, *Anal. Chem.*, 69 (1997) 3015.
167 K.B. Tomer, C.E. Parker and L.J. Deterding, in P.G. Righetti (Ed.), *Capillary Electrophoresis in Analytical Biotechnology*, CRC Press, Boca Raton, 1996, pp. 123–153.
168 J.F. Banks, *Electrophoresis*, 18 (1997) 2255.
169 S. Sabah and G.K. Scriba, *J. Pharmaceut. Biomed. Anal.*, 16 (1998) 1089.
170 S. Ridge and K. Hettiarachchi, *J. Chromatogr. A*, 817 (1998) 215.
171 B.Y. Cho, R. Strong, G. Fate and I.S. Krull, *J. Chromatogr. B*, 697 (1997) 163.
172 W. Voelter, J. Schutz, O.E. Tsitsiloni, A. Weiler, G. Grubler, G. Paulus, S. Stoeva and R. Lehmann, *J. Chromatogr. A*, 807 (1998) 135.
173 Q. Yang, L.M. Benson, K.L. Johnson and S. Naylor, *J. Biochem. Biophys. Methods*, 38 (1999) 103.
174 H. Kaijwara and A.M. Hemmings, *Electrophoresis*, 19 (1998) 1270.
175 E.M. Ban, D. Kim, E.A. Yoo and Y.S. Yoo, *Anal. Sci.*, 13 (1997) 489.
176 S.S. Park, W.L. Hung, D.E. Schaufelberger, N.A. Guzman and J.P. Advis, in G.B. Irvine and C.H. Williams (Eds.), *Neuropeptide Protocols*, Humana Press, Totowa, 1997, pp. 101–111.
177 C.P. Chen, D. Jeffery, J.W. Jorgenson, M.A. Moseley and G.M. Pollack, *J. Chromatogr. B*, 697 (1997) 149.
178 M.E. Roche, M.A. Anderson, R.P. Oda, B.L. Riggs, M.A. Strausbauch, R. Okazaki, P.J. Wttstein and J.P. Landers, *Anal. Biochem.*, 258 (1998) 87.
179 H. Kubota, K. Sato, T. Yamada and T. Maitani, *J. Chromatogr. A*, 803 (1998) 315.
180 Z.K. Shihabi and M. Friedberg, *J. Chromatogr. A*, 807 (1998) 129.
181 R.E. Settlage, P.S. Russo, J. Shabanowitz and D.F. Hunt, *J. Microcol. Sep.*, 10 (1998) 281.
182 N.J. Clarke, A.J. Tomlison and S. Naylor, *J. Am. Soc. Mass Spectrom.*, 8 (1997) 743.
183 A.J. Tomlison, L.M. Benson, S. Jameson, D.H. Johnson and S. Naylor, *J. Am. Soc. Mass Spectrom.*, 8 (1997) 15.
184 J.L.E. Reubsaet, J.H. Beijnen, A. Bult, R.J. Vanmaanen, J.A.D. Marchal and W.J.M. Underberg, *J. Pharmaceut. Biomed. Anal.*, 17 (1998) 955.
185 J.L.E. Reubsaet, J.H. Beijnen, A. Bult, R.J. Vanmaanen, J.A.D. Marchal and W.J.M. Underberg, *J. Pharmaceut. Biomed. Anal.*, 17 (1998) 979.
186 M.A. Hoitink, E. Hop, J.H. Beijnen, A. Bult, J.J. Kettenes van-den Bolt and W.J.M. Underberg, *J. Chromatogr. A*, 776 (1997) 319.
187 M. Lai, D. Skanchy, J. Stobaugh and E. Topp, *J. Pharmaceut. Biomed. Anal.*, 18 (1998) 421.

188 S. Vessillier, J. Bernillon, J. Saulnier and J. Wallach, *J. Chromatogr. A*, 776 (1997) 133.
189 J. Jiskra, V. Pacakova, M. Ticha, K. Stulik and T. Barth, *J. Chromatogr. A*, 761 (1997) 285.
190 Y. Boulis, C. Richet, K. Haupt, S. Hennebicq, B. Soudan, D. Tetaert, P. Degand and M.A. Vijayalakshmi, *J. Chromatogr. A*, 805 (1998) 285.
191 J.J. Pesek, M.T. Matyska, S. Swedberg and S. Udivar, *Electrophoresis*, 20 (1999) 2343.
192 L. Capelli, A.V. Stoyanov, H. Wajcman and P.G. Rigetti, *J. Chromatogr. A*, 791 (1997) 313.
193 P.G. Righetti, C. Gelfi, B. Verzola and L. Castelletti, *Electrophoresis*, 22 (2001) 603.
194 V. Kasicka, *Electrophoresis*, 20 (1999) 3084.
195 V. Kasicka, *Electrophoresis*, 22 (2001) 4139.
196 P.G. Righetti, C. Gelfi, A. Bossi, E. Olivieri, L. Castelletti, B. Verzola and A.V. Stoyanov, *Electrophoresis*, 21 (2000) 4046.
197 E. Szoko, *Electrophoresis*, 18 (1997) 74.
198 M.A. Strege and A.L. Lagu, *Electrophoresis*, 18 (1997) 2343.
199 V. Dolnik, *Electrophoresis*, 18 (1997) 2353.
200 A.L. Lagu, *Electrophoresis*, 20 (1999) 3145.
201 K. Ganzler, N.W. Warne and W.S. Hancock, in P.G. Righetti (Ed.), *Capillary Electrophoresis in Analytical Biotechnology*, CRC Press, Boca Raton, 1996, pp. 183–238.
202 C. Chiesa, R.A. O'Neill, C.S. Horvath and P.J. Oefner, in P.G. Righetti (Ed.), *Capillary Electrophoresis in Analytical Biotechnology*, CRC Press, Boca Raton, 1996, pp. 155–182.
203 F.E. Regnier and S. Lin, in M.G. Khaledi (Ed.), *High-Performance Capillary Electrophoresis*, Wiley, New York, 1998, pp. 683–728.
204 R.M. McCormick, in J.P. Landers (Ed.), *Handbook of Capillary Electrophoresis: A Practical Approach*, CRC Press, Boca Raton, 1993, pp. 287–324.
205 R. Palmieri and J.A. Nolan, in J.P. Landers (Ed.), *Handbook of Capillary Electrophoresis: A Practical Approach*, CRC Press, Boca Raton, 1993, pp. 325–368.
206 V. Dolnik and K.M. Hutterer, *Electrophoresis*, 22 (2001) 4163.
207 J.S. Patrick and A.L. Lagu, *Electrophoresis*, 22 (2001) 4179.
208 R.A. Frazier, *Electrophoresis*, 22 (2001) 4197.
209 S.R. Bean and G.L. Lookhart, *Electrophoresis*, 22 (2001) 4207.
210 T.M. McNerney, S.K. Watson, J.H. Sim and R.L. Bridenbaugh, *J. Chromatogr. A*, 744 (1996) 223.
211 V. Klyushnichenko, V. Tishkov and M.R. Kula, *J. Biotechnol. A*, 58 (1997) 187.
212 J.W. Farchaus, W.J. Ribot, S. Jendrek and S.F. Little, *Appl. Environ. Microbiol.*, 64 (1998) 982.
213 T.P. Roddy, T.E. Molnar, R.E. McKean and J.P. Foley, *J. Chromatogr. B*, 695 (1997) 49.
214 K.E. Asermely, C.A. Broomfield, J. Nowakowski, B.C. Courtney and M. Adler, *J. Chromatogr. B*, 695 (1997) 67.
215 S. Kundu, C. Fenters, M. Lopez, A. Varma, J. Brackett, S. Kuemmerle and J.C. Hunt, *J. Capillary Electrophor.*, 4 (1997) 7.
216 K. Kopp, M. Schluter and R.G. Werner, *Arzneimittelforschung*, 46 (1996) 1191.
217 M.H. Goldman, D.C. James, M. Rendall, A.P. Ison, M. Hoare and A.T. Bull, *Biotechnol. Bioeng.*, 60 (1998) 596.
218 H.P. Bietlot and M. Girard, *J. Chromatogr. A*, 759 (1997) 177.
219 O. Nieto, P. Hernandez and L. Hernandez, *Anal. Commun.*, 33 (1996) 425.
220 G.H. Zhou, G.A. Luo, Y. Zhou, K.Y. Zhou, X.D. Zhang and L.Q. Huang, *Electrophoresis*, 19 (1998) 2348.

221 H.J. Boss, D.B. Watson and R.S. Rush, *Electrophoresis*, 19 (1998) 2654.
222 A. Cifuentes, M.V. Moreno-Arribas, M. de Frutos and J.C. Diez-Masa, *J. Chromatogr. A*, 830 (1999) 453.
223 J.M. Thorne, W.K. Goetzinger, A.B. Chen and K.G. Moorhouse, *J. Chromatogr. A*, 744 (1996) 155.
224 K.G. Moorhouse, C.A. Rickel and A.B. Chen, *Electrophoresis*, 17 (1996) 423.
225 P.G. Righetti, E. Gianazza, A.M. Gianni, P. Comi, B. Giglioni, S. Ottolenghi, C. Secchi and L. Rossi-Bernardi, *J. Biochem. Biophys. Methods*, 1 (1979) 47.
226 P. Comi, B. Giglioni, S. Ottolenghi, M.A. Gianni, G. Ricco, U. Mazza, G. Saglio, C. Camaschella, P.G. Pich, E. Gianazza and P.G. Rigetti, *Biochem. Biophys. Res. Commun.*, 87 (1979) 1.
227 A. Saccomani, C. Gelfi, H. Wajcman and P.G. Rigetti, *J. Chromatogr. A*, 832 (1999) 225.
228 T. Hirokawa, T. Tsuyoshi and Y. Kiso, *J. Chromatogr.*, 408 (1987) 27.
229 J. Pospichal, M. Deml and P. Bocek, *J. Chromatogr.*, 390 (1987) 17.
230 M. Polasek, B. Gas, T. Hirokawa and J. Vacik, *J. Chromatogr.*, 596 (1992) 265.
231 J.L. Beckers, T.H.P.M. Verheggen and F.M. Everaerts, *J. Chromatogr.*, 452 (1988) 591.
232 R.E. Offord, *Nature*, 211 (1966) 591.
233 P.D. Grossman, J.C. Colburn and H.K. Lauer, *Anal. Biochem.*, 179 (1989) 28.
234 B.J. Compton and E.A. O'Grady, *Anal. Chem.*, 63 (1991) 2587.
235 B.J. Compton, *J. Chromatogr.*, 559 (1991) 357.
236 E.C. Rickard, M.M. Strohl and R.G. Nielsen, *Anal. Biochem.*, 197 (1991) 197.
237 C.H. Tanford, *Physical Chemistry of Macromolecules*, Wiley, New York, 1961, Ch. 3.
238 N.J. Adamson and E.C. Reynolds, *J. Chromatogr. B*, 699 (1997) 133.
239 F. Kalman, S. Ma, R.O. Fox and C. Horvath, *J. Chromatogr. A*, 705 (1995) 135.
240 M. Castagnola, L. Cassiano, R. Rabino, D.V. Rossetti and F. Andreasi, *Bassi. J. Chromatogr.*, 572 (1991) 51.
241 M. Castagnola, L. Cassiano, I. Messana, G. Nocca, R. Rabino, D.V. Rossetti and B. Giardina, *J. Chromatogr. A*, 656 (1994) 87.
242 M. Castagnola, D.V. Rossetti, M. Corda, M. Pellegrini, F. Misiti, A. Olianas, B. Giardina and I. Messana, *Electrophoresis*, 19 (1998) 2273.
243 M. Castagnola, I. Messana and D.V. Rossetti, in P.G. Righetti (Ed.), *Capillary Electrophoresis in Analytical Biotechnology*, CRC Press, Boca Raton, 1996, pp. 239–275.
244 I. Messana, D.V. Rossetti, L. Cassiano, F. Misiti, B. Giardina and M. Castagnola, *J. Chromatogr. B*, 699 (1997) 149.
245 V.J. Hilser, Jr., G.D. Worosila and S.E. Rudnick, *J. Chromatogr.*, 630 (1993) 329.
246 S. Meyer, A. Jabs, M. Schutkowski and G. Fischer, *Electrophoresis*, 15 (1994) 1151.
247 M.T.W. Hearn, H.H. Keah, R.I. Boysen, I. Messana, F. Misiti, D.V. Rossetti, B. Giardina and M. Castagnola, *Anal. Chem.*, 72 (2000) 1964.
248 V. Sanz-Nebot, F. Benavente, I. Toro and J. Barbosa, *J. Chromatogr. A*, 921 (2001) 69.
249 G.M. Janini, C.J. Metral, H.J. Issaq and G.M. Muschik, *J. Chromatogr. A*, 848 (1999) 417.
250 C.J. Metral, G.M. Janini, G.M. Muschik and H.J. Issaq, *J. High Resolut. Chromatogr.*, 22 (1999) 373.
251 V.J. Hilser, G.D. Worosila and E. Freire, *Anal. Biochem.*, 208 (1993) 125.
252 R.S. Rush, A.S. Cohen and B.L. Mayer, *Anal. Chem.*, 63 (1991) 1346.
253 M.A. Strege and A.L. Lagu, *J. Chromatogr.*, 652 (1993) 179.
254 K.R. Skelsey and M.M. Bushey, *J. Chromatogr. Sci.*, 34 (1996) 85.
255 F. Kilàr and S. Hjertén, *J. Chromatogr.*, 638 (1993) 269.
256 V.J. Hilser and E. Freire, *Anal. Biochem.*, 224 (1995) 465.
257 Z.H. Fan, P.K. Jensen, C.S. Lee and J. King, *J. Chromatogr. A*, 769 (1997) 315.

258 D. Rochu, G. Ducret and P. Masson, *J. Chromatogr. A*, 838 (1999) 157.
259 D. Rochu, G. Ducret, F. Ribes, S. Vanin and P. Masson, *Electrophoresis*, 20 (1999) 1586.
260 D. Rochu, T. Pernet, F. Renault, C. Bon and P. Masson, *J. Chromatogr. A*, 910 (2001) 347.
261 E. Stellwagen, C. Gelfi and P.G. Righetti, *J. Chromatogr. A*, 838 (1999) 131.
262 B. Verzola, F. Chiti, G. Manao and P.G. Righetti, *Anal. Biochem.*, 282 (2000) 239.
263 N.H.H. Heegard, J.W. Sen and M.H. Nissen, *J. Chromatogr. A*, 894 (2000) 319.
264 B. Verzola, F. Fogolari and P.G. Righetti, *Electrophoresis*, 22 (2001) 3728.
265 P.G. Righetti and B. Verzola, *Electrophoresis*, 22 (2001) 2359.
266 D. Rochu and P. Masson, *Electrophoresis*, 23 (2002) 189.
267 S.B. Prusiner, *Proc. Natl Acad. Sci. USA*, 55 (1998) 13363.
268 F. Chiti, N.A.J. van Nuland, N. Taddei, F. Magherini, M. Stefani, G. Ramponi and C.M. Dobson, *Biochemistry*, 37 (1998) 1447.
269 F. Chiti, N. Taddei, N.A.J. van Nuland, F. Magherini, M. Stefani, G. Ramponi and C.M. Dobson, *J. Mol. Biol.*, 283 (1998) 893.
270 I.L. Medintz, B.M. Paegel, R.G. Blazej, C.A. Emrich, L. Berti, J.R. Scherer and R.A. Mathies, *Electrophoresis*, 22 (2001) 3845.
271 B.M. Paegel, C.A. Emrich, G.J. Wedemayer, J.R. Scherer and R.A. Mathies, *Proc. Natl Acad. Sci. USA*, 99 (2002) 574.

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 16

Chromatography of proteins

ALOIS JUNGBAUER and CHRISTINE MACHOLD

CONTENTS

16.1 PROTEINS: STRUCTURE AND FUNCTIONS

16.1.1 Chemical nature

Proteins play a central role in living organisms. They catalyze all kinds of reactions, act as signal transducers, as transport molecules, and as receptors, where they are coupled to signal transducers. They also function as storage molecules and serve as nutrient for an embryo. Furthermore, they function as contractile elements. The cytoskeleton consists of protein fibers; the collagen network of blood clots and spider webs are composed of protein fibrils [1]. Another important function is host defense, performed by antibodies and T-surface proteins. For investigations of the structure/function relationships of proteins their isolation is a prerequisite. Proteins are also used as analytical tools and therapeutic agents; even their use as semiconductors has been considered. Pure proteins may be required in μg-quantities, but sometimes also on the scale of metric tons [2]. Proteins are natural polymers, consisting of 20 different amino acids, which are connected *via* the peptide bond. The same building blocks are used by all living organisms, from Archea to Man. Often selenomethionine is considered as the 21st building block. The building blocks are unequivocally encoded by the DNA sequence. During protein biosynthesis the information in the DNA is translated into a protein sequence by transcription of DNA into RNA and subsequent translation into proteins. Additional variations in the building blocks, caused by processes occurring after translation are called post-translational modification. The protein chain folds into a very complex three-dimensional structure. Restrictions in the rotation around the peptide

bond exclude certain conformations. Under normal conditions, *i.e.*, ambient temperature and physiological salt concentration, a certain conformation is preferred.

The structure of proteins is defined by the primary structure, *i.e.* the amino acid sequence, the secondary structures *i.e.* certain elements forming characteristic structures (*e.g.*, helices, loops, sheets), and the tertiary structure, which is the overall structure of a protein. Attempts have been made to classify proteins according to the three-dimensional structure and the sub-elements forming that structure. Classification according to the three-dimensional structure plays a central role in understanding the basis of protein function, stability, thermodynamical properties, and evolution. Comparison of protein structures can provide functional details, and X-ray crystallography and NMR spectroscopy can present structural information in advance of biochemical characterization. Knowledge of the structure may also provide deeper insight into the processes during chromatography, when proteins are migrating from the liquid phase through the boundary layer into the porous network of chromatographic sorbents. The protein folds are nearly always described in terms of the type and arrangement of secondary structures (*i.e.*, helices and β-strands). Domains divide protein structures into discrete subunits, which are frequently classified separately. The following criteria define domains:

(a) spatially separate regions of protein chains,
(b) sequence and/or structural resemblance to an entire chain from another protein, and
(c) a specific function associated with a region of the protein structure.

Domains need not comprise single contiguous segments of the polypeptide chain. Classical assignment is usually for domains with predominantly α-helices or β-sheets. They are classified as All-α or All-β. Small elements of secondary structures, such as 3_{10} helices or small β-hairpins are usually ignored in assignment. Protein domains that contain a mixture of α and β are more difficult to classify. α/β proteins are those containing both α-helices and β-sheets. In contrast, α + β define those consisting of segregated regions of helices and sheets.

Numerous databases of structural classification are available, *e.g.*, the Structural Classification of Proteins (SCOP) database and the CATH (Class Architecture Topology Homology) database. It is not yet possible to draw conclusions from similarities in three-dimensional architecture about chromatographic behavior. One reason is that proteins bind to the surface in a highly oriented manner. Often, a certain domain interacts with the surface, *e.g.*, in β-lactoglobulin, which has been studied in detail regarding retention on ion exchangers [3,4]. The two isoforms differ in only one amino acid. The domain containing the variant interacts with the ion-exchange surface, and therefore, both isoforms can be easily separated. The trivial assumption that the average surface charge difference is responsible for the separation of proteins in ion-exchange chromatography cannot explain this property. Databases that link structural homologies or analogies are important. An example is the purification of proteins containing zinc fingers. This structural element is responsible for interaction with DNA. Cibachrom Blue mimicks DNA and has been successfully applied as ligand for DNA-binding proteins. Searching for structural elements with affinity to certain ligands may facilitate the development of a separation process for an unknown protein.

16.1.2 Physical state

The general perception that proteins in a cell are either present in free solution or bound to membranes is not entirely correct. Recent studies have shown that proteins form complex networks in the cell [5,6]. One of the primary methodologies for the large-scale analysis of protein interactions has been the development of the yeast two-hybrid system [7]. This method indicates protein interaction by simple measurement of growth of yeast colonies on a plate. The first large-scale two-hybrid data were generated for the T7 bacteriophage [8], *Caenorhabditis elegans* [9], and *Saccharomyces cerevisiae* [10]. In nature, proteins are not single molecules in solution, and in the process of purification the network is disrupted and their conformation may change. Proteins may start to aggregate and consequently lose their biological activity. Steroid hormone receptors are an example of a class of proteins that are extremely difficult to purify. These important proteins, responsible for the biological response of steroid hormones, are found in the cytosol in an inactive form [11]. For example, the estrogen receptor α is associated with hsp 90 and hsp 70, while both proteins are held together by p60. Upon addition of a hormone, such as 17β-estradiol, the accompanying proteins are shed off, the receptor dimerizes and travels into the cell nucleus, where it binds to the hormone response element and associates with proteins of the general transcriptions complex in order to transact the expression of a gene which is under the control of the hormone response element [12]. In addition, the receptor also interacts with repressor proteins. When such a receptor is extracted from a cell, either in liganded or unliganded form, the protein complex must be disrupted, and this will affect the stability of the receptor proteins [13]. Several additives have been tested to partially substitute for the protective function of the proteins in the complex, but the conditions during chromatographic separation are an artificial environment for the proteins. In the case of membrane-bound receptors, once the lipid bilayer is removed from the protein, the transmembrane domain immediately starts to aggregate. Addition of detergents can prevent this process to some extent.

When recombinant proteins are efficiently expressed in high concentration, they are deposited as inclusion bodies, also called refractile bodies [14]. In inclusion bodies proteins are present in paracrystalline form in a non-native conformation. For protein purification the inclusion bodies must be dissolved by a chaotropic agent or a detergent. In order to regain its native structure, the dissolving agents must be removed, and then the native protein can be further purified by chromatographic methods. Another interesting physical state of proteins is the biological liquid crystal elastomer [15]. There is evidence that the proteins of silk and of spider web are assembled in such a way that they form liquid crystals. A number of dray line silks from the most primitive spiders to advanced orb web spinners have now been partially sequenced [16]. Their principal fibrous proteins, spidroins, like the analogous silkworm fibroins, are largely constructed from highly repetitive block co-polymers showing a regular alteration of hydrophobic and less hydrophobic blocks [15]. In order to separate fibroins or similar proteins by chromatographic methods they must be dissolved in chaotropic agents or detergents.

Proteins form colloidal dispersions, which are composed of particles ranging in size from 1 μm to 1 nm. The radius of an average protein is between 1 and 10 nm [17]. The enormous surface area per unit volume means that surface behavior is a powerful and

frequently dominant property of these systems. Colloids are categorized as either lyophilic or lyophobic. Lyophobic colloids form sols and lyophilic colloids form gels. The cytosolic dispersion and suspensions of many cells have properties similar to those of sols. Typical properties of colloids are scattering of light and migration in the electrical and gravitational fields. Colloids may be very viscous; even a small amount of protein has a significant effect on viscosity. The viscosity of a protein solution is also dependent on the shape of molecule. Globular proteins have a much lower viscosity than fibrous ones. Upon denaturation of a protein its intrinsic viscosity increases (Table 16.1). This effect can

TABLE 16.1

INTRINSIC VISCOSITIES OF A VARIETY OF MACROMOLECULES

Protein	Intrinsic viscosity (C per g)	Ref.
Ovalbumin, native	0.002	[18]
Ovalbumin, heated with mercaptoethanol	0.0449	[18]
Human serum albumin, native	0.0022	[18]
Human serum albumin, heated with mercaptoethanol	0.0296	[18]
Human serum albumin, native	0.0037	[19]
Human serum albumin, random coil	0.052	[19]
Human IgG	0.00206	[19]
Bovine IgG	0.00259	[19]
Fibrinogen	0.027	[19]
Myosin	0.217	[19]

be used for studying conformational changes of proteins. Fully denatured serum albumin has a 13-fold higher intrinsic viscosity than the native protein [18]. The intrinsic viscosity can easily be determined by chromatography, using a continuous differential viscometer [18]. The differential viscometer is very sensitive and provides accurate measurements of minor changes in viscosities of very dilute protein solutions undergoing denaturation. The advantage of using the differential viscometer instead of the conventional glass capillary viscometer is the increased sensitivity, precision, speed and operational ease that permits measurements of solution viscosity of low sample concentrations (up to 1.2 μg of pure proteins). For high protein concentrations other methods must be applied to assess viscosity. While extrapolation from the intrinsic viscosity of low concentrations to extremely high concentrations is not possible, this can be done generally in protein chromatography. Viscosity has a significant effect on chromatographic performance. The diffusion of macromolecules is reduced by high viscosity, and mass transfer is decreased by increasing viscosity.

Weak protein interactions can be characterized in terms of the osmotic second viral coefficient (B_{22}), which is also a measure of the solubility of a protein [20]. Traditional methods for measuring B_{22}, such as static light scattering, often require too much time and protein sample. In *self-interaction chromatography* (SIC), a protein is immobilized on a

stationary phase, and the relative retention of the protein reflects the average protein interaction, which has been related to the second viral coefficient *via* statistical mechanics. The method holds significant promise for the characterization of protein interactions. It requires only commonly available laboratory equipment, little specialized expertise, and not much material. The second viral coefficient correlates well with static light scattering, and this is a valuable method for the determination of solubility and for optimization of phase systems in crystallography or formulation of protein drugs [21]. It can be a substitute for membrane osmometry and light-scattering measurements.

16.2 MICROHETEROGENEITY

16.2.1 Mutations, deletions, and splicing variants

In microorganisms proteins are not always expressed as a single polypeptide chain encoded by a single gene. Many similar proteins with the same function are often observed. Such a heterogeneous population of proteins consists of a number of variants. Although the protein variants may be encoded by the same gene, splicing of RNA leads to translation of different forms, all of which may have biological activity. An example is human growth hormone (hGH), which is present in eight different variants. Several isoforms of growth hormone have been identified in humans [22]. There are many reasons for this heterogeneity. At the genetic level, two genes encode GH: GH-N, expressed in the pituitary, and GH-V, expressed in the placenta. At the mRNA level, GH-N undergoes alternative splicing into 20K and 22K isoforms. Post-translationally, 22K GH undergoes modifications, such as acetylation at its amino terminus, deamidation, and oligomerization. The picture is complicated further in the circulation, where GH binds to two GH-binding proteins, each with different affinities for the GH isoforms. In addition, a highly heterogeneous mixture of GH fragments has been demonstrated [22]. When chromatographic methods are used to purify or detect proteins form natural sources, the heterogeneity of proteins must be taken into account. Splicing variants are often observed in malignant tissues. Splicing or mutation may lead to an altered biological function or even to a loss of activity [23].

Often proteins are expressed by a family of genes located at different sites on the chromosome or at different chromosomes. Such protein families have often been observed in plants [24]. The heterogeneity of proteins can be a normal phenomenon or be an indication of a malignancy or defect in the cells. Either the DNA is affected or the assembly of the mRNA (RNA splicing) is responsible. Chromatographic methods are important tools for the preparation of protein variants. Large deletions facilitate the chromatographic separation, since they change size and shape. Proteins with a small mutation may be difficult to separate, unless it results in a different property of the surface or in a different conformation. Modern genetic techniques, such as real-time polymerase chain reaction (RT-PCR) or RNA chip technology often point to a splicing variant, which can be further investigated later on by other techniques and biological assays. For that purpose small preparations (often less than 10 mg) are required. Chromatography is perfectly suited for such a task.

16.2.2 Post-translational modifications

After the mRNA is translated into the polypeptide chain, the nascent protein can be further modified. This happens either during transport into the different compartments of the cell or upon its pre-destined function. Glycosylation is a typical example of post-translational modification during transport through the Golgi apparatus. Many proteins are activated through phosphorylation by kinases. Post-translational modifications significantly affect the surface properties of a protein, and thus, the chromatographic behavior is also changed. Either the charge is modified when Asp, Glu, or His are involved or the protein may become more hydrophobic when a lipid is added. Some moieties, such as neuraminic acid, are highly charged. Table 16.2, presents a summary of post-translational modifications, including their mass differences and chromatographic behavior. Protein chromatography plays a major role in the detection and study of post-translational modifications. Proteins with the same peptide backbone but different side-chain modifications are called *isoforms*. Often it is impossible to resolve isoforms completely, even with elaborate gradients [25] and separation methods [26]. For many purposes a fingerprint may be sufficient. Chromatographic methods are often preferred over electrokinetic methods, such as capillary electrophoresis or isoelectric focusing, since they are more robust and less sensitive to sample-matrix variations. For preparative separations, chromatographic methods are indispensable. Preparative electrokinetic methods are not so popular, although they may function as alternative methods [27–29].

A good example of the effect of post-translational modification on protein function is *erythropoietin* (EPO). EPO is the main regulator of erythropoiesis. It is a glycoprotein, which is produced primarily in the kidney of adult mammals. The protein acts on bone marrow erythroid progenitor cells to promote development into mature red blood cells. The relative molecular mass of EPO ranges from 34,000–40,000; 40% of this mass is attributable to the carbohydrate structure [30]. The molecule has a peptide backbone of 165 amino acids with a pI of 4.5–5.0 and contains two types of carbohydrates, three *N*-linked complex polysaccharides antennaries and one *O*-linked polysaccharide chain. The efficacy of EPO resides in the extensive sialization at the nonreducing ends of the carbohydrate branch. There is a significant difference between the isoelectric focusing patterns of EPO in urine and in recombinant EPO. A novel generation of rEPO, named darbepoetin [31], has a very closely related pattern, due to only a small mutation in the amino acid backbone. The novel form exerts a much higher biological activity [32]. Electrokinetic methods, such as isoelectric focusing (IEF) and capillary electrophoresis are very popular for characterization of EPO [31,33,34]. In a highly purified EPO preparation, more than fourteen isoforms could be resolved by IEF [35]. Seven fractions of decreasing isoelectric point could be also obtained by DEAE-Sephacel chromatography (Fig. 16.1). The specific activity of the isoforms was different. The isoforms with low and high isoelectric point have significantly lower ($<50\%$) activity than isoforms with pI between 4.1 and 4.8. The composition may play a pivotal role in the biological activity of glycoproteins. The separation method definitely influences the isoform composition, and thus efficacy may vary among different preparations.

Antibodies are glycoproteins secreted by B-lymphocytes. They are the major proteins for host defense. *Immunoglobulins* are divided into five classes, IgG, IgA, IgM, IgD, and IgE.

TABLE 16.2

SELECTION OF FREQUENT POST-TRANSLATIONAL MODIFICATIONS AND THEIR CHROMATOGRAPHIC BEHAVIOR. ↓ DECREASED RETENTION, ↑ INCREASED RETENTION

Modification	Average mass difference	Effect on chromatographic retention
Acetylation	42.0373	Minor for large proteins
Amidation	−0.9847	↓ in IEC
β-Methylthiolation	46.08688	↑ RP-LC, HIC
Biotinylation	226.2934	AC with streptavidin
Carbamylation	43.02502	↑ RP-LC, HIC
Citrullination	0.98476	↑ RP-LC, HIC
C-Mannosylation	162.1424	Difficult to separate
Deamidation	0.9847	↑ IEC
N-Acyl diglyceride cysteine (tripalmitate)	789.3202	↑ RP-LC, HIC
Dimethylation	28.0538	↑ RP-LC, HIC
Flavine adenine dinucleotide	783.1415	↑ RP-LC, HIC
Farnesylation	204.3556	↑ RP-LC, HIC
Formylation	28.0104	Difficult to separate
Geranyl-geranyl	272.4741	↑ RP-LC, HIC
γ-Carboxyglutamic acid	44.0098	↑ IEC
O(*N*-acetyl)-D-glucosamine	203.1950	Difficult to separate
Glucosylation (glycation)	162.1424	AC with boronate
Hydroxylation	15.9994	Difficult to separate
Lipoyl	88.3027	↑ RP-LC, HIC
Methylation	14.0269	↑ RP-LC, HIC
Myristoylation	210.3598	↑ RP-LC, HIC
S-Nitrosylation	28.99816	↑ RP-LC, HIC
n-Octanoate	126.1986	↑ RP-LC, HIC
Palmitoylation	238.4136	↑ RP-LC, HIC
Phosphorylation	79.9799	↑ IEC
Pyridoxal phosphate	229.129	↑ IEC
Phosphopantetheine	339.3234	↑ RP-LC, HIC
Pyrrolidone carboxylic acid	−17.0306	↑ IEC
Sulfation	80.0642	↑ IEC
Trimethylation	42.0807	↑ RP-LC, HIC

The structure common to all antibodies consists of two identical heavy and two identical light chains. These chains are linked *via* disulfide bonds. The polypeptide chains of the light and heavy chain have variable (V_L, V_H) and constant regions (C_L, C_H). Specific enzymatic cleavage of the antibody molecule by papain results in a F_{ab} and

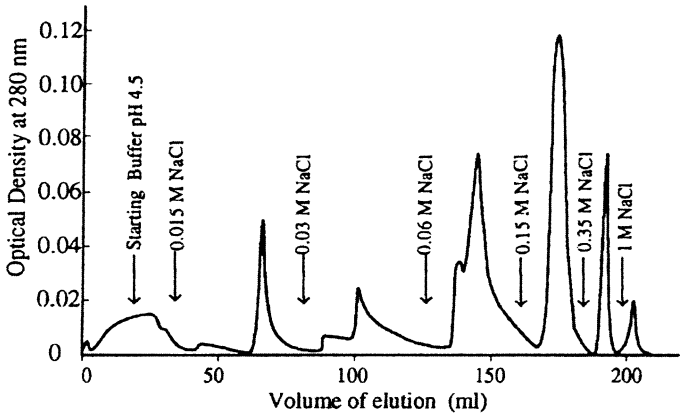


Fig. 16.1. Elution profile of rhEPO, obtained with a stepwise gradient of NaCl (from 0.015 to 1 *M*) after adsorption on DEAE-Sephacel. The material was dialyzed against a 0.04 *M* acetic acid buffer (pH 4.5), containing 2.5 m*M* $CaCl_2$. The column (8.9 × 1.0 cm ID) was run at a flow-rate of 30 mL/h. (Reproduced from Ref. 35 with permission.)

a F_c part. The F_{ab} part (antigen binding) contains the variable region of the light and heavy chain. The actual immune response is triggered by the F_c part upon binding to different receptors. As a consequence, the complement cascade is activated or pathogens are opsonized. The genetically determined glycosylation site for the effector functions is located in the F_c-part. The glycosylation heterogeneity has often been considered responsible for increased or decreased activity. Moreover, glycosylation determines the biological half-life of a glycoprotein. Heterogeneous antibodies, formed by assembly of chains with different length (disulfide heterogeneity) [36], glycosylation of the F_{ab} and the F_c fragment, deamidation, phosphorylation, and sulfatation [37,38], have also been observed. The targeted production of aglycosyl proteins [39] permits a study of the biological significance of glycosylation. One of the effects is the reduced circulation of antibodies when the glycan structure is absent or impaired. Nose and Wigzell [40] have shown that antibodies produced in the presence of tunicamycin, an antibiotic specifically preventing *N*-glycosylation, did show antigen binding, but lost their effector function, namely the ability to activate complement, induce antibody-dependent cellular cytotoxicity (ADCC) and interaction with the F_c receptor of macrophages. Interestingly, these antibodies maintained the ability to bind to the cell-wall protein, staphylococcal Protein A. This means that chromatographic purification of antibodies by Protein A affinity chromatography does not guarantee functional antibodies, since an intact glycan structure is not required for the interaction between the immunoglobulin molecule and staphylococcal Protein A.

16.2.3 Microheterogeneity due to biological processing

Burton [41] has demonstrated that glycosidases and glycosyltransferases have defined specifities. He showed that external conditions, such as the culture medium, and not

non-specific enzyme reactions are responsible for aberrant glycosylation. Glucose deficiency leads to two aberrant glycosylation patterns [42]: addition of an incomplete oligosaccharide precursor and general absence of oligosaccharides at the respective Asn.

Maiorella *et al.* [43] have investigated the influence of different production systems on IgM antibodies, expressed in Chinese hamster ovarian (CHO) cells. When the airlift bioreactor, perfusion bioreactor, and *in vivo* ascites culture were compared, significant differences in glycosylation patterns were found. In chromatographic purification based on ion-exchange chromatography, altered glycosylation may significantly affect binding capacity and resolution. Influence of culture conditions on glycosylation has been also observed with interferon-γ [44] and kallikrein [45]. Wang *et al.* [45] have used hydrophilic-interaction chromatography to separate the tryptic glycopeptides of human interferon. Further characterization was performed by mass spectrometry. James *et al.* [46] separated the isoforms of interferon-γ by cation-exchange chromatography on MonoS (Fig. 16.2), using a linear salt gradient in the presence of 8 *M* urea. Further

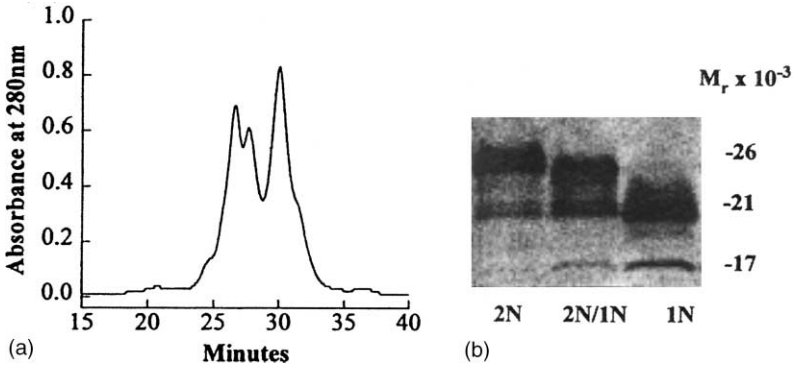


Fig. 16.2. Separation of site-occupancy variants of INF-γ produced in CHO cells by cation-exchange chromatography. (A) Elution profile from Mono S cation exchanger. INF-γ was loaded in 50 m*M* MES, 8 *M* urea (pH 6.5). Elution was effected by a linear salt gradient from 0–0.15 *M* NaCl. (B) Silver-stained SDS-PAGE of peak fractions. 2N, IFN-γ glycosylated at Asn_{25} and Asn_{97}; 2N/1N, IFN-γ glycosylated at both sites and at Asn_{97} only; 1N, IFN-γ glycosylated predominantly at Asn_{97}. (Reproduced from Ref. 44 with permission.)

analysis of the peaks by SDS-PAGE showed three fractions, doubly glycosylated 2N IFNγ (26 kDa), a mixture of 2N and 1N IFNγ (21–26 kDa), and 1N IFNγ (21 kDa). This separation is based upon the difference in sialic acid content of site-occupancy variants; 2N variants are associated with more negatively charged sialic acid than 1N variants. Recent reports indicate that humanized antibody production can be scaled up from 20 to 10,000 L while maintaining the same glycosylation pattern [47]. It seems that under controlled scale-up conditions consistent post-translational modifications can readily be managed.

Cells secrete proteases and glycosidases into the culture medium and may thus modify proteins. Karl *et al.* [48] found an acid protease in the cell-free supernatant of a hybridoma culture. The enzyme had a selectivity similar to a lysosomal aspartate protease and could

be inhibited by pepstatin A. This enzyme extensively degrades albumin and transferrin during short incubations at pH 3 and below. Limited proteolysis of the antibody appears to occur in the culture supernatant. Proteolysis is enhanced at low pH in the presence of urea or 1 *M* acetic acid. This may be critical when the proteinase is not removed, and an acid elution step is used. The proteinase activity accumulates in continuous perfusion and total cell recycle cultures, beginning during exponential growth of the hybridoma. Culture supernatant of cell-free insect cultures contain significant amounts of exoglycosidase activity [49].

C-terminal Lys or Arg residues, whose presence is expected form gene sequence information, are often absent from proteins isolated from mammalian cell culture. The activity of one or more carboxypeptidase is responsible for C-terminal processing [50]. Cation-exchange chromatography of three lots of a recombinant human antibody (HER 2) is shown in Fig. 16.3. In order to improve resolution, the separation was

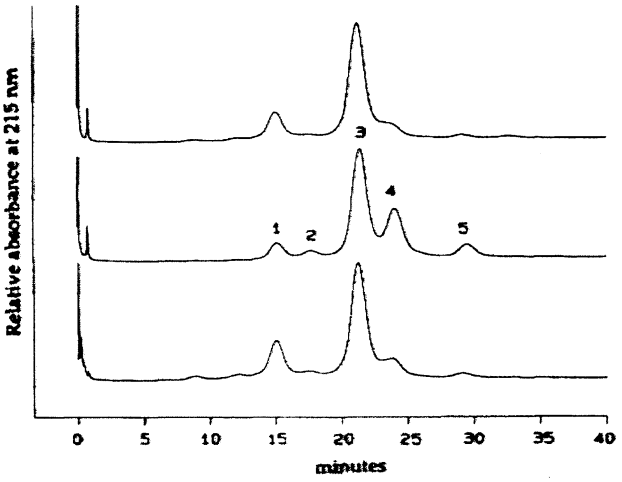


Fig. 16.3. Cation-exchange chromatography of three lots of rH monoclonal antibody HER2. A Mono S (Amersham Biosciences) column (50 mm × 5 mm ID) was equilibrated with 95% Solvent A (20 m*M* sodium phosphate, pH 6.9) and 5% Solvent B (Solvent A + 100 m*M* NaCl) at 40°C at a rate of 76 cm/h. A gradient from 5% to 40% Solvent B was developed within 40 min. Samples of 72 μg of antibody were injected.

performed at 40°C. Variant #2 was not found in one lot. Tryptic peptide maps of two CHO-expressed forms of tissue plasminogen activator are shown in Fig. 16.4. In the two-chain form Arg_{275} is cleaved off, while in the single-chain form this residue cannot be attacked by the carboxypeptidase [50]. Different cleavages of the single chain have been observed with different host cells. While Bowes melanoma cell cleaved the single-chain t-PA, CHO-expressed t-PA was maintained as intact molecule. Further examples of processing C-terminal lysine and arginine residues may be found in the review by Harris [50].

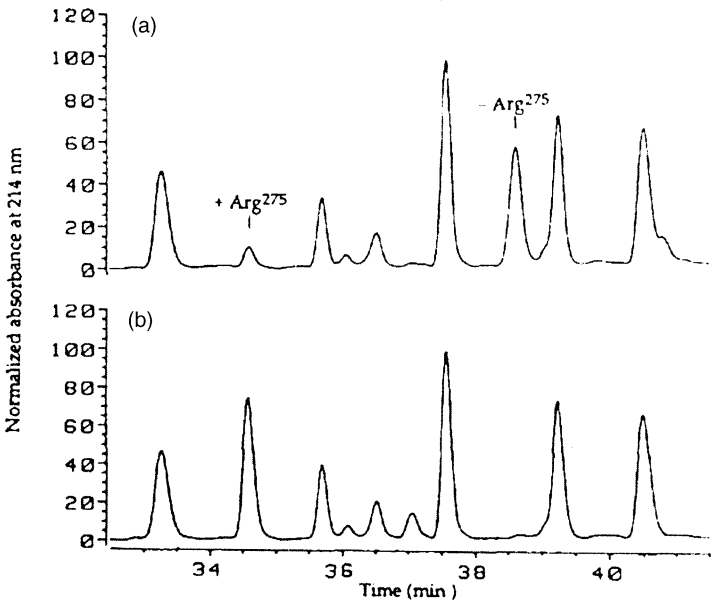


Fig. 16.4. Comparison of tryptic maps of single-chain and two-chain CHO-expressed tPA. (A) Two-chain t-PA digest. (B) Single-chain t-PA. The peaks marked + Arg^{275} and − Arg^{275} contain residues 268–275 (QYSQPQFR) and 268–274 (QYSQPQF), respectively. (Reproduced from Ref. 50 with permission.)

16.2.4 De-amidation

De-amidation is the cleavage of an amide residue in the amino acids Glu and Asn. De-amidation of Asn is pH-dependent and much faster than de-amidation of Gln. In neutral or alkaline media, the de-amidation reaction runs through a cyclic imide as intermediate. The intermediate undergoes spontaneous hydrolysis to Asp and iso-Asp at a ratio of 1:3. Iso-Asp is linked to the next amino acid *via* a γ-carboxyl group. As a side reaction, a D-cyclic amide can be produced by racemization, and a D-aspartyl peptide can be also formed. De-amidation is extremely active in neutral medium when Asp is followed by Gly. Amino acids with hydrophobic character have stabilizing activity. In acidic medium, Asn is directly hydrolyzed to Asp. Amino-terminal Gln can spontaneously cyclize to pyrrolidone carboxylic acid. Many proteins undergo spontaneous de-amidation *in vivo*. Since de-amidation is strongly dependent on the sequence [51], it has been suggested that during evolution sequences adjacent to Gln and Asn have developed and may act as a biological clock [52].

The main reason for microheterogeneity of non-glycosylated proteins is de-amidation. Upon de-amidation, an additional change is introduced into the sequence. De-amidation is always accompanied by a change in the primary structure. Reduction of biological activity as a consequence of de-amidation of Asn has been reported for the adrenocorticotrope hormone [53], calmodulin [54] and human growth hormone (Table 16.2) [55].

De-amidation of Asn to Asp results in the loss of only *ca.* one mass unit but one additional charge is gained, which is useful for the chromatographic separation of isoforms. For consistent determination of de-amidation, sequencing of the protein is required. For the separation of de-amidated proteins electrokinetic methods are preferred, but for preparative purposes chromatographic methods are best. Ion-exchange chromatography can be used for analytical as well as preparative purposes. Since a high resolution is required, a particle size of < 15 μm is strongly recommended. Another type of protein isoform is produced by the de-imination of arginine to citrulline [56]. This has also an effect on protein charge and changes the primary sequence.

16.2.5 Oxidation

Proteins may be oxidized during cultivation or downstream processing. This affects mainly methionine, which is oxidized to methionine sulfoxide. It has been shown that oxidation occurs in the periplasmatic space of *E. coli* cells or intracellularly during fermentation [57]. Recombinant human cystatin C, a cystein protease inhibitor, was oxidized only at positions Met_{14} and Met_{41}, whereas Met_{110} was not affected, indicating a structural preference in oxidation for certain residues. Aerobic growth conditions and a different purification procedure prevented oxidation of Met residues. Un-oxidized cystatin C could be purified by a papain Sepharose. Before immobilization, papain was inactivated by 1-{*N*-[(L-3-*trans*-carboxyoxirone-*Z*-carbonyl)-L-leucyl]amino}-4-guanidinobutane, called E64. When papain was inactivated with *S*-(*N*-ethylsuccinimide), the two variants could not be distinguished. NMR studies corroborated that mono-oxidized cystatin C did not change conformation, in contrast to the form oxidized at positions Met_{14} and Met_{41}. This example demonstrates that oxidation of proteins can be assessed by chromatographic means. It is possible to separate oxidized from native forms and multiply oxidized forms. Oxidation of methionine has been observed not only in *E. coli* but also in all kind of host cells, such as yeast [58,59] and insect [60] cells. Others have reported that oxidation did affect the quality of the recombinant protein [61].

Oxidation also occurs during the storage of proteins. For instance, the recombinant humanized monoclonal antibody HER2 in liquid formulations undergoes oxidation when exposed to intense light and elevated temperature (30°C and 40°C) [62]. Met_{255} in the heavy chain of the F_c region of an antibody is the primary site of oxidation. Met_{431} of the F_c fragment can also be oxidized under extreme conditions. The amount of oxidation was determined by cleaving the F_{ab} and F_c fragments by papain digestion, and the oxidized F_c fragment was detected by hydrophobic-interaction chromatography (Fig. 16.5).

Antioxidants, such as methionine, sodium thiosulfate, catalase, or platinum prevented Met oxidation in rhuMAb HER2, presumably as free radicals or oxygen scavengers. A thiosulfate adduct of rhuMAb HER2 was observed by cation-exchange chromatography. These studies demonstrate that stoichiometric amounts of methionine and thiosulfate are sufficient to prevent temperature-induced oxidation of rh monoclonal antibody HER2, due to free radicals, generated by the presence of metal ion and peroxide impurities in the formulation. One strategy for avoiding protein variants due to oxidative

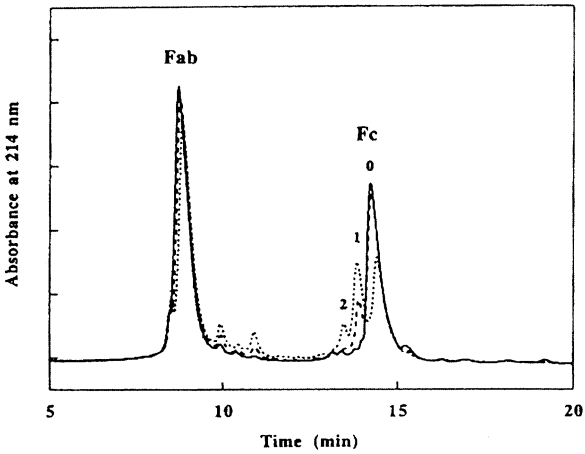


Fig. 16.5. Separation of oxidized F_{ab}-chains by hydrophobic-interaction chromatography, showing the effect of temperature on oxidation of the two methionine residues in the F_c domain of rh monoclonal antibody HER2. Samples were incubated at 5°C (solid line), 30°C (dashed line), and 40°C (dotted line) for two weeks. The three main peaks of the rh monoclonal antibody HER2 F_c domain are non-oxidized F_c (Peak 0), F_c fragment with oxidized Met_{255} (Peak 1), and F_c fragment with oxidized Met_{255} and Met_{431} (Peak 2). After incubation, the 5, 30, and 40°C samples contained 10, 17, and 52% F_c (Peaks 1 and 2), respectively. Samples were digested with carboxypeptidase B, followed by papain. Digested samples were injected into a TSK Butyl NPR column (35 × 4.6 mm ID) from Toso Biosciences and eluted at 0.5 mL/min with a mobile phase consisting of Buffer A (20 m*M* Tris pH 7.0) and Buffer B (2 *M* ammonium sulfate in Buffer A). A linear gradient was formed from 10% to 100% buffer over 37 min at room temperature. (Reproduced from Ref. 62 with permission.)

stress is site-specific mutation of the affected Met [60]. This is not always feasible; therefore appropriate scavengers must be added and separation methods must be developed to separate the oxidized form from the native antibodies.

16.2.6 Expression of proteins in various host cells

Recombinant DNA technology enables access to proteins of low abundance, proteins from human sources, and chimeric proteins. The quality of the heterologous protein is determined by the host cell, the expression system, and type of production. The DNA sequence encoding the desired protein is either inserted into an expression plasmid, integrated into the chromosome of the expression cell, or integrated into a virus genome. There are three possibilities of expression of heterologous proteins: constitutive expression, transient expression, or induction. The cultivation of the host cell can be either batchwise or continuous.

For simple proteins, *E. coli* is still the preferred host [63–65]. Proteins are either secreted in the periplasmatic space or they remain in the cytosol. Often they are deposited

as inclusion bodies [14,66,67]. The general strategy for recovery of active proteins from inclusion bodies involves cell lysis, extraction and washing of inclusion bodies, solubilization of inclusion bodies, and, finally, refolding of the protein into its native conformation [68–70]. After dissolution of inclusion bodies in a buffer containing strong chaotropic agents, such as 8 *M* urea or 6 *M* guanidine hydrochloride, reducing agents, such as dithiothreitol or β-mercaptoethanol, are added to reduce all disulfide bonds. Then, the denatured protein is transferred to a non-denaturating environment in order to shift the folding equilibrium towards its native conformation. This is normally achieved by dilution or dialysis [71]. To prevent aggregation, refolding is usually performed at low protein concentrations (10 to 100 μg/mL) [72,73]. Refolding of recombinant proteins, expressed in *E. coli* is still a bottleneck on an industrial scale, often requiring additional, large refolding tanks. The refolding process is low in productivity, and the yield of native protein is usually low.

In another approach, the *in vivo* folding pathway was emulated by adding chaperones or molecular mimicries of chaperones (minichaperones) [74,75] and enzymes catalyzing disulfide-bond formation or *cis*/*trans*-peptide isomerization in the folding process. These compounds have also been immobilized on a solid phase, and the folding reaction was performed in a packed-bed reactor or stirred tank. While denatured protein passes through the column, it is separated from chaotropic agents and starts to refold, while aggregation is reduced due to interchanges with the immobilized proteins [76,77]. Folding-helper proteins act in a stoichiometric manner, requiring almost the same amount of helper proteins as product. ATP is an essential component of such a refolding system. Thus, this is a very costly strategy, and it is not currently being applied on an industrial scale. Yeast serves as another simple expression system. *Saccharomyces cerevisiae* [78], *Pichia pastoris* [79], and *Hanulea polymorpha* [80,81] are popular host cells. Glycosylation in yeast is markedly different from that in mammalian cells. Despite this difference, recombinant proteins from yeast are successfully used as therapeutic proteins. Nowadays, glycosylation patterns can be controlled by glycoengineering [78]. Comparisons have been made by James *et al.* [46], studying INF-γ, and by Werner *et al.* [82], studying s-ICAM and t-PA.

Proteins can also be produced by transgenic animals [82–86] or transgenic plants [87–89]. These expression systems show post-translational modifications that are different from those in humans. The structures of the *N*-linked glycans, attached to the heavy chains of the monoclonal antibodies in transgenic tobacco plants, are somewhat different. Plantibody has glycans at both *N*-glycosylation sites located on the heavy chain. However, the number of glycoforms is higher in the plant than in the mammalian-expressed antibodies. In addition to high-mannose-type *N*-glycans, 60% of the oligosaccharides *N*-linked to the plantibody have β(1,2)-xylose and α(1,3)-fucose residues linked to the core Man3GlcNAc2. These oligosaccharide linkages, not found on mammalian *N*-linked glycans, are potentially immunogenic. A chromatographic separation process developed for a recombinant protein from a certain host cell must be transferred with caution to a process for the same protein but different host cell. Chromatographic retention will be altered, probably due to different post-translational modification.

16.2.7 Non-enzymatic glycation

Non-enzymatic glycation of proteins was first discovered in human hemoglobin and is now well known for many other proteins. It is believed to impair the biological activity of immunoglobulins [90]. Non-enzymatic glycation can also occur *in vitro* during storage of proteins in the presence of high concentrations of sugar [91]. It is another source of microheterogeneity of proteins. For example, non-enzymatic glycation of human Cu/Zn superoxide dismutase leads to partial inactivation of the enzyme [92]. The non-glycated form has been isolated by boronate affinity chromatography, and incubation with deuterated glucose *in vitro* resulted in gradual accumulation of radioactivity in the enzyme. The sugar is bound through a Schiff base [93]. An increase in the glycation of superoxide dismutase was observed in aged erythrocytes, indicating that the glycation reaction is an age-related change under physiological conditions. It was suspected that the glycated enzyme has a lower stability [92]. Glycation of albumin and lipoproteins has also been observed. Glycation of an humanized IgE antibody was assessed by isoelectric focusing and affinity chromatography [91]. A typical procedure for the separation of glycated proteins is as follows. Plasma or the protein mixture, containing the glycated protein, is diluted or dialyzed against 50 m*M* magnesium chloride/250 m*M* ammonium acetate (pH 8.05), and 50 μL (2 mg protein) is injected onto a Glyco-gel phenylboronate column (50 × 0.60 mm ID) at a flow-rate of 2 mL/min. Non-glycated proteins are eluted in the void volume, while the bound glycated protein fraction is eluted with 0.1 *M* acetic acid. Concentration and volume of the feed may vary with the amount of glycated protein present in the sample.

16.2.8 Analytical artifacts

Sometimes microheterogeneity may be produced by the analytical procedure. Thus, it is necessary to identify isoforms by various analytical methods. However, a chromatographic or electrokinetic separation of the isoforms alone is not sufficient. Mass spectrometry in conjunction with peptide mapping is an efficient way of obtaining further insight into the nature of isoforms. Chromatographic separation of glycopeptides by weak-ion-exchange chromatography with electrochemical detections is also effective in determining the carbohydrate structure of isoforms. It has been reported that cystein is oxidized in polyacrylamide gels to cysteic acid [94], and cysteine acrylamide can also be formed in isoelectric focusing gels. Patterns in isoelectric focusing are often interpreted as microheterogeneity, but caution is indicated: *e.g.*, entrapment of phosphoric acid may maskerade as a protein heterogeneity [95]. Some preparations of a soluble interferon-γ receptor, produced in *E. coli*, appeared as a double band with slightly different mobilities in nonreducing sodium dodecylsulfate and native polyacrylamide gels. Ion-spray mass spectrometry showed that the two forms had a mass difference of 97 ± 2 Da. Evaporation during GC/MS revealed the presence of phosphoric acid in the hydrolyzate and in the intact protein.

16.3 THERAPEUTIC PROTEINS

The stringent requirements for therapeutic proteins are the driving force behind the implementation of chromatographic steps in recovery processes for therapeutic proteins. Chromatography is essential for achieving high purity and the removal of adventitious agents, such as viruses and transmissible spongioform encephalopathies. DNA can be efficiently removed from protein solutions by anion-exchange chromatography. It has a high negative charged and often binds more strongly than the target protein to the ion exchanger. This process is often performed with membrane adsorbers or membrane chromatography. Similar systems are also used for the removal of endotoxins. Endotoxins are heat-stable cell-wall components of gram-negative bacteria. They consist of a lipid anchor (Lipid A), a core region, and an O- or surface antigen, which is highly variable and depends on the strain as well as on the growth conditions. That means that their chromatographic behavior may be different, depending on whether the product is harvested from a batch culture or a continuous culture. Endotoxins form micelles and vesicles in aqueous solutions. A single endotoxin molecule has a molecular mass of 10–20 kDa, while the assembled structures can be extremely large. The endotoxin content of biological fluids or biotechnological feed-streams can vary from less than 1 endotoxin unit (EU) per mL in animal cell cultures to 10^6 EU in high-density cultures of *E. coli*.

At low ionic strength, endotoxins are efficiently removed by ion-exchange chromatography, preferably in the form of membrane adsorbers [96,97]. In the presence of Ca^{++}, endotoxins and proteins can interact and thus be masked. Endotoxins can also directly interact with proteins. Other ligands for endotoxin removal are polymyxin B, poly (L-lysine), DAH:His, and DAH:Doc. Working conditions must be carefully adjusted and conditions must be found that prevent endotoxin masking by proteins. Chromatographic methods allow reduction of the endotoxin content of a bacterial feedstock to a level where it is suitable for the intravenous application of therapeutic proteins. In the production of therapeutic proteins chromatography is often applied for virus removal. Conditions can be found where the viruses bind much more strongly than the protein of interest.

16.4 STABILITY

Although the stability of proteins cannot be directly assessed by chromatographic methods, chromatography has become indispensable in this application. Proteins may undergo a conformational change, the polypeptide chains may be chemically modified or cleaved, or they may aggregate. In all these cases the surface properties and size of the protein will change, and a retention shift between native and denatured protein should be observable. The unfolding of proteins in the presence of chaotropic agents can be thoroughly investigated by SEC. In RP-LC, different conformational variants can also be observed. When proteins are adsorbed on a chromatographic surface, it may mediate a conformational change [98]. Such a process is often reversible. The presence of several peaks in a chromatogram of a pure protein can be an indication of several folding variants, but they could also have been created during chromatography. The evidence for folding

TABLE 16.3

PHYSICOCHEMICAL CONDITIONS APPLIED IN PROTEIN CHROMATOGRAPHY

Chromatography	Adsorption	Elution	Regeneration
Cation-exchange	Low ionic strength, pH below protein pI	Increase of ionic strength, increase pH	Acid/base wash, high salt concn.
Anion-exchange	Low ionic strength, pH above protein pI	Increase of ionic strength, decrease pH	Acid/base wash, high salt concn.
Hydrophobic-interaction	High ionic strength	Ionic strength decrease (ammonium sulfate)	Alkaline wash, organic acids, chaotropics, water-miscible solvents
Hydroxyapatite	Dilute phosphate buffers (pH 6.8)	Increase phosphate buffer concentration	Sodium hydroxide
Size-exclusion	Any ionic strength, any pH, low feed	Isocratic	Salt wash, dilute alkaline wash
Affinity	Close to physiological conditions	pH changes, ionic strength changes, competitive elution	Salt wash, specific displacement treatments
Reversed-phase	Hydro-organic mixtures	Organic additives (MeOH, MeCN)	Solvents, glycols, urea, acidic solutions

variants must be carefully examined by other analytical methods, such as circular dichroism, fluorescence titration, and activity assay of the collected fractions, if possible. Usually, running conditions for protein chromatography are selected to meet the stability requirements of proteins (Table 16.3). Even so, several conditions may be unfavorable, *e.g.*, pH extremes during loading in ion-exchange chromatography, pH extremes during elution in affinity chromatography, high salt concentration during loading in hydrophobic-interaction chromatography, and organic solvents and pH extremes in reversed-phase chromatography. Usually, short exposure (within the period needed for modern chromatography) will not harm the functionality of proteins. It must be noted, however, that chromatographic conditions leading to the destruction of proteins are usually not published. As already mentioned, proteins often occur in the form of a complex and may need additives in the buffer for stabilization during separation.

16.5 NORMAL-PHASE LIQUID CHROMATOGRAPHY

16.5.1 Sorbents

The chemical nature of proteins determines the physical properties of the sorbents. A large surface (10–400 m^2/cm^3) is desirable for a high binding capacity. A large surface

could be obtained with a highly porous material, while small pores would prevent diffusion into the pores. Thus, a compromise has to be found. Chromatographic media for protein chromatography should have a pore size of *ca.* 30 nm. Hindered transport can be neglected when the pore diameter is 10 times the protein diameter. Most proteins have a diameter below 3 nm [17]. In order to allow reversible adsorption, the chromatographic material must be very hydrophilic. Natural polymers, such as cellulose, agarose, and dextran have been used frequently [2]. When highly substituted with hydroxyl groups, they become hydrophilic, and enough functional groups are available to introduce ligands for specific modifications of the surface to suit its intended use. A ligand density below 100 μM is usually sufficient for protein chromatography. A drawback of hydrophilic natural polymers is their soft structure. With increasing flow-rate the medium will be compressed, and the back-pressure will increase [99]. In order to counteract this, the media are cross-linked. The introduction of cross-links makes the medium more hydrophobic and increases non-specific adsorption. The chromatographic sorbents with the lowest non-specific adsorption are cellulose media, such as DE52 (Whatman) or cross-linked agarose and Sephadex media. Ideal features of a sorbent for protein chromatography are: (a) high selectivity, (b) high binding capacity, (c) high mass transfer, (d) low non-specific adsorption, (e) resistance to compression, (f) chemical stability and stable immobilization of ligands, (g) absence of leachable toxins, (h) high reusability, (i) suitability for sanitation under alkaline conditions (only for therapeutic proteins), and (j) cost effectiveness.

Any chemical modification of the surface entails the risk of introducing charged or hydrophobic moieties which will interact non-specifically with the proteins. Sanitation by NaOH is almost mandatory for media suitable for application in the pharmaceutical industry [100]. Proteins are rapidly degradated by alkaline hydrolysis, and lipids are efficiently dissolved in NaOH. Up to 1 *M* NaOH is used for cleaning and sanitization of chromatographic columns, but NaOH does not adequately sterilize a packed bed [101]. An oxidizing agent, such as peracetic acid, is very useful for chemical sanitization [100]. It efficiently destroys bacteria and breaks down into acetic acid and water. Interestingly, NaOCl, a compound with the same oxidizing capacity, is less efficient in sterilization. Presumably, peracetic acid more easily penetrates into bacteria than NaOCl. Moreover, NaOCl forms dioxins upon reaction with organic material containing cyclic hydrocarbons. Many chromatographic materials are not resistant to oxidizing agents, and this precludes the use of this method. Heat sterilization at 100°C or above can be used; packed columns can be put into an autoclave as a whole.

The chemical modifications as well as the immobilized ligand should result in a chemically stable matrix. Absolute stability is impossible and even undesirable. Controlled leakage of ligand is accepted. However, it is important that the leaked material be nontoxic. This requirement excludes a variety of media and ligands from production of therapeutic proteins. Dye ligands have been suspected of being toxic [102], but recent studies showed that they are relatively safe.

The particle size for protein chromatography ranges from 2 to 300 μm. The size distribution varies from monodisperse media to a 30% standard deviation of the size distribution. The choice of sorbent depends on the application, media characterized by small particles and narrow particle-size distributions being preferred for analytical

applications. In preparative applications, resolution can often be obtained by selectivity and performing chromatography in an on/off mode. Conditions are selected where the protein of interest is captured, while the impurities pass through the column. For such applications a larger particle diameter (>50 μm) is advantageous, since the pressure drop is lower. Such sorbents are easier to pack and, since they are easier to manufacture, they are less expensive than media with small particle size and narrow size distribution. Monoliths (Sec. 16.5.1.4) are continuous stationary phases characterized by high mass-transfer efficiency [103–105]. The volumetric flow is so low that these columns can be directly connected to a mass spectrometer.

Sorbents for protein chromatography are characterized by large pore size and compressibility. While the surface area of a rigid sorbent can be determined by nitrogen adsorption (BET isotherm) or mercury intrusion, the soft natural and synthetic polymers must be dehydrated before analysis, but then the material will shrink, making the measurements meaningless. Chromatographic media made from polymethacrylate, *e.g.*, Toyopearls, CIM monoliths, silica beads, and glass can be characterized by the above mentioned methods. The pore size of soft chromatographic media can be measured by inverse size-exclusion chromatography [106]. Dextran molecules with defined size serve as reference material (Chap. 5).

In order to choose the most suitable stationary phase for protein chromatography the nature of the sample and pore size must be considered. Pore sizes of some popular commercial sorbents are compared in Table 16.4. For small proteins below a molecular radius (M_R) of 20,000, 10-nm pores are often sufficient. Molecular-size estimation from SDS-PAGE can be misleading. Aggregates and oligomers are split, and only monomers are shown. For proteins with a $M_R > 20{,}000$, 30-nm pores are recommended. An ideal sorbent should have a pore diameter which is ten times the diameter of the protein molecule to be separated. Especially for large proteins, this requirement cannot be always fulfilled.

The interaction of a protein molecule with a porous sorbent particle can be divided theoretically into several successive processes. The protein in the bulk flow crosses the stagnant layer by diffusion. This process is called film diffusion and has been described in its simplest form by linear driving-force models. The protein enters the pore and diffuses into it until it reaches the site where it is adsorbed, assuming that the pore-size is large enough. If the pore diameter is less than 10 times the protein diameter, hindered diffusion will occur. A problem with large molecules, such as proteins, is that they can be adsorbed at the entrance of the pore and constrict the pore mouth. Also, solid diffusion has been observed when proteins are sorbed in a hydrogel. The protein is not fixed to its site; it can move along within the stationary phase [107]. Solid diffusion can be significant. The driving force is the large concentration gradient. When pore and solid diffusion contribute equally to the mass transport into the porous particle, the diffusional process is modeled as parallel diffusion. For hydrogels, solid diffusion is the dominating mechanism of protein transport. An exact physical explanation for solid diffusion cannot be given, but for all media with high protein binding capacity such a mechanism is assumed. Whereas in classical ion-exchanger chromatography the binding capacity increases when the pH of the loading buffer is far from the pI of the protein, proteins at a pH far from their pI are

TABLE 16.4

COMPARISON OF PORE SIZES OF A SELECTION OF SORBENTS, AS DETERMINED BY INVERSE SIZE-EXCLUSION CHROMATOGRAPHY (ISEC) AND DATA ADOPTED FROM MANUFACTURERS AND FROM REF. 106

Stationary phase	Mean pore diameter (nm)	
	ISEC	Nominal
Amersham Biosciences		
SP Sepharose FF	49.4	–
CM Sepharose FF	54.6	–
TosoH Biosep		
HW 65 F	132.2	100
SP 650 M	153.2	100
CM 650 M	147.8	100
HW 55 F	39.2	30
SP 550 C	17.6	30
EM Industries		
EMD SO_3^- M	33.0	100
EMD SO_3^- M (1 *M* NaCl)	59.3	100
EMD COO^- M	161.0	100
BioSepra		
Silica	136.0	100
SP Spherodex M	68.6	100
SP Spherodex M (1 *M* NaCl)	43.2	100
CM Spherodex M	20.8	100

adsorbed at the outer shell of the sorbent and block the entrance to the pores. At a pH close to the pI, the transport mechanism changes, and solid diffusion becomes possible.

Current models of chromatography are based either on the history of the effluent from the column or on the change in the concentration of free solute in a small, stirred vessel (called "finite batch" by chemical engineers). To visualize intraparticle transport, Firouztale *et al.* [108] incubated sorbent beads with fluorescence-labeled proteins. The beads were removed at intervals and immersed in liquid nitrogen to prevent further diffusion, then cut with a microtome. The fluorescence profile under a fluorescence microscope showed the concentration profile within the beads. Ljunglof and Thömmes [109] and Lenhoff [110] used laser confocal microscopy to visualize the intraparticle protein transport into a porous medium. Proteins were labeled with the fluorochrom Cy 5 (ex: 647 nm, em: 660 nm) and incubated with a sorbent in a infinite bath, removed from the reaction vessel, centrifuged at 14,000*g* for 30 sec, and inspected by confocal microscopic analysis. Lenhoff has constructed an apparatus allowing the visualization of concentration profiles in beads *in situ*. A tiny packed bed is placed on a microscope slide

and perfused with fluorescent-labeled proteins to be analyzed by a confocal microscope. Conclusions about the mechanism of intraparticle transport from effluent history must be drawn with care. Break-through curves are influenced by the non-uniformity of flow and extra-column band-spreading [111]. Zhang *et al.* [112] have tried to calculate isotherms from elution profiles by statistical analysis.

Sorbents can be categorized according to the basic material, according to the transport mechanism, or according to the manufacturing process. Classification according to the transport mechanism is not reasonable, since it may change, depending upon chromatographic conditions and composition of the sample. Classification according to basic material, on the other hand, is straightforward. We can distinguish between beds packed with particles and continuous stationary phases, the so-called monoliths (Sec. 16.5.1.4). The beads can be porous or non-porous, but a monolith must be porous. Packing materials with a solid core, surrounded by a shell of sintered small particles, are also available [113]. The monoliths contain either only macropores, *e.g.*, the CIM disk [104,105], or macro- and mesopores, *e.g.*, the Chromolith [114–117]. The Chromolith was not originally designed for protein chromatography. The basic matrix consists either of natural polymers, such as agarose, dextran, cellulose, chitosan, of synthetic polymers, such as polymethacrylate, polyacrylamide, trisacryl and polystyrol, of inorganic materials, such as silica, zirconium oxide, glass, hydroxyapatite, or of composite material, *i.e.*, beds with gigapores which have been filled with a gel [118,119]. The basic beads are often further modified by coating the surface (silica, polystyrene) or grafting an additional layer onto the surface (Fractogel, Sepharose XL).

16.5.1.1 Polymers

The first application of a natural polymer was reported by Peterson and Sober in 1956 [120]. They derivatized cellulose to produce cellulose beads with ion-exchange functionalities. Three years later, the first dextran-based media, Sephadex G-25 and G-50, became commercially available. The first paper on "gel filtration" was published by Porath and Flodin [121]. Common to all natural polymers used for protein chromatography, *e.g.*, cellulose, agarose, dextran, and chitosan, is their low, non-specific adsorption. These polymers are extremely hydrophilic, and proteins do not adhere to them. Examples of commonly used media made of natural polymers can be found in Table 16.5. The first great success in protein chromatography was achieved with dextran-based and agarose-based beads (Sephadex and Sepharose, respectively, from Pharmacia, now Amersham Biosciences) [122]. Sephadex G-25 and G-50 were introduced in 1959, and ion exchangers based on Sephadex block polymerizates in 1960 and 1962.

The advantage of dextran and agarose beads over cellulose is the better flow behavior. Fibrous cellulose is extremely hydrophilic but difficult to pack. The bed height is usually less than 20 cm. Cellulose beads are also commercially available, *e.g.*, Cellufine from Millipore or the cellulose-based ion exchangers from Whatman [2]. To solidify the soft structure, agarose has been cross-linked. This material is known as Sepharose CL 2B (4B, 6B). Cross-linked agarose beads have been further modified by covalent binding to dextran (Superdex).

TABLE 16.5

EXAMPLES OF SORBENTS MADE FROM NATURAL POLYMERS

Basic material	Physical shape	Example	Manufacturer
Cellulose	Fibrous	DE 32	Whatman
	Micro-pellicular	DE 52	Whatman
	Pellicular	Express-Ion D	Whatman
		Express-Ion Q	Whatman
	Pellicular	Sephacel	Amersham Biosciences
	Pellicular	Cellufine	Millipore
Dextran	Pellicular	Sephadex G-25	Amersham Biosciences
		Sephadex G-50	
		DEAE-Sephadex	
Agarose	Pellicular	DEAE-Sepharose 4B	Amersham Biosciences
	Pellicular cross-linked	DEAE-Sepharose FF	
Agarose-dextran composite	Pellicular	Superdex 30	Amersham Biosciences
		Superdex 200	
		DEAE-Sepharose XL	

To exploit the excellent binding properties of these soft chromatographic media, they are often used in batch contactors. This operation is very popular in blood plasma fractionation. The medium, in the form of a dry powder, is mixed with the plasma in a tank. It swells and adsorbs the proteins. Afterwards, the loaded material is removed by a suction filter, and the proteins are desorbed. In such processes, the sorbent material is used only once. Another way to handle these soft materials is suspended-bed chromatography, introduced by Levison [123]. This technology is a hybrid between chromatography and batch contactor. The equilibrated sorbent is suspended together with the protein solution, and the slurry is then pumped into a chromatographic column, where the subsequent steps are performed. The time-consuming loading phase is minimized by this technology.

Three synthetic polymers are of practical importance: hydrophobic vinyl polymers, polyacrylamide, and polyvinylstyrene. The polyacrylamide polymers were introduced by Hjertén. Polymethacrylate (Spheron) never became widely used. Polystyrene can be used without further modification of the surface for RP-LC, but for other modes of chromatography the surface must be coated with a hydrophilic polymer. A derivate of polyacrylamide is Trisacryl [124]. An overview of common polymeric media for protein chromatography is shown in Table 16.6. Common to all synthetic polymers is their relative hydrophobicity. For many applications they must therefore be coated in order to prevent low recovery. A plethora of other materials has been described, but they are not

TABLE 16.6

EXAMPLES OF SYNTHETIC SORBENTS FOR PROTEIN CHROMATOGRAPHY. THE SURFACE IS FURTHER MODIFIED TO FUNCTIONALIZE IT FOR IEC, AC, HIC, RP-LC, OR SEC. THE EXACT CHEMICAL COMPOSITION IS OFTEN NOT DISCLOSED BY THE MANUFACTURER

Polymer	Trade name	Manufacturer
Polyacrylamide derivate	Trisacryl Hyper D*	BioSepra
Hydrophilic cross-linked vinyl polymer	Toyopearl	TosoH Biosep
Polyacrylamide	Bio-Gel	Bio-Rad
Styrene/divinyl benzene co-polymers	Amberlite** Leweht??	Merck
Polystyrene/divinyl benzene	Source Resource	Amersham Biosciences
Polystyrene/divinyl benzene	PL***	Polymer Labs
Polystyrene/divinyl benzene	Porous****	Perseptive Biosystems
Polymethacylate	CIM-disks*****	BIA Separations

* Hydrogel filled into a porous shell.
** Not optimal for protein chromatography.
*** Coated, highly porous material.
**** Similar to material from Polymer Labs.
***** Also available as monolithic column.

commercially available. The advantage of synthetic polymer-based media is their resistance to extreme conditions, such as pH extremes and oxidizing environments. Some of them can also be sterilized in an autoclave, but *in situ* sterilization is impossible for lack of appropriate equipment. A negative aspect of polymeric columns is swelling at high concentrations of organic solvent, such as acetonitrile, which is widely used in RP-LC of proteins. Swelling leads to increasing back pressure and may damage the column in some instances. In the separation of large proteins, high concentrations of organic solvents must be avoided, since proteins may precipitate under these conditions.

16.5.1.2 Inorganic media

Tiselius introduced hydroxyapatite for protein separation. Although its selectivity was excellent, the original microcrystalline hydroxyapatite had extremely poor flow properties. A breakthrough was achieved with the invention of ceramic hydroxyapatite [125]. It consists of small hydroxyapatite particles, sintered to spherical particles with large pores. The material has excellent flow properties, good selectivity, and high binding

capacity. It is used for large-scale industrial process, such as the production of recombinant antibodies. The prime inorganic support material is silica [126–128]. Since its OH groups can interact with proteins, especially at high pH, it is necessary to modify residual Si–OH groups in order to inactivate them (bonded silica). Peaks with good symmetry are obtained only in RP-LC with fully capped supports, but with the use of partially capped supports peak symmetry can be greatly improved by the addition of amines or quaternary ammonium compounds to the mobile phase (reversed-phase ion-pair chromatography). Silica has also been grafted with dextran [129] or polyvinylpyrrolidone [130] by interaction of a co-polymer of vinylmethyldiethoxysilane and vinylpyrrolidone with Lichrospher Si 300 and Li Chrospher Si 500. The coating procedure retains the wide-pore structure, and the material shows good selectivity for proteins in the HIC mode. Silicas have also been coated with hydrophilic layers [131], cellulose [132], polystyrol [133], dextran [134,135], agarose [135], and poly(alkylaspartamide) [98]. Silica can be treated with zirconium salts and then covalently bonded with a hydrophilic organo-silone (Zorbax Bioseries). The zirconium-treated surface presents no unusual constraints in operating conditions and even permits short-term use of buffered eluents at pH 9.0 [136]. Zirconia beads, which are stable in alkaline solutions, are used for small molecules but not for proteins [137].

Glass has also been used as packing material. The common porous glass is the controlled-pore glass (CPG). It exhibits excellent flow properties, even though the material is not spherically shaped. The surface has reactive hydroxy groups, which can be used for further modification. The use of glass was revived by the introduction of the Protein A adsorbent ProSep A (Millipore). This adsorbent is successfully applied in the large-scale purification of therapeutic antibodies. A proprietary process is used to attach the Protein A ligand. The material has excellent flow and mass-transfer properties [138].

16.5.1.3 Composite materials

Although coated silica could also be regarded as a composite material, the sorbents considered here are made by a combination of inorganic and organic material. Hyper D is a typical representative of such a composite material [118,119]. Having a low degree of cross-linking and a high concentration of functional groups, it offers an extremely high binding capacity. However, the material as such is not suited for chromatography in a packed bed, because it is too soft and could operate only under extremely slow flow-rates. Therefore, the gel has been polymerized into a porous shell, originally made of silica and later of zirconia ("gel in a shell"). It can be used at extremely high velocity without loss of binding capacity. The sorption mechanism was found to be dominated by solid diffusion. This was also confirmed by experiments where a gel was polymerized into a slab and the protein front was observed under a microscope. The solid-diffusion model best describes the moving concentration profile. The high capacity of the composite medium allows direct capture of proteins from a culture supernatant without pre-conditioning [139].

16.5.1.4 Monoliths

Monoliths are a relatively new class of stationary phases, completely different from the conventional stationary phases. The material is cast into a chromatographic column as a continuous block, interlaced with channels [140]. The ramified channels do not have dead ends. Owing to this structure, the transport of the solute to the surface is solely by convection instead of diffusion, as observed in conventional media [103,141]. Monolithic media are characterized by excellent mass-transfer properties and low pressure drop. The large channel diameter makes monoliths an excellent stationary phase for protein chromatography. The first monoliths were developed by Hjertén *et al.* [143–145] and Tennikova *et al.* [145]. Hjertén *et al.* [142–144] compressed polyacrylamide gels and observed excellent resolution. The polymethacrylate monoliths most widely used for protein chromatography are produced as follows. As the polymer chain grows, the solubility decreases, and at a certain polymer chain-length defined particles precipitate and agglomerate to form a homogenous, porous network. Silica monoliths, grown as a single block by a gel/sol process, have been developed for the separation of small molecules [114–116]. These types of monoliths contain also mesopores. Commercially available monoliths are listed in Table 16.7. Numerous designs of monoliths have been published, and this configuration of chromatographic beds promises a bright future for protein chromatography. They are already used for analytical purposes, and although scale-up has been difficult, it was recently accomplished [146]. Polymethacrylate monoliths were scaled up to 800 mL; the conventional, axial flow was replaced by a radial flow.

TABLE 16.7

OVERVIEW OF COMMERCIALLY AVAILABLE MONOLITHS FOR CHROMATOGRAPHY PURPOSES. THE CHROMOLITH AND THE MATERIAL FROM CONCHROM IS DESIGNED FOR SEPARATION OF SMALL MOLECULES

Monolith name	Manufacturer	Material	Pore size
CIM	BIA Separations	Polymethacrylate	1500 nm
CIM RP-SDVB	BIA Separations	PS/DVB	1500 nm
UNO	Bio-Rad	Polyacrylamide	1000 nm
Chromolith*	Merck	Silica	Macropore: 2 μm Mesopore: 13 nm
Seprasorb	Sepragen	Cellulose	Unknown
PS-DVB Monolithic column	LC-Packings	PS/DVB	>1 μm
Conchrom*	Conchrom	Silica	Mesopores: 30 nm Micropores: 5 nm

* Designed for the separation of small molecules.

The production of large-scale monoliths is not easy. In the case of polymethacrylate monoliths, the generated heat is the most critical factor. The pore size is highly dependent on the polymerization temperature. Efficient dissipation of heat during the polymerization process is the key to homogenous, large-scale monoliths. A closer investigation of the polymerization, with the use of differential scanning calorimetry, was undertaken by Mihelic *et al.* [147] in order to determine global kinetic parameters. A multiple heating-rate method was applied to the estimation of the values of the apparent activation energy, pre-exponential factor, and reaction order. Podgornik *et al.* [146] laid the theoretical foundations for the production of monoliths with predictable properties. From the measurement of the heat of reaction released and the thermal conductivity of the monomer mixture the maximal allowed temperature increase that will provide a uniform pore-size distribution can be calculated, and an annular monolith of a specified inner diameter and the thickness can be constructed. In order to minimize the pore-size variation due to the temperature differences in bulky monoliths, the material is polymerized in a tube-like geometry. The different tubes are telescoped, and in this way, a monolithic unit of required volume and uniform pore-size distribution can be constructed. The liquid must be introduced radially, and it is collected by a central tube.

Monoliths have been also used as supports for solid-phase synthesis [148]. The synthesis of peptides on monolithic columns is of great interest. Since the synthesis has been performed on polymethacrylate monoliths, the directly grown peptide can be used as an affinity ligand [149–151]. This strategy provides an excellent screening method for affinity ligands. This can be performed in a microtiter format, and the same resin can be used for screening and large-scale separation. Monoliths are also useful supports for enzyme reactors. The link to protein chromatography is fusion proteins. The fusion partner must be cleaved off, and this can be done either in solution or in an enzyme reactor with immobilized enzymes. The high porosity ensures that the enzymatic process is not mass-transfer limited [152].

16.5.2 Conjoint liquid chromatography

Chromatography performed with media of different separation principles, packed into a single chromatographic column, is named conjoint liquid chromatography. Often, it is desirable to have at least two functionalities in a single column. For instance, the top part should adsorb a contaminant, which could interfere with the resolution of compounds bound to the resin at the bottom. When different conventional sorbents are packed into a single column, they may mix at the interphase [153]. Repacking is extremely difficult since the beads will be mixed up, and unless beads with different functionalities have different densities, the mixture of beads is extremely difficult to separate. Monoliths, on the other hand, are suited for such a column configuration. The individual pieces can be assembled in a column, and the column is just as easily disassembled. This type of chromatography was introduced by Josic *et al.* [154] and named conjoint liquid chromatography (CLC). It allows two-dimensional chromatography to be carried out in a single step without column switching. Recently, Gupalova *et al.* [155] have described a multifunctional fractionation approach to the recovery of IgG and serum albumin (HSA)

form plasma within 15 min. One IgG-binding Protein G disk and one HSA-binding Protein G disk were installed in the same cartridge. IgG and SA were captured simultaneously, but the elution was performed in successive steps. The SA-binding disk was removed first, and the IgG was desorbed, using dilute hydrochloric acid. After reinstallation of the SA-binding disk, SA was eluted. Ostryanina *et al.* [156] combined four disks with different affinity functionalities in the same cartridge, thereby permitting the separation of different antibodies within a few minutes. Branovic *et al.* [157] developed a very fast analysis of impurities in immunoglobulin concentrates by conjoint liquid chromatography on short, monolithic disks. Transferrin and albumin are often present in IgG concentrates. It is important to determine their concentration in order to obtain a well-characterized biological product. Two CIM Protein G and one CIM quaternary amine (QA) monolithic disk were placed in series in one housing, forming a CLC monolithic column. Binding conditions were optimized in such a way that immunoglobulins were captured on the CIM Protein G disks, while transferrin and albumin were bound on the CIM QA disks. Subsequently, transferrin and albumin were eluted separately by a stepwise gradient with sodium chloride, whereas immunoglobulins were released from the Protein G ligands by applying a low pH (Fig. 16.6). A complete separation of all three proteins was achieved in less than 5 min. The method permits the quantification of albumin and transferrin in IgG concentrates and has been successfully validated.

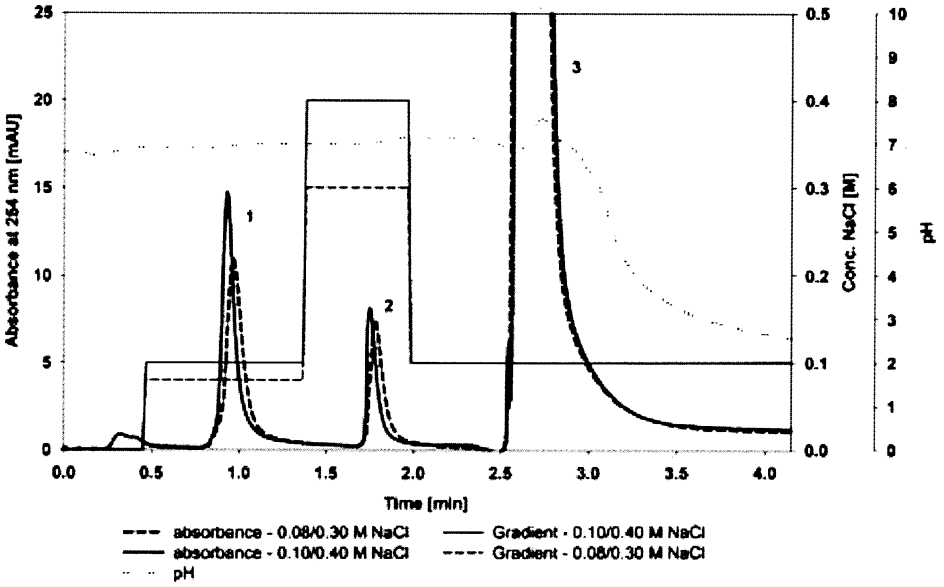


Fig. 16.6. Optimized separation of transferrin, albumin, and IgG on a CIM CLC monolithic column, consisting of two CIM Protein G disks and one CIM QA disk. Chromatograms of two different runs with different step gradients are superimposed. Binding buffer: 20 m*M* Tris–HCl (pH 7.0); Elution Buffer I: 20 m*M* Tris–HCl, 1 *M* NaCl (pH 7.0); Elution Buffer II: 0.1 *M* glycine–HCl, 0.1 *M* NaCl (pH 2.0); flow-rate: 4 mL/min; sample concentration: 2 mg IgG/100 L ("1" indicates the transferrin peak, eluted with 0.08 or 0.1 *M* NaCl; "2" indicates the albumin peak, eluted with 0.3 or 0.4 *M* NaCl; "3" indicates the IgG peak, eluted at low pH).

16.5.3 Perfusion chromatography

In 1990, Afeyan *et al.* [158] introduced a novel chromatographic method for protein separation, claiming that efficiency does not decrease with increasing flow velocity. The phenomenon was explained by through-pores in beads. The diameter of the through-pores was claimed to be large enough for intra-particle convection to take place. By intra-particle convection the solutes are transported to the mesopores, which are reached by diffusion. The diffusional distance is short enough so as not to limit the overall mass transfer. Theoretical considerations showed that an intra-particle Peclet number of over 50 is required to have a significant portion of liquid flowing through a pore instead around the bead. Afeyan *et al.* [158] overestimated the intraparticle Peclet number, and, in calculating the peak-width, they neglected peak tailing. They calculated the peak-width at half-height, which did not change over a wide range of flow-rates. Liapis *et al.* [159] calculated the internal concentration profile of an adsorbed solute during intra-particle convection. Such a profile has never been observed so far. Despite the dispute about perfusion chromatography, the so-called Porous Media are superbly suited for protein chromatography.

16.6 HYDROPHOBIC-INTERACTION AND REVERSED-PHASE LIQUID CHROMATOGRAPHY

16.6.1 Retention mechanisms and adsorption principles

The hydrophobicity of proteins varies with their content of hydrophobic amino acids. This property is used in hydrophobic-interaction chromatography (HIC) and reversed-phase liquid chromatography (RP-LC) as separation criterion. HIC and RP-LC of proteins are discussed in this section in conjunction because of their similar separation mechanisms (Chap. 2). However, chromatographic conditions are different for HIC and RP-LC. HIC is the method of choice for maintaining the native conformation and biological activity of proteins. Owing to its high resolving power, RP-LC is widely used as an analytical method for peptides and proteins, but it is also applied on an industrial scale.

16.6.1.1 Retention mechanism in hydrophobic-interaction chromatography

Shepard and Tiselius [160] first described the adsorption of proteins on hydrophobic surfaces in the presence of sulfate and phosphate solutions under the name *salting-out chromatography*. In a follow up study, Shaltiel and Er-el [161] used the term hydrophobic chromatography or hydrophobic-affinity chromatography and Hofstee [162] described the method as hydrophobic-adsorption chromatography. Hjertén [163] finally called the method hydrophobic-interaction chromatography. Salt buffers of rather high ionic strength are used to effect the adsorption of proteins on a stationary phase by promoting protein/ligand interactions [164]. The salt plays an important role in adsorption. With certain types of salts, protein precipitation can be achieved by increasing the ionic strength.

The Hofmeister series ranks most common cations and anions according to their *salting-in* protein-stabilizing effect and their *salting-out* protein-precipitating effect, respectively.

	←
	Increasing *salting-out*
Anions	PO_4^-, SO_4^{2-}, CH_3COO^-, Cl^-, Br^-, NO_3^-, CLO_4^-, I^-, SCN^-
Cations	NH_4^+, Rb^+, K^+, Na^+, Cs^+, Li^+, Mg^{2+}, Ca^{2+}, Ba^{2+}
	→
	Increasing *salting-in*

Salt precipitation of carboxyhemoglobin with different types of salt showed that the solubility curve increases up to a certain value of ionic strength for all salt types. However, above this value, solubility of the protein decreases strongly for antichaotropic salts, whereas for chaotropic salts, solubility is maintained or even increased [165]. The solvophobic theory, proposed by Green [166] in 1955, described solubility phenomena of proteins in salt solutions. At low ionic strength, electrostatic forces are responsible for the increase in solubility. Proteins are charged molecules, and ions interact with them, thus shielding the protein molecules from each other. This behavior can be described by the Debye equation, including the salting-out term, added by Hückel, in Eqn. 16.1.

$$\log S - \log S_0 = \frac{0.5 Z_1 Z_2 \sqrt{\mu}}{1 + A\sqrt{\mu}} - K_s \mu \tag{16.1}$$

where S and S_0 are the solubility (of a protein in this case) in the presence and absence of electrolytes, respectively, Z_1 and Z_2 are the charges of the salt cations and anions, μ is the ionic strength. K_s is the salting-out constant and A is a constant depending on the mean diameter of the ions in solution and on κ, a reciprocal distance [166]. For higher ionic strength, water activity is drastically increased due to the hydration of the ions, not leaving enough hydrating ions for the proteins. This results in lower solubility and precipitation. The surface tension of the solution increases with the concentration of salt, as described by Eqn. 16.2

$$g = g_0 + s_m \tag{16.2}$$

where g is the surface tension, g_0 the surface tension of pure water, s is the molal surface tension increment and m is the molality of the salt. Table 16.8 shows values of s for selected salts [167]. During solvation of a protein in a solvate, a "cavity" is formed to accommodate the solute. This cavity formation is dependent on the molecular surface area of the protein, the volume and the surface tension of the solvent. Combination of solubility at low ionic strength, expressed by the Debye–Hückel equation and at high ionic strength, described by formation of a cavity, results in Eqn. 16.3 [168].

$$\ln\left(\frac{w}{w_0}\right) = \frac{B(m^{0.5})}{RT[1 + C(m^{0.5})]} + \Lambda m - \Omega \sigma m \tag{16.3}$$

ln w is the solubility of the solute, ln w_0 solubility of the solute in pure water, and B and C are constants from the Debye–Hückel equation. Solubility is thus dependent on two

TABLE 16.8

VALUES OF THE MOLAL SURFACE TENSION INCREMENT (σ) FOR SELECTED SALTS

Salt	$\sigma \times 10^3 \frac{\text{dyn g}}{\text{cm mol}}$	Salt	$\sigma \times 10^3 \frac{\text{dyn g}}{\text{cm mol}}$
NH_4I	0.74	Na_2HPO_4	2.02
$NaNO_3$	1.06	$MgSO_4$	2.1
KBr	1.31	$(NH_4)_2SO_4$	2.16
$FeSO_4$	1.55	Na_3PO_4	2.66
NaCl	1.64	Na_2SO_4	2.58
$CuSO_4$	1.82	$K_3Fe(CN)_6$	4.34

antagonistic effects, the polarity of a protein, described by the salting-in coefficient, Λ, which is dependent on the dipole moment of the protein, and by the salting-out coefficient, $\Omega\sigma$, where Ω is dependent on the surface area of the protein and σ is the surface tension increment, determined by the salt. Melander and Horváth [168] adapted the solvophobic theory to explain retention behavior in HIC. They demonstrated a linear relationship between the logarithm of the normalized retention factor, k', and the ionic strength, I, for high salt concentration in the liquid phase. The capacity factor, k, can be expressed by Eqn. 16.4

$$\ln k = \ln k_0 - \frac{B(m^{0.5})}{1 + C(m^{0.5})} - \Lambda m + \Omega\sigma m \tag{16.4}$$

where k_0 is the capacity factor with pure water and B and C are constants proportional to the net charge of the protein. Retention data from isocratic elution experiments at different salt concentrations can be fitted to Eqn. 16.4. Plotting $\ln k'$ *vs.* the ionic strength gives curves of different shape. The slope and position of the curve depends on the model protein, the salt type, and the stationary phase. At low salt concentrations, k' values decrease with increasing ionic strength, but at higher salt concentrations, k' increases. Eqn. 16.4 can be further simplified by a three-parameter equation, developed by Melander *et al.* in 1989 [169]. It attaches greater value to electrostatic interactions in protein retention on HIC sorbents by applying the counter-ion condensation theory. The theory assumes that proteins are bound through an electric field, which is generated by the fixed charges on the solid support. The logarithm of the retention factor is thus related according to Eqn. 16.5

$$\log k' = A - B \log m_s + Cm_s \tag{16.5}$$

where A is a constant for all characteristic system parameters, B is termed the electrostatic interaction parameter, C is the hydrophobic-interaction parameter, and m_s is the molality of the salt. B is a function of the effective charge of the protein and salt

counter-ion and, indirectly, a function of the charge of the stationary phase. C is dependent on the hydrophobic contact area between the protein and stationary phase and on the molal surface tension increment due to the salt. To investigate the influence of electrostatic interactions on retention in HIC, isocratic elution experiments were carried out with a weak- and strong-ion-exchange resin, operated in HIC mode. Data were fitted to Eqn. 16.5, and the parameters B and C were obtained. It was found that some of the proteins studied were retained on the strong-ion exchanger whereas no retention was possible on the weak resin at a salt molality of 1. Retention mediated through electrostatic interaction was further confirmed by the strong dependency of log k' on pH. For a positively charged protein, log k' increased with lower pH on both, the weak- and the strong-cation-exchange resin. The parameter C was shown to be linearly dependent on σ, the molal surface-tension increment. However, it is an oversimplification to explain specific salt binding effects only by σ. A deeper insight into salt-mediated binding can be provided by preferential hydration values [170,171]. The model assumes that no conformational changes of the protein occur during chromatography.

Another approach to model retention data with respect to the ionic strength of the eluent buffer was provided by Staby *et al.* [172]. They assumed that the chemical potential, μ, of a solute at equilibrium is the same in the mobile phase as in the stationary phase. The chemical potential is defined in Eqn. 16.6.

$$\mu = \mu_0 + RT \ln \gamma x \tag{16.6}$$

where μ_0 is the standard-state chemical potential, γ is the activity coefficient for non-ideal solutes, and x is the mole fraction. Substituting for x the mole fraction, as defined in Eqn. 16.7

$$x = \frac{n\nu}{V} \tag{16.7}$$

where n is the mole number of solute, V is the total phase volume, which is independent on the ionic strength, and ν is the molar volume of the phase, gives Eqn. 16.8.

$$\mu = \mu_0 + RT \ln \frac{n\gamma}{n_0\gamma_0} + RT \ln \frac{\nu}{\nu_0} \tag{16.8}$$

Letters with subscript 0 denote conditions at zero ionic strength and letters without subscript refer to conditions at a certain ionic strength. The capacity factor, k', is defined in Eqn. 16.9

$$k' = \frac{n_s}{n_m} \tag{16.9}$$

where n_s and n_m are the number of moles of a solute in the stationary and mobile phase, respectively. Under the assumption that $\mu_m = \mu_s$, $\mu_{m,0} = \mu_{s,0}$ and that the volume ratio in the stationary phase is 1, several transformations lead to the following expression of the capacity factor, k'

$$\ln k' = \ln k'_0 + \ln \frac{\gamma^m}{\gamma_0^m} - \ln \frac{\gamma^s}{\gamma_0^s} + \ln \frac{\nu^m}{\nu_0^m} \tag{16.10}$$

In Eqn. 16.10, $\ln k'_0$ is the capacity factor at zero ionic strength, γ_0^m, γ_m are the activity coefficients in the mobile phase at zero and I ionic strength, γ_0^s, γ^s are the activity coefficients in the stationary phase at zero and I ionic strength, and ν_0^m, ν_m are the molar volume of the mobile phase at zero and I ionic strength. The molar volume in the mobile phase is, in contrast to the molar volume in the stationary phase, dependent on the ionic strength and cannot be neglected. The ratio of activity in the mobile phase at ionic strength I and zero is modeled by the Debye–Hückel term (Eqn. 16.1) and a term for the salting-out region to give Eqn. 16.11

$$\ln \frac{\gamma^m}{\gamma_0^m} = -\frac{1.5}{a} \frac{\sqrt{I}}{1 + 1.6\sqrt{I}} + 0.15I \tag{16.11}$$

where I the ionic strength and parameter a is a constant for the pH of the mobile phase. The ratio of activity in the stationary phase at ionic strengths I and zero is defined by Eqn. 16.12, which is an empirical expression.

$$\ln \frac{\gamma^s}{\gamma_0^s} = bI - cI^2 \tag{16.12}$$

The parameters b and c must be obtained by fitting experimental retention data. The ratio of the molar volume of the mobile phase at zero and I ionic strength is linearly dependent on I and is defined in Eqn. 16.13.

$$\ln \frac{\nu^m}{\nu_0^m} = 0.016I \tag{16.13}$$

Substitution of the terms of Eqn. 16.10 with the expressions defined in Eqns. 16.11–16.13 results in Eqn. 16.14.

$$\ln k' = \ln k'_0 - \frac{1.5}{a} \frac{\sqrt{I}}{1 + 1.6\sqrt{I}} + 0.15I + bI - cI^2 + 0.016I \tag{16.14}$$

Data for retention on different HIC sorbents were fitted to this model and U-shaped curves were obtained. Only the retention of lysozyme, a protein with high pI (9.1), was investigated. As the operating pH was 8, the protein was positively charged, a factor that was not considered in the model. The retention behavior of negatively charged proteins was different from that in the previously described model [173]. The $\ln k'$ of lactalbumin, lactoglobulin, IgG, BSA, and ovalbumin, which were negatively charged at the operating pH, and of lactoferrin and lysozyme, which were positively charged, was determined for 15 different HIC sorbents of varying hydrophobicity. The shape of curves $\ln k'$ *vs.* I was different, depending on the hydrophobicity of the sorbent and the charge of the model protein. In general, for sorbents with higher hydrophobicity, $\ln k'$ increased more rapidly than for sorbents with lower hydrophobicity. The curves could not be fitted to any of the already described models, because they were either U-shaped, flat, or sigmoid. It could be shown that, for the positively charged lysozyme, curves were U-shaped, whereas for the negatively charged α-lactalbumin, curves were bent downwards at low ionic strength. Lactoferrin showed aberrant behavior in the isocratic elution experiments, indicating a conformational change during chromatography. At zero ionic strength, two broad peaks

were eluted, whereas with a slight increase in ionic strength, the protein could be eluted in a single, sharp peak. Representative chromatograms are shown in Fig. 16.7. Retention increases rapidly with ionic strength; unfolding of the protein may take place, as unfolded proteins are more strongly retained than their native monomers [174].

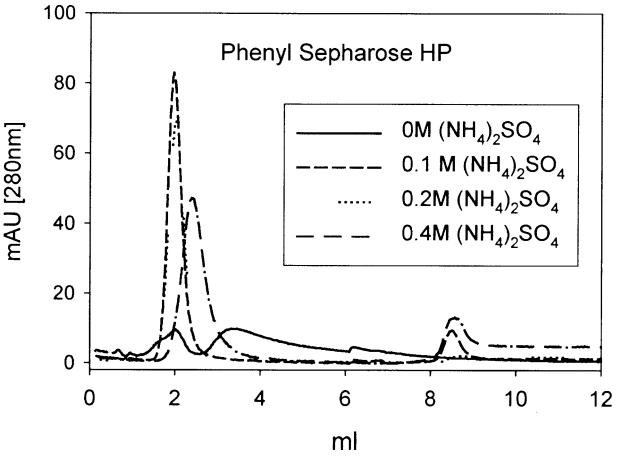


Fig. 16.7. Isocratic elution of lactoferrin (3 mg/mL) from Phenyl Sepharose HP with 0, 0.1, 0.2, and 0.4 *M* ammonium sulfate in 20 m*M* sodium phosphate buffer.

The slope of curves $\ln k'$ *vs.* I is a very useful guide in the interpretation of retention data. The slope varies with increasing temperature and can therefore be a measure of conformational changes of the protein during chromatography. For example, α-lactalbumin is eluted from a mildly hydrophobic sorbent in a native and an unfolded form at low temperature. With increasing temperature, only the unfolded form is eluted [174]. The slope can be interpreted as a combination of changes in preferential hydration and preferential salt interaction of the protein [175]. A quantitative relationship between the salt or additive and protein conformation is given by the preferential-interaction theory [176]. Assuming a protein being dissolved in an aqueous salt buffer, the distribution of ions and proteins is given by the *preferential-interaction* parameter, Γ, expressed in Eqn. 16.15.

$$\Gamma^m_{3,2} = \left(\frac{\delta m_3}{\delta m_2}\right)_{T,\mu_1,\mu_2} = \nu_3 - \frac{m_3}{m_1}\nu_1 \tag{16.15}$$

where m_2 and m_3 are the molal concentrations of salt and protein, m_1 is the molal concentration of water (55.51 *m*) and ν_3, ν_1 are the total number of moles of salt and water interacting with each mole of a protein. For electrolyte solutions, the preferential interaction of cations and anions with a protein can be described by Eqn. 16.16.

$$\Gamma^m_{+,2} + \Gamma^m_{-,2} = (\nu_+ + \nu_-) - n\frac{m_3}{m_1}\nu_1 \tag{16.16}$$

where ν_+ and ν_- are the moles of cations and anions and n is the total number of ions. The preferential-interaction parameter can be applied to the retention of a protein on a

stationary phase. The stoichiometry of an adsorption process can be described as follows.

$$pP + sS \Leftrightarrow cC \tag{16.17}$$

i.e., p mole of protein, P, interact with s moles of a binding site, S, of the surface, forming c moles of a complex, C. The equilibrium binding constant is defined as

$$\ln K = c\,\ln(m_c) - p\,\ln(m_p) - s\,\ln(m_s) \tag{16.18}$$

The expression for the preferential-interaction parameters between cations and anions and the complex, and the protein and the binding surface site, as described in Eqn. 16.16, is the product of the available binding sites, S, and the equilibrium binding constant, K, given by Eqn. 16.19 for electrolyte solutions

$$SK = \left(\frac{\delta \ln K}{\delta \ln a}\right) = (\Delta\nu_+ + \Delta\nu_-) - n\frac{m_3}{m_1}\Delta\nu_1 \tag{16.19}$$

where a is the activity of water; k' is related to K through the phase ratio. Substituting $\delta \ln a$ with $\ln m_3/g$, where g is the activity coefficient as a function of the salt concentration, results in

$$\frac{\delta \ln k'}{\delta \ln m_3} = \ln(k') - c = \left[\frac{\Delta b_+ + \Delta b_-}{g} - \frac{n\Delta\nu_1}{m_1 g}\right]m_3 \tag{16.20}$$

In Eqn. 16.20, Δb is the change in the ion-binding coefficient: $\Delta b = \Delta\nu/m_3$. Thus, the retention factor, $\ln k'$, at a certain salt concentration is related to the number of moles of cations, anions, and water that are released when a protein is adsorbed on a solid phase. Preferential-interaction analysis provides an insight into various structural alteration processes, such as protein stabilization, conformational changes, adsorption, aggregation, and ligand binding. Parameters obtained by this model have a greater physical meaning than parameters estimated by the solvophobic theory. The hydrophobic contact area between the protein and the adsorbent can be estimated. It was found that the number of water molecules released from the adsorbent increased with increasing hydrophobicity of the stationary phase and the size of the protein. Increases in the salt concentration lowers the retention time due to displacement of the ordered water molecules. The model can be used to predict retention behavior.

16.6.1.2 Retention mechanism in reversed-phase liquid chromatography

In analogy to HIC, hydrophobic interactions between hydrophobic surface patches of the protein and the stationary phase are responsible for retention. In contrast to HIC, where interactions are promoted by salt, the strong hydrophobicity of the stationary phase itself causes retention. Proteins can only be eluted by increasing the organic modifier, such as acetonitrile, ethanol, methanol, 2-propanol, or related organic solvents. Two models exist to describe retention mechanism in RP-LC: The first is an adaptation of the solvophobic theory by Horváth *et al.* [177] and the second is the partition theory developed by Dill *et al.* [178–180]. The solvophobic model describes association of molecules in a single aqueous phase; the stationary phase plays a passive role. The retention is due to an adsorption

process. Terminal parts of the ligand or even the whole ligand interact with the solute molecule. The ligand is thought to be rigid and inflexible. The transfer of a solute from one phase to another is the main principle of the partitioning model. The stationary phase contributes actively to retention. The protein is completely embedded in the stationary phase. The bonded alkyl chains, serving as ligand, undergo conformational changes [181], immersing the solute molecule. The selectivity of adsorbents varies with ligand density, and shape selectivity is very different for polyaromatic hydrocarbons on different columns, reflecting an active role of the stationary phase [182–184]. It is still unclear which model is best suited to explain the retention mechanism. Recent studies indicate that retention is mainly governed by a partitioning process rather than adsorption [185]. Analysis of retention mechanisms and factors affecting retention have been evaluated by the determination of the retention factor for various solutes on various stationary phases under different conditions [186–190]. The studies were performed with small solutes of different polarity. Proteins have not been investigated to that extent, but general aspects of the retention mechanism for small solutes can be adapted to large molecules.

Different approaches have been developed for the analysis of retention data. Each has its validity and reveals different aspects of retention data [191]. The retention factor, k', in RP-LC can be related to the mobile-phase composition according to Eqn. 16.21 [192]

$$\log k' = \log k_w - S\varphi \tag{16.21}$$

where k_w is the retention factor with water as the mobile phase, S is a parameter contributing to solute and other chromatographic conditions, and φ is the fraction of organic modifier in the mobile phase. As retention decreases with an increase of organic phase, plots of $\log k'$ *vs.* φ result in linear, decreasing lines. Eqn. 16.21 correlates the retention factor with the mobile-phase composition, but parameter S is not defined physically. Although deviations from linearity are small and Eqn. 16.21 can be applied in most cases, the influence of temperature on the retention should not be neglected. A three-parameter equation, relating the logarithmic retention factor to the mobile-phase ratio and temperature was adapted by Melander *et al.* [193] to a four-parameter equation

$$\log k' = A_1\varphi\left(1 - \frac{T_c}{T}\right) + \frac{A_2}{T} + A_3 + A_4 f(\varphi, T) \tag{16.22}$$

where A_1–A_4 are parameters appropriate to the solute and stationary phase. T_c is the compensation temperature, which is constant in RP-LC, and $f(\varphi, T)$ is a function of the class of solute anions, dipoles, etc., and the mobile-phase composition. It is a semi-empirical function, relating the dielectric constant of the solute and mobile-phase composition. Retention data of small polar and apolar molecules could be best fitted with this four-parameter equation, but there was only a slight decrease in fitting error, compared to the three-parameter equation. However, parameters A_1–A_4 are empirical constants.

A relationship of $\log k$ and possible interactions of the system with the solute is provided by Eqn. 16.23 [188–190].

$$\log k = \log k_{ref} + \eta' H + \sigma' S + \beta' A + \alpha' B + \kappa' C \tag{16.23}$$

The Greek letters refer to properties of the solute, and capital letters describe system properties. k_{ref} is the retention factor for ethylbenzene. $\eta' H$ contributes to hydrophobic

interactions, $\sigma' S$ to steric selectivity, $\beta' A$ should describe hydrogen bonding between uncharged silanol groups and acceptor solutes, $\alpha' B$ stands for interactions between donor groups of the solute and acceptors in the stationary phase, and $\kappa' C$ describes ionic interactions of silanol groups with the solute. Retention data of 67 polar, nonpolar, basic, and acidic small molecules on 10 partly similar, partly different columns were used to determine parameters H, S, A, B, and C for each column. Parameters were determined for all 67 solute molecules. Though all retention data could be predicted with great accuracy, there is no physico-chemical basis for the various terms. The influence of temperature and mobile-phase composition on $\log k'$ was studied [188–190], using the theory described earlier. Relations of solute parameters $\eta, \sigma, \beta, \alpha$, and κ to the molecular structure of the solute and relations of system parameters H, S, A, B, and C to column properties should elucidate the physico-chemical meaning of the parameters [188–190]. As Eqn. 16.23 comprises 5 different possible interactions, it is applicable to many different kinds of solute molecules.

16.6.2 Adsorption and thermodynamics

The adsorption of proteins on hydrophobic surfaces is a complex process. The driving force for the hydrophilic adsorption is reduction of surface. The individual steps are thought to be executed as follows [98]:

(a) a cavity for the protein is formed
(b) the protein fills the cavity
(c) fusion of cavities/aggregation/precipitation
(d) water and ions surround the hydrophobic surface
(e) hydrophobic interaction (Van der Waals forces) between proteins and surface
(f) structural rearrangement of protein
(g) rearrangement of water and ions in the bulk solution

Depending on the nature of the surface, the interaction of the protein can be pure adsorption or some kind of partitioning. When long alkyl chains are present, it is more likely that the protein partitions between both phases. Lin *et al.* [194–196] measured the adsorption process of proteins on HIC sorbents by isothermal titration calorimetry. They found a substantial entropy contribution when proteins interact with surfaces covered with long alkyl chains. This was explained by rearrangement of water molecules. What they did not consider was a conformation change of the proteins. It has also been hypothesized that the long alkyl chain penetrates into the core of the protein. Another way to study the thermodynamics of interaction of proteins with hydrophobic surfaces comprises pulse response experiments at different temperatures, yielding the retention factor, k' [197]. This concept was also applied in studying the adsorption of bovine serum albumin on HIC sorbents [198]. Together with flow-microcalorimeter studies, it was concluded that under linear-isotherm conditions the changes in entropy can drive the adsorption, whereas under nonlinear conditions the changes in enthalpy drive adsorption. The conformational change of the protein was not considered. Differences between linear and nonlinear conditions may be explained by conformational changes.

16.6.3 Stationary phases

16.6.3.1 Hydrophobic-interaction chromatography

In applications of Fast Protein Liquid Chromatography (FPLC), the stationary phase is either based on cross-linked polysaccharides (agarose, cellulose) or on synthetic polymers (polymethacrylate, polystyrene/divinylbenzene). In HPLC, more rigid materials, such as wide-pore silica or polymeric media are used. Ligands of different hydrocarbon content are attached *via* ether linkers to the base matrix (Table 16.9). Although there are many

TABLE 16.9

EXAMPLES OF COMMERCIAL AVAILABLE HIC SORBENTS

Ligand type	Ligand	Semi-synthetic	Synthetic
Short alkyl	Butyl- *tert*-Butyl- Ethyl- Methyl-	Butyl Sepharose 4FF*	Toyopearl butyl** Macroprep *t*-butyl*** Source 15 ETH* Macroprep methyl***
Long alkyl	Octyl- Hexyl-	Octyl Sepharose 4FF*	
-Aryl	Phenyl-	Phenyl Sepharose HP* Cellufine Octyl****	Toyopearl Phenyl**
Thiophilic	*S*-pyridyl- *S*-hexyl- *S*-butyl-	Pyridyl-S-Sepharose* Hexyl-S-Sepharose* Butyl-S-Sepharose*	

* Amersham Pharmacia Biotech.
** Toso Haas.
*** BioRad.
**** Millipore.

examples of purification strategies for proteins on HIC media, the commercially available sorbents are in most cases too hydrophobic to allow elution of proteins in their native state [174]. An ideal sorbent should bind the protein as weakly as possible so that elution can be effected gently [199,200]. Labile proteins, such as antibodies have been purified successfully by hydrophobic charge-induction chromatography [201]. Here, the support carries an ionizable hydrophobic ligand, 4-mercaptoethylpyridine (MEP), which is effective in the separation of immunoglobulins [202]. The protein binds under physiological conditions without the need for increasing the ionic strength. As the mobile-phase pH decreases, both the protein and the ligand become positively charged, and elution is achieved by electrostatic repulsion. The hydrophobic spacer between the

base matrix and the pyridine ring is important for adsorption due to hydrophobic interactions. The sulfur atom contributes to an increase in binding capacity and is necessary for proper interaction with the antibody. Pyridine rings or other heterocycles containing a sulfur and nitrogen atom have also been used in pseudo-affinity chromatography for the purification of IgG [203,204]. Combination of two different types of interaction hydrophobic and electrostatic represents a clever approach to working with low salt concentrations. Not only antibodies have thus been successfully purified, but also endotoxins, host DNA, and viruses.

16.6.3.2 Reversed-phase liquid chromatography

The most common support material for RP-LC is silica. Characteristics of the bonded phase, such as ligand density, surface area, and porosity depend on the base matrix. The silanol groups of silica form the attachment points for anchoring the bonded phase. Silanol groups are weakly acidic and could cause peak tailing and poor resolution of polar compounds. For the preparation of silanol-free bonded phases, re-hydroxylation and de-activation by heat or chloromethylsilane treatment have been successful approaches. The silanol surface must be covered with chloromethylsilane to provide active reaction sites for attachment of the ligand. The extent of surface coverage is an important factor affecting chromatographic performance. Commonly used ligands have saturated hydrocarbon chains of different length. A representative list of commercially available sorbents is presented in Table 16.10. As an alternative to silica, supports based on polymeric material, such as polystyrene/divinylbenzene or polyether, are also available. These materials require no bonded phase, because the resin itself is highly hydrophobic due to phenyl residues of the material. As there are no silanol groups, polymeric materials are stable at lower and higher pH than silica, and no endcapping is necessary. Wide-pore (30-nm) packing material is a superior stationary phase for protein RP-LC; resolution and yield are a function of pore-size and molecular weight [205]. Materials with larger pore size showed higher capacity than materials with smaller pores, both having the same alkyl chain-length. For materials with longer alkyl chains, the capacity is greater [206]. A broad range of proteins with different molecular weight and hydrophobicity could be eluted from wide-pore polystyrene-based sorbents in sharp peaks with satisfactory resolution [207]. Surface diffusion may contribute to mass transfer in RP-LC, at least in the case of small molecules [208]. It is not yet clear to what extent surface diffusion is involved in mass transfer of proteins. It is important to keep in mind that separation principles for small molecules may not apply to proteins. Columns suited for peptide separation are often unsuitable for protein separation. For proteins, a pore size of ≥ 30 nm is recommended, while for peptides a 10-nm pore size may suffice.

16.6.4 Purification strategies

In HIC, the sample must be dissolved in the same buffer of high salt concentration as used for the equilibration of the column in order to retain the protein. This could be crucial in the case of proteins dissolved in buffers of high molarity but without antichaotropic

TABLE 16.10

COMMERCIALLY AVAILABLE RP-LC SORBENTS

Ligand type	Description	Examples	Manufacturers
No	Polymer-based matrix	PolymerX RP-1	Phenomenex
		Phenyl-5PW RP	Toso Haas
		Ambercrom CG (bulk medium)	Toso Haas
		Source 5RPC (bulk medium)	Pharmacia
C1	For polar and multi-functional compounds	Bio-Sil C1	Bio-Rad
		ProntoSIL 120-5-C1	Bischoff
C3	HIC	Ultrapore C3	Beckman/Altex
C4	For non-polar solutes, 300 Å: large proteins	Sephasil C4 (bulk medium)	Pharmacia
		Jupiter C4	Phenomenex
		Vydac 214TP C4	Grace Vydac
		ProntoSIL 300-5-C4	Bischoff
C6	IPC	Spherisorb C6	Waters
C8	Wide applicability, less retentive than C18	Luna C8	Phenomenex
		Hypersil C8 Octyl	Hypersil
		ProntoSIL 300-5-C8 SH	Bischoff
C18	Most retentive for non-polar compounds; wide applicability	Nucleosil C18	Macherey-Nagel
	300 Å: Proteins, large molecules	LiChrospher RP-C18	Merck
		Jupiter C18	Phenomenex
		Discovery C18	Supelco
		ProntoSIL 300-5-C18 H	Bischoff
CN	RP-LC or NP-LC for polar compounds	Apex CN	Jones
		Zorbax C18	Agilent
		ProntoSIL 120-5-CN	Bischoff
Phenyl	For aromatic compounds used in HIC	Cosmosil Phenyl	Nacalai Tesque
		Nucleosil Phenyl	Macherey-Nagel
		ProntoSIL 120-5-Phenyl	Bischoff
Phenylethyl	For extremely polar aromatic compounds		

properties (8 *M* urea). In RP-LC, pretreatment of the sample is necessary; best resolution is obtained when the sample is dissolved in the same mobile phase as the one used as the starting eluent. Elution in HIC is effected by a linear or even stepwise decrease in salt concentration. However, more tightly bound contaminants must be removed by a regeneration step with cleaning agents, such as alcohol, NaOH, or detergents. In RP-LC, regeneration can be simply effected by an increase of the organic modifier up to 95%.

Several labile proteins of medical relevance have been purified with HIC in a single step, *e.g.*, Factor X on Phenyl Sepharose HP [209] and monoclonal antibodies on Phenyl Superose and Phenyl Sepharose (both from AP Biotech) [210,211]. Phenyl Sepharose was also used for the purification of recombinant antibody fragments [212] and for endotoxin removal [213]. The separating power of HIC sorbents was compared by applying the same feed stock to 9 different chromatographic media [173]. As feedstock, whey was prepared from collostral milk by acid precipitation [214]. Although baseline separation of all whey proteins could not be achieved, most of the proteins were eluted in different fractions. Best results were obtained with Phenyl Sepharose. A chromatogram and the corresponding electropherograms of the individual fractions are shown in Fig. 16.8. The elution patterns from the 9 sorbents were quite different, reflecting the importance of the type of ligand and the base matrix. For example, from the strong hydrophobic Octyl Sepharose, all proteins were eluted in a single peak, whereas from Butyl Sepharose, acidic whey was eluted in 4 overlapping peaks. The Butyl Sepharose-based sorbent showed an elution profile different from that of the methacrylate-based sorbent (Tosoh Biosciences), demonstrating the influence of the base matrix.

Peptide mapping is important for ascertaining the composition and conformation of even large proteins (Chaps. 14 and 15). Proteolysis with trypsin, papain, pepsin, etc., cleaves the protein. The digest, consisting of a broad range of peptides, is analyzed by HPLC [215], capillary electrophoresis [216–218], 2D gel electrophoresis, and subsequently, MALDI-TOF [219–222]. The peptide patterns of a single protein can be compared with the patterns of related proteins to investigate structural differences. These are due to, *e.g.*, post-translational modifications, oxidized, de-amidated variants [223] or disulfide bridges [224]. By analysis of peptide sequences, biologically relevant structures, such as active and antigenic sites, receptor-binding areas, or catalytic centers can be identified. By tryptic digestion of cytochrome c and bovine growth hormone, bound to a resin for RP-LC, the relevant binding sites of the model proteins could be determined [225]. The peptide pattern after digestion *in situ* was different from that obtained after digestion in solution. Thus, the protein bound to a stationary phase is protected to a certain extent against proteolytic activity. RP-LC is a convenient technique for peptide analysis because of its reproducibility, speed, and high resolving power. Hydrophobicity and, thus, retention on reversed-phase columns can be directly related to the amino acid composition of the peptide. The retention behavior of peptides with different quantities of hydrophobic amino acids can serve as a basis for the prediction of the retention time of larger proteins [226]. Polypeptide chain-length and the contribution of the α-amino group at the *N*-terminal residue of a peptide to chromatographic behavior must be taken into account [227].

The number of peptides resulting from the digestion of a protein exceeds the resolving power of a single chromatographic column. A combination of two columns of different

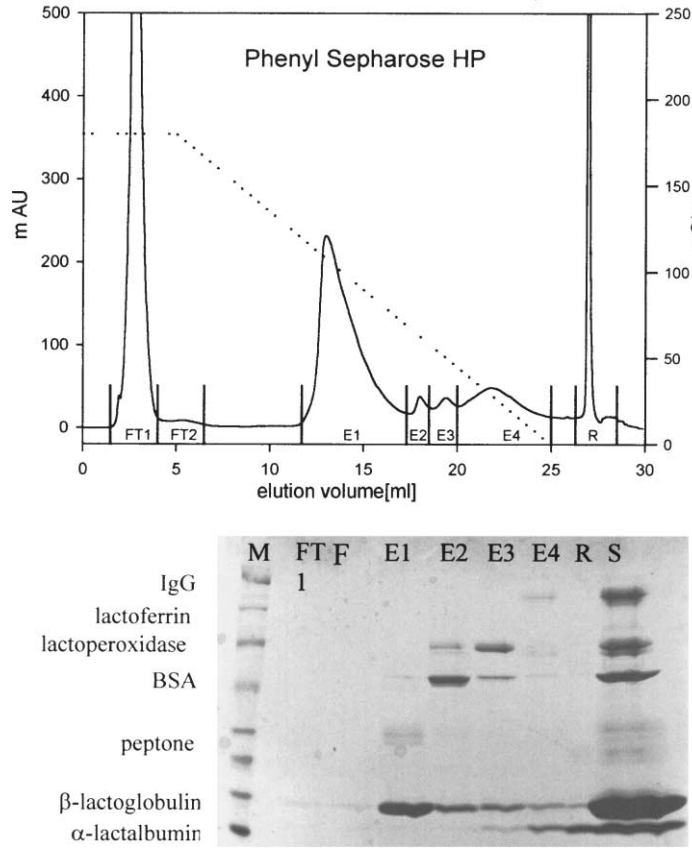


Fig. 16.8. Separation of whey proteins on a column of Phenyl Sepharose HP (100 × 5 mm ID) and SDS-PAGE of the corresponding fractions; M = marker, FT = flow-through, E1–E4 = eluate 1–4, R = regenerate, S = original sample. Equilibration of the column was effected with 2 column volumes (CV) of a 20 m*M* phosphate buffer (pH 7.0), containing 1.5 *M* ammonium sulfate buffer, at a linear flow velocity of 306 cm/h. One mL of the sample was injected at a linear flow velocity of 100 cm/h. Unbound protein was washed out with 2 CV of equilibration buffer. The proteins were eluted by a linear decreasing salt gradient from 1.5 to 0 *M* ammonium sulfate in 20 m*M* phosphate buffer over 10 CV, followed by a regeneration step with 1 mL of 30% 2-propanol. The gradient was prepared from 20 m*M* phosphate buffer (pH 7.0), containing 1.5 *M* ammonium sulfate and a 20 m*M* phosphate buffer (pH 7.0). (Reproduced from Ref. 173 with permission.)

functionality can help to resolve complex peptide mixtures [228,229]. To achieve reproducible results, manual fraction collection and re-injection should be avoided. In the field of proteomics, 2D maps of complex protein mixtures are required. For complex protein samples, such as cell lysates, tissue, serum, or plasma, analysis must be rapid, robust, and reproducible and offer high resolution. In contrast to 2D gel electrophoresis, 2D HPLC can fulfill all of these requirements [230–233].

Recombinant proteins, produced in bacteria cells as inclusion bodies, must be dissolved in buffer solutions containing chaotropic salts (urea, guanidinium hydrochloride) in order to solvate individual protein molecules. For proteins containing cysteine residues, "wrong" disulfide bonds must be broken by addition of reducing agents, such as dithiothreitol (DTT), β-mercaptoethanol, or monothioglycerol. During refolding, conformational changes result from the denatured and reduced to the native state. These are due to re-arrangements of secondary structures, proline isomerization, H-bond and disulfide-bond formation, and association of subunits and domains. Formation of correct disulfide bonds is slow in comparison to the re-arrangement of secondary structures [234]. They can be monitored by RP-LC. Unfolded proteins have a greater hydrophobic contact area than folded proteins and, therefore, they are more strongly retained on a RP column. Folding intermediates can be detected due to differences in their hydrophobicity, which are caused by successive disulfide-bond formation. An example of the applicability of this method is shown in Fig. 16.9 [235]. α-Lactalbumin in its native conformation is eluted

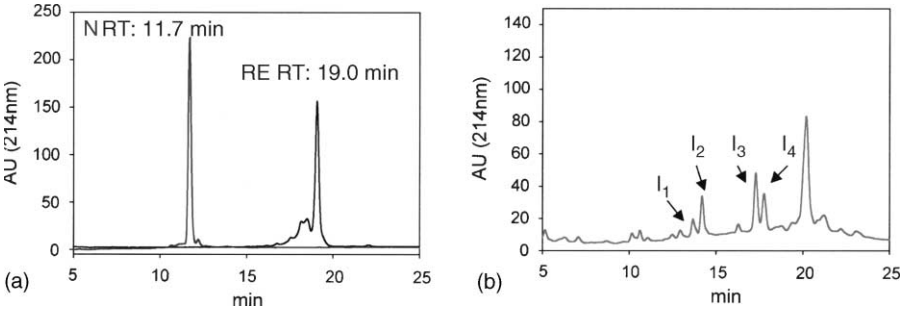


Fig. 16.9. Reversed-phase chromatography of native (N) and denatured, reduced (R) α-lactalbumin (A) and intermediate conformations (I1–I4) (B) on a Vydac C4 column (250 × 4.6 mm ID). Prior to analysis, all samples were centrifuged at 12,000*g* for 3 min to remove insoluble material. RP-HPLC performed on a Vydac C4 column (214TP54), connected to a Agilent 1100 HPLC workstation. Fully denatured α-LA was separated from oxidative folding intermediates and native protein by linear gradient elution from 37% to 45% aq. acetonitrile, containing 0.1% TFA, in 15 min at 1 mL/min and 30°C. The system was calibrated with solutions of native and denatured α-LA, using a molecular extinction coefficient of 23,150 M^{-1} cm^{-1}. (Reproduced from Ref. 235 with permission.)

ahead of the protein in reduced conformation. The peaks eluted in between could be identified by mass spectrometry as folding intermediates, having 1 (*I*1) to 3 (*I*3) disulfide bonds. The peak marked as native conformation is certainly denatured under the harsh conditions of RP-LC. Several proteins that were chromatographed this way show evidence of denaturing conditions [236–241]. However, the proteins are not reduced, and the correct disulfide bonds are conserved. As transition of α-lactalbumin from the denatured to the native conformation occurs within the time span of chromatography [242], the early-eluted peak corresponds to the native conformation.

Proteins can be eluted by *displacement* with a competing agent, having higher affinity for the stationary phase than the protein. Simultaneous concentration and purification of the product in a single step can be achieved. Alternatively to large polyelectrolyte

displacers, low-molecular-weight molecules have been successfully employed in displacement chromatography [243]. They are advantageous with respect to operating conditions. The sample can be eluted in highly concentrated form, while the salt concentration can be kept low. Low-molecular-mass displacers that have been used belong to the classes of dendrimers [244,245], protected amino acids [243], and antibiotics [246]. The design of the displacer molecules depends on stationary-phase characteristics.

16.7 ION-EXCHANGE CHROMATOGRAPHY

16.7.1 Principles and retention mechanism

Ion-exchange chromatography (IEC) exploits the fact that every protein has a different composition of acidic and basic amino acids. Reversible binding occurs to oppositely charged groups, immobilized on an ion-exchange resin. Elution is effected by increasing the ionic strength and, thus, the concentration of salt ions. Salt ions compete with protein molecules for opposite charges on the resin, and depending on the strength of interactions, proteins are removed from the sorbent at a certain salt concentration. The net charge of a protein is dependent on the pH of the buffer system. At a pH far from the isoelectric point (pI) of the protein, binding to an ion-exchange resin is stronger than at a pH near the pI, according to the net charge concept. Theory further postulates that proteins are not retained on an anion or cation exchanger at their pI. Additionally, the protein charge should correlate with its retention time. However, elution behavior deviates from this postulate [247]. For example, it has been observed that proteins can bind to an ion-exchange resin even though they are equally charged [248]. This could be due to an accumulation of differently charged residues at a distinct part of the protein surface. Owing to attraction of protons to cation-exchange groups, the pH in the matrix is about one unit lower than in the surrounding buffer. In the case of anion-exchange groups, hydroxyl ions are attracted, resulting in a pH one unit higher in the matrix than in the buffer. This is the Donnan effect [249]. Bound proteins are thus exposed to a pH one unit lower or higher than the pH of the buffer system [250].

IEC is an attractive method, because the separation principle is straightforward, it is versatile, a broad range of stationary phases and buffer systems are available, the adsorbents have a high binding capacity and resolving power, and the biological activity of the product is often preserved. However, little is known about the fundamental adsorption process. Two different models were developed to explain the retention mechanism. The stoichiometric displacement model (SDM) describes the displacement of ions by the solute [247]. This model has been refined to apply to proteins and was then termed steric mass action (SMA) model [251]. The second model explains retention by electrostatic interactions and additionally, Van der Waals interactions at higher salt concentrations [252,253]. The concept of a stoichiometric model for retention on ion-exchange columns was first formulated by Boardman and Partridge in 1953 [254]. It postulates that ions on the solid support and on the protein in solution are displaced during adsorption, and it is

described by Eqn. 16.24

$$PC_i + ZD_B \Leftrightarrow P_B + ZaD_0 + ZbC_i \tag{16.24}$$

where P is the concentration of protein in solution surrounded by C_i counter-ions, and P_B is the protein concentration bound to the matrix. D_B is the concentration of displacing ions attached to the solid support; Z is the number of charged species, involved in the adsorption/desorption process; D_0 is the concentration of the displacing ions in the mobile phase; and the constants a and b should adjust for valence of the corresponding ions, as the displacing power of these ions is proportional to the ionic strength. The binding constant, K_B, can be expressed as

$$K_B = \frac{[P_B][aD_0]^Z[bC_i]^Z}{[PC_i][D_B]^Z} \tag{16.25}$$

Only a certain fraction, f, of the total proteins, P_m, is bound to the matrix. Fraction f is defined in Eqn. 16.26.

$$f = \frac{P_B}{P_m} \tag{16.26}$$

The concentration of displacing ions attached to the matrix decreases when protein P_B is bound, and D_B can be expressed in Eqn. 16.27.

$$D_B = D_{B,\max} - (1 - f) \tag{16.27}$$

In chromatography, the distribution coefficient, k_i, is defined as the ratio of concentrations in the stationary phase, C_s, and mobile phase, C_m.

$$k_i = \frac{C_s}{C_m} = \frac{fP_m}{PC_i} \tag{16.28}$$

The constants K_b, a^Z, b^Z and $D_{B,\max}$ can be lumped together to a constant, k_y, and Eqn. 16.25 can be simplified to

$$k_y = \frac{fP_m[C_i]^Z[D_0]^Z}{[PC_i]} \tag{16.29}$$

The retention factor, k', is related to the distribution coefficient, k_i, as described in Eqn. 16.30.

$$k' = \frac{k_i A_s}{V_m} \tag{16.30}$$

where A_s is the surface area of the stationary phase and V_m is the volume of the mobile phase. Introducing k_z, as defined in Eqn. 16.31, gives an expression for the retention factor k', expressed by Eqn. 16.32.

$$k_z = k_y \frac{A_s}{V_m} \tag{16.31}$$

$$k' = \frac{k_z}{[D_0]^Z [C_i]^Z} \tag{16.32}$$

The concentration of salt ions displaced upon protein adsorption from the solid support, $[D_0]$, equals the concentration of counter-ions released from the protein $[C_i]$.

$$[D_0]^Z [C_i]^Z = [salt]^{2Z} \tag{16.33}$$

Inserting Eqn. 16.33 into Eqn. 16.32 and expressing the resulting equation logarithmically gives Eqn. 16.34.

$$\log k' = 2Z \frac{1}{[salt]} + \log k_z \tag{16.34}$$

Plotting the retention factor *vs.* the ionic strength gives a straight line. The intercept corresponds to $\log k_z$ and the slope is Parameter Z. Experimental data suggest that the number of charges involved in the adsorption/desorption process (Z) is greater or smaller than the number of charged sites on the protein [247,255]. The charge distribution on the protein surface is not uniform, and there are regions rich in basic or acidic amino acids. The location of charged regions has a major influence on retention behavior. For example, the net charge of cytochrome c was varied by controlled reaction of chloro(dinitro)benzoic acid with lysine residues in the protein. A mono-substituted sample was eluted from a cation-exchange column in four fractions, each having one more lysine residue substituted. Thus, the difference in elution behavior could only be due to a difference in the location of charged regions. The protein orients itself through electrostatic steering of the solid support. Charged surfaces are covered by an electric double layer. The thickness of this layer is dependent on the charge and concentration of ions in the mobile phase. In comparison to the protein size, the layer thickness is small and, therefore, the shielding effect of highly charged regions on the protein is not compensated for by the double layer. Protein conformation also influences the binding capacity of an ion-exchange resin. The SMA model postulates that large molecules, *e.g.*, proteins, can sterically block more ion-exchange sites than they interact with [251,256–258]. A multi-component protein/salt equilibrium can be described with this model. The protein binds to v_i charged sites of the stationary phase. As the density of charged sites on the stationary phase is higher, the bound protein covers these sites without interacting with them. Counter-ions (C_i) remain bound to the shielded binding sites. As long as the protein is attached to the solid support, the shielded charges are not available for other interactions. With the assumption that the protein itself does not carry any counter-ions, the stoichiometric exchange for i number of components is formulated similar to Eqn. 16.24.

$$C_i + v_i \bar{Q}_1 \Leftrightarrow Q_i + v_i C_1 \qquad i = 2...NC \tag{16.35}$$

C_i and Q_i represent the concentration of protein in the mobile and stationary phase, respectively; $\bar{Q}_1$ is the concentration of ions bound to the stationary phase, whereas C_1 is the concentration of ions in the mobile phase. The concentration of sterically shielded salt ions, $\hat{Q}_{1,i}$, is proportional to the protein concentration in the stationary phase, defined in

Eqn. 16.36

$$\hat{Q}_{1,i} = \sigma_i Q_i \qquad i = 2...NC \tag{16.36}$$

where σ_i is the steric factor. The steric factor lumps together all parameters causing a deviation from nonideality in order to avoid working with activity coefficients.

Cramer and coworkers applied the SMA model to different modes of protein adsorption: Linear and nonlinear adsorption of proteins over a wide salt concentration range [259], nonlinear, multi-component adsorption [244], and adsorption equilibrium under nonlinear, isocratic conditions [256]. The elution behavior with a step gradient and linear gradient could be predicted [251,257]. The retention volume, V_r, in isocratic elution is defined as

$$V_r = V_{NA} + B\left(\frac{\Lambda}{z_s c_s}\right)^v \tag{16.37}$$

where V_{NA} is the retention volume at high salt concentration, B is defined in Eqn. 16.38, Λ is the binding capacity of the sorbent, z_s and c_s are charge and concentration, respectively, of the displacing salt ion, and v is the ratio of protein charge and counter-ion charge.

$$B = V_{col}(1 - \varepsilon)\varepsilon_p K_d K \tag{16.38}$$

V_{col} is the column volume, ε is the interparticle porosity, ε_p is the intraparticle porosity, K_d is the exclusion factor, and K is the equilibrium constant. For the retention volume in gradient elution, Eqn. 16.39 applies.

$$V_{r,G} = V_{r,S} + \frac{1}{G}\left[GB\left(\frac{\Lambda}{z_s}\right)^v (1+v) + c_{s,0}^{v+1}\right]^{\frac{1}{1+v}} - \frac{c_{0,S}}{G} \tag{16.39}$$

In Eqn. 16.39, $V_{r,G}$ is the retention volume in gradient elution. $V_{r,S}$ is the retention volume of the salt, G is the gradient slope, and $c_{s,0}$ is the salt concentration at the beginning of the gradient.

In the *electrostatic-interaction model*, the protein as well as the stationary phase are defined as charged bodies, surrounded by an electrical double layer. The simplest geometry between interacting species that can be assumed are two planar slabs. From this assumption, the slab model was derived, correlating the retention time of a macromolecule on an ion-exchange resin with the ionic strength. The solution of the linearized Poisson–Boltzmann equation (Eqn. 16.40) describes the specific work of the system which is expanded to move two differently charged "slabs" together up to a certain distance, L [259].

$$\frac{\Delta G_{es}}{A_p} = \frac{1}{\kappa \varepsilon_0 \varepsilon_r}\left(\frac{(\sigma_p^2 + \sigma_s^2)e^{-\kappa L} + 2\sigma_s\sigma_p}{e^{\kappa L} - e^{-\kappa L}}\right) \tag{16.40}$$

$\Delta G_{es}/A_p$ is the change in free energy per surface area, σ_s and σ_p are the charge densities of the two surfaces, κ is the inverse Debye length, which is defined in Eqn. 16.41, and ε_0 and ε_r are the permittivity of vacuum and the dielectric constant of water, respectively. Eqn. 16.40 is the analytical solution of the second-order differential Poisson–Boltzmann equation under the condition that the interacting surfaces are planar and in contact with an

electrolyte solution. The inverse Debye length is defined as

$$\kappa = \frac{F(2I)^{0.5}}{(\varepsilon_0 \varepsilon_R RT)^{0.5}} \tag{16.41}$$

where F is the Faraday constant, I the ionic strength, R the gas constant, and T the absolute temperature. If the distance, L, is large enough, the electrical double layer is not disturbed. With decreasing L, the double layer begins to overlap and the Gibbs free energy of the system changes. When the free-energy change is negative, due to an increase in entropy upon release of ions from the double layer into the bulk solution, the two surfaces are attracted. For a closer distance, repulsion of the charged surfaces causes an increase in free energy. Repulsion can be explained by a difference in charge density of the two surfaces. For maintenance of electroneutrality, a certain amount of electrolytes is required between the "slabs". With decreasing L, the space for the electrolyte solution becomes smaller and therefore, entropy decreases. In order to account for the spherical geometry of proteins, three independent interaction types between surface and sphere have to be added to the Poisson–Boltzmann equation. However, deviation from the model assuming a geometry of planar surfaces is small. Not only electrostatic forces are involved during the interaction of charged species; Van der Waals interactions must also be considered during the adsorption process of macromolecules on a charged surface.

The combination of electrostatic and Van der Waals interactions can only explain the long-range interactions (>1 nm) in detail. For short-range interactions, the structural behavior of the solvent has to be taken into account. In the case of protein adsorption on a solid support, water molecules must be removed from the stationary phase. The work required for water release depends on the properties of the mobile and stationary phases as well as on the protein molecule. The slab model can be used to correlate the elution behavior of a protein with different salt concentrations under various conditions, such as protein size and net charge, and surface charge density of the solid support. The retention factor, k', can be expressed by Eqn. 16.42, derived by Ståhlberg *et al.* [253]

$$k' = \frac{A_s}{V_0} \int_0^\infty \left(e^{-\left(\frac{\Delta G(L)}{RT}\right)} - 1 \right) dL \sim \frac{A_s}{V_0} d e^{-\left(\frac{\Delta G_m}{RT}\right)} \tag{16.42}$$

where A_s is the surface area of the stationary phase, V_0 is the column dead-volume, d is the thickness of the adsorption layer, and ΔG_m is defined as the minimum value of free energy. For a charge density of the stationary phase higher than that of the protein molecule, the minimum free energy can be defined according to Eqn. 16.43 [260].

$$\frac{\Delta G_m}{A_p} = -\frac{\sigma_p^2}{\kappa \varepsilon_0 \varepsilon_r (1 - K_p)} \tag{16.43}$$

In Eqn. 16.43, K_p is a constant with a value characteristic for a certain protein at given pH and ionic strength of the elution buffer. σ_p is in this case the charge density on the protein surface. Combination of Eqn. 16.42 with Eqn. 16.43 and with expression for κ as in

Eqn. 16.41, gives Eqn. 16.44, an expression for the retention factor.

$$\ln k' = \frac{\sigma_p^2 A_p}{F(2RT\varepsilon_0\varepsilon_r)^{0.5}(1 - K_p)\sqrt{I}} + \ln\left(\frac{A_s}{V_0}\right) \tag{16.44}$$

When the retention factor is plotted against $1/\sqrt{I}$, from the slope of the curve the net charge of the protein, q_{chr} can be estimated. It was found that these values correspond well to those obtained from titrimetric experiments [261]. Though the assumptions of a simple geometry and point charges on the protein are not in good agreement with reality and are oversimplified from a theoretical point of view, the practical use of the slab model could be demonstrated.

16.7.2 Sorbents

There is a great variety of base matrices for ion-exchange sorbents (Chap. 4). These matrices are substituted by covalently bound functional groups, which are either positively or negatively charged. Exchangeable mobile counter-ions, which are displaced upon adsorption of a charged species, are associated with these functional groups. The positively charged functional groups in anion exchangers are associated with negatively charged counter-ions. In cation exchangers, negatively charged functional groups are surrounded by positive, exchangeable counter-ions. The pK_a value of the functional groups determines whether the sorbent is a weak or strong exchanger. Strong exchangers remain charged, regardless of the pH of the mobile phase, because their pK_a values are low or high (depending on whether they are cation or anion exchangers). Strong-anion exchangers have quaternary amines as functional groups, whereas for strong-cation exchangers, sulfonates are used. Weak exchangers are not completely ionized, and their charge density and, consequently, exchange capacity is thus pH-dependent. The advantage of strong exchangers is that their binding capacity does not change at low or high pH and that chromatographic separation is thus more controllable, since the charge of the sorbent does not change with pH. Ion-exchange resins are highly substituted, having 100–500 μmol of functional groups per mL of sorbent [250]. The earliest ion-exchange sorbents were based on a synthetic material, cross-linked polystyrene, with small pores, hydrophobic character, and high ligand density. These materials were suited for adsorption of small ions and used for demineralization, water treatment, and recovery of ions from waste. The first sorbent designed for proteins was based on cellulose. This polymer has a macroporous structure and is therefore accessible to the larger proteins. Cellulose exists in different physical forms (fibrous, microgranular, or beaded). Cross-linking improves its flow properties and mechanical stability. Other frequently used base materials are cross-linked agarose and dextran, because their macroporous structure results in high binding capacities and improved flow properties. Tentacle ion exchangers have functional groups attached to polymer chains, which are grafted onto the support in order to decrease the influence of the base matrix. Materials for high-performance chromatography have silica matrices, coated with a hydrophilic layer of ion-exchange groups. Polymeric media have been developed as alternatives to silica supports. They are available as uniform, monodisperse beads of 10-μm (Mono S and Q, Amersham Biosciences) and 15- to 30-μm

diameter (Source S and Source Q, Amersham Biosciences). Minibeads and monoliths are non-porous materials, used for micro-preparative and analytical separations. The great variety of available ion-exchange resins makes the choice for the best suited material difficult. To compare and evaluate certain characteristics of these materials, factors such as pH-dependence, titration curves, efficiency, binding strength and capacity, as well as particle-size distribution should be determined [262].

16.7.3 Chromatofocusing

Chromatofocusing was developed by Sluyterman and co-workers [263–265] and is most commonly used for the high-resolution separations of proteins. A pH gradient can be produced "externally" by mixing two buffers of different pH in a mixing chamber, or "internally" by taking advantage of the buffering action of the ion exchanger and running a buffer, initially adjusted to one pH, through a column initially adjusted to another pH. The latter method has the advantage of not subjecting the proteins to a pH more extreme than nearly its isoelectric pH value. The method, as typically practiced, employs a weak-anion-exchange bed and a poly-ampholyte elution buffer containing a mixture of polymeric buffering species that buffers over a broad pH range. Owing to the buffering capacity of the column, the pH gradient moves more slowly than the liquid phase. First, the protein sample is bound to the top of the column. When the gradient is close to the pI of the protein, it starts to move, and it moves fastest when the eluent pH is equal to the pI. The protein leaves the column at a pH corresponding to its isoelectric point. The theory of chromatofocusing has been described by Sluyterman [263–265] and further refined by Liu and Anderson [266,267] and Bates *et al.* [268,269]. Van Slyke [270] described the concept of buffering capacity, and showed it to be an additive property of weak acids and bases in solution. The buffering capacity, β, is

$$\beta = \frac{dB}{dpH} \tag{16.45}$$

where dB is the increment of base B, added to the buffer solution, and dpH is the resulting incremental change in pH. The initial assumptions for the eluting pH, outlined by Sluyterman and his co-workers [263–265], are straightforward. They have derived the following equation for the eluting pH, pH_f, when the stationary phase acts as an immobilized acid or base, depending on the type of ion exchanger. The buffering capacity of a defined unit of stationary phase is β_s and that of the mobile phase it is β_m.

$$pH_f = \frac{\beta_s \cdot pH_s + \beta_m \cdot pH_m}{\beta_s + \beta_m} \tag{16.46}$$

Assuming the simple case that β_s and β_m are equal, then

$$pH_f = \frac{pH_s + pH_m}{2} \tag{16.47}$$

The transit time at a certain pH value at which the protein exits from the column is [268]

$$t_{pH} = \frac{L}{u} \cdot \left[1 + \phi \frac{dq_{S,t}}{dC_s} \right] \tag{16.48}$$

where L is the column length, ϕ the phase ratio, u the velocity of the fluid, and dq_{st}/dC_s is the slope of the adsorption isotherm of an inert ion, S, at given pH in a liquid phase, containing the buffering species at their concentrations in the elution buffer. The term dq_{st}/dC_s can be written in following form

$$\frac{dq_{S,t}}{dC_s} = \frac{dq_{St}/dpH_{fluid}}{dC_s/dpH_{fluid}} \equiv \frac{\beta'_{ads}}{\beta_{fluid}} \tag{16.49}$$

where dC_s/dpH_{fluid} is the buffering capacity in the liquid phase (β_{fluid}), and dq_{St}/dpH is the buffering capacity of the adsorbed phase, defined as the change in the concentration of the inert ion in the adsorbed phase per unit change in the pH of the fluid phase (β'_{ads}), where the fluid phase is the elution buffer under consideration. From these equations, the elution profile for several buffering ions could be predicted.

Although the technique of chromatofocusing, as just described, has shown considerable success in separating proteins, the use of poly-ampholyte buffers is perhaps the greatest limitation of the method. In particular, these types of buffers add considerably to the expense of chromatofocusing, and, as obtained from chemical suppliers, they have chemical compositions that tend to vary from batch to batch. In addition, the poly-ampholytes tend to stick to the protein and are difficult to remove by dialysis or SEC [271]. Shallow pH gradients formed with poly-ampholyte buffers also tend to be irregular and irreproducible in shape and may yield poor separations. Due to the limitations of using poly-ampholyte elution buffers, various research workers have investigated methods for replacing these buffers with a chemically well-defined mixture of low-molecular-mass buffering species. For example, Hearn and Lyttle [272] and Hutchens *et al.* [273] have investigated the use of a large number of low-molecular-mass buffering species in the elution buffer. Under these conditions, the effluent pH profile consists of a sequence of discrete pH steps, spanning the elution pH and pre-saturation (*i.e.*, starting) pH, with the number of steps corresponding to the number of adsorbed buffering species in the elution buffer. Nevertheless, if an adequate number of these buffering species is present, the pH profile becomes sufficiently close to a linear shape so that the method operates essentially in the same way as when a polyampholyte elution buffer is employed.

Another approach to generating a linear pH gradient is titration of the *cis*-diol of mannitol with borate, to liberate H_3O^+ in a stoichiometric manner [25]. A stable complex is formed, which does not undergo ageing. The theory of the formation of new acids by association of boric acids and planar diols is based on the fact that the "ionization constants" of such acids often obey an equation in the form

$$K^{**} = C_{2n} K_1 K_n + K_1 \tag{16.50}$$

The ionization constant, K^{**}, varies with the concentration of the ligand, C_{2n}, and is independent of the concentration of boric acid at concentrations below 0.1 M. K_n is the association constant of the boric acid and the diol, and K_1 is the first ionization constant of

boric acid. To generate a linear pH gradient for IEC, a strong-ion exchanger must be used. The disadvantage of conventional chromatofocusing is the low solubility of proteins close to the pI. When using a borate/mannitol gradient, one can add salt to both buffers to help keeping the proteins in solution. In addition, a salt gradient can simultaneously be employed, together with the pH gradient. It was possible to form an extremely stable pH gradient. The dpH/dV was *ca.* 0.23 mL^{-1} for a column with 5 cm × 0.5 ID, and the total elution volume was 12 mL. A linear pH gradient with one pH unit could be established; in an extreme example, a pH gradient of 0.1 mL^{-1} was employed.

Gradient formation is more critical in chromatofocusing than in IEC. Also, chromatofocusing usually yields a much lower resolution than isoelectric focusing. In order to improve the resolution, continuous beds have been developed [274]. A bed activated by epoxy groups was synthesized inside a fused-silica capillary and became at the same time covalently attached to the inner wall of the capillary. A polyethyleneimine (PEI) solution was then pumped through the continuous bed to allow the imine groups in PEI to react with the epoxy groups in the bed. The functionality of the microcolumn and the resolution were checked by separation of hemoglobin variants (Fig. 16.10). The

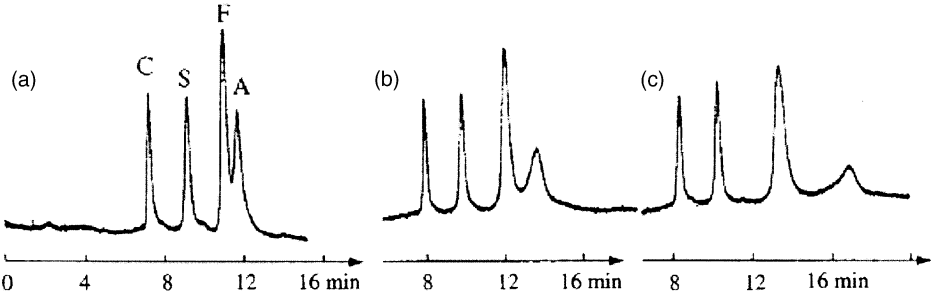


Fig. 16.10. Effect of pH of elution buffer on resolution in chromatofocusing of hemoglobins C, S, F, and A on a PEI-derivatized microcolumn (150 × 0.32 mm ID). Eluent: 1:40 diluted Polybuffer 96 at different pH values: (a) pH 6.6; (b) pH 7.0; (c) pH 7.2. Flow-rate, 10 mL/min. The order of elution of proteins from the column is C, S, F, and A. (Reproduced from Ref. 274 with permission.)

advantage of the chromatographic method over the electrokinetic methods is the scalability. Chromatofocusing has often been used for separating protein isoforms. Recent applications include chitosan isoenzymes [275], human follicle-stimulating isohormones [276], glutathione S-transferase isoenzymes [277], and metallopeptidase isoenzymes [278]. In all applications, chromatofocusing was the last step in a purification sequence. The sample must be purified prior to isoform separation. Newer modifications take advantage of computer algorithms for the generation of exact pH gradients [269,279,280].

16.8 SIZE-EXCLUSION CHROMATOGRAPHY

16.8.1 Principles and retention mechanisms

Compounds of different size show differences in their penetration into the pores of a SEC medium and are therefore retained in the column to a different extent. In 1959, Porath

and Flodin [121] separated proteins according to their size on dextran gels, later marketed as Sephadex (Chap. 5). Proteins are separated according to their diffusivities. For globular proteins there is a relationship between diffusivity, D, and size, which is represented by the Stokes–Einstein equation

$$D = \frac{kT}{6\pi\eta R} \tag{16.51}$$

where R is the radius of the protein. Polson [281] proposed several correlations for estimating the molecular mass of proteins. Based on the Stokes–Einstein equation, a simple relationship of molecular mass to protein diffusion coefficient was suggested [17]

$$D = \frac{A}{M^{1/3}} \tag{16.52}$$

where A is a constant, having a value of 2.85×10^{-5} cm^2 sec^{-1} $g^{1/3}$ $mol^{1/3}$.

Sometimes, proteins of different molecular mass may not be separated in SEC. Owing to their conformation, they may have the same diffusivity. SEC is mainly used for the isolation of proteins from crude mixtures, removal of di- and oligomers, removal of truncated forms, crude estimation of molecular mass, determination of pore-size by inverse SEC, and desalting of protein solutions. SEC can separate proteins under physiological conditions and, therefore, protein aggregates can migrate as intact complex. The association constant and thus the size of the complex can be determined by injecting different concentrations of the reaction partners [282].

The influence of sample concentration and viscosity cannot be described in a simple manner. At high viscosity and density difference between sample and eluent buffer bands may show broadening with the formation of finger-like zones. Less viscous eluents may invade the sample plug and increase the plug volume. The effect of "viscous fingering" has been studied by magnetic resonance imaging [283]. The most commonly used sorbents for SEC of proteins are dextran-based media, known as Sephadex, coated silica, coated polymethacrylate, and polyacrylamide media (Table 16.11). Media with very large pores tend to be soft and must be operated at low velocity, since the diffusion of large molecules is a rather slow process. Therefore, their limited pressure stability seems to present no problem.

16.8.2 Separation of aggregates and complexes

The analysis of albumin and immunoglobulin aggregates is a typical example of the usefulness of SEC in protein chromatography. The method is codified in the European Pharmacopeia. A chromatogram of an immunoglobulin separation is shown in Fig. 16.11. The dimers can be almost baseline-separated from the monomers and oligomers. When the peak areas are added, the sum is often short of the initial amount of injected material. This is due to the slightly different molar absorbance of oligomers, di-, and monomers. The separation of protein aggregates from the monomer by SEC has been performed not only on an analytical scale. It is a very common procedure for purifying sequences of recombinant proteins and plasma proteins. SEC is preferably placed at the end of a purification sequence, because it not only polishes the protein sample but also presents the

TABLE 16.11

SELECTION OF VARIOUS SORBENTS FOR SEC OF PROTEINS

Name	Basic material	Manufacturer
Sephadex	Dextran	Amersham Biosciences
Superose	Agarose	Amersham Biosciences
Sephacel	Cellulose	Amersham Biosciences
Sepharcryl	Dextran cross-linked with N,N'-methylene *bis*-acrylamide	Amersham Biosciences
Superdex 75 and 200	Dextran-grafted agarose	Amersham Biosciences
TSK SW series	Grafted silica	Tosoh
TSK PW series	Coated polystyrene	Tosoh
LiChrosorb Diol	Diol-bonded silica	Merck
Fractogel EMD SEC	Polymethacrylate, grafted with linear polymers chains	Merck
Bio Gel P	Polyacrylamide	BioRad
Trisacryl GF 05 and GF 2000	Modified polyacrylamide	Biosepra
Ultrogel ACA	Agarose/polyacrylamide composite gel	Biosepra
Zorbax GF 250 and GF 450	Silica with zirconia-stabilized surface and diol bonded	Agilent

protein in the required buffer. One drawback of SEC is the dilution of the sample. Thus, often an additional concentration step by ultrafiltration must be added.

The separation of the blood coagulation Factor VIII (F VIII) has been reported by Josic *et al.* [284]. This is an example of the suitability of SEC for separating extremely large proteins. Factor VIII has a molecular mass of 280 kDa, and the dominant form in plasma consists of two chains, a heavy and light chain. The chains are held together by a metal bridge. In plasma, the FVIII molecule is stabilized by the van Willebrand factor (vWF). These multimers have a molecular mass of several million Da. FVIII-substitution products should either consist of FVIII or have a defined FVIII/vWF ratio. The separation of a FVIII product on an analytical scale with a Superose 6 high-performance column (300 × 10 mm ID) is shown in Fig. 16.12A. The main components, all of them in the range of molecular masses between 150,000 and several million Da, were well separated in four peaks. The separation according to molecular masses agrees with the corresponding SDS-PAGE analysis under non-reducing conditions (Fig. 16.12B). In the first peak, a fraction with an apparent molecular mass of several million Da was eluted (Fig. 16.12A, dashed line). The entire FVIII activity was found in this eluate. This is not surprising, as FVIII in this product forms a complex with the vWF multimers, having a molecular mass of at least 4 million. A corresponding SDS-PAGE analysis under nondenaturing conditions confirms that

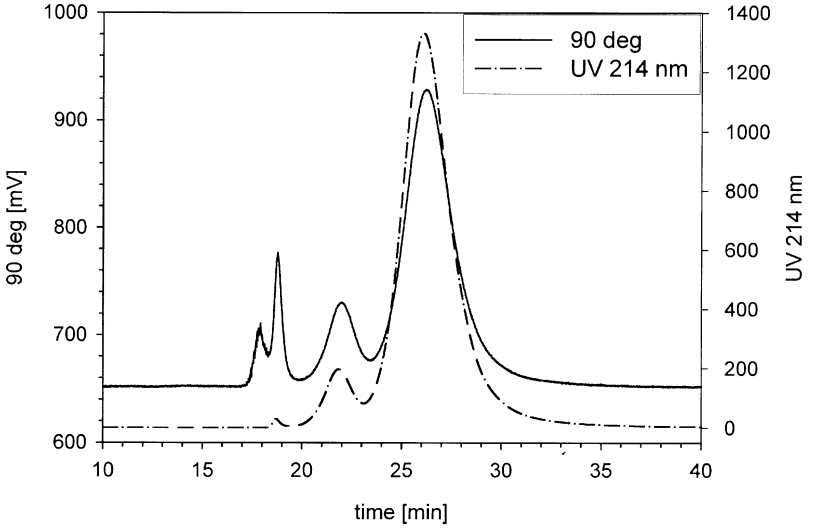


Fig. 16.11. Size-exclusion chromatography of an IgG preparation on a TSK SW 3000 column (60 × 0.75 cm ID) A 20-μL sample was injected. As eluent buffer 20 m*M* sodium phosphate (pH 6.8), containing 0.1 *M* NaCl, was used. The column was operated at 0.6 mL/min. The full line is an on-line static light-scattering signal and the dashed line an on-line UV signal. (Reproduced from Ref. 285 with permission.)

separation occurs according to the size of the molecules (Fig. 16.12B). However, the FVIII/vWF complex which appears in the first peak has such a large molecular size that it cannot even enter the separating gel (Lanes 3 and 4 in Fig. 16.12B). The separation of vWF multimers from Peak 1 in Fig. 16.12A was carried out with the corresponding agarose gel. Fig. 16.12C shows vWF multimers in different fractions from HPSEC. The separation is readily reproducible, provided that the column is regenerated with 0.5 *M* NaOH after each separation in order to remove any residues from the previous sample. This means that, under the given conditions, a part of the sample undergoes non-specific binding with the SEC medium. Therefore, the yield did not exceed 50 to 70%.

Steindl *et al.* [286] have purified monoclonal IgM antibodies by isoelectric precipitation and further purification by SEC, using a Sephacryl S300 column. The IgM antibody precipitated at pH 6.2 and was then redissolved in 0.2 *M* carbonate buffer (pH 8.5) with 0.2 *M* NaCl. A highly purified IgM fraction in a final yield of 40% could be obtained by this procedure. The process was not designed for production of IgM, but addition of a third step would have been sufficient to meet the requirements for a therapeutic protein. In general, preparative SEC is characterized by high recovery of protein and therefore used very frequently.

16.8.3 Desalting

SEC is often used for desalting of protein solutions, for, on a small scale, this is faster and more convenient than dialysis or diafiltration. Flodin [287] characterized the features

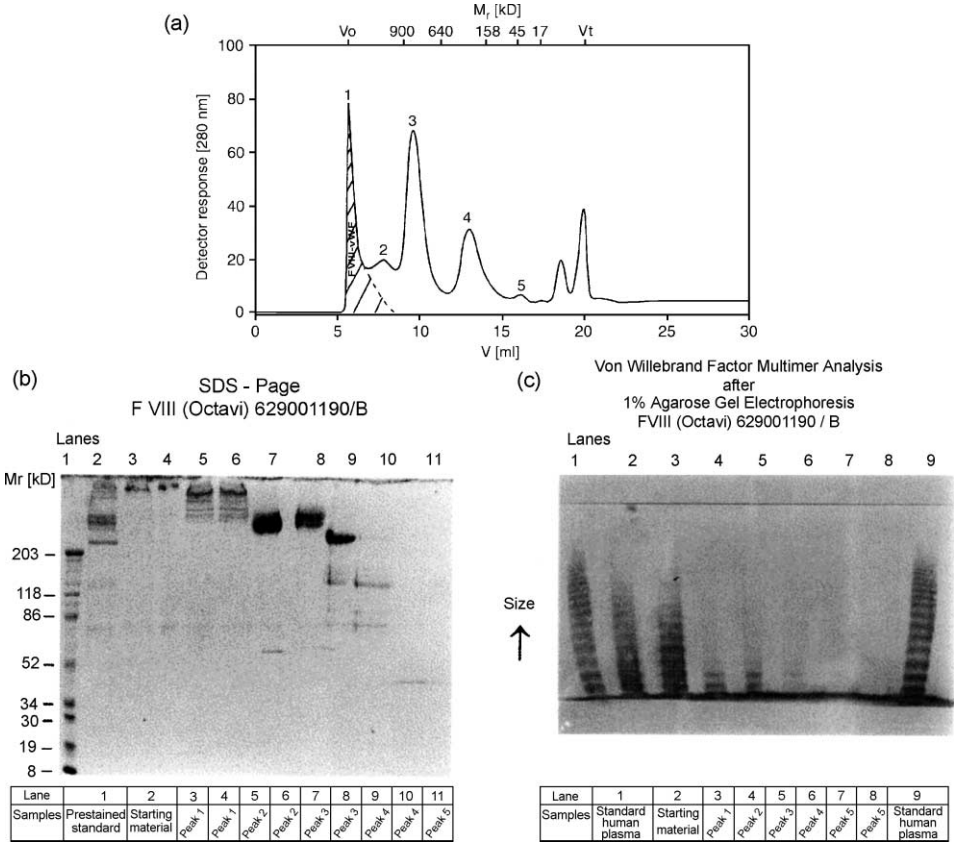


Fig. 16.12. (A) HPSEC of a preparation containing the FVIII–vWF complex. SEC chromatogram under non-denaturing conditions. A Superose 6 analytical high-performance column (300 × 10 mm ID) was used. Chromatographic conditions: 0.9 mg of protein (100 IU of FVIII and 65 IU of vWF Ag) in 0.5 mL; flow-rate, 0.5 mL/min; pressure, 9 bar; room temperature. Standard proteins as molecular size markers (250 g each): Immuoglobulin M (Mr *ca.* 900,000; thyroglobulin (640,000); immunoglobulin G (158,000), ovalbumin (45,000); myoglobin (17,000) and vitamin B_{12} (1300). Mobile phase: 200 m*M* NaCl, 15 m*M* Tris–HCl (pH 7.4); osmolarity, 440–460 mOsmol. After each run, the column was regenerated with 0.5 *M* NaOH for 2 h (flow-rate 0.5 mL/min) and subsequently equilibrated with 2 column volumes of 0.2 *M* Tris–HCl (pH 8.0) and 4 column volumes of mobile phase (flow-rate at equilibration, 1 mL/min). The fractions were collected, and the FVIII activity and the vWF Ag were determined. Recovery, 56% FVIII and 62% vWF Ag. (B) SDS-PAGE under non-reducing conditions of collected fractions from SEC in part (A) of this figure. (C) Multimers of vWF in fractions from SEC. (Reproduced from Ref. 284 with permission.)

of this method as follows:

(a) the column efficiency increases with decreasing particle size and flow-rate,
(b) the viscosity of the sample rather than the concentration is a limiting factor, and
(c) SEC of a protein solution is equivalent to an exhaustive dialysis, but it can be carried out in a much shorter time. For laboratory-scale desalting ready-to-use columns

(PD10, Amersham Biosciences) are commercially available. These columns are packed with Sephadex G10 with a filter plate at the top of the bed, which prevents the column from running dry. The columns are operated at gravity flow. On a larger scale, the columns have to be packed. Diafiltration is often preferred to SEC for large-scale desalting, since the volume can be much better controlled.

16.8.4 Refolding

For the production of proteins from inclusion bodies in *E. coli* cells, they must be refolded into the native form. The proteins are dissolved in a chaotropic agent and then diluted. The refolding process is accompanied by aggregation. The folding into the native protein follows first-order reaction kinetics, while the aggregation reaction follows a higher order. In order to recover sufficient amounts of native proteins, the folding must be performed at a very low protein concentration or aggregation must be strictly prevented. Botas and Choudkuri [288] studied matrix-assisted refolding by SEC. In the presence of chaotropic agents the denatured protein solution was loaded onto a SEC column, which was equilibrated with a buffer promoting refolding of the proteins. From their experimental observations they proposed the following mechanism:

(a) The protein in the unfolded state is characterized by a random-coil configuration with a large hydrodynamic radius. The large size of the protein does not allow it to penetrate into the pores of the SEC medium. The protein is completely excluded from the beads.
(b) Chaotropic agents are more retained than the protein and, thus, the protein gradually migrates to an environment where folding is promoted. As the protein molecule shrinks, it is able to penetrate into pores. Large protein aggregates will be excluded, and intermolecular aggregation will be prevented.
(c) Within the pores, the refolding reaction can be assured, and there is a limited likelihood of aggregation with other proteins.
(d) When the protein is fully refolded, its hydrodynamic radius is constant, and the protein is eluted from the column as an active, native protein.
(e) SEC ensures that aggregation is prevented.

Aggregates which have been formed are eluted first and are therefore separated from the native protein. SEC has been used for continuous refolding by annular chromatography. This process is designed to recycle the aggregates [235].

16.8.5 Light scattering

Light scattering is often used as detection method in conjunction with SEC. Static light scattering is a method for measuring the radius of gyration, r_g, of a protein, which is the radius of a hollow sphere having the same rotation properties as a full sphere. There is a relationship between the radius of gyration of a molecule and the molecule nominal radius. The amount of light scattered is directly proportional to the product of the weight-average molar mass and the protein concentration. At low angles the angular dependence of light scattering depends only on the mean square radius, r_g^2, and is independent of molecular conformation or branching.

The two-detector method is the most convenient one and works well together with SEC [289]. For this method the chromatographic system must be equipped with a light-scattering detector (LSD) and a refractive-index detector (RID). For glycoproteins the concentration cannot be determined by the RI detector, but UV absorbance can be used instead. Fortunately, one can use the UV signal from instead. The UV absorbance is dependent on the concentration, the molecular extinction coefficient, and a detector constant, obtained by calibration of the system. This is the basis of the three-detector method. Dynamic (quasi-elastic) light scattering can also be used for on-line detection of SEC column effluents.

16.9 PROTEOMICS AND MULTI-DIMENSIONAL CHROMATOGRAPHY

16.9.1 Goals of proteomics

The dream of having genomes completely sequenced is now a reality [290]. The complete sequence of several genomes, including the human and mouse genomes are known [291,292]. However, we are still far away from understanding the role of *ca.* half a million human proteins, encoded by some 30,000 genes, and hard work to unravel the complexity of biological systems is still ahead. A new discipline, proteomics, has arisen, which complements physical genomic research. Proteomics can be defined as the qualitative and quantitative comparison of proteomes under different conditions to further unravel biological processes. Proteomics is of interest not only in fundamental life science, but large dividends are also expected in its applications in drug discovery or discovery of new drug targets, and discovery of new biomarkers to improve the predictive value of diagnostics, in animal husbandry, and even in food technology [293].

One objective of proteomics is to find the difference between the proteomes of two different biological states. Thus, we are generally not interested in the whole proteome, but only in a small fraction. The goal is to find a method for detecting these differences in a fairly simple, fast, and reliable fashion. It is beyond our imagination to find a method which can detect proteins in such a vast excess of other proteins. For plasma proteins, there is the problem that the most abundant proteins, albumin and immunoglobulins, interfere with the detection of less abundant proteins. Both proteins overlap proteins with similar physical properties, such as size and surface charge, which are present in minute concentrations. A general strategy involves labeling of proteins and separation by 2D electrophoresis and 2D HPLC or a combination of HPLC and an electrokinetic method with detection and identification by mass spectrometry [294]. Either the mass of the whole protein is detected or a proteolytic digest is further analyzed. Strategies for comparing proteomes comprise:

(a) *In vivo* metabolic labeling. Cells are grown in presence of ^{35}S-methionine and then the cell lysate is separated by a high-resolution method, such as 2D gel electrophoresis (Chap. 15). The changes in protein expression of two different states are determined by autoradiography.
(b) Quantitation by mass spectrometry. One set of cells is grown in a medium containing amino acids labeled with ^{15}N; the other cells are grown in normal

medium, containing the stable isotope (99.6% ^{14}N and 0.4% ^{15}N). Both samples are combined, and then proteins are separated by a high-resolution method, such as 2D gel electrophoresis or 2D HPLC. Identification of proteins is accomplished by mass spectrometry. Also *in vitro* post-translational labeling is used. Two interesting new techniques have emerged, the isotope-loaded affinity tag [295] (Sec. 16.9.2) and the differential gel electrophoresis [296].

16.9.2 Preparation of a subproteome

Preparation of a subproteome is accomplished by either (a) subcellular fractionation, (b) protein fractionation by structural groups, (c) by functional groups, (d) by change, or (e) by size. Subcellular fractionation is achieved by density gradient separation, since the different cellular compartments have different sedimentation properties. From the relevant fraction a protein extract is made, and the subproteome is further separated by high-resolution methods.

Protein fractionation according to structural groups may be targeted to groups, such as carbohydrate chains, phosphate groups, lipid modifications (*e.g.*, acylation, prenylation), or accessible histidine residues. Affinity methods (Chap. 3), such as lectin affinity chromatography and metal chelate affinity chromatography, can be applied to the preparation of a subproteome. Immobilized Fe^{3+} or Ga^{3+} can be used to enrich phosphoproteins [294]. Functionally related families of proteins may be isolated through the use of an appropriate affinity matrix, *e.g.*, immobilized calmodulin for calcium-binding proteins or antibodies that recognize conjugated ubiquitin molecules for poly-ubiquitinated proteins.

Fractionation of subproteomes has been achieved by electrokinetic methods, such as preparative isoelectric focusing in the multi-compartment analyzer or free-flow electrophoresis, or by chromatographic methods, such as IEC, SEC, or chromatofocusing. Aebersold *et al.* [295] have developed a unique set of reagents, named isotope-coded affinity tags (ICAT). A reactive biotin tag contains a linker, which is labeled with either deuterium or hydrogen. The active group is iodoacetamide and is reactive for sulfhydryl groups. Two samples are made to react with either the deuterium- or the hydrogen-labeled affinity tag. Then the samples are combined and digested, and the biotin-labeled peptides are isolated by streptavidin affinity chromatography. Further identification of the peptides is accomplished by mass spectrometry.

16.9.3 Two-dimensional high-performance liquid chromatography

In 2D HPLC, either IEC or SEC is combined with RP-LC. A prerequisite is that the second separation be much faster than the first one. Wagner *et al.* [233] described a system which was composed of an ion-exchange column, a switch port, and two RP-LC columns, operated in parallel. In order to achieve very high speed and high resolution in the second dimension, short columns (14 × 4.6 mm ID with 1.5-μm *n*-octadecyl-bonded non-porous silica packings) were used. The total peak capacity was calculated to be 600. The limit of detection was 300 ng protein on average and 50 ng for ovalbumin. A sample could be analyzed in 20 min, which is equivalent to the running time for the first dimension.

An interesting mapping of the *E. coli* proteome has been reported by Opiteck *et al.* [231]. They connected two TSK G 3000SW$_{XL}$ SEC columns (300 × 7.8 mm ID) in series by a tube with a narrow diameter and analyzed the fractions obtained by RP-LC. The analysis of the fractions was accomplished by two RP-LC columns, run in parallel. While one column was loaded, the other one was eluted. They were able to construct a 2D UV chromatogram analogous to a 2D electropherogram. Chromatofocusing and RP-LC have also been combined. Chromatofocusing separates proteins according to their pI, and RP-LC according to the hydrophobicity and, to some extent, size of the protein. Larger proteins can be considered more hydrophobic than smaller ones, assuming a statistically homogenous distribution of amino acids in proteins.

16.9.4 Protein chips

An emerging technology for studying protein-interaction networks has been developed by Ciphergen. The mass spectroscopy/protein chip technology is based upon the principles of surface-enhanced laser-desorption ionization (SELDI-TOF-MS) [297]. Proteins or ligands are immobilized on a so-called target, a spot on the surface of a small chip. The protein sample is deposited on the spot, and the unbound material is washed off. Then a matrix solution, typically consisting of a saturated solution of sinapinic acid, is added to the spot. The protein chip is analyzed by SELDI-TOF-MS and the bound proteins are identified according to their mass. The chips are coated with some of the sorbents used in protein chromatography (cation or anion exchangers, hydrophobic-interaction or metal-chelate functionalities). The advantage of the technology is that a large number of binding conditions can be studied in a short time with a minimum of material. Furthermore, it is also possible to screen elution conditions. The system has been validated by column chromatography experiments. A crude *E. coli* extract, containing an overexpressed F_{ab} fragment, was separated with a CM-zirconium sorbent. Conditions for loading and elution could be accurately predicted. Another example was the separation of recombinant endostatin-expressed *Pichia pastoris*. In this case also, loading and elution conditions could be accurately predicted.

REFERENCES

1 J.M. Gosline, P.A. Guerette, C.S. Ortlepp and K.N. Savage, *J. Exp. Biol.*, 202 (1999) 3295.
2 A. Jungbauer and W. Feng, in F. Regnier and K. Gooding (Eds.), *HPLC of Biological Macromolecules*, Dekker, New York, 2002, p. 281.
3 S. Yamamoto and T. Ishihara, *J. Chromatogr. A*, 852 (1999) 31.
4 S. Yamamoto, P.K. Watler, D. Feng and O. Kaltenbrunner, *J. Chromatogr. A*, 852 (1999) 37.
5 C.L. Tucker, J.F. Gera and P. Uetz, *Trends Cell Biol.*, 11 (2001) 102.
6 V. Schachter, *Drug Discov. Today*, 7 (2002) S48.
7 S. Fields and O. Song, *Nature*, 340 (1989) 245.
8 P.L. Bartel, J.A. Roecklein, D. SenGupta and S. Fields, *Nature Genet.*, 12 (1996) 72.
9 A.J. Walhout, R. Sordella, X. Lu, J.L. Hartley, G.F. Temple, M.A. Brasch, N. Thierry-Mieg and M. Vidal, *Science*, 287 (2000) 116.

10 T. Ito, K. Tashiro, S. Muta, R. Ozawa, T. Chiba, M. Nishizawa, K. Yamamoto, S. Kuhara and Y. Sakaki, *Proc. Natl Acad. Sci. USA*, 97 (2000) 1143.
11 K. Graumann and A. Jungbauer, *Biochem. J.*, 345 (2000) 627.
12 E. Jisa, K. Graumann and A. Jungbauer, *Biocatal. Biotrans.*, 9 (2001) 427.
13 W. Feng, K. Graumann, R. Hahn and A. Jungbauer, *J. Chromatogr. A*, 852 (1999) 161.
14 J.F. Kane and D.L. Hartley, *Trends Biotechnol.*, 6 (1988) 95.
15 D.P. Knight and F. Vollrath, *Philos. Trans. R. Soc. London B*, 357 (2002) 155.
16 J. Gatesy, C. Hayashi, D. Motriuk, J. Woods and R. Lewis, *Science*, 291 (2001) 2603.
17 M.T. Tyn and T.W. Gusek, *Biotechnol. Bioengng*, 35 (1990) 327.
18 P.K. Dutta, K. Hammons, B. Willibey and M.A. Haney, *J. Chromatogr. A*, 536 (1991) 113.
19 P. Bergethon, *The Physical Basis of Biochemistry*, Springer, New York, 1998.
20 S. Ruppert, S.I. Sandler and A.M. Lenhoff, *Biotechnol. Prog.*, 17 (2001) 182.
21 P.M. Tessier, S.D. Vandrey, B.W. Berger, R. Pazhianur, S.I. Sandler and A.M. Lenhoff, *Acta Crystallogr. D Biol. Crystallogr.*, 58 (2002) 1531.
22 G. Baumann, *Horm. Res.*, 51 (1999) 2.
23 J.L. Hornick, J. Sharifi, L.A. Khawli, P. Hu, W.G. Bai, M.M. Alauddin, M.M. Mizokami and A.L. Epstein, *J. Nucl. Med.*, 41 (2000) 355.
24 B.C. Meyers, A.W. Dickerman, R.W. Michelmore, S. Sivaramakrishnan, B.W. Sobral and N.D. Young, *Plant J.*, 20 (1999) 317.
25 O. Kaltenbrunner, C. Tauer, J. Brunner and A. Jungbauer, *J. Chromatogr.*, 639 (1993) 41.
26 A. Jungbauer, C. Tauer, E. Wenisch, K. Uhl, J. Brunner, M. Purtscher, F. Steindl and A. Buchacher, *J. Chromatogr.*, 512 (1990) 157.
27 E. Wenisch, A. Jungbauer, C. Tauer, M. Reiter, G. Gruber, F. Steindl and H. Katinger, *J. Biochem. Biophys. Meth.*, 18 (1989) 309.
28 E. Wenisch, C. Tauer, A. Jungbauer, H. Katinger, M. Faupel and P.G. Righetti, *J. Chromatogr.*, 516 (1990) 133.
29 E. Wenisch, S. Reiter, S. Hinger, F. Steindl, C. Tauer, A. Jungbauer, H. Katinger and P.G. Righetti, *Electrophoresis*, 11 (1990) 966.
30 P.H. Lai, R. Everett, F.F. Wang, T. Arakawa and E. Goldwasser, *J. Biol. Chem.*, 261 (1986) 3116.
31 D.H. Catlin, A. Breidbach, S. Elliott and J. Glaspy, *Clin. Chem.*, 48 (2002) 2057.
32 J.C. Egrie and J.K. Browne, *Br. J. Cancer*, 84 (2001) 3.
33 A.D. Tran, S. Park, P.J. Lisi, O.T. Huynh, R.R. Ryall and P.A. Lane, *J. Chromatogr.*, 542 (1991) 459.
34 P. Lopez-Soto-Yarritu, J.C. Diez-Masa, A. Cifuentes and M. de Frutos, *J. Chromatogr. A*, 968 (2002) 221.
35 A. Gokana, J.J. Winchenne, A. Ben-Ghanem, A. Ahaded, J.P. Cartron and P. Lambin, *J. Chromatogr. A*, 791 (1997) 109.
36 S. Fasler, F. Skvaril and H.U. Lutz, *Anal. Biochem.*, 174 (1988) 593.
37 Z.L. Awdeh, A.R. Williamson and B.A. Askonas, *Biochem. J.*, 116 (1970) 241.
38 R.C. Grebenau, D.M. Goldenberg, C.H. Chang, G.A. Koch, D.V. Gold, A. Kunz and H.J. Hansen, *Mol. Immunol.*, 29 (1992) 751.
39 J.L. Winkelhake and G.L. Nicolson, *J. Biol. Chem.*, 251 (1976) 1074.
40 M. Nose and H. Wigzell, *Proc. Natl Acad. Sci. USA*, 80 (1983) 6632.
41 D.R. Burton, *Mol. Immunol.*, 22 (1984) 161.
42 A.D. Elbein, *CRC Crit. Rev. Biochem.*, 16 (1984) 21.
43 B.L. Maiorella, J. Winkelhake, J. Young, B. Moyer, R. Bauer, M. Hora, J. Andya, J. Thomson, T. Patel and R. Parekh, *Biotechnology (NY)*, 11 (1993) 387.
44 J. Zhang and D.I. Wang, *J. Chromatogr. B*, 712 (1998) 73.

45 E. Watson, B. Shah, L. Leiderman, Y.R. Hsu, S. Karkare, H.S. Lu and F.K. Lin, *Biotechnol. Prog.*, 10 (1994) 39.
46 D.C. James, R.B. Freedman, M. Hoare, O.W. Ogonah, B.C. Rooney, O.A. Larionov, V.N. Dobrovolsky, O.V. Lagutin and N. Jenkins, *Biotechnology (NY)*, 13 (1995) 592.
47 M.A. Schenerman, J.N. Hope, C. Kletke, J.K. Singh, R. Kimura, E.I. Tsao and G. Folena-Wasserman, *Biologicals*, 27 (1999) 203.
48 D.W. Karl, M. Donovan and M.C. Flickinger, *Cytotechnology*, 3 (1990) 157.
49 M.J. Gramer, C.F. Goochee, V.Y. Chock, D.T. Brousseau and M.B. Sliwkowski, *Biotechnology (NY)*, 13 (1995) 692.
50 R.J. Harris, *J. Chromatogr. A*, 705 (1995) 129.
51 A.A. Kossiakoff, *Science*, 240 (1988) 191.
52 N.E. Robinson and A.B. Robinson, *Proc. Natl Acad. Sci. USA*, 98 (2001) 944.
53 L. Graf, G. Hajos, A. Patthy and G. Cseh, *Horm. Metab. Res.*, 5 (1973) 142.
54 B.A. Johnson, J.M. Shirokawa and D.W. Aswad, *Arch. Biochem. Biophys.*, 268 (1989) 276.
55 B.A. Johnson, J.M. Shirokawa, W.S. Hancock, M.W. Spellman, L.J. Basa and D.W. Aswad, *J. Biol. Chem.*, 264 (1989) 14262.
56 J.M. Boggs, G. Rangaraj, K.M. Koshy, C. Ackerley, D.D. Wood and M.A. Moscarello, *J. Neurosci. Res.*, 57 (1999) 529.
57 P.J. Berti, I. Ekiel, P. Lindahl, M. Abrahamson and A.C. Storer, *Protein Exp. Purif.*, 11 (1997) 111.
58 C. George-Nascimento, A. Gyenes, S.M. Halloran, J. Merryweather, P. Valenzuela, K.S. Steimer, F.R. Masiarz and A. Randolph, *Biochemistry*, 27 (1988) 797.
59 C.T. Przysiecki, J.G. Joyce, P.M. Keller, H.Z. Markus, C.E. Carty, A. Hagopian, M.K. Sardana, C.T. Dunwiddie, R.W. Ellis and W.J. Miller, *Protein Exp. Purif.*, 3 (1992) 185.
60 C. Sandoval, H. Curtis and L.F. Congote, *Protein Engng*, 15 (2002) 413.
61 M.A. Saarinen and D.W. Murhammer, *Biotechnol. Bioengng*, 81 (2003) 106.
62 X.M. Lam, J.Y. Yang and J.L. Cleland, *J. Pharm. Sci.*, 86 (1997) 1250.
63 O. Pines and M. Inouye, *Mol. Biotechnol.*, 12 (1999) 25.
64 P. Balbas, *Mol. Biotechnol.*, 19 (2001) 251.
65 J.R. Swartz, *Curr. Opin. Biotechnol.*, 12 (2001) 195.
66 J.F. Kane and D.L. Hartley, *Bioprocess. Technol.*, 12 (1991) 121.
67 G.A. Bowden, A.M. Paredes and G. Georgiou, *Biotechnology (NY)*, 9 (1991) 725.
68 G. Koller, K. Graumann, W. Kramer, M. Sara and A. Jungbauer, *J. Chromatogr. B*, 664 (1995) 107.
69 H. Lilie, E. Schwarz and R. Rudolph, *Curr. Opin. Biotechnol.*, 9 (1998) 497.
70 E.D. Clark, *Curr. Opin. Biotechnol.*, 12 (2001) 202.
71 B. Fischer, B. Perry, I. Sumner and P. Goodenough, *Protein Engng*, 5 (1992) 593.
72 G. Zettlmeissl, R. Rudolph and R. Jaenicke, *Eur. J. Biochem.*, 121 (1981) 169.
73 M.E. Goldberg, R. Rudolph and R. Jaenicke, *Biochemistry*, 30 (1991) 2790.
74 R.J. Kohler, M. Preuss and A.D. Miller, *Biotechnol. Prog.*, 16 (2000) 671.
75 A.P. Ben-Zvi and P. Goloubinoff, *J. Struct. Biol.*, 135 (2001) 84.
76 M.M. Altamirano, C. Garcia, L.D. Possani and A.R. Fersht, *Nature Biotechnol.*, 17 (1999) 187.
77 X.-Y. Dong, H. Yang and Y. Sun, *J. Chromatogr. A*, 878 (2000) 197.
78 S. Ostergaard, L. Olsson and J. Nielsen, *Microbiol. Mol. Biol. Rev.*, 64 (2000) 34.
79 J.L. Cereghino and J.M. Cregg, *FEMS Microbiol. Rev.*, 24 (2000) 45.
80 R.G. Buckholz and M.A. Gleeson, *Biotechnology (NY)*, 9 (1991) 1067.
81 G. Gellissen and K. Melber, *Arzneimittelforschung*, 46 (1996) 943.
82 R.G. Werner, W. Noe, K. Kopp and M. Schluter, *Arzneimittelforschung*, 48 (1998) 870.

83 A.J. Clark, *J. Mammary Gland Biol. Neoplasia*, 3 (1998) 337.
84 H. Lubon, *Biotechnol. Annu. Rev.*, 4 (1998) 1.
85 D.P. Pollock, J.P. Kutzko, E. Birck-Wilson, J.L. Williams, Y. Echelard and H.M. Meade, *J. Immunol. Meth.*, 231 (1999) 147.
86 S. Soukharev, D. Hammond, N.M. Ananyeva, J.A.M. Anderson, C.A.E. Hauser, S. Pipe and E.L. Saenko, *Blood Cells Mol. Dis.*, 28 (2002) 234.
87 G. Giddings, *Curr. Opin. Biotechnol.*, 12 (2001) 450.
88 J.W. Larrick and D.W. Thomas, *Curr. Opin. Biotechnol.*, 12 (2001) 411.
89 J.W. Larrick, L. Yu, C. Naftzger, S. Jaiswal and K. Wycoff, *Biomol. Engng*, 18 (2001) 87.
90 R. Dolhofer, E.A. Siess and O.H. Wieland, *Biol. Chem. Hoppe–Seyler*, 366 (1985) 361.
91 J.D. Andya, Y.F. Maa, H.R. Costantino, P.A. Nguyen, N. Dasovich, T.D. Sweeney, C.C. Hsu and S.J. Shire, *Pharm. Res.*, 16 (1999) 350.
92 N. Taniquchi, N. Kinoshita, K. Arai, S. Iizuka, M. Usui and T. Naito, *Prog. Clin. Biol. Res.*, 304 (1989) 277.
93 K. Arai, S. Maguchi, S. Fujii, H. Ishibashi, K. Oikawa and N. Taniguchi, *J. Biol. Chem.*, 262 (1987) 16969.
94 M. Chiari, C. Ettori, P.G. Righetti, S. Colonna, N. Gaggero and A. Negri, *Electrophoresis*, 12 (1991) 376.
95 M. Fountoulakis, F. Vilbois, G. Oesterhelt and W. Vetter, *Biotechnology (NY)*, 13 (1995) 383.
96 D. Petsch, T.C. Beeskow, F.B. Anspach and W.-D. Deckwer, *J. Chromatogr. B*, 693 (1997) 79.
97 F.B. Anspach and D. Petsch, *Process Biochem.*, 35 (2000) 1005.
98 M. Hearn, in S. Ahjua (Ed.), *Separation Science and Technology*, Academic Press, San Diego, 2000, p. 71.
99 J.J. Stickel and A. Fotopoulos, *Biotechnol. Prog.*, 17 (2000) 744.
100 H. Lettner, A. Jungbauer, L. Gurrier and E. Boschetti, *BioPharm*, 7 (1994) 37.
101 E. Boschetti, P. Girot and L. Guerrier, *J. Chromatogr. A*, 523 (1990) 35.
102 P. Santambien, S. Sdiqui, E. Hubert, P. Girot, A.C. Roche, M. Monsigny and E. Boschetti, *J. Chromatogr. B*, 664 (1995) 241.
103 R. Hahn, M. Panzer, E. Hansen, J. Mollerup and A. Jungbauer, *Sep. Sci. Technol.*, 37 (2002) 1545.
104 G. Iberer, R. Hahn and A. Jungbauer, *LC–GC*, 11 (1999) 998.
105 D. Josic, A. Buchacher and A. Jungbauer, *J. Chromatogr. B*, 752 (2001) 191.
106 P. DePhillips and A.M. Lenhoff, *J. Chromatogr. A*, 883 (2000) 39.
107 R.K. Lewus and G. Carta, *AIChE J.*, 45 (1999) 512.
108 E. Firouztale, A.P. Scott, S.K. Dalvie and G.M. Von Blohn, *AIChE Symp. Ser.*, 88 (1993) 25.
109 A. Ljunglof and J. Thommes, *J. Chromatogr. A*, 813 (1998) 387.
110 S.R. Dziennik, E.B. Belcher, G.A. Barker, M.J. DeBergalis, S.E. Fernandez and A.M. Lenhoff, *Proc. Natl Acad. Sci. USA*, 100 (2003) 420.
111 O. Kaltenbrunner, A. Jungbauer and S. Yamamoto, *J. Chromatogr. A*, 760 (1997) 41.
112 L. Zhang, J. Selker, A. Qu and A. Velayudhan, *J. Chromatogr. A*, 934 (2001) 13.
113 J.J. Kirkland, F.A. Truszkowski, C.H. Dilks, Jr. and G.S. Engel, *J. Chromatogr. A*, 890 (2000) 3.
114 H. Minakuchi, K. Nakanishi, N. Soga, N. Ishizuka and N. Tanaka, *Anal. Chem.*, 68 (1996) 3498.
115 H. Minakuchi, N. Ishizuka, K. Nakanishi, N. Soga and N. Tanaka, *J. Chromatogr. A*, 828 (1998) 83.
116 K. Nakanishi, H. Minakuchi, N. Soga and N. Tanaka, *J. Sol–Gel Sci. Technol.*, 13 (1998) 163.

117 K. Cabreara, G. Wieland, D. Lubda, K. Nakanishi, N. Soga, H. Minakuchi and K.K. Unger, *Trends Anal. Chem.*, 17 (1998) 50.
118 J. Horvath, E. Boschetti, L. Guerrier and N. Cooke, *J. Chromatogr. A*, 679 (1994) 11.
119 E. Boschetti, L. Guerrier, P. Girot and J. Horvath, *J. Chromatogr. B*, 664 (1995) 225.
120 E.A. Peterson and H.A. Sober, *J. Am. Chem. Soc.*, 78 (1956) 751.
121 J. Porath and P. Flodin, *Nature*, 4676 (1959) 1657.
122 J.C. Janson, *Chromaotgraphia*, 23 (1987) 361.
123 P.R. Levison, A.K. Hopkins, P. Hathi, S.E. Badger, F. Mann, N. Dickson and G. Purdom, *J. Chromatogr. A*, 890 (2000) 45.
124 P. Girot and E. Boschetti, *J. Chromatogr. A*, 213 (1981) 389.
125 T. Kadoya, T. Isobe, T. Okuyama, M. Ebihara, T. Ogawa, M. Sumita, H. Kuwahara, A. Kobayashi and T. Ishikawa, *J. Liq. Chromatogr.*, 9 (1986) 3543.
126 K. Unger, J. Schick-Kalb and K.-F. Krebs, *J. Chromatogr.*, 83 (1973) 5.
127 K. Unger, P. Roumeliotis, H. Mueller and H. Goetz, *J. Chromatogr. A*, 202 (1980) 3.
128 K.K. Unger and R. Janzen, *J. Chromatogr. A*, 373 (1986) 227.
129 M. Petro, P. Gemeiner and D. Berek, *J. Chromatogr. A*, 665 (1994) 37.
130 A. Kurganov, Y. Puchkova, V. Davankov and F. Eisenbeiss, *J. Chromatogr. A*, 663 (1994) 163.
131 F. Ahmed and B. Modrek, *J. Chromatogr. A*, 599 (1992) 25.
132 D. Mislovicova, I. Novak and M. Pasteka, *J. Chromatogr. A*, 543 (1991) 9.
133 V.A. Davankov, A.A. Kurganov and K.K. Unger, *J. Chromatogr. A*, 500 (1990) 519.
134 X. Santarelli, D. Muller and J. Jozefonvicz, *J. Chromatogr. A*, 443 (1988) 55.
135 F.L. Zhou, D. Muller, X. Santarelli and J. Jozefonvicz, *J. Chromatogr. A*, 476 (1989) 195.
136 R.W. Stout and J.J. DeStefano, *J. Chromatogr. A*, 326 (1985) 63.
137 U. Trudinger, G. Muller and K.K. Unger, *J. Chromatogr. A*, 535 (1990) 111.
138 R. Hahn, R. Schlegel and A. Jungbauer, *J. Chromatogr. B*, 790 (2003) 35.
139 R. Necina, K. Amatschek and A. Jungbauer, *Biotechnol. Bioengng*, 60 (1998) 689.
140 A. Jungbauer and R. Hahn, in F. Svec, T. Tenikova and Z. Deyl (Eds.), *Monolithic Materials: Preparation, Properties and Applications*, Elsevier, Amsterdam, 2003, p. 561.
141 R. Hahn and A. Jungbauer, *Anal. Chem.*, 72 (2000) 4853.
142 S. Hjerten and J.L. Liao, *J. Chromatogr.*, 457 (1988) 165.
143 S. Hjerten, J. Liao and R. Zhang, *J. Chromatogr.*, 473 (1989) 273.
144 S. Hjerten, M. Li, J. Mohammed, K. Nakazato and G. Pettersson, *Nature*, 356 (1992) 810.
145 T. Tennikova, F. Svec and B.G. Belenkii, *J. Liq. Chromatogr.*, 13 (1990) 63.
146 A. Podgornik, M. Barut, A. Strancar, D. Josic and T. Koloini, *Anal. Chem.*, 721 (2000) 6693.
147 I. Mihelic, T. Koloini, A. Podgornik and A. Strancar, *J. High Resolut. Chromatogr.*, 23 (2000) 39.
148 A. Jungbauer and K. Pflegerl, in F. Svec, T. Tenikova and Z. Deyl (Eds.), *Monolithic Materials: Preparation, Properties and Applications*, Elsevier, Amsterdam, 2003, p. 725.
149 K. Pflegerl, A. Podgornik, E. Schallaun and A. Jungbauer, *J. Combinat. Chem.*, 4 (2002) 33.
150 K. Pflegerl, A. Podgornik, E. Berger and A. Jungbauer, *Biotechnol. Bioengng*, 79 (2002) 733.
151 K. Pflegerl, R. Hahn, E. Berger and A. Jungbauer, *J. Peptide Res.*, 59 (2002) 1.
152 A. Jungbauer and R. Hahn, in F. Svec, T. Tenikova and Z. Deyl (Eds.), *Monolithic Materials: Preparation, Properties and Applications*, Elsevier, Amsterdam, 2003, p. 699.
153 A. Uretschläger and A. Jungbauer, *Sep. Sci. Technol.*, 37 (2002) 1683.
154 D. Josic, H. Schwinn, A. Strancar, A. Podgornik, M. Barut, Y.-P. Lim and M. Vodopivec, *J. Chromatogr. A*, 803 (1998) 61.
155 T.V. Gupalova, O.V. Lojkina, V.G. Palagnuk, A.A. Totolian and T.B. Tennikova, *J. Chromatogr. A*, 949 (2002) 185.

156 N.D. Ostryanina, G.P. Vlasov and T.B. Tennikova, *J. Chromatogr. A*, 949 (2002) 163.
157 K. Branovic, G. Lattner, M. Barut, A. Strancar, D. Josic and A. Buchacher, *J. Immunol. Meth.*, 271 (2002) 47.
158 N.B. Afeyan, N.F. Gordon, I. Mazsaroff, L. Varady, S.P. Fulton, Y.B. Yang and F.E. Regnier, *J. Chromatogr. A*, 519 (1990) 1.
159 A.I. Liapis, Y. Xu, O.K. Crosser and A. Tongta, *J. Chromatogr. A*, 702 (1995) 45.
160 C.C. Shepard and A. Tiselius, *Disc. Faraday Soc*, Hazell Watson and Winey, London, 1949, p. 275.
161 S. Shaltiel and Z. Er-el, *Proc. Natl Acad. Sci. USA*, 52 (1973) 778.
162 B.H. Hofstee, *Anal. Biochem.*, 52 (1973) 430.
163 S. Hjerten, *J. Chromatogr.*, 87 (1973) 325.
164 J. Porath, *J. Chromatogr.*, 376 (1986) 331.
165 A.A. Green, *J. Biol. Chem.*, 95 (1932) 47.
166 A.A. Green and W.L. Hughes, *Methods in Enzymology*, Academic Press, New York, 1955.
167 M.R. Ladisch, *Bioseparations Engineering: Principles, Practice, and Economics*, Academic Press, New York, 2001.
168 W.R. Melander and C. Horvát, *Arch. Biochem. Biophys.*, 183 (1977) 200.
169 W.R. Melander, Z. El Rassi and C. Horvát, *J. Chromatogr.*, 469 (1989) 3.
170 T. Arakawa and S.N. Timasheff, *Biochemistry*, 23 (1984) 5924.
171 E.S. Parente and D.B. Wetlaufer, *J. Chromatogr.*, 288 (1984) 389.
172 A. Staby and J. Mollerup, *J. Chromatogr. A*, 734 (1996) 205.
173 C. Machold, K. Deinhofer, R. Hahn and A. Jungbauer, *J. Chromatogr. A*, 972 (2002) 3.
174 S.L. Wu, K. Benedek and B.L. Karger, *J. Chromatogr.*, 359 (1986) 3.
175 T. Arakawa and S.N. Timasheff, *Biochemistry*, 21 (1982) 6545.
176 T.W. Perkins, D.S. Mak, T.W. Root and E.N. Lightfoot, *J. Chromatogr. A*, 766 (1997) 1.
177 C. Horvath, W. Melander and I. Molnar, *J. Chromatogr. A*, 125 (1976) 129.
178 K.A. Dill, *J. Phys. Chem.*, 91 (1987) 1980.
179 J.G. Dorsey and K.A. Dill, *Chem. Rev.*, 89 (1989) 331.
180 P.T. Ying, J.G. Dorsey and K.A. Dill, *Anal. Chem.*, 61 (1989) 2540.
181 L.C. Sander and J.B. Callis, *Anal. Chem.*, 55 (1983) 1068.
182 L.C. Sander and S.A. Wise, *Anal. Chem.*, 59 (1987) 2309.
183 K.B. Sentell and J.G. Dorsey, *Anal. Chem.*, 61 (1989) 930.
184 K.B. Sentell and J.G. Dorsey, *J. Chromatogr.*, 461 (1989) 193.
185 J.G. Dorsey and W.T. Cooper, *Anal. Chem.*, 66 (1994) 857A.
186 P.W. Carr, C.T. Lay and J.H. Park, *J. Chromatogr. A*, 724 (1996) 1.
187 A. Tchapla, S. Heron, H. Colin and G. Guiochon, *Anal. Chem.*, 60 (1988) 1443.
188 N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott and P.W. Carr, *J. Chromatogr. A*, 961 (2002) 171.
189 N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder and P.W. Carr, *J. Chromatogr. A*, 961 (2002) 195.
190 N.S. Wilson, J.W. Dolan, L.R. Snyder, P.W. Carr and L.C. Sander, *J. Chromatogr. A*, 961 (2002) 217.
191 A. Tchapla, S. Heron, E. Lesellier and H. Colin, *J. Chromatogr. A*, 656 (1993) 81.
192 L.R. Snyder, J.W. Dolan and G. Cox, *J. Chromatogr. A*, 484 (1989) 437.
193 W.R. Melander, B.K. Chen and C. Horvath, *J. Chromatogr.*, 318 (1985) 1.
194 F.-Y. Lin, W.-Y. Chen, R.-C. Ruaan and H.-M. Huang, *J. Chromatogr. A*, 872 (2000) 37.
195 H.-M. Huang, F.-Y. Lin, W.-Y. Chen and R.-C. Ruaan, *J. Colloid Interface Sci.*, 229 (2000) 600.
196 Y.-S. Tsai, F.-Y. Lin, W.-Y. Chen and C.-C. Lin, *Colloid Surf. A*, 197 (2002) 111.

197 D. Haidacher, A. Vailaya and C. Horvath, *Proc. Natl Acad. Sci. USA*, 93 (1996) 2290.
198 M.A. Esquibel-King, A.C. Dias-Cabral, J.A. Queiroz and N.G. Pinto, *J. Chromatogr. A*, 865 (1999) 111.
199 Y. Kato, K. Nakamura, T. Kitamura, H. Moriyama, M. Hasegawa and H. Sasaki, *J. Chromatogr. A*, 971 (2002) 143.
200 H.P. Jennissen, *J. Bio-Chromatogr.*, 5 (2000) 131.
201 E. Boschetti, *Trends Biotechnol.*, 20 (2002) 333.
202 K.L. Knudsen, M.B. Hansen, L.R. Henriksen, B.K. Andersen and A. Lihme, *Anal. Biochem.*, 201 (1992) 170.
203 A. el-Kak, S. Manjini and M.A. Vijayalakshmi, *J. Chromatogr.*, 604 (1992) 29.
204 T.T. Ngo, *J. Chromatogr. B*, 662 (1994) 351.
205 K.J. Wilson, E.v. Wieringen, S. Klauser and M.W. Berchtold, *J. Chromatogr.*, 237 (1982) 407.
206 D.-R. Wu and H.C. Greenblatt, *J. Chromatogr. A*, 702 (1995) 157.
207 K.A. Tweeten and T.N. Tweeten, *J. Chromatogr. A*, 359 (1986) 111.
208 K. Miyabe and G. Guiochon, *J. Chromatogr. A*, 961 (2002) 23.
209 H. Husi and M.D. Walkinshaw, *J. Chromatogr. B*, 755 (2001) 367.
210 O. Manzke, H. Tesch, V. Diehl and H. Bohlen, *J. Immunol. Meth.*, 208 (1997) 65.
211 I. Tornoe, I.L. Titlestad, K. Kejling, K. Erb, H.J. Ditzel and J.C. Jensenius, *J. Immunol. Meth.*, 205 (1997) 11.
212 K.C. O'Connor, S. Ghatak and B.D. Stollar, *Anal. Biochem.*, 278 (2000) 239.
213 M.J. Wilson, C.L. Haggart, S.P. Gallagher and D. Walsh, *J. Biotechnol.*, 88 (2001) 67.
214 R. Hahn, P.M. Schulz, C. Schaupp and A. Jungbauer, *J. Chromatogr. A*, 795 (1998) 277.
215 K. Kalghatgi and C. Horvath, *J. Chromatogr.*, 443 (1988) 343.
216 Y. He, W. Zhong and E.S. Yeung, *J. Chromatogr. B*, 782 (2002) 331.
217 F. Kilar and B. Visegrady, *Electrophoresis*, 23 (2002) 964.
218 M. Moini, *Anal. Bioanal. Chem.*, 373 (2002) 466.
219 H. Wang, M.T. Kachman, D.R. Schwartz, K.R. Cho and D.M. Lubman, *Electrophoresis*, 23 (2002) 3168.
220 B.A. van Montfort, M.K. Doeven, B. Canas, L.M. Veenhoff, B. Poolman and G.T. Robillard, *Biochim. Biophys. Acta*, 1555 (2002) 111.
221 V. Egelhofer, J. Gobom, H. Seitz, P. Giavalisco, H. Lehrach and E. Nordhoff, *Anal. Chem.*, 74 (2002) 1760.
222 C.L. Nilsson, H.J. Cooper, K. Hakansson, A.G. Marshall, Y. Ostberg, M. Lavrinovicha and S. Bergstrom, *J. Am. Soc. Mass Spectrom.*, 13 (2002) 295.
223 G. Karlsson, P. Gellerfors, A. Persson, B. Noren, P.O. Edlund, C. Sandberg and S. Birnbaum, *J. Chromatogr. A*, 855 (1999) 147.
224 J. Lei, D.A. Chen and F.E. Regnier, *J. Chromatogr. A*, 808 (1998) 121.
225 M.I. Aguilar, D.J. Clayton, P. Holt, V. Kronina, R.I. Boysen, A.W. Purcell and M.T. Hearn, *Anal. Chem.*, 70 (1998) 5010.
226 C.T. Mant, N.E. Zhou and R.S. Hodges, *J. Chromatogr. A*, 476 (1989) 363.
227 T.J. Sereda, C.T. Mant, A.M. Quinn and R.S. Hodges, *J. Chromatogr. A*, 646 (1993) 17.
228 N. Takahashi, N. Ishioka, Y. Takahashi and F.W. Putnam, *J. Chromatogr.*, 326 (1985) 407.
229 M. Stromqvist, *J. Chromatogr. A*, 667 (1994) 304.
230 M.M. Bushey and J.W. Jorgenson, *Anal. Chem.*, 62 (1990) 161.
231 G.J. Opiteck, S.M. Ramirez, J.W. Jorgenson, I. Moseley and M. Arthur, *Anal. Biochem.*, 258 (1998) 349.
232 K. Wagner, T. Miliotis, G. Marko-Varga, R. Bischoff and K.K. Unger, *Anal. Chem.*, 74 (2002) 809.

233 K. Wagner, K. Racaityte, K.K. Unger, T. Miliotis, L.E. Edholm, R. Bischoff and G. Marko-Varga, *J. Chromatogr. A*, 893 (2000) 293.
234 R.H. Pain, *Mechanisms of Protein Folding*, IRL Press, Oxford, 2000.
235 R. Schlegl, G. Iberer, C. Machold, R. Necina and A. Jungbauer, *J. Chromatogr. A*, 1009 (2003) 119.
236 K. Benedek, S. Dong and B.L. Karger, *J. Chromatogr.*, 317 (1984) 227.
237 X.M. Lu, K. Benedek and B.L. Karger, *J. Chromatogr.*, 359 (1986) 19.
238 S. Lin and B.L. Karger, *J. Chromatogr.*, 499 (1990) 89.
239 J.L. McNay, J.P. O'Connell and E.J. Fernandez, *Biotechnol. Bioengng*, 76 (2001) 233.
240 J.L. McNay and E.J. Fernandez, *Biotechnol. Bioengng*, 76 (2001) 224.
241 S.U. Sane, S.M. Cramer and T.M. Przybycien, *J. Chromatogr. A*, 894 (1999) 149.
242 Z.Y. Peng, L.C. Wu, B.A. Schulman and P.S. Kim, *Philos. Trans. R. Soc. London B*, 348 (1995) 43.
243 A. Kundu and S.M. Cramer, *Anal. Biochem.*, 248 (1997) 111.
244 G. Jayaraman, S.D. Gadam and S.M. Cramer, *J. Chromatogr.*, 630 (1993) 53.
245 G. Jayaraman, L. Yu-Fei, J.A. Moore and S.M. Cramer, *J. Chromatogr. A*, 702 (1995) 143.
246 A. Kundu, S. Vunnum and S.M. Cramer, *J. Chromatogr.*, 707 (1995) 57.
247 W. Kopaciewicz, M.A. Rounds, J. Fausnaugh and F.E. Regnier, *J. Chromatogr. A*, 266 (1983) 3.
248 R.K. Scopes and E. Algar, *FEBS Lett.*, 106 (1979) 239.
249 R.K. Scopes, *Protein Purification, Principles and Practice*, Springer, New York, 1982.
250 J.H. Janson and L. Rydèn, *Protein Purification: Principles, High Resolution Methods and Applications*, Wiley-VCH, New York, 1998.
251 S.R. Gallant, S. Vunnum and S.M. Cramer, *J. Chromatogr.*, 725 (1995) 295.
252 J. Stahlberg, B. Jonsson and C. Horvath, *Anal. Chem.*, 63 (1991) 1867.
253 J. Stahlberg, B. Jonsson and C. Horvath, *Anal. Chem.*, 64 (1992) 3118.
254 N.K. Boardman and S.M. Partridge, *Nature*, 171 (1953) 208.
255 M.A. Rounds and F.E. Regnier, *J. Chromatogr.*, 283 (1984) 37.
256 S.R. Gallant, A. Kundu and S.M. Cramer, *J. Chromatogr. A*, 702 (1995) 125.
257 S. Gallant, A. Kundu and S.M. Cramer, *Biotechnol. Bioengng*, 47 (1995) 355.
258 C.A. Brooks and S.M. Cramer, *AIChE J.*, 38 (1992) 1969.
259 S.D. Gadam, G. Jayaraman and S.M. Cramer, *J. Chromatogr.*, 630 (1993) 37.
260 J. Stahlberg, *J. Chromatogr. A*, 855 (1999) 3.
261 J. Stahlberg, U. Appelgren and B. Jonsson, *J. Colloid Interface Sci.*, 176 (1995) 397.
262 A. Staby and I.H. Jensen, *J. Chromatogr. A*, 908 (2001) 149.
263 L.A.A.E. Sluyterman and O. Elgersma, *J. Chromatogr. A*, 150 (1978) 17.
264 L.A.A.E. Sluyterman and J. Wijdenes, *J. Chromatogr. A*, 150 (1978) 31.
265 L.A.A. Sluyterman and J. Wijdenes, *J. Chromatogr. A*, 206 (1981) 441.
266 Y. Liu and D.J. Anderson, *J. Chromatogr. A*, 762 (1997) 207.
267 Y. Liu and D.J. Anderson, *J. Chromatogr. A*, 762 (1997) 47.
268 R.C. Bates and D.D. Frey, *J. Chromatogr. A*, 814 (1998) 43.
269 R.C. Bates, X. Kang and D.D. Frey, *J. Chromatogr. A*, 890 (2000) 25.
270 D.D. Van Slyke, *J. Biol. Chem.*, 52 (1922) 525.
271 J.H. Scott, K.L. Kelner and H.B. Pollard, *Anal. Biochem.*, 149 (1985) 163.
272 M.T.W. Hearn and D.J. Lyttle, *J. Chromatogr.*, 218 (1981) 483.
273 T.W. Hutchens, C.M. Li and P.K. Besch, *J. Chromatogr. A*, 359 (1986) 157.
274 Y.-M. Li, J.-L. Liao, R. Zhang, H. Henriksson and S. Hjerten, *Anal. Biochem.*, 267 (1999) 121.
275 J.Y. Fu, S.M. Wu, C.T. Chang and H.Y. Sung, *J. Agric. Food Chem.*, 51 (2003) 1042.

276 J. Barrios-De-Tomasi, C. Timossi, H. Merchant, A. Quintanar, J.M. Avalos, C.Y. Andersen and A. Ulloa-Aguirre, *Mol. Cell Endocrinol.*, 186 (2002) 189.
277 F. Francis, E. Haubruge and P. Dierickx, *Arch. Insect Biochem. Physiol.*, 49 (2002) 158.
278 D. Fontanini and B.L. Jones, *J. Agric. Food Chem.*, 49 (2001) 4903.
279 X. Kang, R.C. Bates and D.D. Frey, *J. Chromatogr. A*, 890 (2000) 37.
280 X. Kang and D.D. Frey, *Anal. Chem.*, 74 (2002) 1038.
281 A. Polson, *J. Phys. Coll. Chem.*, 54 (1949) 649.
282 J. Schroll, R. Necina, C. Tauer, K. Vorauer, R. Plot and A. Jungbauer, *J. Biochem. Biophys. Meth.*, 27 (1993) 117.
283 L.D. Plante, P.M. Romano and E.J. Fernandez, *Chem. Engng Sci.*, 49 (1994) 2229.
284 D. Josic, H. Horn, P. Schulz, H. Schwinn and L. Britsch, *J. Chromatogr. A*, 796 (1998) 289.
285 K. Ahrer, A. Buchacher, S. Inoue, D. Josic and A. Jungbauer, *J. Chromatogr. A*, 1009 (2003) 89.
286 F. Steindl, A. Jungbauer, E. Wenisch, G. Himmler and H. Katinger, *Enzyme Microbial Technol.*, 9 (1987) 361.
287 P. Flodin, *J. Chromatogr. A*, 5 (1961) 103.
288 B. Batas and J.B. Chaudhuri, *Biotechnol. Bioengng*, 50 (1996) 16.
289 J. Wen, T. Arakawa and J.S. Philo, *Anal. Biochem.*, 240 (1996) 155.
290 R.J. Mural, M.D. Adams, E.W. Myers, H.O. Smith, G.L. Miklos, R. Wides, A. Halpern, P.W. Li, G.G. Sutton, J. Nadeau, S.L. Salzberg, R.A. Holt, C.D. Kodira, F. Lu, L. Chen, Z. Deng, C.C. Evangelista, W. Gan, T.J. Heiman, J. Li, Z. Li, G.V. Merkulov, N.V. Milshina, A.K. Naik, R. Qi, B.C. Shue, A. Wang, J. Wang, X. Wang, X. Yan, J. Ye, S. Yooseph, Q. Zhao, L. Zheng, S.C. Zhu, K. Biddick, R. Bolanos, A.L. Delcher, I.M. Dew, D. Fasulo, M.J. Flanigan, D.H. Huson, S.A. Kravitz, J.R. Miller, C.M. Mobarry, K. Reinert, K.A. Remington, Q. Zhang, X.H. Zheng, D.R. Nusskern, Z. Lai, Y. Lei, W. Zhong, A. Yao, P. Guan, R.R. Ji, Z. Gu, Z.Y. Wang, F. Zhong, C. Xiao, C.C. Chiang, M. Yandell, J.R. Wortman, P.G. Amanatides, S.L. Hladun, E.C. Pratts, J.E. Johnson, K.L. Dodson, K.J. Woodford, C.A. Evans, B. Gropman, D.B. Rusch, E. Venter, M. Wang, T.J. Smith, J.T. Houck, D.E. Tompkins, C. Haynes, D. Jacob, S.H. Chin, D.R. Allen, C.E. Dahlke, R. Sanders, K. Li, X. Liu, A.A. Levitsky, W.H. Majoros, Q. Chen, A.C. Xia, J.R. Lopez, M.T. Donnelly, M.H. Newman, A. Glodek, C.L. Kraft, M. Nodell, F. Ali, H.J. An, D. Baldwin-Pitts, K.Y. Beeson, S. Cai et al. *Science*, 296 (2002) 1661.
291 Y. Okazaki, M. Furuno, T. Kasukawa, J. Adachi, H. Bono, S. Kondo, I. Nikaido, N. Osato, R. Saito, H. Suzuki, I. Yamanaka, H. Kiyosawa, K. Yagi, Y. Tomaru, Y. Hasegawa, A. Nogami, C. Schonbach, T. Gojobori, R. Baldarelli, D.P. Hill, C. Bult, D.A. Hume, J. Quackenbush, L.M. Schriml, A. Kanapin, H. Matsuda, S. Batalov, K.W. Beisel, J.A. Blake, D. Bradt, V. Brusic, C. Chothia, L.E. Corbani, S. Cousins, E. Dalla, T.A. Dragani, C.F. Fletcher, A. Forrest, K.S. Frazer, T. Gaasterland, M. Gariboldi, C. Gissi, A. Godzik, J. Gough, S. Grimmond, S. Gustincich, N. Hirokawa, I.J. Jackson, E.D. Jarvis, A. Kanai, H. Kawaji, Y. Kawasawa, R.M. Kedzierski, B.L. King, A. Konagaya, I.V. Kurochkin, Y. Lee, B. Lenhard, P.A. Lyons, D.R. Maglott, L. Maltais, L. Marchionni, L. McKenzie, H. Miki, T. Nagashima, K. Numata, T. Okido, W.J. Pavan, G. Pertea, G. Pesole, N. Petrovsky, R. Pillai, J.U. Pontius, D. Qi, S. Ramachandran, T. Ravasi, J.C. Reed, D.J. Reed, J. Reid, B.Z. Ring, M. Ringwald, A. Sandelin, C. Schneider, C.A. Semple, M. Setou, K. Shimada, R. Sultana, Y. Takenaka, M.S. Taylor, R.D. Teasdale, M. Tomita, R. Verardo, L. Wagner, C. Wahlestedt, Y. Wang, Y. Watanabe, C. Wells, L.G. Wilming, A. Wynshaw-Boris, M. Yanagisawa et al. *Nature*, 420 (2002) 563.
292 R.H. Waterston, K. Lindblad-Toh, E. Birney, J. Rogers, J.F. Abril, P. Agarwal, R. Agarwala, R. Ainscough, M. Alexandersson, P. An, S.E. Antonarakis, J. Attwood, R. Baertsch, J. Bailey,

K. Barlow, S. Beck, E. Berry, B. Birren, T. Bloom, P. Bork, M. Botcherby, N. Bray, M.R. Brent, D.G. Brown, S.D. Brown, C. Bult, J. Burton, J. Butler, R.D. Campbell, P. Carninci, S. Cawley, F. Chiaromonte, A.T. Chinwalla, D.M. Church, M. Clamp, C. Clee, F.S. Collins, L.L. Cook, R.R. Copley, A. Coulson, O. Couronne, J. Cuff, V. Curwen, T. Cutts, M. Daly, R. David, J. Davies, K.D. Delehaunty, J. Deri, E.T. Dermitzakis, C. Dewey, N.J. Dickens, M. Diekhans, S. Dodge, I. Dubchak, D.M. Dunn, S.R. Eddy, L. Elnitski, R.D. Emes, P. Eswara, E. Eyras, A. Felsenfeld, G.A. Fewell, P. Flicek, K. Foley, W.N. Frankel, L.A. Fulton, R.S. Fulton, T.S. Furey, D. Gage, R.A. Gibbs, G. Glusman, S. Gnerre, N. Goldman, L. Goodstadt, D. Grafham, T.A. Graves, E.D. Green, S. Gregory, R. Guigo, M. Guyer, R.C. Hardison, D. Haussler, Y. Hayashizaki, L.W. Hillier, A. Hinrichs, W. Hlavina, T. Holzer, F. Hsu, A. Hua, T. Hubbard, A. Hunt, I. Jackson, D.B. Jaffe, L.S. Johnson, M. Jones, T.A. Jones, A. Joy, M. Kamal, E.K. Karlsson et al. *Nature*, 420 (2002) 520.

293 F. Kvasnicka, *J. Chromatogr. B*, 787 (2003) 77.

294 T.C. Hunter, N.L. Andon, A. Koller, J.R. Yates and P.A. Haynes, *J. Chromatogr. B*, 782 (2002) 165.

295 S.P. Gygi, B. Rist, S.A. Gerber, F. Turecek, M.H. Gelb and R. Aebersold, *Nature Biotechnol.*, 17 (1999) 994.

296 M. Unlu, M.E. Morgan and J.S. Minden, *Electrophoresis*, 18 (1997) 2071.

297 S.R. Weinberger, E. Boschetti, P. Santambien and V. Brenac, *J. Chromatogr. B*, 782 (2002) 307.

This page is intentionally left blank

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 17

Lipids

ARNIS KUKSIS

CONTENTS

17.1 INTRODUCTION

Since the review [1] in the 5th edition of this book, the practice of lipid chromatography has continued along both established and innovative lines, with the mass spectrometer as the detector of choice. In the present chapter, emphasis has been placed upon the minor phospholipid classes and molecular species, such as the phosphoinositol phosphatides (PtdInsPs), glycosyl phosphatidylinositols (glycosyl PtdIns), and the oxidation products of cholesteryl esters, and neutral and polar glycerolipids, which are now considered along with their parent compounds. In view of the enormous growth of the literature on lipid chromatography with mass spectrometry (LC/MS), references have been selected on the basis of the author's interests and familiarity. For more detailed coverage of recent advances in the chromatography of lipids reference may be made to various critical reviews and monographs about a single or limited number of classes of lipids, *e.g.*, gas liquid chromatography (GLC) of fatty acids [2,3], GLC [4] and high-performance liquid chromatography (HPLC) [5] of prostanoids, GLC of neutral lipids [6], HPLC of neutral and polar lipids [7], thin-layer chromatography (TLC) of neutral and polar lipids [8,9], as

well as gas chromatography/mass spectrometry (GC)/MS, HPLC/MS and MS/MS [10,11] of lipids and related compounds. Other specific reviews are noted in the text.

17.2 PREPARATION OF LIPID EXTRACTS

The method of preparation of the lipid extract still constitutes one of the most critical parts of lipid analysis by chromatographic methods. Traditional solubilization and extraction of neutral and weakly acidic lipids by liquid/liquid partition with chloroform and methanol, or chloroform and 2-propanol for plant tissues, involves large volumes of solvents, which are expensive and hazardous. The less toxic 2-propanol and hexane have not received the attention that they deserve. The conventional extracts are also contaminated to various extent with nonlipid components, which must be removed. Liquid/solid extraction, which greatly reduces the solvent requirements and produces cleaner samples, has received progressively more attention. Since tissues levels of PtdInsPs are relatively low, and post-mortem hydrolysis of PtdInsPs is rapid, accurate analyses of the higher PtdInsPs are difficult to obtain. Recent developments in the lipid extraction techniques have been reviewed by Carrapiso and Garcia [12]. Work on lipid peroxidation products has called attention to the consequences of the general neglect of well-known precautions necessary to avoid lipid peroxidation during sample isolation and chromatographic work-up. Early observations [1] suggested rapid and extensive peroxidative loss of polyunsaturates on TLC. De Long *et al.* [13] have recently quantified by electrospray ionization (ESI)/MS/MS the preferential loss of native glycerophospholipid (GPL) species and formation of new species during both TLC and HPLC. TLC caused a preferential loss of GPL with 0 to 3 double bonds: 20% for choline- and 19.7% for ethanolamine-GPL species, respectively. Selective losses of molecular species having 0 to 3 double bonds caused an apparent enrichment in molecular species having 4 to 6 double bonds. Allowing the phosphatidylcholine (PtdCho) to remain on the plates for an extended period of time caused the oxidation of unsaturated species and formation of lower-molecular-weight species, which were identified as PtdCho core aldehydes by derivatization with 2,4-dinitrophenylhydrazine. The loss of lipid and change in molecular species composition, caused by sample manipulation and TLC separation, were overcome by direct MS analysis of the total lipid extract. Furthermore, Podrez *et al.* [14] have shown that the intra-preparative oxidation of glycerolipids on silica gel plates and columns and even aminopropyl mini-columns may be due to trace levels of contaminant transition metal ions present on the columns. To avoid this problem, all aqueous solvents were cleansed with Chelex-100 prior to use. Further, all columns and filters were pre-rinsed with diethylenetriaminepentaacetic acid (DTPA)-containing solvents (at pH 7), and polar lipids were collected as a flow-through from the C_{18} mini-columns. All lipids were initially extracted in the presence of butylated hydroxy toluene (BHT), and the lipid extracts were concentrated and stored under inert gas (nitrogen or argon) atmosphere. The intra-preparative peroxidative losses may appear minimal when working with large amounts of sample but cannot be neglected because of the formation of new molecular species, which may be mistaken for new natural compounds.

17.2.1 Solvent extraction

Extraction with organic solvents provides a simple method of isolating all major phospholipids, regardless of their fatty acid composition. The isolation of the minor acidic PtdInsPs requires acidification, which causes destruction of plasmalogens unless they are removed first. Good laboratory practice recommends that the lipid extractions be performed in the presence of BHT or other antioxidants, especially when working with small amounts of lipid. The major GPL are readily isolated along with sphingomyelin (SM) by extraction with $CHCl_3$/MeOH (2:1), as described by Folch *et al.* [15], or $CHCl_3$/MeOH (1:1) as proposed by Bligh and Dyer [16], or some variation [17] of these methods. Iverson *et al.* [18] have recently compared the Bligh and Dyer procedure with that of the classic Folch extraction in determining the total lipid content of fish samples, ranging from 0.5 to 26.6% lipid. In samples containing $<2\%$ lipid, the results of the two methods did not differ. However, for samples containing $>2\%$ lipid, the Bligh and Dyer method produced significantly lower estimates of lipid content.

Belle *et al.* [19] compared cell and tissue extraction techniques for the isolation of both aqueous metabolites and lipids simultaneously. Using high-resolution proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy, it was shown that the chloroform/methanol technique was superior to the aqueous metabolite extraction from brain tissue and cells when compared to the perchloric acid method. The simultaneous extraction of aqueous metabolites and lipids by the chloroform/methanol technique was well suited for NMR investigations of both tissues and cells.

Because PtdInsPs and their hydrolysis products are degraded very rapidly, it is essential either to extract the samples immediately after collection or to freeze the samples *in situ* after collection and store them, if necessary, at -70 °C. *In situ* fixation of the samples by freeze-clamping or by microwave irradiation may be helpful in arresting the degradation of the phosphate esters. The recovery efficiency can be determined by means of radiolabelled PtdIns or PtdInsP external standards. Low [20] has described an HPLC purification of the [^{32}P]-labelled PtdIns(4)P and PtdIns(4,5)P_2 from human platelets. This procedure should be adaptable to PtdInsPs from other tissues and cell types. Diethylaminoethyl (DEAE) cellulose columns were used for preparative HPLC of brain lipids. Alternatively, a preparative HPLC separation on an amino column was performed. For the isolation of PtdInsPs from cultured cells for *in vivo* detection of isomeric PtdInsPs, it is necessary to pre-label the cells with inorganic [^{32}P]orthophoshate of myo-[^{3}H]inositol [21]. Auger *et al.* [21] have observed that a load of a few million dpm of [^{3}H]Ins-labeled deacylated lipids is necessary in order to detect the novel minor isomers, as they constitute only a small fraction of the total PtdInsP$_2$ in the cell. Furthermore, PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 are not present in most cells until they are induced by mitogen or when the cells are proliferating. According to Van der Kaay *et al.* [22], the PtdInsPs are extracted in 0.75 mL of $CHCl_3$/MeOH/HCl (40:80:1) for 20 min, and the phases are then demixed by the addition of 0.25 mL of $CHCl_3$ and 0.45 mL of 0.1 *M* HCl. Singh [23] has obtained 50–90% recoveries of PtdIns and InsPs from brain samples.

Zeng *et al.* [24] have systematically investigated phospholipid-subclass-specific alterations in the partitioning of both cationic and anionic amphiphiles to identify the importance of ester, ether and vinyl ether linkages at the sn-1-position on phospholipids in

the partitioning of charged amphiphiles. Substantial differences in the membrane dipole potential between choline GPL subclasses were found, which were thought to be of importance in the selective partitioning of drugs. Costello *et al.* [25] have described the extraction of the acidic glycerolipids of *Trichomonas vaginalis* and *T. foetus*, using sequential extraction with 20 volumes of $CHCl_3$/MeOH/H_2O and $CHCl_3$/MeOH mixtures. The extracts were combined, solvents removed, the residue taken up in $CHCl_3$/MeOH/H_2O (120:70:9), and the nonlipid contaminants were removed by chromatography on Sephadex G-25. Wu *et al.* [26] have recently revised the methodology of acidic GPL extraction, following the observation that changes in phospholipid extractability accompany mineralization of chicken growth-plate cartilage matrix vesicles. The initial extraction (Extract 1) is still being made with $CHCl_3$/MeOH (2:1) using a ratio of 20 mL solvent per mL of aqueous medium, followed by sonication [17]. Wu *et al.* [26] recommend that, after the initial extraction, the collagenase-released matrix vesicle (CRMV) pellets be demineralized with 0.5 *M* sodium salt of EDTA for 20 min at room temperature and sedimented by centrifugation at 3,000 rpm for 12 min. After removal of the supernatant, the decalcified residue is re-extracted, using $CHCl_3$/MeOH/HCl (200/100/1). The second extraction (Extract 2) was found to remove the remaining lipids quantitatively. Finally, the extraction of phospholipids from yeast and neurospora requires grinding of the fungal filaments with glass beads [27] for complete extraction of lipids with chloroform/methanol. Because both methanol and chloroform are toxic, attempts have been made to replace them with less toxic solvents. Eder *et al.* [28] have published a very detailed report on the use of different solutions and extraction times to obtain optimum recovery of different phospholipids from the erythrocyte membranes of rats. However, the equivalence of the chloroform/methanol and other solvent systems has not been adequately demonstrated. Richardson [29] has examined a variety of solvent systems for a pressurized extraction at elevated temperature (10.3 MPa; 80–120 °C). The most successful solvents with regard to speed of extraction, selectivity, and recovery were various mixtures of hexane/dichloromethane (DCM)/methanol and petroleum ether/acetone/ethanol or petroleum ether/acetone/2-propanol for the various dairy products tested. Boselli *et al.* [30] compared pressurized-liquid extraction (15 MPa and 60 °C) with hexane/2-propanol (3:2) with the Folch procedure (a solid/liquid extraction with $CHCl_3$/MeOH, 2:1) for the lipid extraction of egg-containing food. It was found that under the above conditions the pressurized-liquid extraction could replace the Folch extraction. Attempts to improve current methods of lipid extraction further have led to experimentation with automated extraction equipment, microwave irradiation, and supercritical-fluid extraction (SFE) [11]. These techniques, however, have not yet been extensively tested with the variety of lipids present in natural sources.

SFE has received increasing attention as an alternative to conventional extraction methods [31]. Extraction that uses only supercritical carbon dioxide usually yields good recoveries of nonpolar lipids, while polar lipids remain unextracted. Thus, supercritical carbon dioxide extractions with or without ethanol as an entrainer have been carried out to remove lipids and pigments from protein concentrates. Single-step SFE resulted in removal of mostly neutral lipids and part of glycolipids, but phospholipids were not extracted. Addition of ethanol to supercritical CO_2 increased the amount of glycolipids and phospholipids in the extract [32]. Relatively high recoveries of polyunsaturated fatty

acids and essential fatty acids in SFE of algal lipids and protein isolates were observed. Eller and King [33] have reported that the use of ethanol modifier was not necessary before efficient SFE with carbon dioxide of fat from ground beef. However, the ethanol increased the co-extraction of water, which caused a significant gravimetric overestimation of fat. Leray *et al.* [34] have described a microwave-oven extraction procedure for lipid analysis in biological samples, while Yoshida *et al.* [35] reported the effects of microwave heating on acyl lipids in sesame seeds in relation to moisture. The use of microwave irradiation has been suggested as an efficient technique for the extraction of lipophilic substances from biological materials [36] and has been included in the recommended methods of the Association of Official Analytical Chemists (AOAC) for extracting fat from oilseed [37]. Pedersen and Lindholst [38] have described quantification of the xenoestrogens 4-*tert*.-octylphenol and bisphenol A in water and in fish tissue based on microwave-assisted extraction, solid-phase extraction and LC/MS, while Pensado *et al.* [39] have optimized the microwave-assisted extraction of polycyclic hydrocarbons from wood samples. However, the microwave treatment can result in lipid oxidation and quantitative modification in the fatty acid composition [35]. These changes are thought to be minor under controlled conditions, although extensive testing has not been performed. Yaylayan *et al.* [40] used a microwave-assisted extraction procedure in conjunction with GC/MS analysis to investigate the chemical composition of silkworm- and earthworm-generated fatty acids, sterols, and phenol derivatives. Free PtdIns glycans may be extracted from metabolically labeled cells by conventional lipid extraction procedures. According to Menon [41], Naik *et al.* [42], and Santos de Macedo *et al.* [43] a differential extraction procedure with $CHCl_3$/MeOH (2:1) and $CHCl_3$/MeOH/H_2O (10:10:3) is particularly useful in fractionating the lipids into two broad categories on the basis of polarity. Naik *et al.* [42] have provided a detailed description of the isolation of free glycosyl PtdIns from *Plasmodium falciparum*. The nonglycosylated lipids were removed by extraction with $CHCl_3$/MeOH (2:1), and the free glycosyl PtdIns were isolated by extracting five times with $CHCl_3$/MeOH/H_2O (10:10:3).

17.2.2 Purification of extracts

Regardless of the method of solvent extraction, the isolated lipid will contain some nonlipid contaminants, which may need to be removed before further analysis. According to Wurhier [44], the crude lipid extract of tissues is taken up in an appropriate amount of the Folch lower phase, which is transferred to a medium-porosity sintered-glass funnel (to remove precipitated proteins) and filtered directly into the Sephadex columns, soaked overnight, and washed in Folch upper phase. The purified lipid is eluted from the column with Folch lower phase. Costello *et al.* [25] have provided a detailed description of the purification of acidic glycerolipids from *Trichomonas*. The method involved repeated chromatography on Sephadex G-25, DEAE-Sephadex A-25, Sep-Pak C_{18}, and Sephadex G-25, followed by normal-phase HPLC. Specifically, the dried lipid mixture was re-dissolved in $CHCl_3$/MeOH/H_2O (30:60:8) and separated into neutral and acidic fractions by DEAE-Sephadex A-25 column chromatography. The charged anionic lipids were eluted with 5 column volumes of $CHCl_3$/MeOH/0.8 *M* NaOAc (30:60:8) and were taken to dryness. The mixture was desalted, either on a C-18 Sep Pak column or by dialysis

followed by lyophilization. The lyophilized acidic lipid mixture was dissolved in chloroform/methanol/water (120:60:9) and further purified on Sephadex G-25 column. The recovered, charged anionic lipid fraction was dissolved in $CHCl_3$/MeOH/H_2O (120:60:9) and subjected to HPTLC in solvent systems A: $CHCl_3$/MeOH/H_2O (60:35:8) and B: $CHCl_3$/MeOH/0.05% $CaCl_2$ (55:40:10). Individual lipid bands were recovered from silica gel by scraping, and extracted by brief sonication with $CHCl_3$/MeOH (1:1). Individual lipid components obtained by HPTLC were further purified and collected by HPLC, using a 4.6 mm × 150-mm column of Iatrobeads (gRS-8010) (Iatron). The resulting charged GPL classes were subjected to chemical and mass-spectrometric characterization (see below).

Menon [41], Naik *et al.* [42], Santos de Macedo *et al.* [43], and Sevlever *et al.* [45] have described procedures for the purification of the free glycosyl PtdIns, isolated by solvent extraction, using TLC, HPLC, anion-exchange chromatography, and/or hydrophobic-interaction chromatography (HIC). Naik *et al.* [42] purified glycosyl PtdIns extracts on a C_4 reversed-phase Supelcosil LC-304 HPLC column (4.6 × 250 mm, 5 μm particle size), using a gradient of 20–60% aq. propanol, containing 0.1% trifluoroacetic acid (TFA) over a period of 80 min, and held for 30 min, as originally described by Roberts *et al.* [46]. Naik *et al.* [42] also purified glycosyl PtdIns by HPTLC, using $CHCl_3$/MeOH/H_2O (10:10:2.5). Keller and Li [47] have reported a nanoliter-solvent extraction, combined with microspot matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF)MS for the analysis of hydrophobic substances. The method involves the use of a nanoliter droplet, containing organic solvents, at the tip of a small capillary for extraction. After extraction, the droplet is deposited on a MALDI target pre-coated with a thin matrix layer. The performance of this technique was illustrated in several applications, involving the detection of hydrophobic peptides or phospholipids.

17.2.3 Solid-phase extraction

Solid phase extraction (SPE) has emerged as an efficient procedure for the rapid isolation of phospholipids and the resolution of phospholipid classes with limited amounts of solvents. It can be employed in the form of TLC plates or commercially available adsorption cartridges. Several general reviews have appeared on the subject. Penton [48] has described sample preparation for GLC with SPE and solid-phase micro-extraction (SPME). Mayer and Fritz [49] have reported the semi-micro SPE of organic compounds from aqueous and biological samples. Low-molecular-weight phenols, aldehydes, alcohols, esters, ethers, and ketones were included. Pan and Pawliszyn [50] have described a derivatization/SPME for polar analytes. Pentafluorobenzyl (PFB) bromide and (pentafluorophenyl)diazoethane were used to derivatize short-chain fatty acids directly in sample matrices for selective and sensitive NICI-MS. Both normal- and reversed-phase HPLC separation can be performed with these cartridges. Janero and Burghardt [51] have optimized a procedure that selectively removes the major tissue neutral lipids and noncholine-containing phospholipids from complex lipid mixtures and yields an acidic phospholipid fraction made up of phosphatidylinositol (PtdIns), phosphatidylethanol-amine (PtdEtn), phosphatidylglycerol (PtdGro), cardiolipin (Ptd_2Gro), and *ca.* 62% of phosphatidylserine (PtdSer). Singh [23] has described the extraction and analysis of

PtdIns, PtdIns(4)P, and PtdIns(4,5)P_2, using a Sep-Pak cartridge. A 50–90% recovery of PtdIns and InsPs from brain samples was demonstrated. InsP_3 and InsP_4 exhibited the lowest recovery because of poor quantification rather than a poor extraction method. The cartridge methods have not yet been applied to the isolation of the PtdIns(3)P, PtdIns(3,4)P_2 and PtdIns (3,4,5)P_3. Singh [23] has also shown that reversed-phase Sep-Pak cartridges (Accel Plus QMA, Millipore) provide excellent separation of PtdCho, PtdIns, PtdInsP, and PtdInsP_2 from a DEAE eluate.

Agren *et al.* [52] have described a simple method for the separation of serum lipid classes for fatty acid analyses with a single aminopropyl solid-phase glass column. The recoveries of cholesteryl esters, triacylglycerols (TAG), free fatty acids, and phospholipids were all at least 98%. The method differs from previously described procedures, which needed two columns and increased analysis time and consumption of materials. In addition, the acidified solvents used previously eluted considerable amounts of contaminants from commercial, pre-packed, plastic columns. Ingalls *et al.* [53] described a method for the isolation from human plasma of nonesterified fatty acids, cholesteryl esters, TAG, cholesterol, diacylglycerols (DAG), monoacylglycerols (MAG), and some phospholipids by extraction and silica gel column chromatography. All of these lipid classes, except DAG and free cholesterol, were separated cleanly in seven elution steps. The overall recovery of added synthetic odd-carbon-number fatty acids was 80–91%. The technique was considered to be superior to those described previously, especially TLC. Burdge *et al.* [54] have described a method for the separation of phosphatidylcholine (PtdCho), TAG, nonesterified fatty acids (NEFA), and cholesterol esters (CE) from total lipid extracts of plasma by SPE, using aminopropyl silica columns. Following initial separation of polar and neutral lipids, individual classes were isolated by application of solvents of increasing polarity. Recoveries for combined plasma extraction with chloroform/methanol and SPE were: PtdCho 74.2%, NEFA 73.6%, CE 84.9%, and TAG 86.8%. These recoveries were significantly greater for TAG and NEFA than by TLC. Both GLC/flame-ionization detector (FID) and GC/MS analysis of fatty acid methyl esters demonstrated that there was no cross-contamination between lipid classes. Hagerman *et al.* [55] have described a SPE of lipids from *Saccharomyces cerevisiae*, followed by HPLC analysis of coenzyme Q content, while Zhao *et al.* [56] have reported a one-step SPE procedure for F_2-isoprostanes.

Calaf *et al.* [57] utilized an automated cation-exchange extraction of fatty acid esters of 3-(*N*-phenylamino)-1,2-propanediol from oil samples for routine quantification by HPLC/APCI-MS/MS. A SPE procedure was adapted for automatic extraction to avoid the problems associated with the use of the immiscible solvents required for strong-cation-exchange (SCX) extraction. Earlier, Nakamura *et al.* [58] had developed an HPLC procedure for the specific analysis of PtdIns and PtdInsP_2 in the brain. In this method, the lipid extract is first derivatized with 9-anthryldiazomethane to produce (9-anthryl) PtdInsP and di(9-anthryl) PtdInsP_2, and then the derivatized sample is separated by HPLC with UV detection at 245 nm. PtdCho, PdIns, PtdSer, PtdEtn, and SM are not derivatized with this reagent and, therefore, do not interfere with the assay [59]. This method is more sensitive for the analysis of the inositol phospholipids than that for the underivatized compounds with UV detection. Gunnarsson *et al.* [60] used normal-phase HPLC with evaporative light-scattering detection (ELSD) for the analysis of commercial samples of PtdInsPs

along with other phospholipids. Using a linear gradient of (A) $CHCl_3$/MeOH/NH_4 (50:45:3) and (B) $CHCl_3$/MeOH/H_2O/NH_4 (25:55:17:3) well-resolved late-emerging peaks for PtdIns(4)P and PtdIns(4,5)P_2 were obtained.

Vihma *et al.* [61] have reported the quantitative determination of estradiol fatty acid esters in human pregnancy serum and ovarian follicular fluid. After extraction from serum or follicular fluid, estradiol fatty acid esters were separated from nonesterified estradiol by Sephadex LH-20 column chromatography. The HPLC procedures that are commonly used for the separation of deacylated PtdInsP typically utilize salt gradients that cannot resolve the various deacylated phospholipid isomers. However, HPLC systems with a SAX column and a shallow salt gradient can yield baseline separations of the glycerophosphoinositol phosphates (GroPInsP) derived from the PtdIns(3), PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, along with those derived from the more common PtdIns(4)P and PtdIns(4,5)P_2. Jones *et al.* [62] have separated the deacylated PtdInsP by HPLC on a Partisphere SAX column (4.6 × 235 mm, 5 μm, Whatman) with a gradient of 0–1 *M* ammonium phosphate (pH 3.75) over 120 min. Auger *et al.* [21] have described an ion-exchange HPLC method for the identification of PtdIns, PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3, following de-acylation of the glycerophospholipids (GPL), because the HPLC method separated the phospholipids based on the structural differences in the inositol head group. Anderson *et al.* [63] have described a method for the rapid and selective determination of radiolabeled inositol phosphates in cancer cells by SPE with Bond Elut SAX minicolumns. The inositol phosphates InsP, InsP_2, and InsP_3 are selectively eluted with 0.05, 0.3, and 0.8 *M* ammonium formate/0.1 *M* formic acid, respectively. Cancer cells were extracted with 10% perchloric acid, which was then neutralized prior to loading the samples on the mini-columns. Bond Elut SAX does not separate positional isomers, for which HPLC is still required.

March *et al.* [64] have reported a GC/MS method for the determination of phytic acid, based on purification by anion-exchange chromatography, enzymatic hydrolysis of phytic acid to *myo*-inositol, and derivatization to the trimethylsilyl (TMS) derivative, with *scyllo*-inositol as an internal standard. The method was successfully applied to various organs, plasma and urine, and kidney stones. The neutral solvents also extract PtdIns and some PtdInsP, which may be recovered by selective adsorption on neomycin columns, as originally described by Schacht [65] and refined by Palmer [66]. The antibiotic neomycin exhibits a specific affinity for PtdInsP and PtdInsP_2, presumably due to a strong ionic interaction between the cluster of six primary amino groups on the neomycin and the several negatively charged phosphate groups on the lipid molecules. Letcher *et al.* [67] have found that simply using the eluents described by Palmer [66] results in cross-contamination between the three principal inositol lipids. Using a stepwise elution from 200 m*M* to 500 m*M* in 100-m*M* steps, and then taking only the PtdInsP, revealed them to be pure by TLC analysis [68]. Cronholm *et al.* [69] have described the isolation of PtdInsPs from rat pancreas. Each pancreas was immediately homogenized in 3 mL of chloroform/methanol (1:2), and *ca.* 3 MBq of ^{3}H-labelled PtdIns-4-phosphate [PtdIns(4)P or PtdIns-4,5-*bis*-phosphate (PtdIns(4,5)P_2] was added, together with 0.6 mL of 1.2 aq. HCl, prior to extraction with chloroform, as described by Schacht [65].

17.3 GENERAL CONDITIONS OF LIPID CHROMATOGRAPHY

Current methods of identification of neutral and polar lipids in a lipid extract rely largely on chromatographic migration rates in relation to reference standards, but MS and MS/MS methods are also being utilized, as they provide in addition to the identification of the lipid classes also characteristic fragment ions needed for unequivocal identification of the component molecular species. Further characterization of unknown lipids is performed by means of both chemical and enzymatic degradation in combination with further chromatographic, MS, and NMR analyses.

General strategies in chromatographic analyses have been discussed by Myher and Kuksis [6]. Poole and Poole [70] have reviewed the evolution of instrumental TLC, which now offers automation, reproducibility, and accurate quantification for a wide variety of applications. Holm [71] have discussed the mechanism of the FID, including the effect of heteroatoms. Gaudette *et al.* [72] have validated the use of HPLC analysis of radiolabeled PtdInsPs for the estimation of PtdIns(3,4)P_2 mass in agonist-stimulated platelets. Bressolle *et al.* [73] have discussed the validation of HPLC and GLC methods with special emphasis on applications in pharmacokinetics. Abian *et al.* [74] have discussed the practical aspects of the utilization of conventional, narrow-bore, and capillary columns for HPLC/ESI-MS.

17.3.1 Methods of lipid separation

17.3.1.1 Thin-layer chromatography

The one dimensional system of Skipski *et al.* [75] uses $CHCl_3$/MeOH/AcOH/H_2O (25:15:4:2) and silica gel H (without calcium sulfate binder) and has been widely adopted for the resolution of the major phospholipid classes, including PtdIns [75,76], but two-dimensional systems [77] and multiple developments have also been used. However, the system presents difficulties in the separation of the anionic phospholipids and frequently shows an overlap between PtdIns and PtdSer. Mahadevappa and Holub [78] have overcome the problem by altering the proportions of acetic acid and water in the solvent system. The invention of the HPTLC variant of TLC analysis has greatly improved the resolution of PtdIns from phospholipid mixtures. Sun and Lin [79] have described a detailed procedure for a two-dimensional resolution of brain phospholipids, using HPTLC plates and a successive development with $CHCl_3$/MeOH/acetone/16 *M* NH_4OH (70:45:10:10) and $CHCl_3$/MeOH/16 M NH_4OH/H_2O (36:28:2:6) in the same direction. After development with the second solvent system, the plates may be exposed to HCl fumes for 3 min to destroy any plasmalogens, after which the plates are turned through 90° and placed in a third solvent system, consisting of $CHCl_3$/MeOH/acetone/acetic acid/0.1 *M* ammonium acetate (140:60:55:4.5:10), and the spots are located. After development, the plates are dried briefly, and the phospholipids are revealed by spraying with fluorescein or by charring.

Wilson and Sargent [80] have described a comparable separation of PtdIns and PtdSer from each other and from other cellular phospholipids by two-dimensional HPTLC, using

MeOAc/propanol/$CHCl_3$/MeOH/0.25% aq. KCl (25:25:25:10:9) in one direction and hexane/diethyl ether/acetic acid (80:20:2) in the other direction. The dried plates are sprayed with color reagents, including 2′,7′-dichlorofluorescein (0.1%) in methanol/water (7:3), which exhibits fluorescence with most phospholipids; a mixture, containing 37% formaldehyde and conc. sulfuric acid (3:97), which is sprayed on the plate and, after heating at 180 °C for 30 min, causes the lipid compounds to appear as black, charred spots; or a mixture, containing 3% copper acetate in 8% phosphoric acid, which makes phospholipids appear as dark brown spots. Singh and Jiang [81] have reviewed the application of TLC to the resolution of PtdIns and PtdInsPs and have concluded that the recoveries are only about 60% of the applied material. Zhang *et al.* [82] have modified the method of Touchstone *et al.* [83] for the improved separation of PtdIns from PtdSer and other phospholipids in high-density lipoproteins (HDL). More recently, Narasimhan *et al.* [84] and Carstensen *et al.* [85] have reported excellent separation of *scyllo*-PtdIns, *myo*-PtdIns, PtdCho, PtdSer, and PtdEtn from each other and from PtdInsP, which remain at the origin. The separations were performed by HPTLC, using $CHCl_3$/MeOH/NH_4OH (90:90:20) as the developing solvent. However, HPTLC is not well suited for the isolation of the PtdIns for subsequent work-up by methods less sensitive than HPTLC. The PtdInsPs may be resolved by one- [76] or two-dimensional TLC [86] or by HPTLC on silica gel, impregnated with oxalic [87] or boric acid [88].

17.3.1.2 Gas/liquid chromatography

While conventional capillary GLC is still the analytical method of choice for the separation of fatty acids [3,89] and prostanoids [4,90], high-temperature GLC of neutral lipids has been confined largely to the determination of total lipid profiles on nonpolar capillary columns [91,92]. The highly promising GLC separations of molecular species of TAG on polarizable capillary columns [93,94] have found only a few applications [94–96], most investigators choosing to use reversed-phase HPLC instead (see below). Thompson [97] has described simplified fatty acid analyses in food with a standard set of isothermal GLC conditions, coupled with equivalent-chain-length (ECL) determinations, using a Carbowax-20M/fused-silica column. The report provides the ECL elution factors for 25 *trans*- and *cis*-monoethylenic fatty acid methyl esters along with the elution factors of some 59 different fatty acid methyl esters, reported earlier. However, some popular columns have not produced the anticipated completeness of resolution and have required additional sample processing by complementary chromatographic techniques to ensure the correctness of the final result. Thus, the 50-m CP-Sil 88 capillary columns apparently provided unreliable data, compared to previous studies, when used by more than 70 participants in an industry-sponsored study, aimed at obtaining reliable data on the *trans*-unsaturated fatty acid content of several hundred food items. Wolff and Precht [98] point out that this column is known to present several drawbacks, whether operated isothermally or with temperature programming. The latter precludes complete separation of *trans*-unsaturated fatty acids, unless a preliminary separation by silver-ion TLC is performed:

(a) the *trans*-16:1 isomers are not separated from the *cis*-16:1 isomers and also overlap with branched 17:0 acids;

(b) the 50-m CP-Sil 88 column is known to resolve only the Δ^4- and Δ^{11}- *trans*-18:1 isomers, while the other unsaturated *trans*-isomers with Δ^{12}-, Δ^{13}-, Δ^{14}-, Δ^{15}-, and Δ^{16}-double bond are eluted under the main peak of oleic ($\Delta^{cis18:1}$) acid;

(c) *trans*-18-3 isomers that are formed during the deodorization of α-linolenic acid-containing vegetable oils, appear to be to be mixed with 20:0 and/or *cis*- and/or *trans*-20:1 acids. The resolution of these fatty acids is highly dependent on the operating temperature and the length of the CP-Sil 88 column, and it requires special analytical precautions. Wolff and Precht [98] note that methods are presently available to separate clearly, identify, and quantify individual *trans*-unsaturated fatty acids, and also to permit rather accurate estimates of the relative contributions of ruminant fats and partially hydrogenated vegetable oils to the total per capita daily *trans*-unsaturated fatty acid intake [99]. These methods include an obligatory preliminary step, *e.g.*, silver-ion TLC, in order to suppress overlaps of *trans*-UFA with interfering FA, as described above. Wolff and Precht [98] suggest that 100-m or even longer columns should improve the resolution of isomers.

Western *et al.* [100] have described positional and geometric isomer separations of fatty acid methyl esters by comprehensive two-dimensional GLC. The methodology involved the use of two directly coupled capillary columns, providing different retention mechanisms, with a pulsing modulator located near their union. The first column separated the compounds on the basis of boiling point variations, the second based on polarity. A sample of marine oil gave 49 resolved, identified peaks, with at least an additional 20 peaks resolved but not identified. Improved quantification based on FID measurements was also demonstrated. Kramer *et al.* [101] have compared the performance of a 60-m Supelcowax 10 with 100-m CP Sil 88 GC column in the analysis of ruminant milk fat with emphasis on conjugated linoleic acid, the 18:1, 18:2, and 18:3 isomers, along with short- and long-chain fatty acid methyl esters. The results of the study confirmed that 100-m, highly polar GC columns are mandatory for the analysis of milk lipids, and that the 60-m SUPELCOWAX 10 capillary column serves, at best, as a complementary column to provide separations in certain regions, based on intermediate polarity.

17.3.1.3 High-performance liquid chromatography

Rizov and Doulis [102] have reported the use of SPE for the determination of the glycerolipid composition of rice and maize tissues. By manipulating the stationary phase in a solid-phase extraction manifold, efficient and rapid separation of plant membrane lipids was achieved. At different stages of the glycerolipid separation the SPE manifold combined weak-anion-exchange columns, containing aminopropyl, aminopropyl/silica gel, and silica gel/aminopropyl packings. Specifically, the glycerolipids from maize tissues were separated into seven classes (monogalactosyl-DAG, digalactosyl-DAG, PtdEtn, PtdCho, sulfoquinosyl-DAG, PtdIns, and PtdGro). The glycerolipid extract of pigment-containing plant tissues was freed from the pigments on the aminopropyl column. The aminopropyl column with the glycerolipid extract was then connected to a silica gel column, from which monogalactosyl-DAG, PtdEtn, PtdGro, and digalactosyl-DAG were eluted as individual fractions. The silica gel column, containing the remaining glycerolipid

extract, was connected to an aminopropyl anion-exchange column, and individual fractions of sulfoquinovosyl-DAG, PtdCho, and PtdIns were eluted. The separation process was facilitated by ammonium counter-ions and by the polarity gradient of the elution system used. Glycerolipid recovery was 87–95%. Conventional HPLC on normal-phase (NP) columns yields extensive separation of the common phospholipid classes, but PtdIns and PtdCho may overlap, unless the solvent systems have been appropriately selected. One NP-phase HPLC system that has permitted effective separation of PtdIns from other phospholipids utilizes a gradient of (A) hexane/2-propanol (3:3) and (B) water/ Eluent A (27.5:72.5). This solvent system separated neutral lipids from acidic phospholipids but did not resolve PtdIns and individual PtdInsPs [103]. It is nearly transparent in the 200-nm UV range and can be used for quantification. Alternatively, the lipids can be detected by ELSD or by MS, which can be used with most solvent systems. De-acylation of PtdInsPs before analysis may improve the analysis of PtdInsPs. The MS detector also provides an indication of the composition of molecular species (see below). Utilization of reversed-phase (RP)- HPLC with most solvent systems and all detectors provides an indication of the resolution of molecular species (see below).

NP- and RP-HPLC are presently the major routine methods for lipid separation. Except for fatty acid and sterol resolution, they have replaced GLC as the method of choice for resolving neutral lipids. A major advantage of the NP-HPLC column is the possibility of using relatively crude lipid extracts for the final analysis. NP-HPLC has also proven suitable for the resolution of diastereomeric DAG in the determination of the stereoisomeric distribution of fatty acids in natural TAG [104–106]. Conventional NP-HPLC was reported by DeLong *et al.* [13] to lead to peroxidation of the oligounsaturated GPL, as demonstrated by directly analyzing a total cellular lipid extract by MS/MS. Loss of the oligoenoic GPL was three times lower than that experienced with TLC (see above). RP-HPLC with appropriate solvent systems provides excellent separations of the molecular species of intact phospholipids as well as of their diradylglycerol and ceramide moieties. Unless mass spectrometry is used for peak detection, the phospholipid class to be analyzed must first be thoroughly separated from all other phospholipid classes. Most surprisingly, some RP-HPLC columns permit the resolution of many reverse isomers of DAG, following preparation of the dinitrophenylurethane (DNPU) and other nitrogenous derivatives [107]. The separation of these derivatives can be readily monitored by UV, but ES-MS monitoring is superior.

The DAG moieties released from PtdIns by phospholipase C (PLC) are readily quantified by high-temperature GLC, following preparation of TMS derivatives and using tridecanoylglycerol as an internal standard. The peak areas of the molecular species, which are resolved on a nonpolar capillary column according to carbon number, are summed, related to the peak area of a known amount of the internal standard, and the results are expressed on a weight or mole basis. Appropriate correction factors for incomplete recoveries from the column or differences in the detector response may be included in the calculations, as described in detail elsewhere [108]. Dobson *et al.* [109] have described an LC/MS method, in which a particle-beam interface is used to obtain EI-mass spectra of the nicotinate derivatives of DAG mixtures, and thereby obtained maximum structural information on natural GPL. A useful feature of these derivatives is that, in addition to the size and degree of unsaturation of the acyl chains, the positions of the double bonds and

other functional groups can also be ascertained. Dobson and Deighton [110] have subsequently adopted this method for quantifying phospholipid molecular species as nicotinate derivatives by reversed-phase HPLC in conjunction with UV detection and the atmospheric-pressure chemical ionization (APCI)-MS of $[MH\text{-}123]^+$ions. There is continued interest in the identification and quantification of free and bound DAG. MALDI-TOF-MS was found to be a convenient method for the analysis of DAG in organic extracts of cell suspensions. A linear correlation between peak intensity and lipid concentration over one order of magnitude was found. Benard *et al.* [111] performed quantitative analyses of DAGs in a matrix of 2,5-dihydroxybenzoic acid (DHB) into the picomolar range. The 18:0/18:0 species was chosen as internal standard. Chiral-phase HPLC has revolutionized stereoisomer and regioisomer analyses of diradylglycerol moieties of neutral lipids, GPL, and TAG [112,113]. The resolution requires a preparation of the DNPU derivatives of the diradylglycerols. The separation of these derivatives can be readily monitored by UV, but ES-MS detection is superior.

17.3.1.4 Ion-exchange chromatography

HPLC columns containing weak-anion exchangers are generally used for lipid purification, while those containing strong-anion exchangers are used for lipid-class and molecular-species separation of InsP [114]. For the lipid-class separation, the inositol phospholipids must be de-acylated. Thus, baseline separation of all of the PtdInsP is achieved, using a high-resolution 5-μm Partisphere SAX column (Whatman) and a shallow, discontinuous salt gradient (see below). The eluate from the HPLC column is connected, first to an on-line UV monitor, and then directly to an on-line continuous-flow liquid-scintillation detector (Flo-One/Beta CT; Radiomatic Instruments) which can monitor and quantify two different isotopes simultaneously [21]. Alternatively, the radiolabeled phospholipid samples may be analyzed by attaching a fraction collector to the HPLC column and collecting individual fractions for scintillation counting. Spencer *et al.* [115] have used a formate-form Dowex column for a very precise separation of InsP from $InsP_3$ to $InsP_6$. It is also applicable to quenched-tissue samples, and enzyme assays.

17.3.1.5 Supercritical-fluid chromatography

Supercritical-fluid chromatography (SFC) is of interest for the preparative-scale fractionation of vegetable oils, the isolation of fat-soluble vitamins, and of essential oils for the food and cosmetic industry (Chap. 2). The application of SFC to the separation of substances of pharmaceutical relevance has renewed interest in this analytical process in recent years [116,117]. Since pure supercritical CO_2 is a strictly lipophilic solvent, mixtures of organic solvents, especially alcohols can be used to increase its polarity, so that more polar compounds can be chromatographed [118]. Sjöberg *et al.* [119] reported a SFC interface probe for APCI-MS and ESI-MS, having the advantage of convenient switching between ionization modes. Sjöberg and Markides [120] have optimized the design of the spray devise to obtain a stable signal and a low limit of detection (LOD). In the ESI mode, the achievable detection limits were in the 50- to 0.1-pg range. The LOD in

the APCI-/MS mode was improved by a factor of 20–25, compared to an earlier design. Wang *et al.* [121] have designed an automated packed-column semi-preparative SFC/MS system, incorporating mass-directed fraction collection as an alternative to preparative HPLC and preparative HPLC/MS for the purification of pharmaceutical compounds. Garzotti and Hamdan [122] have reported coupling of SFC to a hybrid mass spectrometer (Q-TOf2), equipped with an ESI source, to separate and characterize a wide range of pharmaceutical racemates. Planeta *et al.* [123] have recently described a sample deposition device for off-line combination of SFC with MALDI-TOF-MS. Aro *et al.* [124] have described an on-line SFE/SFC/GLC/MS method for the determination of volatile compounds and co-eluted lipids. Other reports of SFC are discussed under specific applications (see below).

17.3.2 Peak identification and quantification

17.3.2.1 Chromatographic methods

TLC and, especially, HPTLC continue to serve as preliminary methods of chromatographic identification of peaks as well as for preliminary quantification of lipids [6,125]. GLC peaks are commonly identified by retention time, relative to standards analyzed under similar conditions, independently or together with the samples, and the peak areas are derived from FID [71]. High-temperature GLC frequently serves as a preliminary method of separation and quantification of neutral lipids and, in special cases, of total lipids. GLC with FID is also frequently used for the identification and quantification of chromatographic fractions resolved by TLC [6]. For this purpose, the chromatographic fractions recovered from a TLC plate are usually transmethylated, and the resulting fatty acid methyl esters are identified and quantified in the presence of internal standards. Gaudette *et al.* [72] measured the mass of PtdInsP in blood platelets by TLC separation of PtdIns, PtdInsP, PtdIns(4,5)P_2, and PtdIns(3,4)P_2, and quantification of the fatty acid components, derived from them by transmethylation in the presence of an internal standard. For the analysis of the PtdInsP, the lipid extracts from four replicate 1-mL aliquots of platelet suspension were pooled. Several (6 to 12, depending on platelet yield) 2-cm-wide bands were applied to the TLC plates, and the lipids were fractionated with $CHCl_3$/MeOH/conc. NH_4OH (45:35:7:5). Lipids were visualized under UV light, following spraying of the TLC plates with 8-anilino-1-naphthalenesulfonic acid. The system gave good separation of PtdIns(4)P from other lipids. Although PtdIns(4,5)P_2 appeared to be separated from PtdIns(3,4)P_2, HPLC analysis of [^{3}H]Ins-labeled PtdInsP revealed incomplete purification of PtdIns(3,4)P_2 by the single TLC run. The PtdIns(3,4)P_2 was purified by re-chromatographing the TLC fractions. The recovery of the PtdInsP_2 fractions from the silica gel was facilitated by sonication. For quantification, the PdInsP fractions were transmethylated for 2.5 h at 85 °C in 2 mL of acetyl chloride/methanol (5:50) in the presence of an appropriate quantity of internal standard (heptadecanoic acid), and the fatty acids were analyzed by GLC. Appropriate blank areas of the TLC plate were also transmethylated, and the values obtained were subtracted as a background from the corresponding sample peaks.

The GPL fractions recovered from TLC may be subjected to digestion with phospholipase C and the released diradylglycerols and ceramides converted to TMS ethers for identification and quantification by high-temperature GLC. Likewise, HPLC peaks may be identified by relative retention times of standards, analyzed under the same conditions, and quantified by ELSD or UV. Like GLC, HPLC is frequently employed for the identification and quantification of TLC fractions. These methods of peak identification and quantification suffer from peak overlap, lack of uniform detector response to different lipid species, and absence of appropriate standards for all unknown fractions. Nevertheless, discoveries continue to be made by both GLC and HPLC systems. Of special interest are the applications of RP-HPLC to the separation of reverse-isomers of DAG moieties of TAG [107] and of glycerophospholipids [126,127] as the DNPU derivatives. The reverse-isomers of enantiomeric DAG may be resolved by chiral-phase HPLC, using the same DNPU derivatives [112,113]. Ion-exchange chromatography plays an important role in the identification and quantification of the PtdInsP and their deacylation products [114,115] (see below).

17.3.2.2 Mass-spectrometric methods

MS techniques are especially useful for lipid analyses, due to their high sensitivity. However, the effective applicability of MS depends on the proper choice of ionization technique. Only FAB, ESI, and MALDI have played a major role in the field of phospholipid analyses, while APCI has proven rather useful for neutral-lipid analyses. Fast-atom bombardment/mass spectrometry (FAB-MS) was recognized early as being a useful method for establishing the structure of phospholipids. This is due to the unique chemical behavior of these compounds. Their highly lipophilic region enables the molecule to orient itself on the surface of FAB matrices, and their polar functionalities readily accept negative charge sites in the gas phase [128]. FAB-MS/MS of both positive and negative ions is a useful technique for the identification of intact GPL, providing information about polar head group and fatty acid substituents. Kayganich and Murphy [129] utilized FAB-MS/MS for the identification of diacyl, alkylacyl, and alk-1-enylacyl molecular species of GroPEtn in leukocytes. Positive-ion neutral-loss scanning for 141-units was used to confirm the presence of the PEtn head group. This scan was found to discriminate against the abundant subclass of phospholipids having an 1-*O*-alk-1′-enyl linkage (plasmalogens) as well as 1-*O*-alkyl ether species. Using FAB-MS/MS alone, it is impossible to differentiate between plasmalogens and other 1-*O*-alkyl ether molecular species having the same molecular weight. A combination of mild acid hydrolysis, which selectively hydrolyzes the labile 1-*O*-alk-1′-enyl bond, with subsequent FAB-MS/MS, distinguished species of these distinct subclasses. Holbrook *et al.* [130] have compared the FAB-MS spectra of the PtdOH moieties derived from PtdCho, PtdEtn, PtdSer, and PtdIns, extracted from cells and hydrolyzed with phospholipase D. The molecular species of PtdOH can also be readily recognized and quantified by FAB-MS/MS. When ethanol is present in an *in vitro* incubation along with phospholipase D, phospholipids are converted to PtdEt, rather than PtdOH, by transphosphatidylation. Holbrook *et al.* [130] used this

reaction in the formation of PtdEt as a marker for endogenous phospholipase D substrate. The resultant PtdEt was analyzed by FAB-MS.

PtdIns and its phosphates are acidic lipids and therefore produce primarily negative ions in FAB experiments. An abundant $[M-H]^-$ ion indicates the molecular weight of the compound as well as the acyl substituents. Jensen *et al.* [131] have reported a series of charge-remote decomposition ions seen from $[M-H]^-$ of diradyl-GroP. When sufficient sodium is present in the sample, positive ions can be observed corresponding to $[MH]^+$and $[M + 2Na-H]^+$[132]. Collision-induced activation (CID) of the $[M-H]^-$ ions from diradyl GroPIns yield abundant carboxylate anions as well as several phosphoinositol ions at m/z 21, 259, and 299. Sherman *et al.* [132] studied both positive and negative ions, generated from PtdIns(4)P and PtdIns(4,5)P_2. PtdIns(4)P and PtdIns(4,5)P_2 were shown to behave in FAB similarly to PtdIns. GroPIns lipid species behaved similarly to GroPOH lipids, since both are acidic GPL. While positive ions corresponding to $[M + H]^+$can be observed, most FAB ions are negative ions. CID of $[M + H]^+$from GroPIns lipids yields a neutral loss of 260 mu, corresponding to the loss of InsP. This neutral loss is the characteristic transition reported to identify an ion as uniquely derived from GroPIns lipids [128,129]. The negative-ion FAB spectrum of GroPIns lipids contains $[M-H]^-$ ions in low abundance, consistent with the loss of [inositol-H_2O], such as m/z 671. This ion has a structure identical to the $[M-H]^-$ anion of GroPOH lipids. Carboxylate anions from the fatty acyl groups at sn-1 and sn-2 are also observed in the negative-ion FAB mass spectrum. CID of $[M-H]^-$ results in abundant carboxylate anions, but precise assignment of the position requires decomposition of the PtdOH anion (m/z 671.5). Minor ions at m/z 595 and 577 arise from loss of palmitic acid and its ketene analog, respectively. The ions at m/z 392 and 409 likewise result from the losses of the sn-2 substituent from the GroPOH ion formed by loss of inositol. Sherman *et al.* [132] also reported several specific GroPIns-related negative ions at m/z 259 (inositol phosphate), 241 (inositol-phosphate-H_2O), and 297 (diacylglycerophosphoinositol$-R_1COOH-R_2COOH$), which are unique for this class of phospholipid. ES-MS has the advantage that the ionization is "soft." It has been extensively applied for the identification of both neutral lipids and phospholipids. Kerwin and Torvik [133] used negative ESI-MS and ESI-MS/MS to characterize saturated and unsaturated monohydroxy fatty acid metabolites formed following incubation with soybean lipoxygenase. Ions corresponding to $[M-H]^-$ of eicosanoids were readily observed by ESI-MS, but double-bond migration precluded the use of MS to localize double bonds or the position of hydroxyl moieties. However, by following MS analysis with negative-ion (NI)ESI-MS/MS of precursor ions, the position of oxygenation could be determined for picogram quantities of underivatized monohydroxy fatty acids. Spectra of deuterated analogs supported charge-driven vinylic processes as the most common mechanism of fragmentation.

Cheng *et al.* [134] have reported complete structural elucidation of TAG (except for the chirality at the sn-2-position) by ESI-MS/MS. Both ESI- and FAB-produced $[M + NH_4]^+$ and $[M + met]^+$ions (where met = Li, Na, and Cs) of TAG undergo charge-remote and charge-driven fragmentations. ESI-produced $[M + NH_4]^+$ions fragment to produce four types of ions, $[M + NH_4]^-R_nCOONH_4]^+$, $[R_nCO + 128]^+$, $[R_nCO + 74]^+$, and R_nCO^+ ions, from which the carbon number and the degree of unsaturation of each acyl group are

obtained. Furthermore, three series of ions are produced by charge-remote fragmentations (CRF), and analysis of their patterns gives the position and the number of double bonds on the acyl groups. The ESI- and FAB-produced $[M + Na]^+$ions fragment to form eight types of ions that, like those produced by CRF, are structurally informative. In contrast, information about the position of each acyl group on the glycerol backbone is not provided by CID of $[M + NH_4]^+$ions. The new method awaits successful combination with HPLC to make it applicable to analyzing natural mixtures of TAG. Hsu and Turk [135] have described MS/MS of lithiated adducts of TAG species, obtained by ESI-MS with low-energy CID on a triple-stage quadrupole instrument. The spectra distinguished isomeric TAG species and permitted assignment of the mass of each fatty acid substituent and positions on the glycerol backbone on which substituents were esterified. The MS/MS also yielded ions reflecting combined elimination of two adjacent fatty acid residues, one of which is eliminated as the free fatty acid and the other as an α,β-unsaturated fatty acid. Such combined losses always involve the sn-2-substituent, and this feature provides a robust means of identifying that substituent. Determination of double-bond location in polyunsaturated fatty acid substituents of TAG is achieved by source-CID experiments, in which dilithiated adducts of fatty acid substituents are produced in the ion source and subjected to CID in the collision cell [136].

Han and Gross [137] have described a rapid, simple, and reliable method for the quantitative analysis and molecular species fingerprinting of TAG directly from chloroform extracts of biological samples by ESI-MS/MS. By exploiting a rapid loss of phosphocholine from PtdCho, in conjunction with neutral-loss scanning for individual fatty acids, overlapping peaks in the ESI mass spectra were deconvoluted, generating a detailed molecular-species fingerprint of individual TAG molecular species. The new method overcomes the problem of overlapping peaks from PtdCho, which requires chromatographic separation of lipid extracts prior to ESI-MS analyses. The method readily detects as little as 0.1 pmol of each TAG species from chloroform extracts and is linear over a 1000-fold dynamic range. The rapid quantification was facilitated by the development of an algorithm which identifies sensitivity factors. The analysis of molecular species of PtdCho and sphingolipids is presently being performed by admitting the total lipid extracts directly into the mass spectrometer. This was initially thought to be a convenience, permitting rapid analysis of samples [138,139], but, more recently, the detrimental effects of lipid peroxidation observed during sample purification and chromatographic separation have served as a better justification for direct MS/MS analyses [13]. In those instances where comparisons of lipid recovery and molecular species analyses have been made, the direct-liquid-injection MS/MS has been claimed to yield higher recoveries than MS/MS preceded by chromatography [13,14,140], although comparable results from MS/MS and chromatography have also been reported [7,10] (see below).

Han and Gross [138] and Kerwin *et al.* [139] showed that flow-injection ESI-MS with MS/MS offered a rapid method of determining molecular species of phospholipids without prior chromatographic separation. Han *et al.* [140] have identified and quantified (by means of an internal standard) the five major species of PtdIns in total phospholipid extracts from human platelets. ESI-MS of chloroform extracts of human platelets in the negative-ion mode have demonstrated the presence of multiple individual molecular species of PtdIns, *e.g.*, m/z 836 (16:0/18:1 GroPIns); 858 (16:0/20:4 GroPIns);

864 (18:0/18:1 GroPIns); 884 (181/20:4 GroPIns); and 886 (18:0/20:4 GroPIns), which were similar to those previously described, revealing a high proportion of arachidonic acid-containing species. The method of Han *et al.* [140] was used by Williams *et al.* [141] to demonstrate the loss of nuclear membrane phospholipid, after re-perfusion of an ischemic myocardium. Specific comparisons were made between the choline and ethanolamine glycerolipid species from isolated and perfused hearts. Brugger *et al.* [142] have extended these investigations with the goal of a full characterization and quantification of membrane lipids in an unprocessed Bligh-and-Dyer extract from cells or subcellular structures by ESI-MS alone. For this purpose, Brugger *et al.* [142] adopted the use of a nano-ESI source [143], allowing the analysis of picomole or subpicomole amounts of polar lipids, along with the standard MS/MS techniques as parent-ion and neutral-loss scanning. Synthetic analogs with non-natural fatty acid structures served as internal standards. This approach allowed the specific detection of PtdCho, SM, PtdIns, PtdEtn, PtdSer, PtdGro, and PtdOH, including their plasmalogen analogs in sample amounts representing as few as 1000 cells. All individual molecular species within these lipid classes could be detected in one analytical run, using an unprocessed total lipid extract. However, Uran *et al.* [143] have pointed out that because of the ^{13}C isotope effect, it is very difficult or impossible to calculate minor molecular species with chains exceeding $C_{17:0}$ in mixtures of GPL. Prior chromatographic separation of lipid classes avoids or minimizes the problem, which can be further eliminated by an additional pre-fractionation of the molecular species by RP-HPLC.

Zacarias *et al.* [144] have pointed out that quantitative analyses of glycerophospholipids (PtdCho, PtdEtn, PtdSer, PtdGro, PtdIns, PtdOH) are not straight-forward and are confounded by analyte- and mass-discrimination effects, as well as nonlinear dependence of the ion intensity on concentration. This nonlinearity is particularly severe in the negative mode and precludes even comparative measurements of anion concentrations. Zacarias *et al.* [144] suggested measuring the intensity of individual ions at several different concentrations of the total mixture and determining the slope of the double-log plot of sample concentration *versus* intensity for each analyte. The new method allowed facile and accurate comparison of NI spectra of complex mixtures, containing structurally different anions. Fridriksson *et al.* [145] have used positive- and negative-mode ESI Fourier-transform ion cyclotron-resonance MS to compare quantitatively the phospholipid composition of isolated detergent-resistant membranes of RBL-2H3 mast cells. Using the relative intensity of PtdIns in positive- and negative-ion modes to normalize the spectra with respect to each other, the relative sensitivities of the standards were as follows: For the positive-ion mode, 1.00:0.75:0.67 for PtdCho/PtdEtn/SM; and for the negative-ion mode 1.00:1.14:0.46;0.52:1.2 for PtdIns/PtdGro/PtdSer/phosphatidic acid (PtdOH)/PtdEtn.

ESI-MS in the NI mode has provided the molecular masses of the acidic phospholipids [138,145]. In the NI mode, PtdIns yield abundant $[M-H]^-$ ions by ESI. Optimal proof with respect to the identity of the analyte may be obtained by using CID of the molecular related ions, observed in the full mass spectrum. Fragments used in SIM can thereby be correlated with a high degree of certainty to ions from the parent ions. Following CID, the $[M-H]^-$ ions yield three major series of fragment ions that reflect neutral loss of free fatty acid: $(M-H-RxCO_2H]^-)$, neutral loss of ketene $(M-H-Rx'CH{=}C{=}O]^-)$, and fatty

carboxylate anions ($[RxCO_2]^-$), where x = 1,2; Rx=Rx′CH_2. Ions reflecting an inositol polar head-group are also abundant. Samples in chloroform/methanol (1:4) at final concentration of 2 pmol/μL were infused (1 μL/min) into a ESI source with a Harvard syringe pump. The intensities of the ions arising from neutral loss of the sn-2-substituent as a free fatty acid ($[M-R_2CO_2H]^-$) or a ketene ($[M-H-R_2'CH=C=O]^-$) are greater than those of ions reflecting corresponding losses of the sn-1- substituent, and these features have been used to determine the positional distribution of fatty acids. Michelsen *et al.* [146] have demonstrated that PtdInsP and $PtdInsP_2$ give prominent singly and doubly negatively charged deprotonated molecules in ESI-MS. These ions can be used for quantification of PtdInsP and $PtdInsP_2$ in the low-picomole range, without prior chromatographic separation, using SIM and consecutive measurements of the signals from the deprotonated singly charged molecules. The dose/response curves for both compounds are linear. In a complex matrix, consisting of polar lipids (Folch extract), PtdIns and $PtdInsP_2$, monitored at m/z 965.4 and 1045.5 (stearoyl and arachidonoyl), were determined in the low picomole range, at a flow-rate of 100 μL/min. CID of PtdInsP and $PtdInsP_2$ with a mixture of xenon and argon at 25 eV afforded identical high-mass ions, formed by loss of a molecule of water from PtdInsP and a phosphate group and a molecule of water from $PtdInsP_2$. The results indicate that PtdInsP, and biologically relevant changes in their concentrations, can be quantified directly in cells and cellular membranes by SIM with ES-MS. CID of PtdInsP and $PtdInsP_2$ with a mixture of xenon and argon at 25 eV afforded identical high-mass ions, formed by the loss of a molecule of water from PtdInsP and a phosphate group and a molecule of water from $PtdInsP_2$. The dose/response curves for both compounds were linear. Schneiter *et al.* [147] have applied the nano-ESI-MS/MS method to the quantitative analysis of yeast subcellular membrane phospholipids at the picomole level, while Ramanadham *et al.* [148], Hsu *et al.* [149], and Fridrickson *et al.* [145] reported ESI-MS/MS analyses of GPL with the conventional flow-injection mode.

Hsu *et al.* [145] have presented a mechanistic study of the mass-spectrometric characterization of PtdIns, PtdInsP, and $PtdInsP_2$ by ESI-MS/MS. In NI mode, the major fragmentation pathways under low-energy CID for PtdIns arise from neutral loss of free fatty acid substituents ($[M-H-R_xCO_2H]^-$) and neutral loss of the corresponding ketenes ($[M-H-R'xCH=C=O]^-$), followed by consecutive loss of the inositol head-group. The intensities of the ions arising from neutral loss of the sn-2- substitutent as a free fatty acid ($[M-H-R_2CO_2H]^-$) or as ketene ($[M-H-R_2'CH=C=O]^-$) are greater than those ions reflecting corresponding losses of the sn-1-substituent. This is consistent with losses arising from charge-driven processes that occur preferentially at the sn-2-position. Nucleophilic attack of the anionic phosphate on the C-1 or the C-2 of the glycerol, to which the fatty acids are attached, expels sn-1- ($[R_1CO_2]^-$) or sn-2 ($[R_2CO_2]^-$) carboxylate anion, respectively. This pathway is sterically more favorable at the sn-2 than at sn-1. Byrdwell and Emken [150] investigated APCI-MS as a new method for the analysis of TAG mixtures, separated by reversed-phase HPLC. A Finnigan MAT SSQ 710 mass spectrometer, fitted with an APCI source, was used to acquire mass spectral data. The mass spectra obtained for single-acid TAG showed that minimal fragmentation occurred, resulting primarily in DAG $[M-RCOO]^+$ions and $[M+1]^+$ protonated molecular ions. The degree of unsaturation within acyl chains had a marked effect on the proportion of

DAG ions *versus* the $[M\text{-}1]^+$ions formed in the APCI source. Byrdwell [151] has recently reviewed the application of APCI to the analysis of molecular species of native and peroxidized TAG. In a separate report, Byrdwell *et al.* [152] discussed the quantitative aspects of TAG analysis by APC-MS, while Mottram and Evershed [153] discussed the structure analysis of TAG positional isomers by APCI-MS.

Laakso and Voutilainen [154] used APCI/MS for the characterization of TAG rich in α- and/or γ-linolenic acids, following separation by silver-ion HPLC. The APC-MS spectra were obtained on-line. Mass spectra of most TAG exhibited abundant $[M + H]^+$ and $[M-RCOO]^+$ions, which defined molecular weight and molecular association of fatty acyl residues in the TAG molecules. Silver-ion HPLC/APCI-MS provided extensive separation and structure identification of seed-oil TAG from various plants. More recently, HPLC with APCI-MS was employed by Mu *et al.* [155] to identify DAG and TAG in a structured lipid sample. The ammonia-adduct molecular ion was the base-peak for TAG containing polyunsaturated fatty acids, whereas the DAG fragment-ion was the base-peak for TAG containing saturated and monounsaturated medium- and long-chain fatty acids. The most abundant ion for DAG was the molecular ion $[M\text{-}17]^+$. These distinctive differences between the DAG and TAG spectra were utilized for rapid identification of the acylglycerols in the sample of structured lipid.

Kallio and Currie [156,157] showed that MS by ammonia NICI-MS/MS is an efficient method for the analysis of TAGs. The abundance of $[M-H]^-$ ions define unambiguously the number of acyl carbons and the number of double bonds present in the TAG of most fats and oils, as well as the proportions of different molecular species. Furthermore, the daughter-ion spectrum provides information on the fatty acid constituents and their distribution between sn-1- and sn-1,3-positions of a TAG species. Laakso and Kallio [158] have optimized the MS conditions for the analysis of seed oils by NICI. Samples were introduced *via* direct-exposure probe into the ion source without chromatographic separation before MS. The effects of the ion-source temperature and reactant-gas pressure, as well as the rate of probe heating on the quality of ammonia NICI-MS/MS of TAG was investigated. Like ES-MS, MALDI-TOF-MS does not require sample derivatization. Unlike ES-MS, MALDI-TOF-MS cannot easily be combined with HPLC. However, MALDI-TOF-MS has the advantage that all measurements can be performed in a single organic phase, since both the lipid as well as the matrix [*e.g.*, dihydroxybenzoic acid (DHB)] are readily soluble in organic solvents. This provides extremely homogeneous matrix/analyte crystals and leads to excellent reproducibility. MALDI is more sensitive than ESI and is not affected by impurities to such a high extent. Harvey [159] and Marto *et al.* [160] used this technique successfully for the analysis of lipids in model membranes. Schiller *et al.* [161] have demonstrated that these properties allow the efficient and convenient detection of lipids by MALDI-TOF-MS, while Schiller and Arnold [162] used it in the analysis of organic-solvent extracts of cells. Schiller *et al.* [161] have investigated the application of MALDI-TOF methodology for the determination of the molecular species of GPL. PtdCho can be easily analyzed by MALDI-TOF-MS. The sample is cationized by the addition of inorganic ions. This process is strongly dependent on the corresponding ion concentration. In biological samples various cations are present (mainly H^+, Na^+, and K^+) and, therefore, a mixture of different adducts is formed. Since phospholipids exhibit a wide distribution of different fatty acid residues, a considerable

peak overlap may occur. Schiller *et al.* [161] have demonstrated that this problem may be overcome by mixing the analyte with cesium chloride. This yields Cs^+ adducts which do not occur naturally and are apparently due to the large shift of the molecular mass. The proposed method has been applied to the analysis of natural GPL. Schiller *et al.* [161] and Petkovic *et al.* [163] have applied MALDI-TOF-MS to the structural characterization of PtdIns and PtdInsP. However, these techniques have not been perfected to the same extent as the older FAB-MS and ESI-MS methods discussed above. All of the MS/MS methods provide DAG fragmentations from which the molecular species of the PtdIns can be identified (see below).

While PtdIns bears one negative charge at the dissociated phosphodiester group, additional phosphomonoester groups at the inositol ring lead to higher negative charges for PtdInsP ($z = -3$) and $PtdInsP_2$ ($z = -5$) at pH 7.4. The charge of $PtdInsP_3$ is still higher ($z = -7$) at pH 7.4. Furthermore, PtdInsP possess a higher molecular mass than all other known phospholipids containing two acyl chains. Muller *et al.* [164] have recently investigated the detectability of PtdInsP by MALDI-TOF-MS. Using the signal-to-noise (S/N) ratio to describe the quality of a given spectrum and the detectability of a certain PtdInsP, they defined the minimum amount of analyte necessary to obtain a reasonable S/N ratio as detection threshold. Using DHB as matrix, the detection limit for PtdIns is seven times higher than for PtdCho, and it further increases with increasing phosphorylation or in mixtures with other well-detectable phospholipids. For $PtdInsP_3$ in a mixture with PtdCho, the limit is about 20 times higher than for PtdIns. It is therefore advisable to separate PtdInsPs from biological lipid mixtures prior to the application of MALDI-TOF-MS. Muller *et al.* [164] acquired the spectra on a Voyager Biospectrometry DE workstation (PerSeptive Biosystems). All measurements were made under delayed extraction with an extraction voltage of 20 kV. The samples were prepared in a matrix of 0.5 M DHB in methanol. The lipid amounts of the plate were 113 pmol of PtdIns; 261.8 pmol of PtdInsP; 478 pmol of $PtdInsP_2$, 572 pmol of dipalmitoyl $GroPInsP_3$, and 444.3 pmol of stearoyl/arachidonoyl $GroPInsP_3$. Spectra were the average of 128 laser shots. According to Muller *et al.* [164], MALDI-TOF-MS spectra cannot be analyzed quantitatively, if only the measured signal intensity is taken into account, because the signal intensity is influenced by factors that cannot be effectively standardized. Therefore, Muller *et al.* [164] have introduced the use of the S/N ratio as a quantitative measure. This can be done semi-automatically, using the standard software packages available for commercial MALDI-TOF mass spectrometers.

The signal intensity in MALDI-TOF-MS is influenced by sample preparation, inhomogeneities of the laser intensity and ionization and desorption efficiency of different molecules. The signal intensity also depends on the substance of interest and the method of estimation of relative concentrations of the individual components in a mixture from the respective relative peak intensities [163]. The calculated S/N ratio increases continuously over a wide concentration range with the sample concentration, which also provides an exact criterion for the estimation of the detection limit. Muller *et al.* [164] have observed that the detection limits for PtdInsP are about 20 times higher than for PtdCho, while Petkovic *et al.* [163] have noted that the presence of other easily ionized substances in the mixture may alter the MS response to PtdInsP. It is therefore advisable to separate PtdInsP from biological lipid mixtures prior to the application of MALDI-TOF/MS.

17.3.2.3 Combined methods

Both indirect and direct (on-line) combinations of chromatography and mass spectrometry have been routinely explored. Nakamura *et al.* [58] reported FAB studies of the 9-anthryl derivatives of natural PtdInsP, isolated by preparative TLC. The mass spectra showed di- and tri-sodiated molecules of the anthryl derivatives. Others have used one or more TLC steps for the isolation of phospholipids for subsequent MS and MS/MS [10]. Direct combinations of TLC with MS have been pursued for some 20 years. Development of this method is driven by the ultimate goal of obtaining direct two-dimensional scanning of entire TLC plates, using mass-spectrometric detection without compromising spatial TLC resolution and MS sensitivity [165,166]. Somsen *et al.* [165] have reviewed the various approaches taken to combine planar chromatography or LC with MS techniques. The utility of TLC for separation, followed by MS with a variety of ionization techniques, was illustrated with reference to a wide range of compounds, including lipid-soluble drugs. Mehl *et al.* [166] have reported the investigation and optimization of a TLC/MALDI coupling procedure. TLC/MALDI takes advantage of the high sensitivity (femtomole to attomole range) of MALDI, and its ability to ionize fragile low- and high-molecular-weight compounds without significant fragmentation. The fundamental coupling parameters that influence sensitivity and lateral analyte spreading were: extraction solvent selection, extraction time, and pressure. For maintaining chromatographic resolution and maximum signal intensity, extraction solvents with R_F values between 0.4 and 0.6 were optimal. The TLC/MALDI coupling procedure has been described elsewhere [167].

GLC peak identification is greatly facilitated by on-line MS. Usually, the EI mode is utilized, although it may yield little or no molecular ion. The formation of pseudo-molecular ions and fragment ions must then provide the information necessary for peak identification and quantification. CI approaches provide high-abundance peaks for pseudomolecular ions, from which the mass of the parent molecule can readily be determined, as well as abundant fragment ions for structural characterization. The peak identification and quantification in GC/MS is greatly simplified by the inclusion of stable-isotope labelled internal standards. Both total- and fragment-ion response in GC/MS may differ significantly among the different molecular species, and appropriate calibration is necessary for quantitative work. Since the FID response provides a highly accurate indication of the carbon mass of the GLC peaks, it is usually employed for the quantification of all resolved peaks. The sensitivity and uniformity of the GC/MS response may be improved by preparation of special derivatives and by analysis in either positive- or negative-ion mode, as required. Couderc [168] has described a GC/MS/MS investigation of fatty acid mixtures, using the charge-remove fragmentation process of collisionally activated carboxylate anions, generated in the gas phase by electron capture of fatty acid pentafluorobenzyl (PFB) esters. Sherman *et al.* [132] earlier reported several specific GroPIns-related negative ions at m/z 259 (inositol phosphate), 241 (inositol-phosphate$-H_2O$), and 297 (diacylglycerophosphoinositol$-R_1COOH-R_2COOH$), which are unique for this class of phospholipid.

Haroldsen and Murphy [169] have developed a rapid and sensitive procedure for analyzing DAG dinitrobenzoates by NICI-MS. The DAG released from the GPL were

derivatized with 3,5-dinitrobenzoyl chloride. The dinitrobenzoates were resolved by reversed-phase HPLC, and the collected peaks were subjected to direct-probe MS, which gave mass spectra characterized by an intense molecular anion. From this molecular anion the total carbon number and degree of unsaturation of the fatty chains could be determined. Analysis of the fatty acid content of the molecular species allowed the unequivocal assignment of a structure of the DAG. Likewise, Kuypers *et al.* [170] have analyzed the benzoate derivatives of DAG, released from glycerophospholipids by PLC, using RP-HPLC in combination with thermospray ionization (TSI)-MS. Molecular species showed as base peaks the salt adducts of the molecular ion, and this permitted easy deduction of the overall fatty acid composition. In addition, the DAG fragment of each species was found at $[M\text{-}122]^-$, and two fragments formed by the loss of the fatty acyl groups (R) in the sn-1- and sn-2-position were found at $[M-R_1]^-$ and $[M-R_2]^-$, respectively. Cronholm *et al.* [69] determined the quantitative composition of the molecular species of the PdInsP of rat pancreas by FAB/MS, following isolation of the phospholipid subclasses by neomycin affinity chromatography. Ravandi *et al.* [171] used NP-HPLC in combination with ESI-MS for the identification of the glycerophospholipids and their ozonides and core aldehydes, with the $CHCl_3$/MeOH/NH_4OH system of Becart *et al.* [172].

Michelsen *et al.* [146] investigated the suitability of LC/MS for the quantification of the major PtdInsP and PtdInsP$_2$. Three consecutive chromatograms of a brain extract, obtained by using $CHCl_3$/MeOH/H_2O (10:20:8) at a flow-rate of 100 μL/min, produced signals in SIM at m/z 965.4 and 1045.3, corresponding to concentrations of PtdIns and PtdInsP$_2$ of 7.4 ± 0.2 and 7.2 ± 0.2 pmol/μL, respectively. The results indicate that PtdInsP, and biologically relevant changes in their concentrations, can be quantified directly in cells and cellular membranes by SIM with ESI-MS. Michelsen *et al.* [146] have found that the Folch extract used contains 10–20% of PtdInsP, of which *ca.* 1% constitutes PtdInsP and PtdInsP$_2$. The results show that PtdInsP and PtdInsP$_2$ are present in nearly equal amounts and that each of the analytes can be quantified also, even though they constitute only *ca.* 0.05% of the total phospholipids in a complex mixture. Gunnarsson *et al.* [60] have compared the relative response of PtdIns, PtdIns(4)P, and PtdIns(4,5)P$_2$ of a Folch Type I brain extract by ESI-MS and HPLC with ELSD. The area under each peak in the mass chromatograms (m/z 885, 965, and 1045), representing PtdIns, PtdIns(4)P, and PtdIns(4,5)P$_2$ reflected the relative abundance of the PtdInsP in the mixture, in agreement with an earlier report [146]. Approximately equal amounts of each phosphatide were present in the synthetic PtdInsP preparation, while the Folch extract contained very little PtdIns(4)P and PtdIns(4,5)P$_2$, relative to PtdIns. Use of SIM allowed the quantification of less than 200 pmol of PtdInsP [146]. Earlier, HPLC identification of glycerolipids was greatly facilitated [173,174] by on-line MS with TSI, but recently TSI has been replaced to a large extent by ESI-MS and APCI-MS procedures, either as independent analytical systems or in on-line combination with HPLC [175–177]. Unlike ESI, TSI, and APCI usually produce some degree of fragmentation that is useful for structural characterization.

In principle, MS/MS is MS of a specific ion, selected from the initial mass spectrum and subjected to CID. The resulting fragments (daughter-ions) are analyzed by a second mass spectrometer, which provides the MS/MS spectrum. This approach can be extended to a

similar, further examination of the daughter-ions by MS/MS/MS [10,11]. In pseudo-MS/MS LC/ESI-CID-MS is used in place of ESI-MS/MS [178,179]. The ionization in this instance is effected with a dual-stage ESI source. When analytes are present in the sprayed solution, molecular adducts, typically protonated parent-ions $[M + H]^+$, are formed and guided into the reaction region of the mass spectrometer between the exit of the glass capillary and the first skimmer. At low voltage (50–120 V) of the capillary exit, the molecular ionic species remain intact, and the molecular mass of the analyte is obtained. At higher capillary exit voltages, extensive and reproducible fragmentation of the molecular adduct-ion can be effected. The fragmentation arises by means of a CID process which is identical to that in conventional MS/MS systems. However, the product-ions or fragment-ions produced in the ESI/CID region are more effectively transferred into the quadrupole mass analyzer with much less off-axis scatter than the transfer of fragment-ions from an MS/MS collision process with the ESI lens system. Voyksner and Pack [179] have shown that CID daughter-ion yield to the detector for a single quadrupole system is nearly 90%, as compared to only a few percent for the MS/MS system. It has been demonstrated that HPLC is a better means of separation than the use of a mass filter, particularly for isomeric compounds, such as positional isomers, *cis*/*trans* isomers, *syn*/*anti* isomers, diastereomers, and enantiomers, where MS/MS spectra are not sufficiently different to distinguish their individual identities. Voyksner and Pack [179] have concluded that the claim that MS/MS has greater specificity than single MS does not apply when ESI-MS/MS is compared with LC/ESI-CID-MS. LC/ESI-CID-MS with a single quadrupole mass spectrometer has been successfully applied to the analysis of a variety of glycerolipids [178,180,181]. Byrdwell and Neff [152] have employed simultaneously APCI-MS/MS and ESI-MS/MS/MS for the analysis of TAG in canola oil, trioleoylglycerol oxidation products, and of TAG positional isomers, separated by RP-HPLC, which resulted in the isolation and identification trioleoylglycerol dimers.

17.3.2.4 Nuclear magnetic resonance and infrared spectroscopy

NMR can be used for both identification and quantification of phospholipid classes and molecular species. Malewicz and Baumann [182] have shown that ^{31}P-NMR can be used to distinguish and to quantify the alk-1-enylacyl, alkylacyl, and diacyl GroPEtn subclasses, in their native form without prior degradation or derivatization, provided the phospholipids are observed in the non-aggregated state. ^{31}P NMR spectra were recorded at 121.42 MHz on a Varian Unity 300 instrument, using a 5-mm variable-temperature probe. Spectra were recorded at 25 ± 1 °C on freshly prepared samples, dissolved in $CDCl_3$/CD_3OD/D_2O (50:50:15), using $CDCl_3$, CDOD, and D_2O that had been purged of dissolved oxygen by a stream of nitrogen to minimize line-broadening. Nouri-Sokhabi *et al.* [183, 184] reported quantitative ^{31}P-NMR analysis of the phospholipids of erythrocyte membranes with the use of a detergent. ^{31}P-NMR spectra of animal and human erythrocyte lysates, dissolved in sodium cholate, were acquired. The narrow resonances of phospholipids were mostly well resolved, allowing identification and accurate quantitative analysis of phospholipid classes of the erythrocyte membranes. Identification of the ether-linked ethanolamine phospholipid components of the erythrocyte membranes was

based on the removal of plasmalogens by acidolysis and of the diacyl GPL by degradation with phospholipase A_1. It was also shown that that the introduction of double bonds on the acyl chains of PtdCho shifted the ^{31}P-NMR resonance to lower frequencies. The recoveries of phospholipids from erythrocytes were significantly higher than those in conventional extraction procedures. ^{31}P-NMR spectra were recorded on a Bruker AMX-400 wide-bore spectrometer, operating at 161.98 MHz in the Fourier-transform mode, with the probe temperature controlled at 25 ± 1 °C. The concentrations of phospholipids were calculated from the spectra by comparing the peak areas (intensities) of phospholipid resonances with those obtained for known concentrations of authentic compounds. Standard samples were prepared by adding a known mass of lyso-PtdCho in D_2O to the samples that were treated with sodium cholate. The effective molecular weight of lyso-PtdCho was determined by comparison of the peak area of lyso-PtdCho with that of a known amount of inorganic phosphate (dried at 110 °C overnight).

Capuani *et al.* [185] have described an improved resolution of ^{31}P-NMR of phospholipids from brain. Proton decoupled ^{31}P-NMR spectra were measured on a Varian XL-300 spectrometer, operating at 121.4 MHz with an acquisition time of 1.5 sec, pulse length of 10 microsec, and pulse delay of 1.5 sec. The chemical shifts of the phosphorus signals were measured relative to phosphonitrilic chloride (trimer) in benzene as an external reference. The lipids were dispersed as micelles in aqueous sodium deoxycholate solution, and the spectral resolution was further optimized by adjusting pH and removing ions from the solution. Wissing and Behrbohm [186] employed the improved ^{31}P-NMR method to confirm the structure of DAG pyrophosphate, which showed two peaks with identical intensities at about −9 and −11.5 ppm, typical for a pyrophosphate group. Dioleoyl GroPOH showed a peak at about +1.4 ppm, typical for a phosphomonoester group, as established by other laboratories [185]. Seijo *et al.* [187] measured ^{31}P-NMR spectra of meningioma phospholipids. The NMR spectrometer used was a GE 500NB system (General Electric), operating at 202.4 MHz for ^{31}P. Chemical-shift data were reported relative to 85% inorganic orthophosphoric acid, but a primary reference standard was PtdCho (chemical shift, −0.84 ppm) or trimethylphosphate. Phospholipid signals in the ^{31}P-NMR spectra were sequentially identified by adding known quantities of individual phospholipid standards.

Differences in the chemical shifts of C-2, olefinic carbon, carbonyl carbon, and n-3 carbons of saturated TAG isomers in the ^{13}C-NMR spectra have been used to deduce the position of acyl groups in TAG. Henderson *et al.* [188] used ^{1}H–^{13}C-correlated NMR spectroscopy to assist quantitative one-dimensional ^{1}H and ^{13}C evaluation of positional isomers in an interesterified TAG mixture. Kalo *et al.* [189] studied the distribution of short-chain acyl groups between primary and secondary positions of TAG of milk fats by ^{1}H-NMR. ^{1}H-NMR spectra were recorded in $CDCl_3$ with a Varian Unity 500 (11.74T) spectrometer and referenced to internal tetramethylsilane. Proton-NMR, ^{2}H-NMR, ^{13}C-NMR, as well as ^{31}P-NMR have been utilized for the characterization of PtdIns and their derivatives with varying success. PtdIns, isolated from natural sources, are heterogenous in chain length and unsaturation, and synthetic long-chain PtdIns require solubilization with organic solvents or dispersion in water. This complicates interpretation of their spectra. While a ^{2}H-NMR study provides possible structures, there are not enough constraints to orient the inositol ring with respect to the glycerol backbone or acyl chains.

Hansbro *et al.* [190] used ^{2}H-NMR to determine the conformational behavior of PtdIns in model membranes. Computational modeling has identified six possible "tilted" orientations, all of which are consistent with the ^{2}H-NMR data.

In order to use the full power of high-resolution NMR for structural analyses, monomeric or micellar PtdIns are required. Lewis *et al.* [191] have synthesized the D- and L-Ins isomers of short-chain GroPIns and PtdIns analogs, including 2-methoxy derivatives, and have used ^{31}P-NMR to establish the structural requirements of PtdIns-specific PLC. The ^{31}P-NMR parameters were based on those previously used by Volwerk *et al.* [192]. Zhou *et al.* [193] have presented a detailed NMR analysis of the conformation of these short-chain PtdIns molecules in both monomeric and micellar states. Coupling-constant analysis and nuclear Overhauser-effect data addressed the differences between D- and L-Ins isomers as well as the similarity of 2-methoxy-PtdIns to diheptanoyl GroPIns. QUANTA computational modeling provided a likely orientation of the Ins ring with respect to the interface. ^{13}C-relaxation times characterized the internal motion of the PtdIns molecule and highlighted differences between monomers and aggregated PtdIns. Leondaritis and Galanopoulou [194] used ^{1}H-NMR analysis to identify PtdIns in *Tetrahymena* PtdIns. PtdIns exhibits characteristic features in both ^{31}P-NMR and in ^{1}H-NMR, and the NMR methods can be used for quantification of PtdIns. However, the NMR methods are most accurate with relatively large amounts of pure sample. Nevertheless, both methods have been successfully employed for this purpose in the past [190,192,194].

17.4 ISOLATION OF INDIVIDUAL LIPID CLASSES

The various chromatographic methods for resolution and isolation of specific neutral lipid classes were extensively discussed [1] in the 5th Edition of this book. Subsequent work has confirmed the utility of these systems as well as reported improvements and modifications. Although modern mass-spectrometric analyses can provide more or less complete profiles of lipid classes and molecular species of both neutral and polar lipids in total lipid extracts, isolation of individual lipid classes is necessary for preparing pure standards, determining radioactivity, and resolving isobaric isomers of molecular species not resolved by MS.

17.4.1 Free fatty acids and neutral lipids

In the course of experiments on the oxygenation of polyunsaturated fatty acids, *bis*-allylic hydroxy or hydroperoxy products have been found as enzymatic products. Recently, *bis*-allylic products were detected as hydroxy derivatives in cytochrome P450 reactions and in the myoglobin-catalyzed mono-oxygenation of linoleic acid. The *bis*-allylic 11-hydroperoxylinoleate derivative was found to be stable under chromatographic and other analytical conditions, provided the sample was not subjected to low pH [195–197]. Brash [198] has identified the *bis*-allylic 11-hydroperoxide in vitamin E-controlled autoxidation of methyl linoleate. The 11-hydroperoxy derivative was the next-most prominent primary peroxidation product after the 9- and 13-hydroperoxides.

It was present in approximately 5–10% of the abundance of the 9- or 13-hydroperoxide. The structures of 11-hydroperoxylinoleate and its 11-hydroxy derivative were established by HPLC, UV spectroscopy, GC/MS, and ^{1}H-NMR. HPLC of autoxidized methyl linoleate was performed on a 5-μm silicic acid column with hexane/2-propanol (100:1 or 100:0.5) as solvent. This resolved the 13-hydroperoxy-18:2 and 9-hydroperoxy-18:2, with the 11-hydroperoxy derivative well isolated between the two peaks. RP-HPLC and purification of the 11-hydroperoxy-18:2 was carried out on a Beckman Ultrasphere 5-μm ODS column (25 × 0.46 cm) with an Upchurch guard column and a solvent system of MeOH/H_2O (85:15). For final purification SPE/HPLC on a Beckman analytical silica column was used with hexane/2-propanol 1900:1 as solvent. GC/MS spectra were obtained on the hydrogenated 11-hydroxylinoleate methyl ester TMS ether derivative by chromatography on a SPB-1 fused-silica capillary column of 5 or 15 m × 0.25-mm ID, using temperature programming (150 to 300 °C at 10 or 20 °C/min). Mass spectra were recorded in the EI mode (Hewlett-Packard Model HP5980 at 70 eV).

Nourooz-Zadeh [199] has described an improved procedure for the isolation of isoprostanes from plasma or isolated lipoprotein fractions, based on earlier work [200, 201]. The improvement involves the following changes:

(a) Recovery of PGF_2-like compounds during the C_{18} chromatography step;
(b) The Si cartridge and the TLC steps are replaced with an aminopropyl (NH_2) cartridge. The NH_2 sorbent functions as an ion exchanger, selectively binding organic compounds, containing a carboxylate function. The overall recovery of PGF_2-like compounds, following chromatography on C_{18}- and NH_2-cartridges, was 75%;
(c) Improved GLC separation of PGF_2-like compounds.

For the SPE, the solvent extracts were loaded onto C_{18} cartridges (Waters, Millipore), conditioned with methanol and water (pH 2). The cartridges were washed with water, followed by MeCN/H_2O (15:85) to remove nonlipid materials. The lipids were eluted by washing the cartridge with 5 mL of hexane/ethyl acetate/2-propanol (85:10:5). The final eluates from the C_{18} cartridges were applied to NH_2-cartridges, pre-conditioned with hexane. The NH_2-cartridges were sequentially washed with 10 mL of hexane/ethyl acetate (15:85) and MeCN/H_2O (90/10), and acetonitrile. Isoprostanes were eluted by washing the NH_2-cartridges with 5 mL of ethyl acetate/methanol/acetic acid (85:10:5). Hirano and Takahashi [202] reported an improved resolution of low-melting-point TAG molecular species on a chilled column by isocratic liquid chromatography. The method was applied to the isolation of TAG, rich in eicosapentaenoic (EPA) and docosahexaenoic (DHA), prepared by enzymatic synthesis. Various column temperatures, from −20 °C to 20 °C, were used. A RP-Wakosil 5C18N HPLC column (300 × 4 mm, 5 μm, Waco Pure Chemicals) and Supersphere RP-18 column (250 × 4 mm, 4 μm, Merck) were used in tandem. The solvent was acetone/acetonitrile (3:2, 3:1, or 7:1). The TAG peaks were detected by a mass detector (Applied Chromatographic Systems). It was found that a critical column temperature provided optimum column selectivity.

Yoshida *et al.* [203] have reported the isolation of tocopherols by NP-HPLC on a column (Shim-Pack CLC-SIL(M), 5 μm, 250 × 4.6 mm ID, Shimadzu), protected by a 20-mm guard column. A mixture of hexane/1,4-dioxane/ethanol (490:10:1) was used as mobile phase at a flow-rate of 2.0 mL/min. The tocopherols were monitored by a

fluorescence detector (Shimadzu RF-535). Liu *et al.* [204] have reported the application of NP-HPLC with ELSD to the determination of MAG and DAG in vegetable oils and emulsifiers. The 1,3-DAG were separated from 1,2-DAG positional isomers, although some 1,3-DAG of low molecular weight interfered with the 1,2-DAG of high molecular weight. A mobile-phase gradient was generated, using hexane (Channel A) and hexane/2-propanol/ethyl acetate/10% formic acid (80:10:10:1) (Channel B). The samples were dissolved in a hexane/2-propanol mixture and were analyzed without derivatization. The results obtained by this method for the MAG agreed well with those derived from GLC and SFC. Dreyfus *et al.* [205] have reported an effective separation of neutral lipids (TAG, 1,3-DAG, 1,2-DAG, Chol, FFA, and MAG) by HPTLC, using a CAMAG ATS3 automatic TLC sampler. The neutral lipids were separated with chloroform/acetone (96:4) as migration solvents during a 25-min run. The HPTLC plate was then submerged for 20 sec in a copper sulfate/orthosphosphoric acid mixture and placed in an over at 130 °C for 10 min order to visualize the neutral lipid classes. The relative amounts of the lipids were determined by densitometric scanning.

Dreyfus *et al.* [205] also reported excellent TLC resolution of the non-sialylated sphingolipids and of gangliosides. Many nonsialylated sphingolipids migrated as several bands, due to the presence of hydroxylated and nonhydroxylated species, *e.g.*, cerebrosides [monohexyl ceramide (CMH), dihexyl ceramide (CDH), and trihexyl ceramide (CTH)], globuside (GLOB), and sphingosine (SPH)]. The use of a single solvent mixture led to overlapping fractions. As a result, it was necessary to use several different solvent combinations to discriminate among the eight different subclasses of these lipids. Separation of the non-sialylated lipids [ceramide (CER), CMH, CDH, CTH, SPH, GLOB, sphingomyelin (SM)], spotted with the Camag automatic TLC sampler III (ATS3), was achieved by HPTLC with $CHCl_3$/MeOH/aq. 0.4% $CaCl_2$ (69:31:3) as developing solvents in a 40-min run. The different non-sialylated sphingolipids (NSL) were visualized, using a 0.2% orcinol/24 *N* H_2SO_4 spray, and their relative abundance was estimated at 577 nm. The separation of GLOB and SM, which overlapped in this system, was accomplished by HPTLC with $CHCl_3$/MeOH/acetone/acetic acid/water (52:8:8:18:4) in a 60-min run. Further definition of some of the lipids was obtained by two-dimensional HPTLC. Dreyfus *et al.* [205] also reported excellent separations of gangliosides (GM3, GM2, GM1, GD3, GD1a, GD1b, GT1b, and GQ1) by HPTLC. The major brain monosialoganglioside, GM1, has the following abbreviated structure: galactosyl-(1 → 3)-*N*-acetylgalactosaminyl-(1 → 4)-[(2 → 3)-*N*-acetylneuraminyl]-galactosyl-(1 → 4)-glucosyl-(1 → 1)-[2-*N*-acyl]-sphingosine. In the other major brain ganglioside, modifications of the same oligosaccharide chain occurs – the chain is incomplete in some (GM2, GM3) and contains more sialic acid residues in others (GD1a, GD1b, GT) – as well as other modifications (GQ). Aliquots of *ca.* 3.5–6.5 μg ganglioside-*N*-acetyl neuraminic acid (NeuAc) were spotted with the ATS3 on plates previously washed in $CHCl_3$/MeOH (1:1). Three successive developments were performed, using three different mobile phases: $CHCl_3$/MeOH (2:1) for 70 min; $CHCl_3$/MeOH/H_2O (65:25:4) for 40 min.; and $CHCl_3$/MeOH/0.25% aq. $CaCl_2$ (50:37:10) for 40 min, the plates being thoroughly dried for 10 min between developments. The gangliosides were then visualized with a resorcinol-HCl reagent, and the chromatographic patterns were scanned densitometrically at 577 nm.

Bergqvist and Herslof [206] reported the isolation of an enriched (*ca.* 60% digalactosyl-DAG) fraction of galactoglycerolipids from commercial oat flakes, which Hauksson *et al.* [207] purified further by SPE and preparative HPLC on a polar DIOL column. This procedure gave a fraction containing >99% digalactosyl-DAG. The structure of the lipid was determined by high-resolution ^{1}H- and ^{13}C-NMR spectroscopy. The lipid was found to be 1,2-diacyl-3-*O*-[α-galactosylpyranosyl-1(1-6)-β-galactopyranosyl]-glycerol [204]. Bergqvist and Kaufmann [208] reported the molecular species composition of this galacto-DAG. Hamberg *et al.* [209] isolated and determined the structure of a new galactolipid from oats, which contained a new hydroxy fatty acid (avenoleate). The new compound was isolated by TLC and HPLC and identified by chemical degradation and MS, and its structure was confirmed by NMR. TLC was carried out on pre-coated plates of Kieselgel 60 (Merck), using $CHCl_3$/MeOH/H_2O/HOAc (50:10:1:1). RP-HPLC was performed on a Nucleosil C_{18} column, using methanol as the mobile phase. Degradation of the new galactolipid by chemical and enzymatic methods demonstrated the location of the acyl chains, *i.e.*, linoleate at sn-1 and linoleoyl-avenoleate at sn-2. Luquain *et al.* [210] reported the HPLC determination of (*bis*-MAG) phosphate (BMP) and other lysophospholipids in cell cultures. Total phospholipids from cells, labelled or not with trace amounts of [^{3}H]DHA, were extracted, and phospholipid classes were separated by TLC. After extraction of the gel corresponding to the BMP area, a known amount of internal standard was added. The free hydroxyl groups of the phospholipid were totally derivatized with 6-methoxy-α-methyl-2-naphthaleneacetic acid (naproxen). Derivatized phospholipids were separated by NP-HPLC and quantified by UV absorption at 231 nm. Maier *et al.* [211] reported the isolation and structure of glucosylceramides from the starfish. From the water-insoluble lipid fraction of the DCM/MeOH extract of the starfish, two new glucosylceramides, together with a known glucosylceramide, ophidiacerebroside E, were isolated by chromatographic procedures and characterized by spectroscopic (^{1}H- and ^{13}C-NMR, MS) methods. NP-TLC was performed on silica gel F254 (Merck) with DCM/MeOH/EtOAc/H_2O (8.5:1.5:2:0.1) while RP-TLC was performed on preformed C_{18} silica gel 60 F254 with MeOH and preparative HPLC on a Phenomenex Ultracarb ODS 20 column, eluted with absolute MeOH.

Sjövall *et al.* [212] used RP-HPLC to determine the elution factors of synthetic oxo-TAG as an in identification of peroxidized natural TAG. The oxo-TAG elution factors were determined in relation to a homologous series of saturated TAG, ranging from 24 to 54 acyl carbons on a Supelcosil LC-18 column, using a gradient of 20–80% 2-propanol in MeOH, with ELSD or on-line ESI-MS as detectors. It was shown that the elution times varied with the nature of the functional group and its regio-location in the TAG molecule. A total of 31 incremental elution factors were calculated from the chromatography of 33 oxygenated and nonoxygenated TAG species, ranging in carbon numbers from 36 to 54 and double-bond numbers from 0 to 6. The oxygen functions included hydroperoxy, hydroxy, epoxy, keto, and aldehyde groups. The aldehyde and ketone functions were derivatized as the dinitrophenylhydrazones (DNPH). Sjövall *et al.* [213–215] reported the isolation and purification of *tert*-butyl hydroperoxide (*tert*-BOOH) oxidation products of unsaturated TAG. Silica gel H plates (Merck) were prepared in the laboratory, and heptane/isopropyl ether/acetic acid (60:40:4) was used as the mobile phase [215] to resolve the hydroperoxides, the DNPH derivatives of the core aldehydes (prepared by

treating the oxidation mixture with 2,4-dinitrophenylhydrazine), along with the *tert*-BOOH adducts. The DNPH derivatives of the core aldehydes were seen as yellow bands on the chromatoplates [192–194]. The compounds were recovered from the silica gel scrapings by extraction with $CHCl_3$/MeOH (2:1) and, after the extracts had been dried with anhydrous sodium sulfate, they were subjected to HPLC/LSD and LC/ESI-MS analysis [213]. A total of 113 core aldehydes and their derivatives were generated from corn and sunflower oil TAG by rapid oxidation with *tert*-BOOH and ferrous ions [216].

Spitzer and Aichholz [217] reported the isolation of naturally occurring TAG, containing acetic acid. Extracts of the leaves of *Maytenus ilicifolia*, which have been utilized in folk medicine, were found to contain major amounts of monoacetyl-DAG. The acetyl-DAG were identified by GLC and CI-MS. Limb *et al.* [218] isolated monoacetyl-DAG from bovine udder by conventional solvent extraction and repeated silica gel column chromatography and HPTLC. The progress of the isolation and purification of the acetyl-DAG fraction was followed by NMR analysis. Monoacetyl-DAG were identified by a variety of 1-D and 2-D NMR techniques and CID-MS/MS coupled with FAB (see below).

17.4.2 Glycerophospholipids

Wang and Gustafson [219] reported a modified procedure for one-dimensional TLC separation of PtdSer, PtdEtn, PtdIns, (PtdCho and SPH) and lysoPtdSer, lysoPtdEtn, and lyso PtdCho from tissue lipid extracts. This was achieved by simple inclusion of 0.4% ammonium sulfate in silica gel H and of acetone in the developing solvent, *e.g.*, $CHCl_3$/MeOH/HOAc/acetone/H_2O (40:25:7:4:2). The weakness of this method is the partial degradation of PtdEtn to lysoPtdEtn, but this can be avoided by applying 20–59 μL of glacial acetic acid to the sulfate-containing plate at the site of lipid application before sampling. Silica gel plates, immersed in 100 m*M* ammonium sulfate and dried 1.5 h at 120 °C were also used by Lin *et al.* [220] for the separation of phospholipid classes. Samples were applied to the plates in a spotting chamber. Separation was performed with the solvent system 1-propanol/chloroform/propanoic acid/0.1% aq. KCl (3:2:2:1). Glycolipids were separated on a silica gel plate in the solvent system $CHCl_3$/MeOH/NH_4OH (13:5:1). Bands containing lipids to be analyzed were routinely localized by iodine staining. Mallinger *et al.* [221] performed HPTLC separations of complex mixtures of GPL, using two-dimensional HPTLC on silica gel plates (200-μm thickness, 5-μm particle size) and an inert organic binder. Prior to being developed, the plates were activated by heating to 110 °C for 15 min. Samples of lipid extract, containing 1.8–2.0 μg P, were applied to the HPTLC plate with a 10-μL Hamilton syringe. The plates were developed at room temperature in tanks that were lined with filter paper. The lipid was applied in successive, superimposed drops, at a location 1.7 cm from the lower edge. The first development was initially performed with $CHCl_3$/MeOH/4.3 *M* NH_4OH (90:65:20) to resolve the inositol phosphatides. After the plate had been dried, it was developed in a second dimension, using $CHCl_3$/MeOH/HOAc/H_2O (100:30:35:1). The method is suitable for quantification of even small amounts of specific classes, when scanned by densitometry.

Orgambide *et al.* [222] analyzed the phospholipid composition of *Rhizobium leguminosarum* by two-dimensional TLC, FAB-MS and GLC/FID. The phospholipids

included PtdEtn, *N*-methyl PtdEtn, *N*, *N*-dimethyl PtdEtn, PtdGro, Ptd_2Gro, and PtdCho. Individual phospholipid classes were resolved by two-dimensional TLC on silica gel plates with $CHCl_3$/MeOH/NH_4OH (70:35:5) as solvent for the first dimension and $CHCl_3$/acetone/MeOH/HOAc/H_2O (10:4:2:2:1) for the second dimension, and characterized by FAB-MS. The phospholipid classes were quantified by densitometry, using a high-resolution charge-coupled device camera and Ambis Optical Imaging System, following staining of the TLC plates with phosphomolybdic acid or sulfuric acid/methanol (25:75), followed by heating at 150 °C. The fatty acid composition of the phospholipids classes was determined by GLC/FID of the methyl esters. Wilson and Sargent [80] used two-dimensional TLC to resolve lipid classes from human brain. Total phospholipid, sulfatide, cerebroside, and neutral lipid fractions were resolved by HPTLC with by one-dimensional double development and quantified by charring or after phosphate staining. The plates were developed for 6 cm with methyl acetate/2-propanol/$CHCl_3$/MeOH/0.25% aq. KCl (25:25:5:10:9) [223]. After they had been dried, the plates were fully developed in hexane/diethyl ether/acetic acid (80:20:2) to separate neutral lipids and cholesterol, and dried again. The plates were sprayed with 3% copper acetate in 85% phosphoric acid until saturated, and stained by charring at 160 °C for 20 min. Ethanolamine plasmalogens were measured by first treating isolated ethanolamine phospholipids with HCl vapor to convert plasmalogens into lysophosphatides, then the products were separated by HPTLC, using methyl acetate/2-propanol/$CHCl_3$/MeOH/0.25% aq. KCl (25:25:25:10:9), and the separated lysoPtdEtn were quantified by phosphate staining. The major phospholipid classes were further separated by two-dimensional HPTLC, using methyl acetate/2-propanol/$CHCl_3$/MeOH/0.25% aq. KCl (25:25:25:10:9) in the first dimension, then $CHCl_3$/acetone/MeOH/HOAc/H_2O (10:4:2:2:1) in the second dimension [224].

Gorges *et al.* [225] synthesized a non-hydrolyzable fluorescent diether analog of PtdCho (1-*O*-hexadecyl-2-*O*-pyrenedecanyl-sn-glycero-3-phosphocholine, and analyzed it by TLC, using $CHCl_3$/MeOH/25% NH_4OH (65:35:5) as developing solvent. Fluorescent spots were scraped off the wet TLC plate, and the lipids were eluted with chloroform/methanol (2:1). This derivative has been used subsequently in studies of PtdCho transfer from low- and high-density lipoproteins to human platelets [226]. Dreyfus *et al.* [205] have reported an excellent separation of Ptd_2Gro, PtdOH, PtdEtn, PtdSer, PtdIns, PtdCho, and SM by TLC on LK5 plates, previously sprayed with H_3BO_3 (2.3% in ethanol), dried, and activated at 100 °C for 15 min. Aliquots of *ca.* 15 μg lipid P were spotted with the ATS3, using a concentration zone. The phospholipids were separated with $CHCl_3$/MeOH/EtOH/H_2O/triethylamine (30:35:7:35) as developing solvent for 3 h and visualized by a primuline spray. Small *et al.* [227] used the method of Rouser *et al.* [228] for TLC separation of HL-60 cell phospholipids and the antineoplastic ether lipid 1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphocholine (ET-18-OCH_3). The TLC plates were developed with $CHCl_3$/MeOH/HOAc/H_2O (50:25:8:2) as the solvent system. Wu *et al.* [26] used HPTLC for qualitative analysis and NP-HPLC for quantitative analysis of GPL and SM to demonstrate changes in phospholipid extractability and composition accompanying mineralization of chicken growth-plate cartilage-matrix vesicles, as previously described by Wu *et al.* [229]. The HPTLC plates were developed to 5 cm from the origin with ethyl acetate/1-propanol/$CHCl_3$/MeOH/0.25% aq. KCl (25:25:20:15:9), dried at room temperature for about 15 min, and then re-developed full-length (about 9.5 cm) with

n-hexane/diethyl ether/acetic acid (75:21:4). After the plates had dried, the bands were treated with cupric/phosphoric acid charring reagent (10% $CuSO_4$ in 8% H_3PO_4).

Numerous HPLC methods have been described for the separation of phospholipid classes. Silica gel has been most frequently used as the stationary phase [230]. Different solvent mixtures have been utilized, including hexane/2-propanol/water, acetonitrile/methanol/water, or $CHCl_3$/MeOH/NH_4OH. The problem of detecting phospholipids has been resolved to a large extent by the use of ELSD and on-line MS. Caboni *et al.* [231] have used the HPLC system of Becart *et al.* [172], consisting of a binary gradient of $CHCl_3$/MeOH/30% NH_4OH (80:19.5:0.5) and $CHCl_3$/MeOH/H_2O/30% NH_4OH (60:34:5.5:0.5) for the separation and quantification of phospholipids in biological systems. A Sedex Model 45 ELSD was used for peak detection. The method of Becart *et al.* [172] has been extensively utilized for the resolution of GPL classes and their oxidation products in combination with on-line ESI-MS [171,232–236]. Wiley *et al.* [237] obtained an excellent resolution of neutral lipids, PtdEtn, PtdIns, PtdSer, Ptd_2Gro, PtdCho, SM, and lyso-PtdCho by NP-HPLC on a column (10 × 250 mm), packed with LiChrospher Si 100 (EM Science), using a modification of the method of Hax and Van Kessel [238]. The solvent mixtures used for eluting the lipids contained 2-propanol/hexane/ethanol/1 m*M* ammonium phosphate/acetic acid, combined in specific proportions to provide Solvents A and B for HPLC with "small" and "large" columns. Bonanno *et al.* [239] have reported that the basic NP-HPLC separation of GPL can be improved by introducing a limited contribution of solvophobic retention. For this effect, an additional alkyl silica (C_{18}) column of variable length was coupled in series with another silica column. With increased proportion of reversed phase in this system, the retention of PtdGro increased. PtdIns and PtdSer were separated into molecular species. The selective retention defined in this study permits an evaluation of the solvophobic retention of phospholipids in the coupled system.

Bayon *et al.* [240] resolved and quantified the common phospholipid classes, using a method described by Juaneda *et al.* [241]. The separation was achieved by NP-HPLC on a 5-μm Lichrosorb Si60 silica column (250 × 7.4 mm, Merck) with a ternary elution gradient made up of Solvent A (hexane), Solvent B (2-propanol/chloroform, 4:1), and Solvent C (2-propanol/water, 1:1). Arnoldsson and Kaufmann [242] reported a general method of analysis of lipid classes by HPLC, using a multivariate optimization strategy. Detection and quantification were optimized by a factorial design in the ELSD parameters to ensure maximum detector response. Bernhard *et al.* [243] described an isocratic HPLC system for the separation of PtdCho, SM, lysoPtdCho, PtdGro, PtdEtn, PtdIns, and PydSer. Phospholipids were detected by UV at 205 nm and subsequent fluorescence monitoring. Fluorescence of the phospholipids (ex: 340 nm; em: 460 nm) was achieved by post-column formation of mixed micelles with 1,6-diphenyl-1,3,5-hexatriene. Quantification of PtdCho and PtdEtn by HPLC with *N*-monomethyllysoPtdEtn (dioleoyl) as an internal standard gave the same results as phosphorus quantification after TLC.

Ingvardsen *et al.* [244] have published a method for the resolution of phospholipid classes, based on micellar electrokinetic chromatography (MEKC). Sodium cholate was found suitable as the micellar phase, and 1-propanol was important in obtaining an efficient separation into individual phospholipids, including PtdCho, PtdEtn, PtdIns, and PtdSer. The separation conditions included the use of a fused-silica capillary and detection

performed at 530 mm from the place of on-column measurements of ultraviolet absorption at 200 nm. The total analysis time was 25 min, and only small amounts of reagents and sample were required. Szucs *et al.* [245] have adopted this system for the analysis of phospholipids in lecithins from plant sources. The resolution of phospholipid classes in plant lecithins by normal-phase HPLC was compared with MEKC. MEKC conditions were optimized to provide a robust analytical method. It offers the advantage of a much higher peak capacity, and this results in improved resolution, especially for PtdSer. However, the repeatability of the MEKC results was somewhat lower than for HPLC.

Schaefer *et al.* [246] separated red cell membrane phospholipids by NP-HPLC on a Lichrosorb Si-1000 column (25 cm × 4 mm ID, 5 μm) with an isocratic system of hexane/2-propanol/ethanol/25 m*M* potassium phosphate/acetic acid (376:585:100:56.2:0.3), as originally described by Patton *et al.* [247], except that after mixing and filtering (to remove precipitated potassium phosphate), 75 mL water was added to the mobile phase. This addition resulted in elution of a PtdEtn peak free of neutral lipids and other phospholipids, within 4.0–7.5 min. Vaghela and Kilara [248] have reported the quantitative analysis of phospholipids from whey protein concentrates by HPLC with a narrow-bore column and ELSD. Cerebrosides, PtdIns, PtdEtn, PtdSer, PtdCho, SM, and lysoPtdCho were base-line resolved. A gradient elution with a pH modifier was necessary for good resolution of acidic phospholipids. A binary solvent system, consisting of (A) ($CHCl_3$/MeOH, 80:20) and (B) ($CHCl_3$/MeOH/H_2O/20% NH_4OH, 60:34:6:0.25), was used. Analysis time was 36 min. Arduini *et al.* [249] reported a NP-HPLC resolution of phospholipid classes and long-chain acylcarnitine on a Nucleosil 100-7 column (250 mm × 4 mm ID) with isocratic elution by acetonitrile/hexane/methanol/conc. phosphoric acid (918:30:30:17.5). A total lipid extract of erythrocytes, obtained according to Rose and Oaklander [250], was used, and the peaks were monitoring at 206 nm with a variable-wavelength UV detector.

Otten *et al.* [251] employed the method of Seewald and Eichinger [252] for the resolution of the major phospholipid classes, PtdCho, PtdEtn, PtdSer, PtdIns, Ptd_2Gro, SM, lysoPtdCho, and lysoPtdEtn, and the neutral lipids and the collection of fractions for subsequent GLC analysis. The solvent system for elution required the simultaneous use of pH and polarity gradients. It consisted of the following progression of solvent changes: (1) elution with acetonitrile for 5 min; (2) elution with acetonitrile, containing 0.2% phosphoric acid, which was changed to methanol containing 0.2% phosphoric acid between 15–35 min. The lysoPtdCh and lyso-PtdEtn fractions also represented the plasmalogen content of the respective phospholipid, as confirmed by treatment with HCl fumes. The individual fatty acids in the lipid fractions were transesterified to fatty acid methyl esters. Abidi [230] has presented an extensive review of the purification of PtdSer from complex matrices, including its separation from other GPL classes by TLC and NP- and RP-HPLC, along with selected chemical derivatization procedures, useful for enhancing the separation efficiency and detection sensitivity and specificity. The review includes tabulation of various solvent systems for NP-HPLC of PtdSer from other GPL. The observed elution sequence is dramatically affected by variation in the mobile phases. NP-HPLC with $CHCl_3$/MeOH/NH_4OH produced two different elution patterns for PtdEtn, PtdIns, and PtdSer, depending on the exact solvent gradient. In contrast, HPLC with MeCN/MeOH/H_2O/HOAc gave the same order of elution for PtdIns > PtdSer > PtdEtn > PtdCho > SP, regardless of the gradient or isocratic conditions employed.

Costello *et al.* [25] used HPTLC and NP-HPLC for the resolution of the acidic glycerophospholipids of *Trichomonas* by using a column of Iatrobeads 6RS-8010 (Iatron). For PtdGro and PtdIns, the column was equilibrated and eluted isocratically with $CHCl_3$/MeOH/H_2O (180:19:1). The flow-rate was 1 mL/min, and fractions were collected at 2-min intervals for 30 min. For acyl PtdGro, the column was equilibrated and eluted isocratically with $CHCl_3$/MeOH/H_2O (485:14:1) at a flow-rate of 1.5 mL/min. For accurate quantitative results, the lipids were analyzed by HPLC on a Lichrosorb SI-100 column with ELSD monitoring (Alltech Varex MKIII ELS detector system). Two mobile phases (A) MeOH/H_2O (80/20, v/v) and (B) $CHCl_3$/MEOH/1% formic acid (80:20:0.1) were used to generate a complex gradient. The total running time was 33 min.

Lin *et al.* [220] separated lipid classes according to Singleton and Stikeleather [253] on a HPLC silica column (25 × 0.46 cm, 5 μm, Spherosorb S5W, Phase Separations) with a linear gradient from 2-propanol/hexane (4:3) to 2-propanol/hexane/water (4:3:0.75) in 20 min, then isocratic elution for 15 min. A Pre-Sat silica saturation column (25 × 0.46 cm; Alltech Associates) was installed between the pump and the injector to saturate the mobile phase with silica before it reached the analytical column. Free PtdGro, Ptd2Gro, PtdIns, and PtdOH standards were obtained by acidification of their salts with HCl. Karlsson *et al.* [254] have used normal-phase LC/ESI-MS and LC/APCI-MS for class separation and identification of GPL, including PtdIns. For APCI, the response was lower than for other GPL classes. With HPLC class separation, the tedious task of correctly identifying a phospholipid class is avoided. If the unknown sample consisted of only a few phospholipid classes, MS/MS could be employed directly without HPLC separation, bearing in mind, however, that suppression effects in the ion source can occur and make quantification difficult. Deng and Solomon [255] have reported the total synthesis of the (11E)-9-hydroxy-13-oxotridec-11-enoate ester of 2-lysoPtdCho (HOT-PC) and its characterization by chromatographic, mass-spectrometric, and NMR methods. HOT-PC was purified by flash chromatography on a silica gel column ($CHCl_3$/MeOH/H_2O, 15:9:1) as a diastereomeric mixture of epimers at the allylic hydroxyl. It gave an R_F value of 0.21 in TLC with $CHCl_3$/MeOH/H_2O (15:9:1). High-resolution MS (FAB, CsI/NaI/glycerol) gave m/z 852.3802, compared to a calculated value of 852.3791 for $C_{37}H_{70}NO_{11}PCs$ (MCs^+).

17.4.3 Phosphatidylinositol phosphates

Palmer [66] separated PtdIns, PtdInsP, and PtdInsP_2 by one-dimensional TLC, using pre-coated silica gel HR plates (Analtech) which were dipped in 1% potassium oxalate, dried, and reactivated by heating at 120 °C for 40 min. These plates were developed with $CHCl_3$/acetone/MeOH/HOAc/H_2O (40:15:13:12:8) and yielded excellent separations of the PtdInsP. The phospholipids were located with the molybdate spray reagent or by charring with 3% cupric acetate in 8% phosphoric acid. Jolles *et al.* [76] have reported a one-dimensional technique to yield chemically pure PtdIns(4,5)P_2 in a single step. Although this separation also produced radiochemically pure PtdIns(4)P, the phosphate-containing compound(s) ran sufficiently close together to make an assay of total PtdIns(4)P impossible [256]. Consequently, the samples were chromatographed twice to obtain uncontaminated PtdIns(4)P. Firstly, they were resolved in a two-dimensional

system, leaving the PdInsP at the origin. Secondly, the origin was scraped from the plate and the PtdInsP were eluted with $CHCl_3$/MeOH/HCl (20:40:1), containing 5% water, applied to a new plate, and PtdIns(4)P was separated by the one-dimensional separation method described above. Recoveries were of the order of 85%. Some ^{32}P-labelled PtdIns(4,5)P_2 spots were scraped off the plates, the silica gel was placed in the deacylating mixture of Clarke and Dawson, and the resulting GroPIns(4,5)P_2 was purified by their procedure [257].

A simple, rapid two-dimensional TLC system, which resolves the four PtdIns cyclic phospholipids as well as all commonly encountered major and minor phospholipids, has been described by Mitchell *et al.* [258]. Ca^{2+}-free lipid samples were loaded onto silica gel H plates and developed, first, in $CHCl_3$/MeOH/H_2O/conc. NH_4OH (48:40:7:5) and, then, at an angle of 90° in $CHCl_3$/MeOH/HCOOH (55:25:5). Development with a basic system in the first dimension and an acidic system in the second dimension resulted in an effective resolution of all major and minor phospholipid classes. After appropriate staining and outlining the spots, the zones could be scraped off and the resolved compounds recovered for further analysis. PtdInsP and PtdInsP$_2$ migrated as two distinct spots, well separated from the origin. The method was successfully applied to various cells. Scored 20 × 10-cm, 250-μm silica gel H plates (Analtech) were broken into 10 × 10-cm plates and used without activation or pre-washing. The PtdInsP [PtdIns(3)P and PtdIns(4)P], the PtdInsP$_2$ [PtdIns(3,4)P_2 and PtdIns(4,5)P_2], and PtdInsP$_3$ can cleanly be separated by TLC, as noted above, but the resolution of the D-3/D-4 isomers of PtdInsP and PtdInsP$_2$ is critical [259, 260]. A few procedures for accomplishing this have been published. Some of them provide separation of PtdIns(3)P from PtdIns(4)P [261,262], and others of PtdIns(3,4)P_2 from PtdIns(4,5)P_2 [262,263].

Sun and Lin [79] have provided a detailed description of the separation by HPTLC of phospholipids in brain lipid extract, equivalent to 1–5 mg protein, as an example. The silica Gel 60 HPTLC plates (10 cm × 10 cm, Merck) were impregnated by dipping them in a solution consisting of 1% (w/v) potassium oxalate 2 m*M* Na_2EDTA, mixed with methanol in the ratio of oxalate/methanol 2:3, then dried overnight before use. PdInsP standards (100 μg) were added to the sample as carriers for visualization purposes. The lipid sample was applied to the lower left corner of the HPTLC plate and the plate was dried by a stream of dry nitrogen to remove any residual moisture. The plate was then developed with $CHCl_3$/MeOH/acetone/16 M NH_4OH (70:45:10:10) to a height of 9 cm. After development, the plate was dried under a stream of nitrogen for 5 min. The first system displaced all phospholipids from the origin, except PtdInsP. The HPTLC plates were subsequently developed in the same direction with a second solvent system, consisting of $CHCl_3$/EtOH/16 *M* NH_4OH/H_2O (36:28:2:6). The solvent was allowed to move up to about 7 cm. The second solvent served to separate the PdInsP and PtdInsP$_2$ from the origin. After development with the second solvent system, the HPTLC plates were thoroughly dried by a stream of dry nitrogen for 6–8 min. They were then exposed to HCl fumes for 3 min, and the excess HCl fumes were removed from the plates with dry nitrogen for 8 min. The plates were then turned through 90° and placed in a third solvent system, consisting of $CHCl_3$/MeOH/Me_2CO/HOAc/1 *M* NH_4OAc (140:60:55:4.5:10). Finally, the plates were dried briefly with dry nitrogen, and were then visualized by spraying the plate with 2′,7′-dichlorofluorescein (1% in methanol) and viewed under an ultraviolet lamp. The fluorescent

dye procedure is used to recover the PtdInsP for analysis of molecular species and fatty acids by GLC. For measuring radioactivity, the lipid spots are identified by exposing the plate to iodine vapor. The brown lipid spots are scraped into counting vials for the measurement of radioactivity. Sun and Lin [79] have demonstrated effective separations of PtdInsP from brain homogenates and myelin preparations by two-dimensional HPTLC. The brain samples were spiked with PtdInsP standards (100 μg) prior to spotting the plates. After development, the lipids were visualized by charring with a solution containing 3% (v/v) copper acetate in 8% phosphoric acid and subsequent heating for 20 min at 140 °C. For radiochromatography, an unstained plate was exposed to Kodak OMATAR film at − 70 °C overnight. It is possible to reveal radioactive spots that cannot be visualized by the charring procedure.

The discovery of PtdIns 3-kinase has led to the need for distinguishing between isomeric $PtdInsP_2$ and $PtdInsP_3$ [259]. The so-called PtdIns 3-kinase phosphorylates PtdIns, PtdIns(4)P, and $PtdIns(4,5)P_2$ to form PtdIns(3)P, $PtdIns(3,4)P_2$, and $PtdIns(3,4,5)P_3$, respectively. The simple two-dimensional TLC systems used to resolve the commonly encountered major and minor phospholipids cannot resolve them. The detection of the novel polyphosphoinositol phosphatides *in vivo* is hampered by their presence in relatively low abundance, although they have been found to occur in all cells thus far investigated. TLC separation of the D-3/D-4 isomers is generally performed with alkaline mixtures of chloroform and methanol, which result in a slightly lower migration of PtdIns(3)P [263] or of $PtdIns(3,4)P_2$ [264], relative to the respective 4-D isomer. Other workers have shown that inositol lipids can be separated by methods based on the ability of boric acid to form complexes with 2,3-*cis*-diols, present in the inositol ring of PtdIns [88]. In the 3-D isomers, the 3-OH is substituted by a phosphate group, thus inhibiting complex formation. The chromatographic behavior of $PtdIns(4,5)P_2$ seems to be unaffected by boric acid; it thus retains its slower migration relative to $PtdIns(3,4)P_2$ present in the boric acid-free TLC system.

Hegewald [88] has described the first procedure for the separation of D-3 and D-4 PtdInsP lipids. Chromatography is performed on two different types of HPTLC plates under otherwise identical conditions and depends on the ability of the D-4, but not D-3, isomers to form complexes with boric acid. For this purpose, pre-coated HPTLC plates of Silica Gel 60F254 (Art 1.056641) and HPTLC plates NH_2 (Art 1.12572), were obtained from Merck. Reference standards, ^{32}P-labeled PtdIns(4)P, $PtdIns(4,5)P_2$, and PtdOH were produced by *in vitro* incubation of human erythrocyte membranes with Mg-[γ-^{32}P]ATP, and the lipids were extracted with hexane/2-propanol/HCl mixtures, essentially as described [264]. ^{32}P-labeled PtdIns(3)P, $PtdIns(3,4)P_2$, and $PtdIns(3,4,5)P_3$ were synthesized by incubation of a sonicated mixture of the erythrocyte lipids with Mg-[γ-^{32}P]ATP and the recombinant PtdIns-3-kinase γ. Cultured A431 cells were incubated for 24 h with [^{32}P], and the lipids were extracted as described [88]. Hegewald [88] impregnated HPTLC plates of Silica Gel 60 by dipping them upside down into 5% boric acid (w/v) solution in methanol and then drying them for 5 min in an air current (System A). Samples containing *ca.* 0.01 to 0.1 mg lipid were streaked with a CAMAG-Linomat IV as 10-mm lanes, 10 mm above the bottom edge of the plate. The plates, which did not need to be activated, were then developed in 1-propyl acetate/2-propanol/absolute ethanol/6% aq. ammonia (3:9:3:9), in a well-equilibrated paper-lined twin-trough

chamber (CAMAG). The mobile phase was allowed to reach the top of the plates (*ca.* 2 h). Following chromatography, the dried plates were dipped in charring reagent (5% $CuSO_4$ solution in 8% aq. H_3PO_4) and heated at 180 °C for 15 min. The radioactivity of the ^{32}P-containing phospholipids was visualized and quantified, using the GS-250 Molecular Imager System (Bio-Rad Laboratories). Occasionally, the optical densities of the charred lipid bands were scanned at 366 nm in the reflectance mode, with a DESAGA CD 60 densitometer. Sometimes weakly radioactive bands were detected below PtdIns(4,5)P_2, PtdIns(4)P, and PtdOH. Treatment of sonicated ^{32}P-labeled erythrocyte lipids with phospholipase A_2 from *Vipera ammodytes* resulted in three additional radioactive bands with R_F values identical to the above-mentioned weak bands. Thus, they are thought to consist of the lyso-compounds of the 4-D phosphoinositol phospholipids and of PtdOH. TLC separation of erythrocyte phospholipids, labeled additionally by means of Mg-[γ-^{32}P]ATP in the presence of the PtdIns-3-kinase γ led to the appearance of at least three new radioactive bands. The identity of the bands was revealed by co-migration of radiolabeled PtdIns(3)P, PtdIns-(3,4)P_2, and PtdIns(3,4,5)P_3, which were synthesized by incubation of the corresponding precursor PtdIns, PtdIns(4)P, or PtdIns(4,5)P_2 with PtdIns 3-kinase γ and Mg-[γ-^{32}P]ATP. Also, the lyso compounds of the 3-D inositol phosphatides were sometimes detectable [88]. Thus, the TLC System A (see above) separates PtdInsP_3, lysoPtdIns(3,4)P_2, lyso-PtdIns(4,5)P_2, PtdIns(3,4)P_2, PtdIns(4,5)P_2, lyso PtdInsP, PtdIns, lyso PtdIns, and PtdEtn. TLC System B (boric acid-impregnated HPTLC plates NH_2, developed under identical conditions) is able to separate lysoPtdInsP_2, PtdInsP_2, lysoPtdIns(4)P, lysoPtdIns(3)P, PtdIns(4)P, PtdIns (3)P, lysoPtdIns, PtdIns, PtdOH, and PtdSer. SM co-migrates with cardiolipin.

The above HPLC methods have been designed to separate the inositol phospholipids that exhibit phosphorylation at the 4-OH position of the inositol ring, such as PtdIns(4)P and PtdIns(4,5)P_2. However, it is now known that PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 also occur in natural systems, albeit at low levels. Detection of PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3 *in vivo* has been difficult, due to the fact that these lipids are present in relatively low abundance in almost all cells. To detect and identify these novel phospholipids in intact cells, it is necessary to incorporate high levels of radioisotope into the total cellular PtdInsP. Further complications have arisen from the failure of conventional methods to separate these novel isomers from the well-known and more abundant PtdInsPs. Thus, PtdIns(3)P and PdIns(4)P are unresolved in nearly all one- and two-dimensional TLC systems thus far investigated. PtdIns(3,4)P_2 and PtdIns(4,5)P_2 also migrate unresolved in conventional TLC systems. The PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns (3,4,5)P_3 are usually recovered from incubations with PtdIns 3-kinase. According to Auger *et al.* [21], the PtdIns 3-kinase reaction may be performed in a total volume of 50 μL and is initiated by the addition of 10–50 μCi of [γ-^{32}P]ATP, 3000 mCi/mmol) in a carrier of 50 μM unlabeled ATP, 10 mM $Mg^{2+}$and 20 mM Hepes (pH 7.5) to the washed immune complexes that have been pre-incubated at room temperature for 5–10 min. After stopping the reaction with 80 μL of 1 M HCl and extracting the lipids with 160 μL of chloroform/methanol (1:1), the ^{32}P-labelled phospholipid products in the (bottom) organic phase are collected after a brief centrifugation and stored at − 70 °C until further use. It has been found that such samples can be best analyzed and resolved on TLC plates (Silica Gel G, 0.2-mm thickness, Merck) that have been precoated with 1% (w/v) potassium oxalate

and baked at 100 °C for 30 min and again 1 h immediately before use. Unlabeled phospholipid standards are run in parallel to monitor lipid migration and are visualized by exposure to iodine vapor. The highly phosphorylated PtdIns(3,4,5)P_3 is separated from the radioactivity remaining near the origin and from any [γ-^{32}P]ATP and [^{32}P]phosphate, carried over with the organic phase during lipid extraction of incubation mixtures, by using an acidic solvent system of 1-propanol/2.0 *M* acetic acid (13:7) instead of the more commonly used $CHCl_3$/MeOH/2.5 *M* NH_4OH (9:7:2) solvent system. To achieve maximum separation of the phospholipids from each other and from the material close to the origin, the solvent is allowed to migrate nearly to the top of a 20-cm TLC plate, a process that is routinely accomplished in 5 to 6 h [21].

Stein *et al.* [266] have described a method for the acetylation of PtdIns(4)P and PtdIns(4,5)P_2 with [^{3}H]acetic anhydride and for the separation of the products from each other and from unchanged starting material. The principle of the method is to acetylate the lipid and then isolate the lipid acetate from unchanged lipid by TLC. The addition, before acetylation, of a sample of lipid, labeled with a second isotope (^{14}C or ^{32}P is convenient) allows a ^{3}H/^{14}C or ^{3}H/^{32}P ratio to be obtained for the acetylated lipid by comparison with the ratio obtained for a set of lipid standards treated in an identical fashion. The amount of lipid in the unknown sample can then be calculated. This value (a ratio) is not affected by losses in extraction and processing, counting efficiencies, or exactness of specific activity, because the addition of a known number of counts of the ^{14}C- or ^{32}P-labeled lipid to both unknown and standard samples at the start of the procedure means that the initial concentration of the lipid present may be determined.

An early report by Nakamura *et al.* [58] described a simple and direct HPLC method for the separation and quantification of PtdIns(4)P and PtdIns(4,5)P_2, derivatized with 9-anthryldiazomethane. The derivatives were separated on a reversed-phase column, using isocratic elution, and detected with a UV detector. The detection limits of PtdInsP and PtdInsP_2 were 0.25 μg, which was low enough for sensitive measurement in rat brain. Gunnarsson *et al.* [60] combined NP-HPLC of PtdInsPs with ESI-MS. For this purpose, a Gilson HPLC system was combined with a Quattro II mass spectrometer (Micromass), equipped with pneumatically assisted ESI and APCI sources. The HPLC columns (100 × 2 mm ID) were packed in the laboratory with 5-μm silica (Spherosorb) at 950 bar. The columns were developed with a linear gradient of (A) $CHCl_3$/MeOH/NH_4OH (50:45:3) and (B) $CHCl_3$/MeOH/H_2O/NH_4OH (25:55:17:3). The HPLC effluent entered the mass spectrometer through an ESI capillary set at − 2.6 kV and 120 °C. The PtdInsP in the HPLC eluate were directly admitted to the ESI-MS system. The Folch-type brain extract gave mass chromatograms for the ions of m/z 885, 965, and 1045, representing PtdIns, PtdIns(4)P, and PtdIns (4,5)P_2, respectively. Nagatsuka *et al.* [267] isolated a new phosphoglycerolipid, "phosphatidylglucose", found in umbilical cord red cells by affinity chromatography on a column of multi-reactive monoclonal anti-cold agglutinin, mA GL-1-1/GL-2. Secondary-ion mass spectrometry/CID-MS analysis of this lipid gave a main molecular peak at m/z 885, coresponding to phosphatidylhexose. The antigen was susceptible to PLA_2, C and D, but resistant to PtdIns-specific PLC. Two-dimensional NMR spectroscopy confirmed that glucose is linked to the sn-glycerol 3-phosphate residue *via* a β-anomeric configuration.

17.4.4 Glycosyl phosphatidylinositols

Sevlever *et al.* [268] purified the GlcN(acyl)PtdIns by a two-step procedure, involving LC on octyl-Sepharose and TLC. Octyl-Sepharose separated phospholipids according to their total number of hydrocarbon chains: one in 2-lysoPtdIns, two in PdIns, and three in Glc(acyl)PtdIns. The GlcN-inositol head-group in purified GlcN(acyl)PtdIns was confirmed by a number of procedures, including radiomethylation, following which an equal molar ratio of $GlcN(Me)_2$/inositol was measured. Taguchi *et al.* [269] have taken advantage of the improved sensitivity and soft-ionization characteristics of ESI-MS/MS and (MALDI)-TOF-MS to analyze the glycosylPtdIns-anchored C-terminal peptide derived from 5′-nucleotidase. In the CID dissociation spectrum, single-charge ions, such as m/z 162 (glucosamine), 286 (mannose-phosphate-ethanolamine), and 447 [(mannose-phosphate-ethanolamine)glucosamine] were clearly detected as characteristic fragment ions of the glycosyl PtdIns-anchored peptide. The FA composition was not discussed or determined.

17.4.5 Sphingomyelins and glycosphingolipids

Natomi *et al.* [270] reported a systematic analysis of glycosphingolipids of the human gastrointestinal tract by means of TLC, TLC-immunostaining, and negative FAB/MS. Individual glycosphingolipids were chromatographed on Silica Gel 60 HPTLC plates, using the solvent systems (A) $CHCl_3$/MeOH/H_2O (65:35:8) and (B) $CHCl_3$/MeOH/0.5% aq. $CaCl_2$ (55:45:10) and were detected with orcinol/H_2SO_4 reagent for neutral glycosphingolipids and sulfoglycosphingolipids, and with resorcinol/HCl reagent for gangliosides. The major neutral glycosphingolipids were ceramide monohexosides (GalCer, GlcCer, LacCer, Gb3Cer, Gb4Cer, and more polar ones with more than four sugars), whereas neither Cg3Cer nor Cg4Cer were present. The acidic glycosphingolipids consisted of sulfatides and gangliosides, such as GM3, GM1, GD3, and GD1a. The structure of the isolated sulfatides was analyzed by NI-FAB-MS, using triethanolamine as the matrix solvent. Ruwisch *et al.* [271] have described a two-step lipid extraction procedure to separate sphingosine-1-phosphate from most interfering phospholipids and sphingolipids. Following extraction, SPP and dihydro-SPP were converted to fluorescent isoindol derivatives by *o*-phthaldialdehyde (OPA) and separated by HPLC, using a gradient program with methanol and 0.07 *M* K_2HPO_4 as eluents. Dihydro-SPP, which is not detectable in cultured cells and biological samples, was used as internal standard. Although not a glycerolipid or a sphingolipid, the chromatographic behavior and ESI-MS properties of certain proprietary detergents are also of interest, as they may contaminate sample preparations obtained with these detergents as membrane or lipoprotein solubilizers. Yang-Boja *et al.* [272] have described the ESI-MS properties of three proprietary detergents, M-PER, Y-PER, and B-PER, which they identified as 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, *N*-tradecyl-*N*,*N*-dimethyl-3-ammonio-1-propanesulfonate, and *n*-octyl-β-D-thioglucopyranoside, respectively. The detergents are lipid-soluble and may be extracted along with polar natural lipids. They yield positive ESI-MS spectra with protium, sodium and/or potassium adducts.

17.5 RESOLUTION OF MOLECULAR SPECIES

17.5.1 Fatty acids and neutral lipids

17.5.1.1 Fatty acids

Craske [273] reported on a collaborative study, which attempted to separate instrumental and chemical errors in the analysis of TAG oils by GLC. A primary standard mixture of fatty acid methyl esters (FAME) was used to determine how well the participants had optimized their gas chromatographs, while a primary standard of the equivalent of TAG was used to determine the total error of analysis. The experimental error was calculated as the difference between the two absolute errors. Wolff and Bayard [274] reported that doubling the length of a CP-Sil 88 capillary column (Chrompack) from 50 to 100 m remarkably improves the resolution of individual *trans*-18:1 isomers from either ruminant fats or partially hydrogenated oils. Specifically, the *trans*-9, *trans*-10, and *trans*-12 18:1 isomers were almost baseline-separated from other isomers. However, it was not possible to separate the critical pairs of *trans*-13 and *trans*-14 18:1 acids. Sattler *et al.* [275] have described a simple, accurate, and fast procedure for the quantitative analysis of fatty acids in simple lipid classes from various biological specimens. Lipid extracts from isolated plasma lipoproteins and macrophages were separated by TLC on Silica Gel 60 plates. Bands migrating together with authentic lipid standards were scraped off under argon and subjected to direct, *in situ* trans-esterification with BF_3/methanol in the presence of the TLC adsorbent. The FAME were subsequently quantified by capillary GLC. A comparison of the fatty acid content present in total lipid extracts and in lipid subclasses, separated by TLC, showed recoveries ranging from 93–99.7%.

Ichihara *et al.* [276] have reported an improved procedure for the determination of the fatty acid composition of glycerolipids. The procedure includes KOH-catalyzed trans-esterification and high-speed GLC. The fatty acid methyl esters in hexane were analyzed on 10% SP-2340 at 240 °C. The free fatty acids obtained on saponification were methylated with 15% methanolic BF_3. Benistant *et al.* [277] reported the fatty acid composition of brain cells by analyzing the fatty acid methyl esters and dimethylacetals on a 60 m × 0.25-mm capillary column, coated with SP-2380 liquid phase. The fatty acids were derived from the GPL, following resolution of the lipid classes on TLC with the solvent system $CHCl_3$/MeOH/$MeNH_2$ (60:20:5). Weylandt *et al.* [278] determined the polyunsaturated fatty acid composition of the phospholipids from myocyte cell cultures. Following TLC isolation of the phospholipids, the FAME were prepared by treating the gel scrapings with 14% BF_3 in 1.5 mL methanol and 2 mL hexane and heating the mixture for 1 h at 100 °C. The methyl esters were analyzed by GLC on a 30-m Omegawax TM column (Supelco) with hydrogen as carrier gas and temperature programming. Zang *et al.* [279] described an improved method of fatty acid analysis with optimum recovery of highly polyunsaturated fatty acid methyl esters in biological samples. The method was based on trans-esterification of phospholipids and TAG to FAME with a commercially available reagent, Methyl-Prep II (Alltech). The modified conditions virtually eliminated

the methylation of BHT, which ordinarily may reach 50% of the BHT added to prevent oxidation of polyunsaturates. Methylated BHT is eluted close to the 14:0 fatty acid and no longer functions as an antioxidant. A chelator (DTPA) was also added to prevent peroxidation of metal-catalyzed free-radical chain-reactions.

Brekke *et al.* [280] developed a highly sensitive method for assaying the agonist-induced release of endogenous fatty acids from cells in culture by HPLC with fluorescence detection. Fatty acids were selectively derivatized with 1-pyrenyldiazomethane and separated on a 5-μm LC18 Supelcosil column (250 × 4.6 mm ID) with a Pelliguard precolumn (4.6 × 20 mm, Supelco). A gradient from water to acetonitrile was used. The fatty acids were identified by collecting the fractions and re-analyzing them by a combination of TLC and GLC. For GLC, the fatty acid fractions were converted to methyl esters. Agren *et al.* [281] reported the fatty acid composition of the various glycerolipids and glycerophospholipids of serum and red blood cells of strict vegetarians. Serum lipids were separated in a single aminopropyl solid-phase column. FAME were prepared with 14% BF_3/methanol, but the contribution of plasmalogens was not reported. Borch-Jensen *et al.* [282] used SFC and GLC to determine the content of vernolic acid (12,12-epoxy-9-octadecenoic) in the oil of *Euphorbia lagascae*.

Others have used silver-ion HPLC for the effective segregation of unsaturated fatty acids prior to GLC. Juaneda *et al.* [283] obtained a complete separation of the geometric isomers of linolenic acid by HPLC with a silver-ion column, using a gradient of dichloromethane and methanol. The silver nitrate was immobilized on a Nucleosil 5SA column. Adlof *et al.* [284] used silver-ion HPLC (ChromSpher) with isocratic solvent conditions (0.08% acetonitrile in hexane) to maximize 18:1 positional isomer separations, while 0.4% acetonitrile in hexane was used to elute the polyunsaturated FAME. Nikolova-Damyanova *et al.* [285] resolved a series of positionally isomeric octadecenoic acids as the allyl, butenyl, pentenyl, and hexenyl alcohol esters by means of HPLC on an ion-exchange column, loaded with silver ions. Monchilova *et al.* [286] later reported the effect of the ester moiety and mobile-phase composition on the HPLC resolution of isomeric *cis*- and *trans*-octadecenoic acids. Clear resolution of *cis*- and *trans*- positional isomers of 18:1 in partially hydrogenated sunflower oil was achieved in 22 min after conversion to *p*-methoxyphenacyl esters on a silver-ion column, operated under isocratic elution conditions with hexane/dichloromethane/acetonitrile (60:40:0.2). George [287] proposed HPLC of fatty acids without derivatization. The fatty acids were detected by their refractive index after isocratic RP-HPLC. The mobile phase consisted of acetonitrile/tetrahydrofuran/water/glacial acetic acid (45:20:34.5:0.5). The TAG were saponified and acid-hydrolyzed to free fatty acids. Alasnier and Gandemer [288] have reported the fatty acid and dimethylacetal composition of individual phospholipid classes of rabbit skeletal muscle in relation to the type of dietary fiber. The dimethylacetals were generated from the plasmalogens during acid-catalyzed transmethylation of the individual GLP classes.

Zirolli and Murphy [289] reported a novel method for the analysis of branched-chain fatty acids, based on low-energy CID of the molecular ions of FAME obtained by EI-MS/MS, yielding a regular, homologous series of carbomethoxy ions. Unlike the fragment ions observed in the ion-source following EI, CID of the molecular ion yielded only one series of ions, corresponding in mass to the carbomethoxy series. GC/MS analyses of simple esters of unsaturated fatty acids have been confounded by double-bond migration.

To overcome this problem, on-site derivatization techniques had been used in the past, but not very successfully. More successful has been the reaction of the carboxyl group with nitrogen-containing compounds, known as remote-site derivatization. Fatty acid derivatives, such as picolinyl, piperidyl, and morpholinyl esters, and pyrrolidides, triazolopyridines, and 2-alkenylbenzoxazoles, have been investigated and their MS properties determined [290,291]. Another type of *N*-containing derivatives for GC/MS analysis of fatty acids, the 2-alkenyl-4,4-dimethyloxazolines (DMOX), was introduced by Zhang *et al.* [292,293]. Dobson *et al.* [294] characterized 16 cyclic dienoic fatty acids, formed from linolenic acid in linseed oil heated at 275 °C, by GC/MS of the picolinyl esters and DMOX derivatives, before and after hydrogenation and deuteration. GC/MS analyses were performed on a capillary column (0.25 mm × 25 m, 0.25 μm), coated with 5% phenylmethyl silicone (CP-Sil 8CB, Chrompak), and picolinyl esters were injected on-column at 70 °C with a linear temperature increase to 220 °C at 60 °C/min. The gas chromatograph was attached to a Model 5970 Mass-Selective Detector. DMOX derivatives of selected isomer fractions were analyzed on a BPX70 column [0.22 × 50 m, 0.25 μm (SGE)]. The samples were injected at 80 °C, and the temperature was held constant for 3 min before rising to 160 °C at 20 °C/min and then to 260 °C at 2 °C/min. The column was connected directly to the ion source of the mass spectrometer. Berdeaux and Wolff [295] prepared the DMOX derivatives of Δ^5-unsaturated polymethylene-interrupted fatty acids from conifer seed oils. In the mass spectra, all showed an intense peak at m/z 153, which is a diagnostic ion of fatty acid derivatives with Δ^5-ethylenic bond. Christie [296] proposed an improved method of preparation of DMOX derivatives.

Tanaka *et al.* [297,298] developed a method for purification and structural elucidation of several non-methylene-interrupted fatty acids (NMIFA) from plant and animal tissues. Later, Tanaka *et al.* [299] used the purified NMIFA (podocarpic, pinolenic, and columbinic acids) and cultured-cell systems to evaluate the ability of these acids to substitute for arachidonic acid in the PtdIns fraction of HepG2 cells. The total lipid extracts from HepG2 cells were subjected to methanolysis, and the FAME were purified by TLC with petroleum ether/diethyl ether/acetic acid (80:30:1) and subjected to Ag-TLC with petroleum ether/diethyl ether/acetic acid (70:20:2) as solvent. The trienoate was partially hydrogenated with hydrazine monohydrate, and the resulting monoenoates were converted to the dimethyl disulfide adducts for analysis by GC/MS, as described by Tanaka *et al.* [299]. The FAME were analyzed by GC on a capillary column (0.22 mm × 30 m), coated with polar CBP 20 (Shimadzu). Slater *et al.* [300] reported an improved method for the analysis of 1-^{13}C-palmitic acid in stool samples by GC/EI-MS. The improvement was due largely to the use of *tert*-butyldimethylsilyl (*tert*-BDMS) ester derivative, which allows quantification of the pseudomolecular ion $[M-57]^+$.

Li *et al.* [301] used GC/NICI-MS for a sensitive measurement of free and esterified arachidonate (AA), which was converted to its PFB ester. A Finnigan SSQ 7000 mass spectrometer, linked to a Varian gas chromatograph, was used to inject a 1-μl volume of the sample solution on-column into a 1-m retention gap, attached to a fused-silica capillary column (25 m × 0.25 mm ID, 0.25-μm film thickness, Rtx-1, Restek). Helium was used as a carrier gas at 10 psi. The starting temperature of 210 °C was increased to 310 °C at a rate of 20 °C/min. Arachidonic acid-d_8 was used as internal standard, and the masses of both

acids were quantified by selected-ion monitoring of the two ions at m/z 303.2 (AA) and 311.2 (d_8-AA). Lagerstedt *et al.* [302] have developed a capillary GC/NICI-MS method for the quantitative determination of C_8-C_{26} total fatty acids in plasma. Following hydrolysis, hexane extraction, and derivatization with PFB bromide, fourteen saturated and 25 unsaturated fatty acids were quantified by selected-ion monitoring in a ratio to ^{13}C stable isotope-labelled internal standards. Recoveries ranged from 76 to 106%. Pragst *et al.* [303] have developed a method for the analysis of ethyl myristate, ethyl palmitate, ethyl oleate, and ethyl stearate from hair, based on the extraction of the hair sample with a dimethylsulfoxide (DMSO)/*n*-hexane mixture, separation and evaporation of the solvent, and application of headspace solid-phase micro-extraction (SPME) in combination with GC/MS to the extract.

Metges *et al.* [304] determined the enrichment of selected serum fatty acids of human volunteers after a small oral dosage of (1-^{13}C)- and (8-^{13}C)triolein by GLC/combustion isotope-ratio (IR)-MS. It was concluded, that although GC/IR-MS is approximately 1000 times more sensitive than GC/MS, the dilution of unlabeled oleic acid from the liquid diet is probably too high to detect an enrichment. Ruiter *et al.* [305] have reported simultaneous GLC quantification and isotopic enrichment of fatty acids in human plasma by FID and MS detection. In quantification, intra-assay variation ranged from 1.5 to 4.9%, inter-assay variation ranged from 3 to 11%, and intra- and inter-assay variations of the enrichment determination of palmitic acid were 1.4 and 0.9%, respectively. Adlof [306] used a combination of reversed-phase and silver chromatography to separate *cis*-9, *trans*-11- and *trans*-9, *trans*-11-isomers of conjugated linoleic acid (*cis*-9-, *trans*-11-octadecadienoic acid), labeled with deuterium atoms on the 17- and 18-carbon atoms (17,17,18,18-d_4). GC/MS was used to quantify the deuterated and non-deuterated FAME, using a 30 m × 0.25-mm Supelcowax 10 capillary column and a HP Model 5889 quadrupole mass spectrometer in positive-CI mode.

17.5.1.2 Prostanoids and oxo-fatty acids

GC/MS provides the most sensitive and specific method for the detection of eicosanoids, but it is limited to a small number of samples per day, due to the lengthy sample preparation [307]. Recent improvements in the GC/MS method have increased the throughput of this method to as many as seven prostaglandins per sample in a 15- to 30-min period [308,309]. Margalit *et al.* [310] have described a new method for the detection and quantification of eicosanoids in biological samples that utilizes ESI-MS/MS in the multiple-reaction monitoring (MRM) mode. This method is quantitative, capable of high throughput, and does not require prior derivatization or chromatographic separations. An application of this method was described to the analysis of eicosanoids in biological fluids. Ferretti and Flanagan [311] have reported the mass spectra of piperidide and TMS ester derivatives of the major metabolite of prostaglandin F, 9α,11α-dihydroxy-15-oxo-2,3,4,5,20-pentanor-19-carboxyprostanoic acid [312]. Powell *et al.* [313] have compared the chromatographic behavior of three groups of eicosanoids: prostaglandins, leukotriene B_4 and some of its metabolites, and monohydroxy-eicosanoids and their oxo-metabolites. Powell *et al.* [313] found that in reversed-phase HPLC the retention times of

oxo-eicosanoids were longer than those of the corresponding hydroxy-eicosanoids with mobile phases containing acetonitrile as the major organic component, whereas the reverse was true for mobile phases containing methanol. NP-HPLC with mobile phases containing hexane, 2-propanol, and acetic acid, gave excellent separation of oxo- and hydroxy-eicosanoids. Increasing the concentration of acetic acid in the mobile phase selectively reduced the retention times of oxo-eicosatetraenoic acids compared to monohydroxy-eicosatetraenoic acids, whereas the reverse was true for 2-propanol. John and Schlegel [314] have described an improved RP-HPLC method for the separation and detection of both hemiacetalic 11-hydroxy anomers of thromboxane B2 (TXB2). Water/acetonitrile mixtures served as the mobile phases. By diminishing the temperature of the chromatographic system stepwise from 40 °C to 0 °C, the UV-absorbance profile of TXB_2 changed from one broad peak to two clearly separated narrow peaks, corresponding to the two anomers, existing in equilibrium. Increase of the mobile-phase pH from 1.6 to 6.9 (at 0 °C) resulted in different concentration ratios of the anomers. The intermediate open-aldehyde form of TXB_2 was unstable and, therefore, could not be observed by HPLC nor by ^{1}H-NMR measurements. Sajiki and Kakimi [315] identified the eicosanoid metabolites of AA in red algae by HPLC, interfaced with ESI-MS. Prostaglandin E_2, 15-keto-PGE, and 8-hydroxyeicosatetraenoic acid were detected as major eicosanoids, and PGA and leukotriene B_4 as minor ones.

Whelan and Murphy [316] have developed a RP-LC/MS/MS method for the quantification of leukotriene B_4 (LTB_4), the 5-lipoxygenase product, 5-hydroxyeicosatrienoic acid (5-HETE), as well as the ω-oxidation metabolites of LTB_4, 20-hydroxy-LTB_4, and 20-carboxy-LTB_4. ESI-generated carboxylate anions were collisionally activated and decomposed to specific and abundant product-ions. MRM was used to analyze LTB_4 (m/z 335 to 195), 20-hydroxy-LTB_4 (m/z 351 to 195), 20-carboxy-LTB_4 (m/z 365 to 195), and 5-HETE (m/z 319 to 115). F_2-isoprostanes are complex metabolites of AA, generated *via* nonenzymatic free-radical oxidation, and are isomeric with prostaglandin F2α, which is enzymatically produced by prostaglandin H_2 synthase [4,317, 318]. Depending upon which of the labile hydrogen atoms is first abstracted by free-radical attack, up to 64 isomers in four structural classes can be generated [319]. Waugh and Murphy [320] found that eight synthetic $PGF_{2\alpha}$ isomers were separable by capillary GLC and RP-HPLC. ESI-MS/MS was used to detect the elution of these isomers from the HPLC column by monitoring the characteristic loss of 44 mu (C_2H_4O) from the 1,3-diol cyclopentane ring. Catalytic reduction, derivatization, and ESI/MS techniques were used to obtain definitive information as to the location of the side-chain hydroxy position in these isomers through abundant α-cleavage ions. Free-radical oxidation of AA was used to generate a complex mixture of F_2-isoprostanes, which were separated by HPLC and capillary GLC and identified by ESI-MS/MS.

Nourooz-Zadeh *et al.* [199,321] have presented evidence for the formation of F_3-isoprostanes during peroxidation of EPA. After conversion to PFB ester TMS derivatives, 3-isoprostanes were analyzed by NICI-MS, using tetradeuterated $PGF_{2\alpha}$ as the internal standard. Quantitative analysis was carried out by SIM of the carboxylated anion $[M\text{-}10]^-$ at m/z 567 and 573 for the PGF_3-like compounds and PGF_2-d_4, respectively. Prostanoids were separated, using a BPX-70 column (50 m × 0.25 mm ID, 0.25 μm) and temperature programming. EPA, oxidized by 2,2′-azo-*bis*(2-amidinopropane) hydrochloride (AAPH)

or by copper gave rise to a family of F_3-isoprostanes, with 8-*epi* $PGF_{3\alpha}$ as a minor product. Free-radical attack at the 4-carbon centers, located between adjacent double bonds in EPA, yielded pentadienyl radicals, which subsequently reacted with molecular oxygen to form eicosanoid peroxyl radicals. These products subsequently undergo endocyclization, insertion of molecular oxygen to give 6 sub-families of F_3-isoprostanes, which are only partially resolved. The GLC separations were performed on a SPB-1701 column (30 m × 0.25 mm ID) with temperature programming from 175 to 289 °C at 30 °C/min. The GLC column was linked to VG70SEQ (Fison Instruments), using NICI with ammonia as reagent gas. Morrow *et al.* [322] have described quantification of the major urinary metabolite of 15-F_{2t}-isoprostane (8-iso-PGF2α) by stable-isotope dilution MS assay. Specifically, a method was developed to quantify 15-F_{2t}-isoprostane, 2,3-dinor-5,6-dihydro-15-F_{2t}-IsoP (2,3-dinor-5,6-dihydro-8-iso-$PGF_{2\alpha}$), by GLC with NICI-MS. The metabolite was chemically synthesized and converted to an $^{18}O_2$-labeled derivative for use as an internal standard. Burke *et al.* [323] described the detection of dinor-dihydro-isoprostane $F_{2\alpha}$-III (2,3-dinor-isoprostane $F_{1\alpha}$-III), a metabolite of isoprostane $F_{2\alpha}$-III and an oxidation product of γ-linolenic acid, in human plasma and urine. Utilizing an ^{18}O-labeled homologous internal standard, a GC/MS assay was developed for the determination of the 2,3-dinor-5,6-dihydro (dinor-dihydro) metabolite of isoprostane $F_{2\alpha}$-III. The isoprostanes were analyzed by GC/MS as the PFB esters/TMS ester derivatives. Walter *et al.* [324] have reported a further refinement of these methods for F_2-isoprostane analysis by replacing SPE and TLC with HPLC on an amino column with a hexane/2-propanol gradient. PFB esters of F_2-isoprostanes were prepared and purified by HPLC, silylated, and then analyzed by GLC with NICI-MS. This method, particularly the substitution of the TLC step common to these methods with HPLC, resulted in a more sensitive and reproducible assay.

Bessard *et al.* [325] determined isoprostaglandin $F_{2\alpha}$ type III in urine by GLC with EI/MS and compared the results obtained with those derived from enzyme immunoassay. Isoprostaglandin $F_{2\alpha}$ type II concentrations determined by GC/EI-MS did not agree with those of the immunoassay, apparently because the two assays do not measure the same compounds. Compounds analogous to the F_2-isoprostanes may be formed from other fatty acid substrates [321,326]. Thus, Roberts *et al.* [326] and Reich *et al.* [327] reported the formation of isoprostane-like compounds (neuroprostanes) *in vivo* from DHA, while Nourooz-Zadeh *et al.* [321] reported the formation of F3-isoprostanes during peroxidation of EPA. Eicosanoids with E/D-type prostane rings are unstable and are dehydrated to cyclopentenone-containing compounds possessing A-type and J-type prostanoate rings, respectively. Fam *et al.* [328] have shown that cyclopentenone neuroprostanes (A4/J4-neuroprostanes) are formed from dehydration of E_4/D_4 neuroprostanes. The A4/4-neuroprostanes were identified by chemical approaches and LC/ESI-MS/MS methodology. Jones *et al.* [329] have reported the analysis of mercapturic acid conjugates of lipid peroxidation (*trans*-4-hydroxy-2-hexenal and *trans*-4-hydroxy-2-nonenal) products by FAB-MS in the NI mode. Deprotonated molecular ions for mercapturic acids gave simple daughter-ion spectra, the dominant mode of decomposition involving cleavage of C–S bonds to give a characteristic neutral loss of 129 Da. Chance *et al.* [330] reported the GC/MS separation of hydroxy fatty acids as their methyl ester *tert*-BDMS ethers. The *tert*-BDMS ethers of secondary hydroxy fatty acid methyl esters produce

stable derivatives amenable to GC/MS. The derivatives produced prominent molecular-mass-minus-57 $[M-57]^+$fragment ions and unique fragment ions indicating the location of the secondary hydroxyl group along the aliphatic chain from the ω-2- carbon to C-5 from the carboxyl terminus, in addition to yielding information regarding carbon-chain-length, and degree of unsaturation. The GC/MS analyses were performed on 30 m × 0.25-mm-ID capillary columns, coated with a bonded 0.25-μm film of 100% methyl silicone (Quadrex). The carrier gas was helium, and the mass spectra were obtained with a Kratos MS-25 double-focusing instrument. Jira *et al.* [331,332] reported a strong increase in hydroxy fatty acids, derived from linoleic acid in the low-density lipoproteins (LDL) of atherosclerotic patients. The hydroxy fatty acids were isolated and identified. Folcik and Cathcart [333] compared the profile of oxidized linoleic acid products, formed by oxidation of LDL by $CuSO_4$ and soybean 15-lipoxygenase under conditions that yielded levels of oxidation similar to those found after monocyte-mediated modification. The oxidized lipids were analyzed by RP-HPLC of the native and saponified lipid esters of oxidized LDL. The major fatty acid oxidation product was esterified hydroperoxy-octadecadienoic acid (HPODE), the oxidized product of the most abundant polyunsaturated fatty acid in human LDL, linoleic acid. About 13.5% of the cholesteryl linoleate was converted to cholesteryl HPODE and cholesteryl hydroxyoctadecanoic acid (HODE) ester at the end of the 24-h incubation.

Yamane *et al.* [334] have described a method for the simultaneous detection of epoxy-polyunsaturated fatty acids (EpPUFA), epoxyhydroxy-polyunsaturated fatty acids (EpHPUFA), and dihydroxymetabolites (DiHPUFA), corresponding to each precursor fatty acid in rat tissue homogenate, by LC/TSI-MS with SIM. The method was applied to the analysis of EpPUFA in incubates of rat tissues, which were suggested to contain a highly active cytochrome P450 system or nonenzymatic oxidative reactions in aged rat tissue homogenate. Van Rollins and Knapp [335] identified the epoxide regio-isomers of arachidonic acid (EET) and their hydrolysis products, dihydroxyeicosatrienoic acids (DHET) as the PFB ester derivatives, and their properties were compared with those of the methyl esters. Yurawecz *et al.* [336] used GC/EI-MS to identify furan fatty acids among the oxidation products of conjugated octadecadienoic acid. Products identified included the following furan fatty acids: 8,11-epoxy-8,10-octadecadienoic; 9,12-epoxy-9,11-octadecadienoic; 10,13-epoxy-10,12-octadecadienoic; and 11,14-epoxy-11,13-octadecadienoic acid. The separations were performed on a CP Sil 88 (Chrompack) capillary column (50 m × 0.25 mm ID), with He carrier gas and FID. The column was temperature-programmed from 75 °C to 185 °C and held at 185 °C for 33 min, then raised to 225 °C at 4 °C/min. GC/EI-MS analyses were performed with a Hewlett-Packard 5890 Series II gas chromatograph, coupled to a Fissons VG Autospec Q mass spectrometer. The epoxides were eluted in the order of 8,11-epoxy-8,10-, 9,12-epoxy-9,11-, 10,13-epoxy-10,12- and 11,14-epoxy-11,13-octadecadienoic acids. Kerwin and Torvik [133] used NI-ESI-MS and NI-ESI-MS/MS to characterize saturated and unsaturated monohydroxy fatty acid metabolites, formed following incubation with soybean lipoxygenase. Ions corresponding to $[M-H]^-$ of eicosanoids were readily observed by ESI-MS, but double-bond migration precluded the use of MS to localize double bonds or the position of hydroxyl moieties. However, by following MS analysis with NI-ESI-MS/MS of precursor ions, the position of

oxygenation could be determined for picogram quantities of underivatized monohydroxy fatty acids.

Hou [337] used GC/MS to characterize 10-hydroxy-12(*Z*)-octadecenoic acid as the product of microbial oxidation of linoleic acid in 55% yield. GC/EI-MS spectra were obtained on-line with a Hewlett-Packard Model 5970 Series mass-selective detector. Separations were performed with the methyl ester (prepared by diazomethylation) isothermally at 200 °C on a nonpolar column (15 m × 0.25 mm) with a temperature gradient of 8 °C/min from 160 to 250 °C, after initially holding the temperature at 160 °C for 3 min. The structure was confirmed by ^{1}H- and ^{13}C-NMR spectroscopy. Laudert *et al.* [338] have developed an analytical technique for the direct analysis of nanogram amounts of the enantiomers of 12-oxo-phytodienoic acid, as its methyl ester, in plant tissues by capillary GC/MS on β- and γ-cyclodextrin stationary phases. In the species analyzed, endogenous *cis*-12-oxo-phytodienoic acid is the (+)-enantiomer. Schneider *et al.* [339] have developed a strategy, based on LC/ESI-MS/MS, for the detection of fatty acid hydroperoxides in complex samples, followed by the identification of the corresponding regio-isomers. Localization of fatty acid hydroperoxides in complex mixtures was achieved by monitoring the loss of hydrogen peroxide by constant neutral-loss scanning. Low-energy CID of precursor ions $(M + -NH_4]^+$ led to characteristic product-ions, formed from both the 9- and 13-regio-isomers. The chromatographic separation was performed on a Eurosphere 100 C18 column (5 μm, 2 × 100 mm; Knauer) with a binary gradient of Solvent A [THF/MeOH/H_2O/HOAc (25:30:44.9:0.1)] and Solvent B [(MeOH/H_2O (9:1)]. Both solvents contained 5 m*M* NH_4OAc.

Brash [198] has identified the *bis*-allylic 11-hydroperoxide in vitamin E-controlled autoxidation of methyl linoleate. The 11-hydroperoxy derivative was the next-most prominent primary peroxidation product after the 9- and 13-hydroperoxides. It was present in *ca.* 5–10% of the abundance of the 9- or 13-hydroperoxide. The structures of 11-hydroperoxylinoleate and its 11-hydroxy derivative were established by HPLC, UV spectroscopy, GC/MS, and ^{1}H-NMR. HPLC of autoxidized methyl linoleate was performed on a 5-μm silicic acid column with hexane/2-propanol (100:1 or 100:0.5) as eluent. This resolved the 13-hydroperoxy-18:2 and 9-hydroperoxy-18:2, with the 11-hydroperoxy derivative well separated between the two peaks. Reversed-phase HPLC and purification of the 11-hydroperoxy-18:2 was carried out on a Beckman Ultrasphere 5-μm ODS column (25 × 0.46 cm) with an Upchurch guard column and a solvent system of MeOH/H_2O (85:15). Final purification was achieved by SPE-HPLC on a Beckman analytical silica column with hexane/2-propanol (1900:1) as eluent. GC/MS spectra were obtained on the hydrogenated 11-hydroxylinoleate, methyl ester TMS ether derivative by means of a SPB-1 fused-silica capillary column (5 or 15 m × 0.25 mm ID) and a temperature program (150 to 300 °C at 10 or 20 °C/min). Mass spectra were recorded in the EI mode (Hewlett-Packard Model HP5980 at 70 eV). The 11-hydroperoxide was not detectable in the absence of α-tocopherol, indicating that efficient trapping of the 11-peroxy radical as the hydroperoxide is critical to permitting its accumulation. Wang *et al.* [340] have determined the isomeric hydroperoxides of 18:2 by HPLC with a post-column diphenyl-1-pyrenylphosphine (DPPP) fluorescence detection system, described earlier [341]. Geometric isomers of hydroperoxides were separated on a Supelcosil LC-Si silica column (2.1 mm ID × 250 mm, 5 μm, Supelco) with a mixture of 500 mL of

n-hexane and 34 mL of diethyl ether as a mobile phase at a flow-rate of 0.6 mL/min. The separated hydroperoxides were mixed with DPPP solution, pumped at a flow-rate of 0.3 mL/min to form DPPP oxide in the postcolumn reaction coil, and the fluorescence intensity was measured (em: 340 nm, ex: 352 nm). The hydroperoxides were eluted in the order: methyl 13-hydroperoxy-*cis*-9, *trans*-11-, methyl 13-hydroperoxy-*trans*-9,*trans*-11-, methyl 9-hydroperoxy-*trans*-10, *cis*-12- and methyl 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoate.

17.5.2 Neutral glycerolipids and ceramides

17.5.2.1 Diradylglycerols and ceramides

Chromatographic resolution of molecular species of intact diradylglycerols was first accomplished by high-temperature GLC [1]. More recently, reversed-phase HPLC and combinations of HPLC with MS or MS/MS have largely taken over this task. Nevertheless, there have been notable applications of high-temperature GLC to the resolution of acylglycerols. Falardeau *et al.* [342] described a method for quantifying DAG as their 1-PFB-2-acyl-3-acetylglyceryl derivatives by capillary GC/NICI-MS. The method was designed to permit the detailed analysis of the molecular species composition of the free tissue DAG, which may contain high proportions of polyunsaturated fatty acids in the sn-2-position. It involved the sequential treatment of DAG with acetic anhydride, pancreatic lipase, and PFB chloride. In between treatments, the derivatives were purified by TLC. The final product was monitored by capillary GC/NICI-MS in SIM mode (m/z 614 and 604 for 2-arachidonoyl and 2-nonadecadienoyl internal standard, respectively). Zollner *et al.* [343] prepared the 1- and 2-monoacylglycerols and 1,2- and 1,3-DAG of saturated and unbranched fatty acids with 8 to 18 carbon atoms and subsequently derivatized them with nicotinic acid hydrochloride. The resulting nicotinyl derivatives were examined as pure substances by EI-MS and as mixtures by GC/MS. EI-MS enabled differentiation between MAG- and DAG and between the two positional isomers of MAG and DAG. Zollner and Lorbeer [344] have shown that these spectra provide rules for structure elucidation of partially acylated acylglycerols and probably also for mixed DAG by means of GC/MS, including the detection of double bonds and methyl-branch positions at the fatty acid alkyl chains. Specifically, an accurate determination of double-bond positions in MAG and DAG was possible by characteristic spacings between abundant diagnostic ions in this fragmentation pattern. Zollner and Schmid [345] have also demonstrated the utility of nicotinoyl derivatives in structural studies of MAG and DAG, containing methyl branchings and epoxy and cyclopropyl rings in their chains.

Liepkalns *et al.* [346,347] performed detailed chromatographic analyses of the molecular species of the major GPL and of free sn-1,2-DAG from neuroblastoma cells, following incubation with LiCl. For this comparison, the inositol, choline, ethanolamine, and serine GPL were dephosphorylated with phospholipase C, and the released sn-1,2-DAG, along with the free DAG, was subjected to high-temperature GLC on polar and nonpolar capillary columns as their TMS and *tert*-BDMS ethers. It was shown that the free DAG originated mainly from PtdIn, as indicated by the high levels of characteristic

18:0/20:4 and 18:0/20:3 species in both, and to a lesser extent from choline GPL, which contained mainly oligo-enoic species. Alkenylacylglycerols were not detected among the free DAG. Woodard *et al.* [348] determined the molecular species of platelet-activating factor (PAF) in saliva by GC/MS analyses of the PFB of the DAG. Choline-containing sn-2-acetylated GPL with sn-1-ether- or ester-linked fatty alcohol/acid moieties (alkyl-PAF or acyl-PAF, respectively) were dephosphorylated by treatment with PFB anhydride. Individual species of PFB-derivatized PAF were separated by GLC prior to MS analyses. NICI-MS analysis of PFB after direct derivatization with PFB anhydride revealed at least 6 different species in normal human saliva. Jeong *et al.* [349] isolated the choline and ethanolamine GPL of ascidians and sea urchins and hydrolyzed them with PLC to obtain the corresponding 1,2-diradylglycerols. These were purified and acetylated, and the acetylated derivatives were separated into alkenylacyl, alkylacyl, and diacyl subclasses by preparative TLC. The individual diradylglycerol subclasses were saponified with 1 *N* KOH in ethanol at 85 °C for 1 h. The 1-*O*-alk-1′-enylglycerols and 1-*O*-alkylglycerols thus obtained were extracted and converted to their TMS ether derivatives. The fatty-chain composition of the 1-*O*-alk-1′-enyl-2,3-diTMS glycerols and 1-alkyl-2,3-diTMSGro derivatives were resolved by GLC on a SUPELCOWAX-10 fused-silica wall-coated open-tubular capillary column (30 m × 0.25 mm ID). The injector and column temperatures were held at 250 °C and 210 °C, respectively. The TMS ethers of the monoradylglycerol diTMS ethers were identified by on-line CI-MS.

Hubbard *et al.* [350] quantified endogenous 1-stearoyl-2-arachidonoyl-sn-3-glycerol in human basophils *via* GC/NICI-MS. DAG standards and biological extracts were treated with PFB chloride in the presence of dimethylaminopyridine (DMAP), and the resulting PFB derivatives were analyzed on a 7.5-m nonpolar capillary column (Quadrex), using He as a carrier gas. The temperature was programmed from 145 °C to 185 °C at 40 °C/min, followed by an additional increase in temperature to 313 °C at a rate of 3 °C/min. Monitoring m/z 838 for detection of 18:0/20:4 and m/z 841 for detection of trideutero 18:0/20:4, added as internal standard, provided an analytical basis for GC/MS quantification of the endogenous DAG in human basophils. Thyssen *et al.* [351] assayed various molecular species of PAF and lyso-PAF in ulcerative colitis, using GC/MS to separate the derived DAG as the PFB derivatives. For quantification of lyso-PAF, the lipid extract was concentrated, derivatized with propionyl chloride, and the product was extracted along with PAF, and subjected to hydrolysis with PLC. The resulting DAG were converted to the PFB derivatives by reaction with PFB chloride. GC/MS was performed on 8-m Ultra-performance capillary columns, coated with methyl silicone (0.17-μm thickness, Hewlett-Packard). The column was connected to a Hewlett Packard Model 5988 quadrupole mass spectrometer, operated in the NICI-MS mode with He as a carrier gas. The temperature was programmed from 85 °C to 300 °C at 40 °C/min. SIM of the following ions was performed (m/z): 552 (16:0 PAF); 566 (2-propionyl-lyso 16:0 PAF); 580 (18:0 PAF); 578 (18:1 PAF) and 555 ($^2H_3$-labeled 16:0 PAF, internal standard).

Antonopoulou *et al.* [352] reported the separation within 55 min of the main neutral lipids, including MAG, DAG, and TAG, into classes and molecular species by reversed-phase HPLC with UV detection. A step-gradient elution with methanol/water, MeCN/MeOH, MeCN/THF, and 2-propanol/MeCN was performed on a C_{18} column with UV detection at 206 nm. Lin *et al.* [353] developed RP-HPLC methods for the separation of

molecular species of 45 synthetic TAG and DAG. They used linear gradients of methanol/2-propanol and UV detection at 205 nm as well as radioactive flow detection. Takagi and Ando [354] separated molecular species of MAG, as their di-3,5-dinitrophenylurethane (DNPU) derivatives by NP-HPLC on a nitrile-bonded phase. Upon elution with hexane/1,2-dichloroethane/ethanol (40:10:1) the MAG emerged in the order: 20:0 < 18:0 < 16:0 < 18:1 < 16:1 < 18:2 < 18:3.

Recent studies have demonstrated the presence of small amounts of both alkylacyl [355,356] and alkenylacyl [355] in addition to the DAG moieties of PtdIns in erythrocytes. The presence of both alkylacyl and alkenylacylglycerol moieties was established among the molecular species of the glycosyl PtdIns anchor of acetylcholinesterase [357]. Butikofer *et al.* [357] separated the molecular species of the diradylglycerol moieties of membrane PtdIns as the benzoates. Like the glycosyl PtdIns anchor of the acetylcholinesterase, they were made up exclusively of the diacyl species. Lee *et al.* [355] prepared the benzoate derivatives of the diradyl GroPIns of erythrocytes and, after a fractionation into diacyl, alkylacyl, and alk-1-enylacyl types, identified 2–2.4% alkenylacyl, 4.8–9.5% alkylacyl, and 93–88% diacyl GroPIns as subfractions, prepared from inositol-containing phospholipids. It was suggested that the alkyacylGroPIns could represent precursors of the diradyl GroPIns found in glycosyl PtdIns. Using a similar analysis of the diradylglycerolbenzoate derivatives, Butikofer *et al.* [357] detected 1.5–3.5% alkylacyl GroPIns in human and 2.5–4.8% alkylacyl GroPIns in bovine erythrocytes.

Brautigam *et al.* [358] have reinvestigated the plasmalogen content of the plasma lipoprotein phospholipids in normolipidemic donors and patients with hypercholesterolemia. The phospholipid classes were resolved by two-dimensional TLC, and the plasmalogen content was established by exposure to HCl vapor. Alternatively, the subclasses of the choline and ethanolamine phopholipids were resolved, following digestion with phospholipase C, preparation of 3,5-DNB derivatives, and TLC resolution of the diacyl-, alkenylacyl-, and alkylacyl-glycerols by established techniques. Individual molecular species were resolved by RP-HPLC (ODS Hypersil, 200 × 2.1 mm) with acetonitrile/2-propanol (8:2) as mobile phase, as previously described by Engelmann *et al.* [359]. Approximately 4.4–5.5% choline and 53–60% of ethanolamine GPL consisted of plasmalogens. Scott *et al.* [360] used previously established methods for the analysis of molecular species of choline- and ethanolamine-GPL in ruminant muscle in response to feeding fish with oil supplements, containing 17% 20:5(n-3) and 11% 22:6(n-3). Individual phospholipids were dephosphorylated with phospholipase C, as described by Louie *et al.* [361], and the released diradylglycerols were extracted to prepare benzoate derivatives. The recovered benzoate derivatives were resolved into the diacyl (R_F 0.26), alkylacyl (R_F 0.37), and alkenylacyl (R_F 0.46) subclasses by TLC on Silica Gel G in a solvent system of toluene/hexane/diethyl ether (50:45:5). The DAG were recovered and resolved into molecular species by RP-HPLC, using either acetonitrile/2-propanol (80/20) or methanol/2-propanol (95:5) [362].

Zhu and Eichberg [363] used the benzoates of DAG, derived from PtdIns of nerve tissue. Sn-1,2-DAG moieties of purified PtdIns were released by PLC and converted to benzoates by reaction with benzoyl chloride, as described by Blank *et al.* [364]. Molecular species of DAG benzoates were separated on a C_{18} 4.6 × 250-mm Microsorb

reversed-phase HPLC column, using a linear gradient of acetonitrile/2-propanol from 80:20 to 44:56 from 0 to 70 min at a flow-rate of 1 mL/min. The most prominent molecular species of PtdIns was 18:0/20:4. Lee and Hajra [365] have given details of a method, based on combinations of the above HPLC procedure, in which DAG is first derivatized with a chromophoric group (benzoyl), and different molecular species are then separated according to hydrophobicity by chromatography on a RP-HPLC column. The benzoates were also used for the analyses of the molecular species of choline- and ethanolamine-GLP in erythrocytes by Connor *et al.* [366]. The phospholipids were hydrolyzed with PLC, and the released DAG were converted to the benzoates, resolved into diacyl-, alkylacyl-, and alkenylacyl-glycerol sub-classes, and subjected to molecular-species separation on a reversed-phase column, using various ratios of acetonitrile/2-propanol for elution.

Schmid *et al.* [367] have reported excellent resolution of the molecular species of the DAG dinitrobenzoate derivatives, prepared from the DAG moieties of PtdCho and PtdEtn of rat hepatocytes, supplemented with various polyunsaturated fatty acids. The separations were performed as previously described [368–370]. One half of each sample was fractionated on a RP-HPLC column, using a solvent system of methanol/2-propanol/glacial acetic acid (80:20:0.01), and the peaks were collected in 7-mL scintillation vials. The other half of each sample was resolved in the same manner, and the peaks were collected for subsequent resolution of unresolved species in a solvent of acetonitrile/2-propanol (70:30). McKeone *et al.* [371] investigated alterations in the molecular species of serum PtdCho, caused by ingestion of the ethyl esters of 20:5n3 and 22:6n3 fatty acids, by using HPLC of the DAG moieties, following derivatization with 7-methoxycoumarin-3-carbonyl azide. The DAG were released by hydrolysis of the serum PtdCho with PLC. The coumarin derivatives were prepared by adding 7-methoxycoumain-3-carbonyl azide (Molecular Probes), dissolved in dry DCM, to the DAG at a 2:1 weight ratio. The tube was capped tightly and sealed with parafilm. It was then refluxed in a 60 °C heating block for 90 min, cooled in an ice bath, and the fluorescent coumarin derivatives were isolated by TLC, using toluene/ethyl acetate (4:1) as developing solvents. The molecular species of the derivatized DAG were resolved on a 4.5 mm × 25-cm ODS-2 C_{18} column (Phase Separations) at 50 °C with acetonitrile/2-propanol/water (64:25:11) as eluent and fluorescence detection (ex: 340 nm, em: 379 nm).

Bell and Dick [372] identified the molecular species of PtdCho, PtdEtn, and PtdSer as the DNB derivatives of DAG, released by PLC hydrolysis from the parent phospholipids, by using RP-HPLC with methanol/2-propanol (95:5) and acetonitrile/2-propanol as the developing solvents [373]. Peaks were identified by extrapolation of retention times from standards. A minimum of 69 diacyl species were identified. Bayon *et al.* [240] separated the sn-1,2-DAG moieties, released by PLC from the GLP of human placenta, by reversed-phase HPLC on 5-μm Ultrasphere ODS columns, following preparation of the 3,5-DNB derivatives. The species were resolved by isocratic elution with acetonitrile/2-propanol (90:10). A similar method of resolution of the molecular species was applied by Bayon *et al.* [374] to the sn-1,2-DAG, released by PLC from human platelet membrane GPL. Xu and Siegenthaler [375] identified the molecular species of PtdGro of photosynthetic membranes by analyses of the sn-1,2-DAG moieties, released from the PtdGro by hydrolysis with PLC, by RP-HPLC of the dinitrobenzoates. A new equivalent carbon number value (ECN), which takes into consideration the number of *cis*-(nc) and *trans*-(nt)

double bonds per molecular species was defined as ECN=CN-2nc-nt, where CN is the number of carbon atoms in the aliphatic residues. Thevenon *et al.* [376] resolved the molecular species of GPL by applying fluorometric detection (ex: 360 nm, em: 460 nm) of anthroyl derivatives of diradylglycerol species. DAG species were obtained by PLC treatment of PtdCho sub-classes and PtdOH, extracted from rat thymocytes. Prior to RP-HPLC separation, the 9-anthroyl derivatives of the diradylglycerols were separated into the acyl, alkyl, and alkenyl sub-classes by TLC. The anthroyl DAG species were separated by RP-HPLC with acetonitrile/2-propanol (90:10) and detected by fluorescence at 460 nm (ex: 360 nm). The 9-anthroyl derivatives of the alkylacyl- and alkenylacyl-glycerols were resolved into molecular species with a similar HPLC column and solvent system.

Normal-phase HPLC provides special advantages for the resolution of diastereomeric diradylglycerol naphthylethyl urethanes [104]. These derivatives yield excellent ESI spectra in the form of strong signals for the parent ions, which are adequate for the identification of molecular species of the resolved sn-1,2-diradylglycerol derivatives [106]. Mixtures of racemic diradylglycerols have been resolved by NP-HPLC as the diastereomeric napthylethyl urethanes by Santinelli *et al.* [377]. The diastereomeric DAG derivatives were prepared by reaction of rac-1,2-DAGs with (*S*)-(+)-1-(1-naphthyl)ethyl isocyanate and 4-pyrrolidinopyridine. The diastereomers were resolved on two columns of silica gel (Hypersil, 25 cm × 4.6 mm ID) in series, using 0.4% *n*-propanol (containing 2% water) in hexane as mobile phase. Complete separations were obtained for the X-1,3-DAG and sn-1,2- and sn-2,3-DAG, which were eluted in that order. Agren and Kuksis [106] have combined the resolution of the diastereomeric DAG on NP-HPLC with an on-line ESI-MS analysis of the peaks for a complete determination of the molecular species of the sn-1,2- and sn-2,3-DAG moieties of plasma and liver TAG, from which the molecular species of the original TAG could be calculated. The separations of the rac-1,2-DAG were carried out with both (*R*)-(−) and (*S*)-(+)-1-(1-naphthyl)ethylurethane derivatives. The diastereomeric sn-1,2- and sn-2,3-DAG were resolved on two Supelcosil LC-Si (5 μm, 25 cm × 4.5 mm ID) columns in series by isocratic elution with 0.37% 2-propanol in hexane at a flow-rate of 0.7 mL/min. The DAG were detected by UV absorption at 280 nm and identified by ESI/MS in the positive-ion mode, following post-column addition of chloroform/methanol/30% ammonium hydroxide (75:24.5:0.5) at 0.6 mL/min. Kim *et al.* [378] reported a general method for the determination of the optical purity of 1,2- (or 2,3-) di-*O*-acyl-sn-glycerol, which involves the chemical transformation to the key compound, 3- (or 1-) *O*-*tert*-BDMS-*O*-(+)-TBMB-sn-glycerol. [TBMB = (*S*)-(+)-2-*tert*-butyl-2-methyl-1,3-benzodioxole]. The chiral di-*O*-acylglycerols were first silylated and the acyl groups were removed by Grignard degradation to 3-(or 1-)*O*-*tert*-BDMS-sn-glycerol. Subsequent fluorescent labeling by (+)-TBMB−COOH gave the key compound without causing acyl migration. The diastereomers were separated by silica gel TLC or NP-HPLC with fluorescence detection. This was found to be an extremely sensitive method for determining the stereo-selectivity of lipase reactions. Lin *et al.* [220] resolved the molecular species of acylglycerols (TAG and DAG) by the use of a RP-C_{18} column (25 × 0.46 cm, 5 μm, Ultrasphere C_{18}, Beckman) with a linear gradient, from 100% methanol to 100% 2-propanol in 40 min. Another gradient from 100% methanol to 100% 2-propanol in 40 min was used for the identification of 1,2-dioleoyl-sn-glycerol. Unexpectedly, certain reverse isomers of the sn-1,2-DAG may be effectively resolved

by RP-HPLC as the DNPUderivatives. Itabashi *et al.* [107] reported baseline resolution of several pairs of synthetic DAG, made up of fatty acids with unequal chain lengths: *e.g.*, 1-18:1-2-20:4 and 1-20:4-2-18:1 glycerols, or 1-16:0-2-22:6 and 1-22:6-2-16:0 glycerols. Itabashi and Kuksis [126,127] have since reported similar resolutions of reverse isomers of individual molecular species of DAG, derived from natural GPL.

Hartvigsen *et al.* [181] reported the regio-specific analysis of neutral ether lipids by LC/ESI-MS, using a single quadrupole instrument. The 1-*O*-alkyl-sn-glycerols were analyzed as the diacetoyl derivatives, and showed $[M-\text{acetoyl}]^+$ion as an important diagnostic fragment. The diagnostic ions of directly analyzed 1-*O*-alkyl-2-acyl-sn-glycerols and 1-*O*-alkyl-3-acyl-sn-glycerols were the $[M-\text{alkyl}]^+$, $[M+H-H_2O]^+$, and $[M+H]^+$ions. Regio-specific characterization of the fatty acid position was evident from the relative intensities, as the sn-2 species had relatively high $[M+H]^+$ion intensities compared with $[M+H-H_2O]^+$, whereas the reverse situation characterized the sn-3- species. The diagnostic ions of directly analyzed 1-*O*-alkyl-2,3-diacyl-sn-glycerols were $[\text{M-alkyl}]^+$, $[\text{M-sn-2-acyl}]^+$, and $[\text{M-sn-3-acyl}]^+$ions. RP-HPLC of the ether compounds was performed on an HP ODS Hypersil column (5 μm, 200 × 2.1 mm ID), using an elution system adapted from Kim *et al.* [379]. Reference compounds were eluted isocratically with 100% Solvent A for 3 min, followed by a linear gradient to 100% Solvent B in 25 min, and held for 6 min before returning to 100% Solvent A. The composition of Solvents A and B varied with the exact nature of the alkylglycerol. However, 30% ammonium hydroxide (0.5%, by vol.) was added to all solvents made up of methanol/water and methanol/hexane. RP-LC/ESI-MS was performed, as described by Ravandi *et al.* [171]. Itabashi *et al.* [380,381] have combined chiral-phase resolution of rac-DAG as DNPU derivatives with high-temperature GLC resolution of the molecular species of the enantiomeric DAG, following silolysis of the DNPU derivatives. In parallel studies, the molecular species of the enantiomeric DAG, resolved on the chiral columns, were identified and quantified by on-line ESI-MS [382].

Marai *et al.* [383] reported the detection of the DNPU derivatives of DAG as the negative chloride-attachment fragment-ions, which increased the sensitivity and specificity of analysis of the solutes about 100 times over PCI/MS detection. For this purpose, a chiral column, containing the *R*(+)-naphthylurethane polymer was eluted with an isocratic mixture of solvents containing 1% DCM, which provided the chloride ions. Norman *et al.* [384] have described techniques for the semi-preparative isolation of sulfoquinovosyl-DAG from plant leaf-tissue lipid extracts, their resolution, and analysis of component molecular species. Sulfoquinovosyl-DAG was separated from phospholipids in a polar fraction by isocratic NP-HPLC with detection at 208 nm. Using heptane/2-propanol/0.001 *M* KCl (40:52:8) as the eluent, the molecular species of the purified sulfoquinovosyl-DAG were separated by RP-HPLC on a C_{18} column. Kim *et al.* [385] have described the structural characterization of sulfoquinovosyl, monogalactosyl, and digalactosyl DAG from *Cyanobacteria* by FAB-CID-MS/MS. The lipids were isolated by conventional solvent extraction and were purified by two-dimensional TLC, using $CHCl_3$/MeOH/H_2O (65:25:4) as first dimension and $CHCl_3$/acetone/MeOH/HOAc/H_2O (10:4:2:2:1) as the second dimension. The analyses were performed on a JMS-HX 110/110A MS/MS instrument with an E1B1E2B2 configuration. The resolution of MS-1 was so adjusted that only the 12C-species of the precursor ions was transmitted while MS-2

was operated at a resolution of 1000. Kim *et al.* [386] have followed up this work by describing direct and rapid structural identification of the molecular species of the major glycerolipids in the total extract of a *Cyanobacterium*, including sulfoquinosyl DAG, monoagalatosyl DAG, digalactosyl DAG, and PtdGro. In addition, four major lysoglycerolipids, corresponding to their MAG types, were isolated, and structural analyses were performed.

Closely related to the separation and quantification of molecular species of diradylglycerols are the separations of ceramides. Thus, esterification with 6-methoxy-α-methyl-2-napthaleneacetic acid and anthroyl, and the subsequent detection of the fluorescent or UV-absorbing products after HPLC permitted quantification [387,388]. Gu *et al.* [389] performed a precursor-ion scan for a specific ceramide fragment, resulting from CID. However, the addition of a sole unnatural ceramide species as internal standard did not allow quantification. Liebisch *et al.* [390] have overcome the problem of using multiple internal standards in flow-injection ESI-MS/MS by the use of a constant concentration of the not naturally occurring internal standard C_8-CER, together with a calibration curve, established by spiking different concentrations of naturally occurring ceramides.

17.5.2.2 Triacylglycerols

Mixtures of natural TAG have been resolved by high-temperature GLC on nonpolar capillary columns, based on the molecular weight or carbon number of the species, and by RP-HPLC on the basis of carbon number and degree of unsaturation [1]. Subsequently, TAG, in the range of 24 to 66 carbons, have been resolved by high-temperature GLC on nonpolar capillary columns [391,392]. For optimum carbon-number resolution, the TAG mixtures were hydrogenated. The TAG peaks representing odd carbon numbers generally overlapped with alkyldiacylglycerol peaks of the same carbon number [393]. Oku *et al.* [394] have reported the accumulation of 1-*O*-alkyl-2,3-diacylglycerols in cultured rat keratinocytes by TLC and GLC of nicotinylidene derivatives of deacylated products of the alkyldiacylglycerol. Kawashima *et al.* [395] used a DB-1ht capillary column (0.25 mm × 5 m, J&W Scientific) to analyze medium-chain-length TAG species. The column temperature was programmed from 120–270 °C at 25 °C/min and from 270–360 °C at 4 °C/min. Santinelli *et al.* [377] employed silver-ion HPLC to fractionate the TAG of olive oil according to the degree of unsaturation, the fractions being collected *via* a stream splitter and used for stereospecific analysis *via* their diastereomeric naphthylethyl urethanes. Winter *et al.* [396] used HPLC and GLC techniques in combination with silver-ion HPLC to resolve human milk TAG into 14 fractions by silver-ion HPLC with ELSD. Subsequent fractionation by RP-HPLC/ELSD resulted in 75 subfractions. The major 48 fractions were analyzed by GLC for their intact TAG and FA composition. Using a constrained nonlinear optimization computer program, the FA compositions and abundances of 170 different FA combinations in the TAGs were calculated. Kemppinen and Kalo [397] fractionated the TAG of lipase-modified butter oil into saturated, monoenoic, dienoic, and trienoic fractions by silver-ion HPLC and examined the recovered fractions by high-temperature GLC, using polarizable liquid-phase

(65% phenylmethylsilicone, OV-22, Quadrex) columns (25 m × 0.25 mm ID). The column temperature was programmed from 200 °C to 360 °C with He as carrier gas. Near-baseline resolution was obtained for the complex molecular species making up each unsaturation class. Ruiz-Gutierrez *et al.* [398] used a short 400 65 HT aluminum-clad silica capillary column, coated with OV-22, to separate the TAG of human very-low-density lipoproteins (VLDL) on the basis of molecular weight and degree of unsaturation. The molecular association of the FA in the TAG and alkyldiacylglycerols of primate milk fat were determined [393] by GLC on a short (25-m) capillary column (coated with OV-22). Similar GLC columns have been used for the resolution of the molecular species of human plasma TAG [95]. Evershed [96] reported the high-resolution analysis of TAG mixtures by high-temperature GC/MS with a polarizable stationary phase and NICI.

Demirbuker *et al.* [399] characterized the molecular species in TAG of a seed oil by RP-HPLC, micropacked argentation SFC, direct-inlet MS, and GC/MS of the picolinyl esters of the constituent FA. Analytical RP-HPLC with ELSD of the TAG was performed on Ultrasphere 100 (ODS, 5 μm, 150 × 4.6 mm). A binary solvent gradient from (A) acetonitrile to (B) acetonitrile/ethanol/*iso*-octane (2:2:1) within 60 min was applied. Preparative RP-HPLC was performed on Lichrosphere 100 RP 18 (5 μm, 250 × 10 mm) with methanol/acetone (1:1) as the mobile phase. SFC with UV detection at 210 nm was carried out on a fused-silica column (330 mm × 250 μm), packed with Nucleosil 5 SA and impregnated with $AgNO_3$. Carbon dioxide/acetonitrile/2-propanol (92.8:6.5:0.7) was used as the mobile phase. The study demonstrated an excellent selectivity of packed, fused-silica, argentation SFC for the separation of TAG. Sundin *et al.* [400] reported the presence of chlorinated TAG in fish lipids and performed MS studies on model compounds.

Fabien *et al.* [401] achieved quantitative separation of TAG from C_{24} to C_{54} by HPLC with refractive-index detection (Model 7512, Erma Inc.) and an eluent composed of propionitrile and butyronitrile (80:20). Chromatography was performed on three columns in series [one Phenomenex Ultremex C_{18} (3 μm, 150 × 4.6 mm) and two Waters Novapak C18 (4 μm, 150 × 3.9 mm)]. The validity of the response-factor calculations were tested by analysis of primary standard mixtures of saturated TAG. Bergqvist and Kaufmann [402] reported a statistical experimental design and multivariate optimization of a RP-HPLC method for the analysis of TAG molecular species in natural oils. The optimal conditions found were: a C_{18} column eluted in a negative exponential gradient from acetonitrile/*iso*-octane (90:10) to acetonitrile/ethanol/*iso*-octane (40:35:25), at a flow-rate of 1.5 mL/min and a column temperature of 50 °C. A version of the equivalent-carbon-number concept, based on the Snyder polarity index, was used to identify the molecular species. Lamberto and Saitta [403] performed a principal-component analysis by FAB-MS of TAG in edible oils in order to differentiate among the most common edible oils by their spectra. Cationized TAG gave characteristic spectra for various oils, and the data were analyzed with the aid of chemometrics by using principal-component-analysis (PCA) to evaluate the differences. Twelve variables were reduced to two principal components, accounting for 82% of the total variance. A two-dimensional plot of 97 PCA scores allowed the differentiation of the edible oils.

Kawashima *et al.* [395] performed a HPLC analysis of synthetic TAG on a C_{18} silica column (4.6 cm × 200 mm), packed with Cosmosil 5C18-A2 (Nacalei). The mobile phase

was acetone/acetonitrile (1:1) at 40 °C, and the peaks were detected by a refractometer. Positional isomers of dicaproyl-γ-linolenoyl glycerol were analyzed on a Chrompack silver-ion column (4.6 × 250 mm), as described by Irimescu *et al.* [404]. Kermasha *et al.* [405] performed RP-HPLC separation of butterfat TAG, using two Spherisorb-ODS-2 columns (150 × 4.6 mm ID, Alltech) in series and eluting the TAG with a linear gradient, ranging from 20 to 50% $CHCl_3$/MeCN. The peaks were located by ELSD (Varex). To isolate TAG for positional analysis, semi-preparative runs were made on a Spherosorb-ODS-2 column (300 × 10 mm ID, pore size 5 μm, Alltech). Elution was performed isocratically with $CHCl_3$/MeCN (30:70) at a flow-rate of 3 mL/min for 60 min. Itabashi *et al.* [406] determined the positional distribution of short-chain fatty acids in bovine milk fat by chiral-phase separation of the derived sn-1,2- and sn-2,3-DAG DNPU. The short-chain TAG were isolated by NP-TLC from a molecular distillate of butter oil. The molecular species composition of the sn-1,2- and sn-2,3-DAGs was established by on-column LC/ES-MS in the chloride-attachment mode. The results demonstrated the exclusive association of the acetic and butyric acid residues with the sn-2,3- and X-1,3-DAGs of short and long chain-length. Regio-specific analyses of TAG with the use of allyl magnesium bromide as a Grignard reagent, followed by boric acid TLC of the sn-1,2(2,3)-DAG and sn-2-MAG were reported by Becker *et al.* [407].

Taylor *et al.* [408] performed detailed stereospecific analyses of the TAG from high-erucic-acid Brassicaceae seed oils, using Grignard-based deacylation, conversion of the sn-1, sn-2, and sn-3-MAGs to their di-DNPU derivatives, resolution of the di-DNPU-MAG by HPLC on a chiral column, transmethylation of each sn-di-DNPU-MAG fraction, and analysis of the resulting FAME by GLC. Taylor *et al.* [409] combined direct-probe EI-MS and ammonia-CI-MS/MS of TAG from *Arabidopsis* with stereospecific analysis, based on the chiral-phase HPLC resolution of the di-DNPU derivatives of the sn-2 and [sn-1 + 3] MAG, generated by Grignard degradation. Kawashima *et al.* [395] used the method of Becker *et al.* [407] to determine the regio-distribution of fatty acids in synthetic TAG. Hori *et al.* [410] reported molecular species analysis of polyunsaturated fish oil TAG by HPLC with FAB-MS. Baiocchi *et al.* [411] adopted capillary SFC with supercritical CO_2 and FID to separate and determine TAG in vegetable and fish oils. A standard mixture of 13 TAG was used to optimize the procedure, and their retention times were used as reference to identify peaks in real samples. SFC with FID has also proven superior to other methods for estolide analysis. Hayes and Kleiman [412] have reported a detailed TAG analysis of *Lesquerella* oil by silica column fractionation, followed by SFC. The oil, which is rich in C_{20} hydroxy fatty acids, was first separated into nine fractions on the basis of overall polarity, using hexane/ethyl acetate. SFC of the fractions was performed on a nonpolar 10 m × 50-μm SB-Methyl 100 (Dionex) column. An elution program that simultaneously increases column pressure and temperature was used to obtained extensive fractionation of the mono-, di-, and tri-hydroxylated and non-hydroxylated TAG species.

Manninen *et al.* [413] reported the separation of γ- and α-linolenic acid-containing TAG by capillary SFC, while Manninen *et al.* [414] used SFC to characterize TAG and fat-soluble vitamins in edible oils and fats. Myher *et al.* [393] combined reversed-phase HPLC analysis with stereospecificity investigations of TAG rich in long-chain polyunsaturated fatty acids. RP-HPLC of the polyunsaturated oils was performed on Supelcosil C-18 columns with ELSD. The eluent was a linear gradient of 10 to 90%

2-propanol in acetonitrile over a period of 70 min. The stereospecific analyses of the TAG were performed by chiral-phase HPLC, following Grignard degradation and preparation of DNPU derivatives [415]. Ruiz-Sala *et al.* [416] used RP-HPLC with gradient elution and ELSD to separate the TAG of milk fat. Quantification of partially resolved peaks in the chromatogram was accomplished by applying a peak deconvolution program. HPLC fractions were collected every 40 sec at the outlet of the column after 14 min, and the fatty acids were identified by GLC. Lin *et al.* [353] used a gradient of methanol/2-propanol for RP-HPLC with UV detection (205 nm) in the separation of synthetic TAG ranging from triricinoleoylglycerol to tristearoylglycerol. The method was applied in the investigation of TAG biosynthesis in the castor microsomal fraction. Myher *et al.* [417] used reversed-phase HPLC with direct CI-MS to identify and quantify the less common (isobaric) short-chain TAG in the most volatile 2.5% molecular distillate of butter oil, while Marai *et al.* [418] reported the use of RP-HPLC in combination with direct-inlet CI-MS for the identification of the uncommon TAG structures generated by randomization of butter oil. Spanos *et al.* [419] explored the utility of RP-HPLC in combination with desorption CI-MS and CI-MS/MS for the characterization of milk fat TAG. TAG of anhydrous bovine milk fat were separated by two C_{18} RP-HPLC columns, and eluates were monitored with ELSD. Fifty-eight fractions were resolved and analyzed by positive-ion *iso*-butane desorption CI-MS. The formation of protonated molecules and of major fragments, corresponding to the random loss of any one of the constituent fatty acids, readily identified acyl carbon numbers and the number of double bonds within each fatty acid. Byrdwell and Emken [150] coupled the APCI-MS method to RP-HPLC analysis of soybean oil TAG, various seed oils, and to the TAG of genetically modified canola varieties [151]. In a separate report, Byrdwell *et al.* [152] discussed the quantitative aspects of TAG analysis by APCI-MS, while Mottram and Evershed [153] discussed the structure analysis of TAG positional isomers by APCI-MS. Laakso and Voutilainen [154] used APCI-MS for the characterization of TAG rich in α- and/or γ-linolenic acids, following separation by silver-ion HPLC. The APCI-MS spectra were obtained on-line. Mass spectra of most TAGs exhibited abundant $[M + H]^+$and $[M-RCOO]^+$ions, which defined molecular weight and molecular association of fatty acyl residues in the TAG molecules. Silver-ion HPLC/APCI-MS provided extensive separation and structure identification of seed oil TAG from various oils.

More recently, HPLC with APCI-MS was employed by Mu *et al.* [155] to identify DAG and TAG in a structured lipid sample. The ammonia-adduct molecular-ion was the base-peak for TAG containing polyunsaturated fatty acids, whereas the DAG fragment-ion was the base-peak for TAG containing saturated and monounsaturated medium- and long-chain fatty acids. The most abundant ion for DAG was the molecular ion $[M\text{-}17]^+$. These distinctive differences between the DAG and TAG spectra were utilized for rapid identification of the acylglycerols in the sample of structured lipids. Kallio and Currie [156,157] showed that MS in the ammonia NICI-MS/MS mode is an efficient method for the analysis of TAG. The abundance of $[M-H]^-$ ions unambiguously defined the number of acyl carbons and the number of double bonds in the TAG of most fats and oils, as well as the proportions of different molecular species. Furthermore, the daughter-ion spectrum provides information on the fatty acid constituents and their distribution between the sn-2 and sn-1,3-positions of a TAG species. Laakso and Kallio [158] have optimized the MS

conditions for analysis of seed oils by NICI. Samples were introduced *via* direct-exposure probe into the ion source without prior chromatographic separation. Laakso *et al.* [159] used SFC with CO_2 and FID to obtain the carbon-number distribution of milk fat TAG on a nonpolar column. In parallel, ammonia NICI-MS/MS of TAG was performed as described [158].

Han and Gross [137] have described a rapid, simple, and reliable method for the quantitative analysis and molecular-species fingerprinting of TAG by ESI-MS/MS directly from chloroform extracts of biological samples. By exploiting a rapid loss of phosphocholine from choline GPL, in conjunction with neutral-loss scanning for individual fatty acids, overlapping peaks in the ESI mass spectra were deconvoluted, generating a detailed molecular-species fingerprint of individual TAG molecular species. The new method overcomes the problem of overlapping peaks from choline GPL, which requires chromatographic separation of lipid extracts prior to ESI-MS analyses. The method readily detects as little as 0.1 pmol of each TAG species from chloroform extracts and is linear over a 1000-fold dynamic range. A rapid quantification was facilitated by the development of an algorithm that identifies sensitivity factors. Bernard *et al.* [111] used MALDI-TOF-MS to resolve the molecular species of DAG in a synthetic mixture. All DAG produced mixtures of the corresponding sodium $[M + 1 + 23]^+$and potassium $[M + 1 + 39]^+$, whereas the molecular species itself, $[M + 1]^+$, could not be detected. The DAG were displaced from a matrix of DHB in ethyl acetate. Limb *et al.* [218] reported structural analyses of the molecular species of the monoacetyl-DAG, isolated from bovine udder, NMR techniques, and CID-MS/MS, coupled with FAB. CID of sodium-adduced molecular ions ($[M + Na]^+$) generated numerous types of product ions, providing information on the double-bond position of fatty acyl groups as well as fatty acid compositions. MS/MS analyses were carried out, using a JMS-HX110/110A tandem instrument with the $E_1B_1E_2B_2$ configuration previously described in detail by Kim *et al.* [386].

17.5.2.3 Oxo-acylglycerols

Neff *et al.* [420] have studied the autoxidation of soybean oil TAG by RP-HPLC. These authors reported that the peaks in chromatograms of autoxidized soybean oil represented the monohydroperoxides and hydroperoxy epidioxides, which were eluted in the order of their polarity. Kuksis *et al.* [421] identified the mixed hydroperoxide and core aldehyde derivatives of synthetic and natural TAG, following reaction with *tert*-BOOH and Fe^{3+}ions. The oxidation products were extracted with $CHCl_3$/MeOH, resolved by NP-TLC, and identified by RP-LC/MS, using a NICI-TSI and NICI-ESI interface. The TLC separations were performed with heptane/*iso*-propyl ether/acetic acid (60:40:4) producing a total of nine closely-spaced yellow bands. LC/MS was performed with a HP-1090 liquid chromatograph, equipped with a C_{18} Supelcosil RP-column (4.6 mm × 25 cm), which was developed with a gradient of 20–80% 2-propanol in methanol over 30 min. The HPLC column was connected to a HP Model 5985 quadrupole mass spectrometer, and a post-column stream of 0.2 *M* ammonium acetate/methanol (1:1) was added at a rate of 0.2 mL/min. The major TAG after a 2- to 4-h peroxidation of 18:2/18:1/16:0 and corn oil

contained one unmodified fatty acid in combination with 9-oxononanoic acid and a 9- or 13-hydroperoxy derivative of linoleic acid.

Neff and Byrdwell [422,423] characterized the autoxidation products of model TAG [OOO, LLL, and trilinolenoylglycerol (LnLnLn)] by HPLC, coupled with APCI-MS. They employed simultaneously APCI-MS/MS and ESI-MS/MS/MS, not only for the analysis of TAG in canola oil, but also of trioleoylglycerol oxidation products, and TAG positional isomers, separated by RP-HPLC, which resulted in the isolation and identification of trioleoylglycerol dimers. The same authors [423] then modified the HPLC and MS/MS conditions to permit isolation and identification of the trimers and tetramers of trioleoylglycerol as well as the dimers of tristearoylglycerol, formed during heating of the corresponding TAG. Viinanen and Hopia [424] modified the RP-HPLC method of Neff *et al.* [420] by using MeCN/DCM/MeOH (80:10:10) as the liquid phase and applied it to the analysis of TAG autoxidation products with UV and ELS detectors. The oxidation products of trilinolenin amd trilinolein gave similar profiles with both detectors, but triolein, the oxidation products of which were detected only by ELSD, did not produce conjugated double bonds that could be detected in the UV. By analyzing oxidized synthetic TAG, Sjövall *et al.* [212] established the HPLC retention times for many oxygenated TAG, including the core aldehydes. TAG and oxidation products were resolved by RP-HPLC on a Supelcosil LC-18 column (250 × 4.6 mm ID) with a linear gradient of 20–80% 2-propanol in methanol in 30 min. For HPLC with ELSD, a Hewlett-Packard Model 1050 liquid chromatograph was combined with a Varex II ELSD. Later, Sjövall *et al.* [213] employed these retention factors to identify *tert*-BOOH oxidation products of unsaturated vegetable oils. Previously unidentified peroxide-bridged *tert*-butyl adducts were found to accompany the oxidation products in significant amounts (5–50% of total oxidation products). Sjövall *et al.* [214,215] have since proceeded to identify and quantify a large number of the oxygenated TAG, including the core aldehydes, which they specifically detected as the DNPH along with the *tert*-BOOH adducts.

Steenhorst-Slikkerveer *et al.* [425] analyzed the nonvolatile lipid oxidation products in vegetable oils by NP-LC/MS. This resulted in a separation into classes of TAG oxidation products, such as epoxy-TAG, oxo-TAG, hydroperoxy-TAG, hydroxy-TAG, and "21/2 glycerides" (core aldehydes), which were identified by SIM. LC/MS was performed on a HP 1100 LC/MSD instrument, which was operated with a binary high-pressure gradient pump, an auto-injector, a thermostated column, and a diode-array detector (DAD). Downstream of the DAD, 0.15 m*M* NaI in EtOH/MeOH (1:1) was added at the rate of 100 μL/min to the eluate by means of another HPLC pump. The separations were performed on a Waters Diol column (200 × 3 mm ID), with mobile phases of hexane/methyl *tert*-butyl ether (MTBE) or hexane/2-propanol. When these lipid oxidation products were analyzed by RP-HPLC, the various species present were separated according to class [OOH-TAG, hydroxy-TAG (OH-TAG), epoxy-TAG, etc.] and size [OOH-LLL, OOH-LOL, OOH-OOL, etc.] [426]. For the analysis of oxidation products originating from a single polyunsaturated parent TAG species, RP-HPLC yielded excellent separations, while oxidation products of mixed TAG gave complex chromatograms. Kusaka *et al.* [427] reported compositional analysis of normal plant TAG and hydroperoxidized rac-1-stearoyl-2-oleoyl-3-linoleoyl-sn-glycerols by HPLC in combination with APCI-MS. TAG irradiated with a tungsten lamp (40 W) gave

characteristic fragment ions $[M-H_2O_2+H]^+$, $[M-H_2O+H]^+$, and $[M-R_1(R_3)COOH-H_2O+H]^+$, which could be used to discriminate between fatty acids in sn-1-(or sn-3-) and 2-positions by this method. Myher *et al.* [216] used NP-HPLC with on-line ESI-MS for the sensitive detection of oxygenated GPL and TAG. Ravandi *et al.* [171] analyzed synthetic lipid ester ozonides and core aldehydes of GPL and TAG by RP-HPLC with NI-TSI-MS and NP-HPLC with positive-ion ESI-MS.

Kurvinen *et al.* [428] prepared the core aldehydes of MAG by ozonization and reduction with triphenylphosphine, using the general method of Ravandi *et al.* [171]. The aldehydes were separated by TLC, and aldehyde-containing bands were visualized by spraying a sample strip on the plate with a Schiff base reagent. Two bands, corresponding to sn-1(3)- (R_F 0.07) and sn-2- (R_F 0.12) isomers of 9-oxo-nonanoylglycerol, were detected as a result of isomerization during ozonization. The structure of the 2-MAG aldehyde was further confirmed by preparing its DNPH derivative and analyzing the free aldehyde and the DNPH derivative by HPLC/ESI-MS. Kurvinen *et al.* [428] prepared Schiff bases from 2-[9-oxo]nonanoyl-glycerol (2-MAG-ALD) and various amino compounds. Schiff bases were synthesized in over 50% yield by reaction of 2-MAG-ALD with a two-fold molar excess of valine, lysine, and selected tripeptides. This reaction and reduction were carried out as described for the preparation of the PtdCho core aldehyde aducts with selected amino acids and peptides (see below). The Schiff bases of amino acids were resolved by NP-HPLC/ESI-MS in a HP 1090 liquid chromatograph, which was connected to a HP 5989A quadrupole mass spectrometer, equipped with a nebulizer-assisted ESI interface. The solvent system consisted of (Solvent A) $CHCl_3$/MeOH/30% NH_4OH (80:19.5:0.5) and (Solvent B) $CHCl_3$/MeOH/H_2O/30% NH_4OH (60:34:5.5:0.5) [428]. The column was eluted by a gradient of 0% B to 100% B in 14 min, and then holding. Reduced Schiff bases of 2-MAG-ALD and peptides were analyzed on a RP-column (ODS Hypersil, 5 μm, 100 × 2.1 mm ID) with a flow-rate of 0.5 mL/min of mobile phase, which was changed from 0.5% NH_4OH in H_2O/MeOH/hexane (12:88:0) to (0:88:12) in 17 min and holding for 3 min.

17.5.2.4 Sterols, steryl esters and their oxidation products

Kuksis [429] has recently reviewed the chromatographic analysis of non-cholesterol plasma sterols, including their oxidation products. Kritharides *et al.* [430] used a C_{18} RP-HPLC column and the isocratic solvent system of acetonitrile/2-propanol/water (44:54:2) with detection at 234 nm to identify cholesterol, cholesteryl arachidonate, cholesteryl linoleate, cholesteryl oleate, cholesteryl palmitate, and cholesteryl linoleate hydroperoxide, along with several unidentified oxidation products, two of which were later recognized as 7-ketocholesterol and cholesteryl linoleate hydroxide. Johnson *et al.* [431] have described a rapid screening procedure for cholesterol and dehydrocholesterol by ESI-MS/MS. For this purpose, they used the mono-(dimethylaminoethyl)succinyl (MDMAES) ester, which is a new derivative for rapid, mild, and sensitive ESI-MS/MS analysis of cholesterol and dehydrocholesterol. It is one order of magnitude more sensitive than the previous, most practical alternative, the *N*-methylpyridyl ether derivative. [25,26,26,26,27,27,27-$^2H_7$]-7-dehydrocholesterol was used as an internal standard for

quantification. Both free and total (following saponification) sterol measurements were made. Korytowski *et al.* [432] performed lipid hydroperoxide analysis by HPLC with on-line mercury cathode electrochemical detection. The authors prepared standard cholesterol hydroperoxides by dye-sensitized photo-oxidation of parent lipids, followed by HPLC separation of the products: 3β-hydroxy-5α-cholest-6-ene 5-hydroperoxide (5α-OOH); 3β-hydroxycholest-4-ene 6α-hydroxyperoxide (6α-OOH); 3β-hydroxycholest-4-ene 6β-hydroperoxide (6β-OOH); 3β-hydroxycholest-5-ene 7α-hydroperoxide (7α-OOH); 3β-hydroxycholest-5-ene 7β-hydroperoxide (7β-OOH); and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine hydroperoxide (POPC–OOH). Chromatography was accomplished on an Ultrasphere XL-ODS column (4.6 × 70 mm; 3-μm particles), using an Isco integrated HPLC system, interfaced with an EG&G-Princeton Model 420 electrochemical detector. The detector was equipped with a hanging-mercury-drop electrode (operating potential set at − 150 mV *vs*. Ag/AgCl). The mobile phase consisted of MeOH/MeCN/2-propanol/aq. 1 m*M* sodium perchlorate plus 10 m*M* ammonium acetate (72:11:8:9), which was spurged continuously with high-purity argon that had been passed through an OM-1 oxygen scrubber to reduce the O_2 concentration to <10 ppb, and then through a pre-saturating mobile-phase scrubber. The chromatogram of cholesteryl ester hydroperoxides from photo-oxidized cells showed the presence of 7α,7β-OOH, 5α-OOH, 6α-OOH, 6β-OOH, and the PtdCho-OOH family.

Plat *et al.* [433] used GC/MS to analyze oxidized forms of plant sterols in serum. They identified 7β-hydroxysitosterol and 7β-hydroxycampesterol along with 7β-hydroxycholesterol. Other oxosterols identified were: 7-ketositosterol, 5α,6β-epoxysitosterol, and 3β,5α,6β-sitostanetriol. The TMS ethers of the oxo-phytosterols in cyclohexane were analyzed on a Trace GC2000 (Thermoquest CE Instruments) gas chromatograph, equipped with a RTX5MS column (30 m × 0.25 mm ID, 0.25 μm), coupled to a GCQ-plus ion trap (Thermoquest CE Instruments). Using He as carrier gas, the oven temperature was programmed from 150 °C to 320 °C. It was shown that interference of oxycholesterols and oxyphytosterols in GC/MS identification was not a problem.

17.5.3 Glycerophospholipids

For the purpose of this discussion, PtdCho, PtdEtn, PtdSer, PtdIns, and PtdOH have been designated as neutral glycerophospholipids in distinction from PtdInsPs and glycosyl PtdIns, which have been considered as acidic GPL.

17.5.3.1 Neutral glycerophospholipids

Until recently, the main methods of resolving the molecular species of intact GPL were those based on the pioneering work of Patton *et al.* [434]. Beckman *et al.* [435] separated molecular species of choline-, ethanolamine-, and inositol-GPL and PtdOH by RP-HPLC, using the method originally described by Patton *et al.* [434], and compared them to the molecular species of the free DAG, isolated from a stem-cell line. Similar results had been obtained earlier by Liepkalns *et al.* [346,347] who compared high-temperature GLC profiles of the DAG moieties of the GPL and of free DAG. Bernhard *et al.* [436] resolved

the molecular species of PtdCho from gastric mucosa by RP-HPLC on a 25 cm × 4.6-mm-ID Apex II ODS column (Jones Chromatography) at 50 °C, using a mobile phase of MeOH/H_2O (92.5:7.5), containing 40 mmol/L choline chloride, at a flow-rate of 1 mL/min. Eluted species were quantified by UV absorbance at 205 nm and subsequent fluorescence detection (ex: 340 nm, em: 460 nm), after formation of mixed micelles in the presence of 1,6-diphenyl-1,3,5-hexatriene. An improved RP-HPLC method for separating and quantifying molecular species of intact PtdCho, PtdEtn, PtdSer, and PtdIns has been reported by Wiley *et al.* [237]. Tissue phospholipids were isolated by conventional solvent extraction and were resolved into lipid classes by chromatography on silica gel (LiChrosorb Si 100) with elution by 2-propanol/hexane/ethanol/1 m*M* ammonium phosphate/acetic acid (490:370:1165:25:0.4 or 490:370:60:80:0.4). The PtdEtn, PtdCho, and PtdIns fractions were re-dissolved in 0.5 to 2 mL MeOH/H_2O/MeCN (90.5:7.0:2.5) per g wet weight of tissue, and a portion of the resulting solution (100–500 μL) was injected into a 4.5 × 250-mm column, packed with octadecyl silica (5-μm particle size, Ultrasphere ODS; Rainin). The species were resolved by eluting the column with methanol/286 m*M* aq. choline chloride/acetonitrile (90.5:7.0:2.5), using a flow-rate of 1 mL/min instead of the 2 mL/min flow-rate recommended by Patton *et al.* [434]. For molecular species resolution of PtdSer, the column was eluted with methanol/25 m*M* KH_2PO_4/acetonitrile/phosphoric acid (90.5:7.0:2.5:0.1), to which 0.42 g choline chloride per 100 mL was added. The mixture is similar to that used by Patton *et al.* [434], except that phosphoric acid is used instead of acetic acid. The molecular species were identified on the basis of the retention time of standards. The fatty acid composition of each peak, in all cases resembled closely the composition determined previously by GLC and HPLC for the derived DAG moieties.

McHowat *et al.* [437] synthesized 37 different molecular species of diradyl GroPCho and determined their UV absorbance response factors at 203 nm in the 2- to 100-nmole range. The molecular species were resolved by RP-HPLC, using a modification of the gradient elution technique developed by Patton *et al.* [434]. Sample phospholipids were eluted for 30 min with a mobile phase of MeOH/H_2O/MeCN (87:6:7), containing 20 m*M* choline chloride (Solvent A), followed by a linear increase, over 60 min, to a mobile phase of MeOH/H_2O/MeCN (76:4:20), containing 20 m*M* choline chloride (Solvent B). The solvent composition was held constant at 100% Solvent B for an additional 30 min, after which the solvent composition was returned to 100% Solvent A in a linear fashion over 10 min. The stationary phase consisted of a 4.6 mm × 250-mm, 5-μm Ultrasphere-ODS (C_{18}) column with a C_{18} cartridge pre-column (Alltech). Kim and Salem [438] had shown earlier that a resolution of molecular species of PtdEtn and PtdSer can be obtained on a RP-HPLC column, along with a sensitive detection (342 nm), following preparation of the UV-absorbing trinitrophenyl (TNP) derivatives. Using an ODS column in combination with a solvent gradient of MeOH/10 m*M* NH_4OAc (84:16) and MeOH/10 m*M* NH_4OAc (87:13) over a period of 40 min, the molecular species of erythrocyte PtdSer as the TNP derivatives were resolved in the order of decreasing polarity. Kim *et al.* [379] later reported a combined RP-HPLC/ESI-MS procedure for analyzing aminophospholipid molecular species, following preparation of the DNP and TNP derivatives. Using 0.5% aq. NH_4OH/MeOH/hexane and a C_{18} column, a complex mixture of phospholipid molecular

species was fractionated, and mainly protonated or natriated molecular species were detected.

Abidi and Mounts [439] have demonstrated the advantages of a RP ion-pair HPLC technique for the separation of PtdSer molecular species. In this method, tetraethylammonium phosphate (TEAP), dissolved in a mobile phase of MeCN/MeOH/H_2O served as an ion-pairing reagent for the negatively charged PtdSer species. Without addition of TEAP to the mobile phase, the polar PtdSer species passed through an ODS column without retention on the stationary phase nor separation of its subcomponents. Using MeCN/MeOH/H_2O (70:22:8), containing 5 m*M* TEAP at pH 7, in combination with an ODSA column (NovaPak C_{18}, 300 × 3.9 mm ID, 4 μm). The peaks were detected at 208 nm in the low-UV-absorbing solvent medium. Abidi and Mounts [440] later showed that separations of molecular species of PtdSer may also be obtained by using a buffered mobile phase (*e.g.*, ammonium acetate) and other than conventional ion-pairing reagents. Abidi and Mounts [441,442] described RP-HPLC separations of glycerol esters of phosphatidic acids. A RP + ion-pair HPLC technique was developed to resolve molecular species of PtdGro and Ptd_2Gro. Homologs of quaternary ammonium phosphates (QAP) were used as counter-ions in a solvent system of acetonitrile/methanol/water. Retention characteristics of the lipid analytes were dramatically affected by varying the concentration and the structural type of QAP. An increase in concentration and size of the QAP resulted in proportional enhancements in solute retention.

Brouwers *et al.* [443] reported a quantitative analysis of PtdCho molecular species by RP-HPLC with ELSD. Isocratic resolution of molecular species was obtained by using two 5-μm end-capped Lichrosphere 100-RP18 columns (Merck) in series and a solvent composed of acetonitrile, methanol, and triethylamine in varying ratios (*e.g.*, 3:2 plus 1% triethylamine) at a flow-rate of 1 mL/min. The ELSD instrument was a Varex MKIII (Alltech). A combination of HPLC with MS or MS/MS allowed improved analyses of the molecular species of both the derived DAG moieties and intact GPL. Itabashi and Kuksis [444] used chiral-phase HPLC and ESI-MS to re-determine the stereochemical configuration of some natural and synthetic PtdGro. For this purpose, the synthetic and natural PtdGros were converted to their DNPU derivatives, which were separated by HPLC on two columns having chiral phases of opposite configuration, [(*R*)-(+)- and (*S*)-(−)-1-(1-naphthyl)ethylamine polymers]. The molecular species were identified by on-line NI-ESI-MS. Absolute configurations of the resolved peaks were assigned by comparison with the elution order of the corresponding 1(3)-monoacyl-sn-glycerol enantiomers as bis-DNPU derivatives on the same column. The results clearly showed that the PtdGro from plant phospholipids consisted of single *R*, *S*- isomers (1,2-diacyl-sn-glycero-3-phospho-1′-sn-glycerols), despite the presence of non-stereospecific phospholipase D.

Myher *et al.* [216] and Myher and Kuksis [178] have demonstrated that molecular species of natural and oxygenated GPL could readily be detected by NP-HPLC and on-line ESI-MS. The solvent system was a gradient of $CHCl_3$/MeOH/NH_4OH. Under the mild ESI conditions, protonated and sodiated molecular ions were seen as the ionization products without fragmentation. Subsequently, this technique was extensively applied to the analysis of the molecular species of PtdCho, lysoPtdCho, and sphingomyelin of plasma lipoproteins [171,232–236]. Rizea Savu *et al.* [445] combined NP-HPLC and APCI-MS

to develop a quantitative method for acyl-PAF, PAF, and related phospholipids. Positive mass spectra showed intense $[M + H]^+$ions, while CID of protonated molecular ions gave characteristic daughter-ions corresponding to a polar head-group. Detection limits of 0.1 to 0.3 ng injected were obtained by MRM. Antonopoulou *et al.* [446] reported the identification of a hydroxyl-PAF analog with a relative molecular mass of 703. The molecule was isolated by HPLC with 0.01 *M* NH_4OAc in $MeOH/H_2O$ (70:30) as eluent, and identified by ESI-MS/MS in combination with chemical and enzymatic treatments. Li *et al.* [447,448] analyzed molecular species of intact phospholipid classes, using capillary HPLC/continuous-flow liquid secondary-ion mass spectrometry (CFLSI-MS) on a JEOL HX110A double-focusing mass spectrometer. The HPLC column was a KAPPA Hypersil BDS C_{18} capillary column (100 mm × 0.30 mm ID). Ions were produced by bombardment with a beam of Cs^+ions (10 keV for the positive-ion mode and 15 keV for the negative-ion mode), with an ion-source accelerating voltage of 10 keV. Differentiation between the 1-*O*-alkyl-2-acyl and 1-*O*-alk-1′-enyl-2-acyl (plasmalogen) phospholipid species was accomplished by acid hydrolysis (2 *M* HCl at room temperature for 30 min). Li *et al.* [448] determined intact PtdIns molecular species, including the arachidonoyl-containing phospholipid species, using capillary HPLC/(CFLSI-MS). CFLSI-MS analyses were performed on a JEOL HX110A double-focusing mass spectrometer. The phospholipid classes isolated by HPLC were dried under vacuum and dissolved in 1 mL of methanol/2-propanol (80:20), containing 1.5% glycerol. The capillary HPLC flow was derived by splitting the main flow from a Waters 600MS HPLC pump to 3 μL/min, using an open split at a VALCO tee. The flow was directed through a VALCO injector with 10-μL loop and then through a KAPPA Hypersil BDS C_{18} capillary column (100 mm × 0.30 mm ID). The mass spectrometer was operated in the LSI-MS mode. Ion was produced by bombardment with a beam of Cs^+ions (10 keV for the positive-ion mode and 15 keV for the negative-ion mode), with an ion-source accelerating voltage at 10 kV.

Ramanadham *et al.* [449] used ESI-MS of intact phospholipid molecules to demonstrate that the most prominent components of all major GPL head-group classes in islet cells are arachidonate-containing species. Heikinheimo and Somerharju [450] used four different methods to determine the molecular-species composition of cellular PtdSer and PtdEtn: (1) PtdSer and PtdEtn were converted to trinitrophenyl (TNP)-derivatives, and the molecular species were separated by RP-HPLC, as described by Hullin *et al.* [451]. (2) PtdEtn was hydrolyzed to diradylglycerols by incubation with phospholipase C, and the diradylglycerols were converted to benzoyl derivatives, which were separated into diacyl, alkylacyl, and alkenylacyl subclasses. These were subjected to RP-HPLC for the resolution of molecular species, as described by Patton *et al.* [452]. (3) The isolated PtdSer and PtdEtn classes were subjected to ESI-MS/MS analysis, which allowed both qualitative and quantitative analysis [453]. (4) GLC analysis of the fatty acid methyl esters of the total PtdEtn and PtdSer fractions was performed. The identity and relative abundance of PtdSer and PtdEtn molecular species were confirmed by EI-MS/MS and, in the case of PtdEtn, also by RP-HPLC of diacyl, alkenylacyl, and alkylacyl subclasses as benzoyl diradylglycerols.

Hsu *et al.* [454] reported that lithiated adducts of PtdCho species are readily formed by adding lithium hydroxide to the solution in which phospholipid mixtures are infused into the ESI source. CID of $[MLi^+]$ ions of PtdCho yields MS/MS profiles that contain

prominent ions, representing losses of the fatty acid substitutents. These ions and their relative abundances can be used to assign the identities and positions of the fatty acid substituents of PtdCho species. Similar scans for monitoring parents of specific product-ions can be used to identify the fatty acid substituents of PtdCho species, and this facilitates identification of distinct isobaric contributors to ions observed in the ESI-MS total-ion current. Hvattum *et al.* [455] used NP-LC/NIESI-MS/MS to determine individual molecular species of PtdIns in salmon kidney. In all instances, 18:0/20:4 species made up 36 to 55% of total, with 18:0/22:6, 16:0/22:6, and 16:0/20:4 providing the other major molecular species, none of which exceeded 10% of the total. The intensity ratio of sn-1/sn-2 fragment-ions increased with increasing number of double bonds in the sn-2 acyl-chain, but was not affected by an increasing number of double bonds in the sn-1- acyl-chain of the species examined. The relative distribution of molecular species was determined by MRM of the carboxylate anion fragment from the sn-1-position. The assignment of the fatty acids to the sn-1- and sn-2- positions for PtdIns was easier than for other phospholipid classes. All identified species contained fatty acids with a chain-length of 16 or 18 carbons, paired with fatty acids having chain-lengths of 20 or 22. The sn-1 to sn-2 abundance ratio of the 16:0/20:4 GroPIns species was *ca.* 1.

Kurvinen *et al.* [456] used NP-LC/ESI-MS on a single-quadrupole spectrometer to determine the effect of feeding n-3 and n-6 fatty acid esters to guinea pigs on the molecular species of GPL in their brain, using a previously described method [232]. Dietary deficiency of n-3 fatty acids mainly affected the molecular species of alkenylacyl and diacyl GroPEtn and GroPSer, while PtdCho was affected less and PtdIns not at all. Uran *et al.* [143] reported quantitative analysis of PtdIns species in human blood by NP-LC/ESI-MS/MS. Chromatographic base-line separation between PtdIns and other GPL was obtained by HPLC on a diol column with a gradient of chloroform/methanol mixtures, containing 0.1% formic acid and titrated to pH 5.3 with ammonia and added 0.05% triethylamine. The species from each phospholipid class were identified by using MS^2 or MS^3, which gave characteristic lyso-fragments. Khaselev and Murphy [457] used RP-LC with on-line ESI-MS and MS/MS to characterize various oxidation products of the arachidonate plasmenyl phosphocholine, 16:0p/20:4-GroPCho, where p indicates an alk-1-enyl fatty chain. The HPLC analyses were performed on a 5-μm Columbus C-18 100 A (1.0 × 150 mm) column (Phenomenex), connected to a UV-PDA monitor on-line, just ahead of the ESI interface. HPLC was operated at a flow-rate of 50 μL/min, with a mobile phase consisting of MeOH/H_2O/MeCN (60:20:20) and containing 1 m*M* NH_4OAc as Solvent A, and 1 m*M* methanolic NH_4OAc solution as Solvent B, by elution with 0% to 100% B in 40 min, followed by isocratic elution with 100% B for 20 min. The Sciex AP III + triple-quadrupole mass spectrometer was operated in the positive-ion mode, with the orifice at +70 V, as previously described [458]. The oxidation products involved the sn-1-position alone (1-formyl-2-arachidonoyl lipids and lysophospholipid), oxidation products involving the sn-2-position alone (chain-shortened ω-aldehyde radyl substituents at sn-2), as well as products oxidized at both the sn-1- and sn-2-positions. The results suggested that oxidation with the radical initiator 2,2′-azo-*bis*(2-amidinopropane) hydrochloride undergoes unique and specific free-radical oxidation at the 1′-alkenyl position as well as oxidation of the double bond closest to the ester moiety at sn-2.

Taguchi *et al.* [459] used capillary LC/ESI-MS to analyze phospholipid mixtures from cultured cells. Analyses at four different cone voltages were combined with the HPLC retention times to identify different molecular species. Among them, negative fragment-ions at high cone voltage in negative-ion mode gave ions originating from FA and PCho, PEtn, and cyclic InsP, detected at specific retention times. Positive fragment-ions at high cone voltage in the positive-ion mode indicated ions, such as the diradylglycerol derivative of 1-alkyl or 1-alkenyl cyclic PtdOH from PtdEtn, and PCho from choline-containing phospholipids. The ions produced from positive molecular ions indicated choline-containing phospholipids, such as PtdCho, SM, lysoPtdCho, and PtdEtn. The negative molecular ions effectively indicated acidic phospholipids, such as PtdIns. By this method, more than 500 molecular species of phospholipids were obtained within a few hours immediately after extraction from culture cells with a chloroform/methanol (2:1) mixture. Baker *et al.* [460] have described a direct quantitative analysis of lysophosphatidic acid (LPA) molecular species by stable-isotope dilution LC/ESI-MS. This method utilizes a deuterium-labeled internal standard, LPA (18:0-d_{35}), and a single liquid/liquid extraction with acidic butanol that allows $>95\%$ recovery of LPA, followed by on-line NP-LC/MS. This protocol afforded the accurate, sensitive, and reproducible analysis of the individual 1-acyl-LPA species present in biological samples. Recently, molecular species of GPL have been successfully determined by flow-injection MS/MS. Han and Gross [138] and Kerwin *et al.* [139] showed that flow-injection ESI-MS with MS/MS offered a rapid method of determining molecular species of phospholipids without prior chromatographic separation. Han *et al.* [140] have identified and quantified (by means of an internal standard) the five major species of PtdIns in the total phospholipid extracts from platelets. ESI-MS of chloroform extracts of platelets in the negative-ion mode demonstrated the presence of multiple individual molecular species of PtdIns, *e.g.*, m/z 836 (16:0/18:1 GroPIns); 858 (16:0/20:4 GroPIns); 864 (18:0/18:1 GroPIns); 884 (181/20:4 GroPIns); and 886 (18:0/20:4 GroPIns), revealing a high proportion of AA-containing species. The method of Han *et al.* [140] was used by Williams *et al.* [141] to demonstrate loss of nuclear membrane phospholipid after re-perfusion of an ischemic myocardium.

Brugger *et al.* [142] have extended these investigations with the goal of a full characterization and quantification of membrane lipids in an unprocessed Bligh-and-Dyer extract from cells or subcellular structures by ESI-MS alone. For this purpose, Brugger *et al.* [142] adopted the use of a nano-ESI source, allowing the analysis of picomole or subpicomole amounts of polar lipids, along with the standard MS/MS techniques, such as parent-ion and neutral-loss scanning. Synthetic analogs with non-natural FA structures served as internal standards. This approach allowed the specific detection of PtdCho, SM, PtdIns, PtdEtn, PtdSer, PtdGro, and PtdOH, including their plasmalogen analogs in sample amounts, representing as few as 1000 cells. All individual molecular species within these lipid classes in unprocessed total lipid extracts could be detected in one analytical run. Chen [461] characterized the major molecular species of PtdSer in plasma by NI-FAB-MS/MS as 1-stearoyl-2-oleoyl-sn-glycero-3-phosphoserine and 1-stearoyl-2-arachidonyl-sn-glycero-3-phosphoserine. Kayganich and Murphy [129] have determined the time-dependent increase in incorporation of [$^{13}C_{17}$]-arachidonic acid into the arachidonate-containing GPL. Isotope incorporation values were obtained from the ratio

of ion intensity for the transitions of the major anions (A- to m/z 303) compared to the resultant labeled species ([$^{13}C_{17}$]A- to m/z 320). The apparent level of isotope incorporation was different for each of the major phospholipid classes.

Costello *et al.* [25] determined the molecular-weight distribution of the PtdGro and *O*-acyl PtdGro in *Trichomonas* by negative-ion liquid secondary-ionization mass spectrometry (LSI-MS). The fatty acyl groups within each moleclar species were assessed by CID-MS/MS. The LSI-MS spectra were recorded with MS-1 of a JEOL HX110/HX110 tandem mass spectrometer. Both species were found to contain primarily oleic acid in the sn-2-position. Other acidic lipids included inositol phosphosphingolipids and inositol diphosphosphingolipids. Koivusalo *et al.* [462] concluded that quantitative determination of molecular species of phospholipids, including PtdIns, can be obtained by ESI-MS only if proper attention is paid to experimental details, particularly the choice of internal standards. The molecular species of PtdIns were measured in cells with an ion-trap instrument (Esquire-LC, Bruker-Franzen Analytik) and a triple quadrupole instrument (Perkin-Elmer Sciex API 300). The lipids were dissolved in $CHCl_3$/MeOH (1:2) with or without NH_4OH (0.25–1%) or 0.1 m*M* NaCl, and were infused into the ESI source *via* a 50-μm-ID fused-silica capillary, using a syringe pump, at a flow-rate of 5 μL/min. With the ion trap, nitrogen was used as the nebulizing (at 5–6 psi) and drying gas (5–7 L/min at 200 °C). The most important novel finding of the study was that unsaturation of phospholipid acyl chains can have a major effect on instrument response, which was found to vary by as much as 40%, depending on the total number of double bonds and the lipid concentration. The structure of the polar head-groups was also found to have a major effect on the instrument response. In the negative-ion mode, the acidic phospholipids, including PtdIns and PtdOH, gave a much higher response than PtdEtn and PtdCho. The variation in instrument reponse, depending on phospholipid acyl chain-length, had been observed previously [142,145,463].

Karlsson *et al.* [254] used NP-LC/ESI-MS to analyze the major molecular species of PtdIns of gastric juice. The parent ions of the major species submitted to MS/MS analysis revealed combinations of 16:0, 18:0, 18:1, 18:2, and 20:4 fatty acids. McPherson *et al.* [464] isolated PtdEtn, PtdCho, PtdSer, PtdIns, SM, and Ptd_2Gro by HPLC. The major phospholipid classes PtdEtn, PtdCho, PtdSer, and PPtdIns were collected, and ESI-MS was used to determine the molecular-species composition, including the alkylacyl and alkenylacyl components. LysoPtdOH represents a potential biomarker for certain cancers. To improve the accuracy and increase the sensitivity and specificity of the assay, Xiao *et al.* [465] have developed an ESI-MS-based mass spectrometric analysis of PtdOH, lysoPtdIns, lysoPtdSer, and lysoPtdCho, detected with high sensitivity (in the low pmol range) by this method. PtdOH and closely related lysophospholipids, isolated by TLC, were analyzed directly by ESI-MS. This test allowed assays in the 5- to 300-pmol range. The test was at least 50 times more sensitive when MRM was used. Gunnarsson *et al.* [466] used HPLC with ELSD or on-line ESI-MS to determine PtdEt formed in the presence of ethanol in human plasma. Separation was performed on a diol column with a NP-binary gradient system. The molecular species of PtdEtOH were the same as those of PtdCho found in the same blood sample. The ELSD instrument was a Sedex Model 45.

Schiller *et al.* [161] have applied positive MALDI-TOF to the analysis of the molecular species of PtdCho from egg yolk and plasma lipoproteins, recorded in the presence of

CsCl. The mass spectrum could be divided into two sub-spectra, since all peaks between *ca.* m/z 750 and 820 are caused by proton and sodium adducts, whereas the peaks at higher masses are due to the cesium adducts. The spectra indicated that 1-stearoyl-2-arachidonoyl GroPChol (m/z 942.5) is only a minor component in the egg yolk PtdCho and that the higher estimates result from the sodium adduct (m/z 810.6). Analogous changes were reflected in the peaks attributed to 18:0/18:2 and 18:0/18:1 GroPCho. Schiller *et al.* [161] have also recorded the MALDI-TOF mass spectra of the PtdCho of LDL and HDL and have shown that they are composed mainly of 16:0 and 18:0 acid as saturated residues, whereas 18:2, and, to a smaller extent, 18:1 and 20:4 acid residues contribute to the unsaturated fatty acids. The preparation of the Cs adducts avoided ambiguous assignments and yielded mass spectral data in complete agreement with FA analyses. Marto *et al.* [160] had shown earlier that different species of phospholipids can easily be investigated by MALDI-TOF-MS by using Fourier-transform ion-cyclotron resonance spectrometry.

17.5.3.2 Acidic glycerophospholipids

Only PtdInsP and glycosylated PtdIns are discussed under acidic glycerophospholipids. Until recently, no TLC system was available for the simultaneous resolution of all known isomers of the inositol phosphatides and PtdInsP_3. Auger *et al.* [467] resolved GroPInsP by anion-exchange HPLC on a Partisphere SAX column (Whatman), using a method adapted from Whitman *et al.* [468]. [^{32}P]GroPInsP, generated *in vitro* from anti-phosphotyrosine immunoprecipitates of platelet-derived growth factor (PDGF)-stimulated cells were de-acylated and analyzed as described. Baseline separations of all of the GroPInsPs were achieved by HPLC on a high-resolution 5-μm Partisphere SAX column (Whatman) with a shallow, discontinuous salt gradient [21]. Dried, de-acylated samples were re-suspended in 0.1–05 mL of 10 m*M* $(NH_4)_2HPO_4$ (adjusted to pH 3.8 with H_2PO_4) and applied to the column for analysis. The HPLC column was equilibrated with H_2O prior to sample loading and was eluted with a discontinuous gradient of up to 1 *M* $(NH_4)_2HPO_4.H_2PO_4$ (pH 3.8) at a flow-rate of 1 mL/min. The gradient was established from 0 to 1.0 *M* $(NH_4)_2HPO_4.H_2PO_4$ over 115 min. To develop the shallow gradient required for these separations, dual pumps were used. Pump A contained H_2O; Pump B contained 1.0 *M* $(NH_4)_2HPO_4.H_2PO_4$.

Jones *et al.* [62] have been able to achieve baseline separation of the three radiolabelled deacylated isomers of PtdInsP_2 {[^{32}P]GroPIns(3,5)P_2, ([^{32}P]GroIns(3,4)P_2, and [^{3}H]GroPIns(4,5)P_2}, known at this time. Separation of all deacylated phospholipids was performed by HPLC on a Partisphere SAX column (4.6 × 235 mm, 5 μm, Whatman) with a gradient of 0 to 1 *M* ammonium phosphate (pH 3.75) over 120 min. The gradient consisted of 0 to10 min 100% for Pump A, 10–70 min linear rise to 2% for Pump B, 70–120 min steep linear rise to 100% for Pump B. The pump and column rinse was from 120 to 130 min with 100% for Pump A (H_2O). Radiolabeled de-acylated phospholipids were detected by on-line radiochemical monitoring (Beckman Instruments and Berthold). Nasuhoglu *et al.* [27] have described the quantification of de-acylated PtdInsP by anion-exchange HPLC with suppressed-conductivity measurements. The major anionic head-groups could be quantified in single runs with practical detection limits of about

100 nanomol, and the D3 isoforms of PtdInsP and $PtdInsP_2$ were detected as shoulders on peaks. The HPLC system and columns were from Dionex. The NaOH gradient was optimized to separate the major anionic phospholipid head-groups in single runs. After injection, the elution was carried out in four stages: (1) 1.5 m*M* to 4 m*M* NaOH (0–7 min.); (2) 5 to 16 m*M* NaOH (7 to 12 min.); (3) 16 to 86 m*M* NaOH (12 to 30 min.); and finally (4) isocratic 86 m*M* NaOH (10 min.).

Michelsen *et al.* [146] have demonstrated that PtdInsP and $PtdInsP_2$ give prominent singly and doubly negatively charged de-protonated molecules in ESI-MS. These ions could be used for quantification of PtdInsP and $PtdInsP_2$ in the low-picomole range, without prior chromatographic separation by means of SIM and consecutive measurements of the signals from the de-protonated singly charged molecules. The dose/response curves for both compounds are linear. In a complex matrix, consisting of polar lipids (Folch extract), PtdIns and $PtdInsP_2$, monitored at m/z 965.4 and 1045.5 (stearoyl and arachidonoyl), were determined in the low-picomole range, at a flow-rate of 100 μL/min. CID of PtdInsP and $PtdInsP_2$ with a mixture of xenon and argon at 25 eV, afforded identical high-mass ions, formed by loss of a molecule of water from PtdInsP, and a phosphate group and a molecule of water from $PtdInsP_2$. Gunnarsson *et al.* [60,466] combined NP-HPLC separation of PtdInsP with LC/ESI-MS. For this purpose, a Gilson HPLC system was combined with a Quattro II mass spectrometer (Micromass), equipped with pneumatically assisted ESI and APCI sources. The HPLC columns (100 × 2 mm ID) were packed in the laboratory with 5-μm silica (Spherosorb) at 950 bar. The columns were developed with solvent mixtures consisting of (A) $CHCl_3$/MeOH/NH_4OH (50:45:3) and (B) $CHCl_3$/MeOH/H_2O/NH_4OH (25:55:17:3). A linear gradient was run. The HPLC effluent entered the mass spectrometer through an electrospray capillary, set at −2.6 kV and 120 °C. The PtdInsP in the HPLC eluate were directly admitted to the ESI-MS system. The mass spectra of a Folch-type brain extract showed m/z 885, 965, and 1045, representing PtdIns, PtdIns(4)P, and PtdIns (4,5)P_2, respectively.

Schiller *et al.* [162] have recently reported the application of MALDI-TOF-MS for the analysis of PtdInsP. It was shown that in a matrix of DHB, the molecular ions (M + 1) of the different PtdInsP are easily detected, even in complex mixtures, and thus, detailed data on the FA composition are provided. PtdIns(4,5)P_2 (triammonium salt from bovine brain) was obtained from Calbiochem as a lyophilized powder. It was dissolved in chloroform in a concentration of 30 g/L (*ca.* 30 μM). Two μL of 1 *M* HCl was added to a total volume of 10 mL PtdIns(4,5)P_2 solution to give a final concentration of 0.2 m*M* HCl. For all samples a 0.5 *M* DHB solution in methanol, containing 0.1% TFA was used (acetoacetate was also found to be suitable as solvent for the matrix compound). Schiller *et al.* [162] employed a Voyager Biospectrometry workstation (PerSeptive Biosystems). For all lipids under investigation, the spectra recorded in the negative mode contained a lower number of peaks, and all peaks were shifted by one (PtdIns) or two mass units (PtdInsP and $PtdInsP_2$) to masses lower than the positive-ion mass spectra. Spectra of negative ions also exhibit an enhanced signal-to-noise (S/N) ratio for PtdInsP and $PtdInsP_2$, whereas in the case of PtdIns, the positive-mode spectrum gave a better S/N ratio. The differences between negative- and positive-ion mass spectra can be explained by the different number of hydrogen or sodium ions added to compensate for the negative charges. Naik *et al.* [42] purified GPIs of *Plasmodium* to homogeneity by TLC and HPTLC, and they determined

the detailed structures by biochemical degradation and MS. MALDI-TOF-MS was used, and spectra were acquired with a time-delayed extraction, as the avarage of 50 laser shots. The glycosyl PtdIns of the parasite differed from those of the host in that they contained palmitic (major) acid and myristic (minor) acids at C-2 of inositol, predominantly 18:0 and 18:1 at sn-1 and sn-2-, respectively.

Mueller *et al.* [164] investigated the PtdInsP from bovine brain, including PtdInsP, $PtdInsP_2$, dipalmitoyl $GroPInsP_3$, and stearoyl/arachidonoyl $GroP\text{-}InsP_3$. The spectra were acquired on a Voyager Biospectrometry DE workstation. The ion-chamber pressure was held between 1×10^{-7} and 4×10^{-7} torr. All measurements were made under delayed extraction with an extraction voltage of 20 kV. The samples were prepared in a matrix of 0.5 M DHB in methanol. The lipid signals were observed to decrease in lipid mixtures. Thus, the detectability of $PtdInsP_2$ was reduced by the presence of PtdCho. According to Muller *et al.* [164], MALDI-TOF-MS spectra cannot be analyzed quantitatively, if only the measured signal intensity is taken into account, because the signal intensity is influenced by factors that cannot be effectively standardized. However, internal standards of the same chemical nature as the substance of interest can be used to compensate for the external influences. Muller *et al.* [164] have introduced the use of the S/N ratio as a quantitative measure. This can be done semi-automatically, using the standard software packages available for commercial MALDI-TOF mass spectrometers. Andersson *et al.* [469,470] demonstrated the occurrence of monoacyl-diglucosyl-DAG and monoacyl-bis-glycerophosphoryl-diglucosyl-DAG in membrane of *Acholeplasma* by HPLC, using a modified version of the method described by Arnoldsson and Kaufmann [242], and by TLC on Silica Gel 60 to effect the polar head-group separation. The analyses were performed on a Lichrosphere 100 DIOL column (250 mm × 4.6 mm, 5-μm particle size) column (dihydroxypropyl-silica, Merck). The column was eluted at 75 °C with a linear gradient of 100% Solvent A (hexane/2-propanol/butanol/tetrahydrofuran(THF)/*iso*-octane/water, 64:20:6:4.5:4.5:1 to 100% Solvent B (2-propanol/butanol/THF/*iso*-octane/water, 75:6:4.5:4.5:10, plus 180 mg of NH_4OAc/L). The peaks were detected by ELSD (Sedex 45). The following lipids were resolved: DAG, MAMGlcDAG, MGlcDAG, MADGlcDAG, DGlcDAG, PG, MABGPDGlcDAG, and GPDGlcDAG.

17.5.3.3 Oxo-phospholipids

Zhang *et al.* [471] reported the first method for the analysis of intact hydroxy-eicosatrienoyl-sn-glycero-3-phosphocholine species by FAB/MS. They subsequently described a LC/TSI-MS technique for the analysis of phospholipid hydroperoxides [472]. Several investigators [216,232,473] have used TSI-MS and ESI-MS in combination with NP-HPLC for the identification of the major hydroperoxides and core aldehydes of PtdCho from oxidized plasma lipoproteins and atheroma tissue. The hydroperoxides and core aldehydes were resolved by NP-HPLC, using a gradient of $CHCl_3$/MeOH/NH_4OH [171, 172]. The isolation and chromatographic separation of PtdCho hydroperoxides, isoprostanes, and core aldehydes has been further described by Ahmed *et al.* [235,236], who used comparable LC/ESI-MS methods. Ravandi and Kuksis [474] have reviewed

these and other separations. Further applications of ESI-MS to the analysis of phospholipid hydroperoxides have also been reported [475–477].

Ponchaut *et al.* [478] used FAB-MS/MS to identify the molecular species of PtdCho of heart mitochondria. Detailed analysis of the negative-ion mass spectrum of PtdCho, isolated from mitochondrial fractions, revealed the presence of six carboxylate ions at m/z 267, 269, 271, 295, 297, and 299, which corresponded to the hydroxyhexadecadienaote, hydroxyhexadecenoate, hydroxyhexadecanoate, hydroxyoctadecadienaote, hydroxyoctadecadecenoate, and hydroxyoctadecanoate, respectively, as indicated by their fragmentation patterns. The presence of PtdCho, containing hydroxy fatty acids was attributed to a free-radical-initiated mechanisms. Detailed examination of the NI-FAB mass spectrum, combined with an analysis of product-ion spectra, obtained by CID of the high-mass ions that correspond to M-15, M-60, and M-86 ions of the PtdCho molecular species, allowed the determination of the FA association in the PtdCho molecule. Hall and Murphy [476] reported that NI-ESI-MS also gives information about the position of oxidation in the side-chains when a high-orifice potential is applied during the ionization process. However, the high potential causes in-source fragmentation of the phospholipid species, so that the parent-ion, as well as daughter-ions that correspond to the m/z for the carboxylate anions of the FA side chains, are observed. As an alternative to protonation, Teesch and Adam [479] have suggested the use of cations, such as Li^+, Na^+, or Ag^+. This ionization technique involves the formation of charged analyte complexes, formed by the addition of a suitable coordinated ion to the analyte [480]. Such coordinated-ion spray mass spectrometry (CIS-MS) has already proven useful for the analysis of complex mixtures of cholesteryl ester peroxides [481]. Milne and Porter [482] have reported an improved method for the separation of phospholipid hydroperoxides by RP-HPLC, which, along with CIS/MS, for the first time separates some of the hydroperoxide isomers. CIS-MS can be coupled with RP-HPLC by the addition of $AgBF_4$ to the mobile phase or to the column effluent, thus allowing powerful HPLC/MS techniques to be used for the identication of complex mixtures of phospholipid hydroperoxides. HPLC was performed with a Discovery ODS column (4.6×250 mm, 5 μm, Supelco) and operated with a mobile phase of MeOH/H_2O (95:5) at a rate of 1 mL/min. For LC/CIS-MS experiments, the hydroperoxides were isolated from the unoxidized phospholipid, using analytical HPLC (MeOH/H_2O, 95:5). The oxidized fraction of 16:0/18:2 GroPCho (13.5–15.5 min) or 16:0/20:4 GroPCho (20–28 min) was collected, concentrated, and analyzed.

Schlame *et al.* [483] have developed a novel technique for the quantitative analysis of phospholipids with oxidized acyl chains. The method involves the chromatographic enrichment of phospholipids with short acyl chains, derivatization with 9-(chloromethyl) anthracene and RP-HPLC with fluorescence detection. The method was suitable for measuring dicarboxylate-containing PtdCho. The plasma concentration of short-chain oxidized PtdCho, calculated from the fluorescence yield, was between 0.3 and 1.0 μmoles. Frey *et al.* [484] have improved this method by utilizing NP-HPLC after derivatization. This greatly enhanced the selectivity of the attached fluorescent label. Frey *et al.* [484] concluded that the fragmented PtdCho from human plasma contained a palmitoyl residue and a short fragment (C_4 or C_5), derived from either arachidonoyl or docosahexaenoyl chains, respectively. In other studies, the fragmented PtdCho, recovered from lipoprotein peroxidation, has been identified as the aldehyde, 1-palmitoyl 2-(5-oxo)valeroyl

GroPCho [226,473,485]. Podrez *et al.* [14,486] have recently reported the characterization of a structurally conserved family of oxidized PtdCho that serve as novel high-affinity ligands for cells stably transfected with CD36, mediating recognition of multiple oxidized forms of LDL. Specifically, four major, structurally related phospholipids with CD36-binding activity were identified from oxidized 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-sn-glycero-3-phosphocholine, and four corresponding structural analogs with CD36 binding-activity were identified from oxidized 1-hexadecanoyl-2-octadecadi-9′,12′-enoyl-sn-glycero-3-phosphocholine. Each was synthetically prepared and its structure confirmed by multilammellar NMR and high-resolution MS. LC/ESI-MS/MS studies demonstrated that oxidized PtdCho are formed during LDL oxidation by multiple, distinct pathways. The lipids were resolved with a ternary (MeCN/MeOH/H_2O) gradient, generated by a Waters 600 E multi-solvent delivery system, and monitored by ELSD. Mass-spectrometric analyses were performed on a Quatro II triple-quadrupole mass spectrometer (Micromass) equipped with anESI probe and interfaced with an HP-1100 HPLC instrument. Lipids, both free and derivatized, were resolved on a Luna C_{18} column (250 m × 4.6 mm ID, 5 μm, Phenomenex). A discontinuous gradient was prepared by mixing Solvent A (MeOH/H_2O, 85:15) with Solvent B (MeOH). A key methodological issue was the need to avoid intra-operative peroxidation. Quantification of the various oxidized PtdCho species was performed by LC/ESI-MS/MS in positive-ion mode, using MRM. Formic acid (0.1%) was included in the mobile phases.

Kamido *et al.* [473,487] used LC/TSI-MS to resolve and identify GPL core aldehydes, generated by OsO_4 oxidation of egg yolk PtdCho, followed by $NaIO_4$ cleavage. Both 16:0/9:0ALD and 18:0/9:0ALD GroPCho were generated. The core aldehydes were purified by TLC prior to LC/TSI-MS. Ravandi *et al.* [171] used NP-HPLC with on-line positive-ion LC/ESI-MS for the separation and identification of core aldehydes [16:0(18:0)/9:0 and 16:0(18:0)/5:0 GroPCho and GroPEtn], prepared from egg yolk PtdCho and PtdEtn by ozonization and reduction with triphenylphosphine. The HPLC column (Spherisorb, 3-μm, 100 × 4.6 mm ID, Alltech) was eluted with a linear gradient of 100% Solvent A ($CHCl_3$/MeOH/NH_4OH, 80:19.5:0.5) to 100% Solvent B ($CHCl_3$/MeOH/H_2O/NH_4OH, 60:34.5:5.5:0.5) in 14 min, then at 100% B for 10 min. The flow-rate was set at 1 mL/min. Itabe *et al.* [488] have reported a similar preparation of the core aldehydes, using ^{14}C-labeled and non-labeled 16:0/18:1 and 16:0/18:2 and 16:0/20:4 GroPCho, which yielded the 16:0/9:0 and 16:0/5:0 GroPCho, respectively. The TLC-purified C_9 and C_5 core aldehydes were subjected to RP-HPLC (Lichrosorb RP-18) and eluted with a gradient of Solvent A (MeOH/MeCN/H_2O, 616/264/120) and Solvent B (MeOH/MeCN, 70:30). The structures of the synthetic compounds were confirmed by MALDI-TOF-MS, recorded for the $[M + H]^+$and $[M + Na]^+$ions. Ou *et al.* [489] also have reported the identification and quantification of choline GPL that contain aldehyde residues by fluorometric HPLC.

17.6 TOTAL LIPID PROFILING

The complex nature and large number of molecular species with closely similar physico-chemical properties has made chromatographic separations the exclusive means of lipid profiling. Recently, this prerogative has been challenged by the soft-ionization

mass-spectrometric methods, which can provide the masses of the molecular species of various lipid classes without derivatization and prior chromatographic separation. Nevertheless, TLC, HPLC and high-temperature GLC continue to provide useful separations of total lipid extracts along with quantification, which, unexpectedly, continues to plague mass-spectrometric analyses.

17.6.1 Thin-layer chromatography and thin-layer chromatography/ time-of-flight-mass spectrometry

In the past, TLC has provided highly effective resolution of the most complex mixtures of lipids, and in combination with FID systems has rivaled the quantifications achieved with high-temperature GLC. However, serious limitations have been recognized, including lower sensitivity, nonlinear calibration curves, and variations between Iatroscan (Chap. 6) TLC rods, which have restricted the usefulness of the method [490]. Hydrogenation of the sample and automated sample spotting are recommended in order to improve quantification with the Iatroscan system. The most frequently utilized separation scheme involves several steps: the first two steps serve to develop and quantify the neutral lipids, the third to develop and quantify the more polar lipids with acetone as developer, and the fourth to develop phospholipids [491,492]. Improved TLC/FID procedures for neutral lipids have since been reported by Okumura *et al.* [493] and Striby *et al.* [494]. Okumura *et al.* [493] developed the Chromarods with three solvent systems in a four-step development technique. The first development was carried out in a solvent system of $CHCl_3$/MeOH/H_2O (57:12:0.6) until the solvent front had migrated about 2.5 cm, when the rods were air-dried at room temperature and then subjected to the second development in 1,2-dichloroethane/chloroform/acetic acid (46:6:0.05) until the solvent front had migrated about 9 cm. The second development was repeated (third development). The fourth development was carried out in *n*-hexane/diethyl ether/acetic acid (98:1:1) until the solvent front had migrated *ca.* 11.5 cm. The Chromarods were dried at 50 °C, and then scanned in the Iatroscan MK-5. Baseline resolutions were obtained for cholesteryl acetate, triolein, oleic acid, cholesterol, 1,2-diolein, and ceramides. Striby *et al.* [494] improved the procedures of Parrish *et al.* [491,492] by adding two elution steps: hexane/diethyl ether/formic acid (70:30:0.1) after TAG and free fatty acid (FFA) scan, and 100% acetone, followed by chloroform/acetone/formic acid (99:1:0.2) after the 1,2-DAG. This resulted in the separation of 1,2- and 1,3-isomers of DAG from each other and from free sterols in standards and in marine lipids. Complete separation of lipid classes was obtained with the new elution scheme, followed by a development of phospholipids with $CHCl_3$/MeOH/NH_4OH (50:50:5) to separate PtdGro/Ptd_2Gro, PtdEtn, and PtdCho. As many as 17 lipid classes could be identified and quantified in samples using the proposed six-step development. Kawashima *et al.* [395] have recently used TLC/FID to analyze structured (synthetic) triacylglycerols. A development with a mixture of hexane/ethyl acetate/acetic acid (90:10:1) permitted resolution and quantification of MAG, DAG, TAG, and FFA of medium and long chain-lengths.

Peyrou *et al.* [495] have proposed a TLC/FID method for the separation of MAG, DAG, TAG, and free oleic acid, which does not require chemical derivatization. Good separation of the mixture was obtained with hexane/diethyl ether/formic acid (65:35:0.04).

Cholesterol was used as internal standard. Hudson *et al.* [496] have described a new multi-dimensional chromatographic method in which material, separated into lipid classes on silica-coated quartz TLC rods, is desorbed and, after pyrolysis, introduced directly into a GC/MS system for molecular-species analysis. Steryl esters, wax esters, hydrocarbons, ketones, and FAME are thermally desorbed without pre-treatment. Free sterols, MAG, aliphatic alcohols, and FFA are converted to TMS derivatives on the rod. TAG and GPL are converted to FAME by thermochemolysis with NMe_4OH. HPTLC and, especially, two-dimensional HPTLC have greatly increased the resolution of lipid classes and molecular species, but quantification has been difficult [490]. Recent attempts to combine TLC with TOF-MS promise to revolutionize the quantification of the components resolved by TLC and HPTLC [169–171].

17.6.2 Gas-liquid chromatography and gas chromatography/mass spectrometry

It was recognized early in the development of high-temperature GLC that natural mixtures of neutral lipids consist of lipid classes and molecular species that are distributed over a wide range of molecular masses, well suited to separation on nonpolar columns. Using short columns with thin-film silicone packings and FID, it was possible to demonstrate quantitative recoveries of lipid classes, ranging from FFA to TAG. The high-temperature GLC method was especially well suited for the profiling of plasma neutral lipids, including free cholesterol and cholesteryl esters. Furthermore, by pre-digesting the total lipid extract with phospholipase C, the DAG and ceramides released from the phospholipids could also be accommodated in the total-lipid profile, the recording of which was automated. This methodology was later adopted to separations on short, nonpolar capillary columns and subsequently automated [497]. Yang *et al.* [92] extended its application to hydrogenated neutral lipids from marine samples. The use of relatively large solvent plug sizes (2–3 μL), fast injection rates (4–8 μL/sec), hydrogen carrier gas with high flow-rates (20 mL/min), and short capillary columns (5.5–7.5 m × 0.32 mm; 5% phenylmethylsilicone, 0.25-μm film thickness, J&W Scientific) enhanced recoveries of high-molecular-weight neutral lipids. Automated high-temperature GLC of hydrogenated samples was found to offer a precise tool for the measurement of highly unsaturated neutral lipid compounds (see also [498]). Evershed *et al.* [499] improved the usefulness of high-temperature lipid profiling by combining it with MS to derive detailed compositional information directly from extracts without chemically degrading them. Quantitative information was obtained by including an internal standard at the extraction stage [500], with processing of several thousand samples for the presence of lipids in organic residues [501].

Mondello *et al.* [502] have reported a multi-dimensional tandem capillary GLC system for the analysis of complex samples. A fully automated tandem GLC system was developed for multi-transfer operations. Although multi-dimensional GLC has been used for many years, the method had not been adopted for high-temperature use. An effective application of high-temperature GLC to the analysis of natural TAG on polarizable capillary columns by Geeraert and Sandra [93] prompted Kuksis *et al.* [94] to utilize such columns for plasma total-lipid profiling. The additional resolution provided by the column at higher temperatures allowed the inclusion in the total-lipid profile of the DAG and TAG

peaks, resolved on the basis of both molecular weight and degree of unsaturation. Evershed [96] has adopted high-temperature resolution of TAG mixture analysis on polarizable capillary columns to on-line MS with NICI. The use of NICI at an ion-source block temperature of 300 °C overcomes problems with interpretation of EI spectra, produced during high-temperature GC/MS. The NICI spectra of TAG, produced under these condition, contain abundant $[RCO_2]^-$, $[RCO_2\text{-}18]^-$, and $[RCO\text{-}19]^-$ ions, believed to be produced by nucleophilic gas-phase ammonolysis, which was used to identify the individual fatty acid moieties associated with peaks in the TAG total-ion chromatogram.

17.6.3 High-performance liquid chromatography and liquid chromatography/mass spectrometry

Separation and quantification of lipid fractions by HPLC and mass detection have been attempted by many laboratories. Of the various methods tried, only FID and ELSD have shown promise. Christie *et al.* [503] have reported that HPLC lipid profiling can be further improved by substituting for the silica gel phases chemically bonded stationary phases, such as propionitrile or polymeric vinyl alcohol as the functional moiety. Adsorption chromatography with silica gel gave disappointing results for plant glycolipids. The separations were obtained with a column (100 × 3.2 mm ID), packed with 3-μm Spherisorb S3CN (Hichrom) and another column (250 × 4.6 mm ID), packed with 5-μm YMC-Sil (Hichrom), using optimized gradient elution with Solvent A (*iso*-hexane/methyl *tert*-butyl ether, 98:2), Solvent B (2-propanol/chloroform/acetic acid, 82:20:0.01) and Solvent C (2-propanol/water/triethylamine, 47:47:6). Effective separations were obtained for steryl esters, TAG, sterols, acylsterol glycosides, steryl glycosides, monogalactosyl-DAG, cerebrosides, digalactosyl-DAG, PtdIns, PtdEtn, and PtdCho.

Marcato and Cecchin [504] have reported direct analyses of mixtures containing FFA, MAG, DAG, and TAG by HPLC, coupled to ELSD. The procedure was based on the use of a ternary-gradient HPLC instrument, equipped with ELSD and a RP-8 end-capped column. The mobile phase consisted of two consecutive binary gradients: MeCN/H_2O plus 0.1% HOAc and MeCN/DCM. Base-line separations were obtained for complex mixtures of FFA, MAG, DAG, and TAG. The method was fast and showed high sensitivity and selectivity, enabling the resolution of positional isomers of both MAG and DAG. Silversand and Haux [505] reported an improved HPLC method for the separation and quantification of lipid classes from fish lipid extracts. The separations were obtained on a NP-column (Lichrosphere 100 DIOL, 5 μm, 250 × 4 mm) and the lipids were detected by ELSD (Sedex 45). Polar lipids were resolved, following injection of a total lipid extract, by using two solvents in a binary gradient as follows: (A) hexane/2-propanol/acetic acid (82:17:1.0) and (B) 2-propanol/water/acetic acid (85/14/1.0). Triethylamine (0.08%) was added to the solvents. The neutral lipids, separated from the polar lipids by this solvent system, were collected and re-analyzed in a neutral solvent system: (A) hexane/acetic acid (99:1.0) and (B) hexane/2-propanol/acetic acid (84:15:1.0). The lipid peaks were identified by chromatography with added standards.

Liu *et al.* [204] reported a quantitative determination of MAG and DAG by NP-HPLC with ELSD. The 1,3-DAG were generally separated from the 1,2-DAG, but some 1,3-DAG of low molecular weight interfered with the 1,2-DAG of high molecular weight.

For MAG, the separations of isomers were optimized only between those pairs with identical fatty acyl groups. The results obtained by HPLC agreed well with those derived from GLC and SFC. Lin *et al.* [506] have reported non-aqueous RP-HPLC systems for the separation of 45 synthetic TAG and DAG. For these methods, linear gradients of methanol/2-propanol were used with UV detection at 205 nm. The elution order corresponded closely with chain-length, degree of unsaturation, and presence of polar groups. Murphy *et al.* [507] have described a quantitative HPLC separation of neutral lipid classes with a high degree of resolution and reproducibility. Specifically, an isocratic separation on a 5-μm Phenomenex Selectosil silica column was used, along with 1.2% 2-propanol in hexane, containing 0.1% acetic acid (90%) and *n*-hexane (10%) as the eluting solvent. The column temperature was maintained at 55 °C, which was critical for baseline resolution of 1,3-DAG and cholesterol.

17.6.4 Tandem mass spectrometry

Kim *et al.* [508] have reported a direct and rapid method for the structural determination of individual molecular species of each glycerolipid class by FAB-MS and FAB-MS/MS. The lipids in each class were quantified in comparison with the two internal standards of that class, using a correction curve extrapolated from the standards. The lipids chosen as internal standards were not present in measurable quantities in representative plant lipid samples. Molecular species of the following lipid classes were analyzed: PtdIns, PtdGro, PtdEtn, PtdCho, PtdOH, MGDAG, DGDAG, as well as lysoPtdEtn and lysoPtdCho. The relative abundance of the fatty acyl ions was used to designate the position of the acyl chain, because the acyl group in the 2-position generally produces the more abundant ion [458]. To verify the quantification of the MS/MS data, the amount and fatty acid composition of each head-group class, as determined by ESI-MS/MS, were compared with those determined previously, using traditional lipid analyses. Welti *et al.* [509] have established an automated ESI-MS/MS strategy for profiling quantitatively the molecular species of plant lipids and for determining the lipid changes in *Arabidopsis* after cold acclimation and freezing at sublethal temperature. From 1 mL of plant lipid extract, two aliquots were taken for MS analysis: one sample for phospholipid analysis and one for galactolipid analysis. For phospholipid and galactolipid analysis the samples were combined with appropriate internal standards, such that the ratio of $CHCl_3$/MeOH/300 m*M* NH_4OAc in water was 300:665:35 and of CHCl3/MeOH/50 m*M* NaOAc in water was 300/665/35. The samples were analyzed on a triple-quadrupole tandem mass spectrometer (Micromass Ultima), equipped for ESI. Precursors of lipid head-group-derived fragments were detected using appropriate scans. The mass analyzers were adjusted to a resolution of 0.6 atomic mass units full-width at half-height. For each spectrum, 12–42 continuum scans were averaged in multiple-channel analyzer mode. The phospholipid scanning modes were as described by Brugger *et al.* [142].

Ekroos *et al.* [510] have employed a hybrid quadrupole time-of-flight mass spectrometer with ion-trapping capability for quantitative profiling of total extracts of phospholipids. Simultaneous acquisition of precusor-ion spectra of multiple fragment-ions allowed detection of major classes of phospholipids in a single experiment. Relative changes in their concentration were monitored, using a mixture of isotopically labelled

endogeous lipids as a comprehensive internal standard. Precursor-ion scanning spectra were acquired simultaneously for acyl anions of major fatty acids in negative-ion mode and identified the fatty acid moieties and their relative position at the glycerol backbone in individual lipid species. The methodology is based on the ion-trapping and -bunching technology introduced in the QSTAR Pulsar mass spectrometer. Fragment ions of a given m/z can be trapped temporarily in the collision cell and then released as a short ion packets into the TOF analyzer. In this way, a 100% duty cycle can be achieved for any fragment ion, with significant improvement for ions with m/z close to the selected ion. This makes QSTAR Pulsar instruments attractive for lipid analysis, because inherent features of the TOF analyzer, such as high mass accuracy, resolution, and non-scanning acquisition of spectra, can help to overcome the limitations of conventional quadrupole mass filters. Profiling of a total extract of *E. coli* phospholipids (PtdEtn, PtdGro, and Ptd_2Gro) was reported.

17.7 CHEMICAL AND ENZYMATIC CHARACTERIZATION OF LIPIDS

It is frequently necessary to complement the chromatographic and mass-spectrometric analyses by chemical and enzymatic modification of the solutes. These transformations have proven to be especially important in the analysis of PtdIns phosphates.

17.7.1 Chemical methods

17.7.1.1 Complete hydrolysis

Chemical characterization of an unknown glycerolipid is likely to start with acid or alkaline hydrolysis of a purified chromatographic fraction. The water-soluble moieties, such as choline, ethanolamine, glycerol, serine, and inositol in phosphatides and plasmalogens may be determined by classical methods [265]. Modern chemical characterization of nitrogenous bases involves chromatographic isolation of the bases or appropriate derivatives and subsequent quantification by electrophoretic, spectrometric, or mass-spectrometric methods. Zhang *et al.* [511] have described capillary electrophoresis (at low pH) of free choline in plant leaves, following its conversion to the benzoyl ester. A well-resolved peak in the electropherogram was obtained. Wise *et al.* [512] have described electrochemical measurement of choline by capillary electrophoresis in the presence of an internal standard (butyrylcholine). The method may be adapted for the determination of ethanolamine and serine. Olsson *et al.* [513] have reported a procedure for the preparation of long-chain base residues of egg yolk, bovine milk, and bovine brain sphingomyelin. The bases were converted to *N*-acetyl-*O*-TMS derivatives before being subjected to GLC and MS. The chromatographic profile of the milk sample was complex, with thirteen peaks, whereas the profiles of brain and egg yolk long-chain bases were simple and remarkably similar.

Mitchell *et al.* [258] described a micro-adaptation of the method of Bartlett for the assay of phosphorus in TLC spots. However, the method described by Stull and Buss [514] for protein-bound phosphate may be preferable. Others [23] have employed the method of

Lanzetta *et al.* [515] for the quantitative determination of organic phosphate in PtdIns or PtdInsP. The glycerol content of lipids can now be readily determined by various commercial kits and by automated equipment. The commercial kits are based on enzymatic oxidation of free glycerol, which is released from the PtdIns by alkaline hydrolysis, as described for release of InsPs. Routine procedures for the quantification of TAG are currently based on enzymatic methods. By means of lipase (sometimes combined with esterase), TAG are hydrolyzed, and the amount of liberated glycerol is monitored by the indicator reactions. The commercial kits are intended for analysis of serum, in which the TAG are bound to lipoproteins. When trying to measure the amount of TAG in lipid extracts from cells directly or after TLC separation, by means of a commercial kit (Sigma TAG, GPO-Trinder) reagents, difficulties have been encountered, due to inadequate dissolution of the dried substrate, as revealed by the use of triolein standard. Van Veldhoven *et al.* [516] have observed that the use of detergents to solubilize the dried lipids can improve the lipase specificity and the colorimetric or fluorescent procedure. These authors recommend an initial separation of the lipid extracts by TLC. To allow for efficient hydrolysis by *Pseudomonas* lipase, the eluates are dried in presence of Thesit [dodecylpoly(ethylene glycol ether)] (Boeringer).

The Ins content of a purified PtdIns preparation can also provide a good indication of the mass of the sample. Myo-Ins is commonly detected by colorimetric methods of high sensitivity [517,518]. The chromatographic methods offer the opportunity to assay more than one inositol isomer at a time. Rubin *et al.* [519] have reviewed this methodology. Myo-Ins for quantitative analysis by GC/MS methods may be released from PtdIns by saponification, and the resulting InsP is de-phosphorylated with alkaline phosphatase. Heathers *et al.* [520] de-phosphorylated the isomeric InsP fractions, obtained by concentration of the HPLC column eluates. To de-phosphorylate the samples (5–10 nmol), $MgCl_2$ (5 m*M*) and alkaline phosphatase (type VIIS, 35–50 units, Sigma) were added to each tube. Samples were incubated overnight at 37 °C, followed by boiling for 3 min to terminate the reaction. Following de-phosphorylation, 1 nmol of chiro-inositol was added to each sample to serve as an internal standard for GLC analysis. The isomeric Ins were resolved by GLC, following the preparation of hexa-TMS ethers. According to Pak and Larner [521], the PtdIns, purified by TLC, is hydrolyzed in sealed vials with 6 *N* HCl at 110 °C for 48 h. The acid hydrolyzates are analyzed for inositol by HPAA-Dionex HPLC on an Aminex HPX-87C column (Bio-Rad). GC/MS analysis of the hydrolyzates is performed on a DB-5 column (0.32 mm × 30 m), after preparation of the hexa-TMS derivatives. Ostlund *et al.* [522] have described a chiral-phase GLC method for the separation and quantification of enantiomeric D- and L-chiro-inositols in urine and plasma. For GLC, the inositol samples were converted to their hexakis(pentafluoropropionic) esters by an overnight reaction at 65 °C with 150 μL of a 1:1 solution of pentafluoropropionic acid anhydride and acetonitrile. Most of the derivatizing reagent was removed prior to GLC analysis in order to avoid damage to the column. The peaks were quantified by GC/MS with reference to HFB_6-*myo*-inositol, measured by NICI monitoring, using m/z 1036 for HFB_6-*myo*-inositol and m/z 1041 for HFB_6-d_6-*myo*-inositol.

The fatty acid content of the glycerolipid and GPL samples is determined by GLC, following alkaline or acid transmethylation, as described above. The positional

distribution of FA in GPL is determined by hydrolysis with enzymes specific for either the sn-1- or sn-2-position of the phosphatide. The released free fatty acids may be diazomethylated or converted to methyl esters or other derivatives for GLC or HPLC. Liu [523] has reviewed the preparation of FAME for GLC analysis of lipids in biological materials. Major and Wolf [524] reported the preparation and GLC analysis of the pentafluorobenzyl (PFB) esters of fatty acids. The GLC peaks were determined by electron-capture detection (ECD) with picomole sensitivity. Kramer *et al.* [525] have evaluated acid and base catalysts in the methylation of fatty acids with special emphasis on conjugated dienes and total *trans*-fatty acids. The recent availability of long, polar capillary columns (50 to 100 m) for GLC has improved the resolution of many positional and geometricaI FA isomers. Kramer *et al.* [525] have reported the separation of about 180 peaks from milk lipids, using a 100-m SP-2560 fused-silica capillary column (100 m × 0.25 mm ID, 0.2-μm film thickness; Supelco) with H_2 as carrier gas, a 1:15 split mode, and a FID. The fatty acid methyl esters were also analyzed on a CP-Sil 88 wall-coated open-tubular (WCOT) fused-silica column (100 m × 0.25 mm ID, 0.2-μm film thickness, Chrompack). Both columns were operated at 70 °C for 2 min, the temperature programmed at 13 °C to 175 °C in 8 min, held there for 27 min, programmed at 4 °C/min to 215 °C, and finally held there for 31 min; the total run-time being 80 min. Prior separations with argentation chromatography still showed several regions with overlapping peaks, particularly in the mono- and di-unsaturated FA region. All acid-catalyzed procedures resulted in decreased *cis*/*trans* ($\Delta^{9c,11t}$-18:2) and increased *trans*/*trans* ($\Delta^{9t,11t}$-18:2) conjugated dienes and in the production of allylic methoxy artifacts.

Hallaq *et al.* [526] have reported that the use of acetyl chloride and methanol for assumed selective methylation of plasma nonesterified fatty acids results in significant methylation of esterified fatty acids. It was therefore necessary to first isolate the nonesterified plasma FA by TLC. To minimize oxidation, the spotting and scraping off of the polyunsaturates was done under an atmosphere of nitrogen. Although some FA have presented problems in the preparation of the derivatives, the dimethyloxazoline (DMOX) derivatives are now generally considered the method of choice for structure determination. The use of the picolinyl esters or DMOX derivatives has been reviewed in detail [291–295]. Christie [296] has introduced a mild treatment for the preparation of the DMOX derivatives from methylene-interrupted ene-yne systems, which minimizes artefact formation. Rubino and Zecca [527] have published an improved derivatization procedure for the preparation of nicotinate and 3-picolinyl esters from mixtures of fatty alcohols and acids. The conversion takes place under very mild conditions by employing N,N'-carbonyldiimidazole as the condensing agent in a modification of previously reported methods. Dobson *et al.* [294] have characterized 16 cyclic dienoic fatty acids, formed from linolenic acid in linseed oil upon heating to 275 °C, by GC/MS of the picolinyl esters and DMOX derivatives, before and after hydrogenation and deuteration. The stereochemistry of the double bonds was elucidated by GC/Fourier-transform infrared spectroscopy. All possible combinations of configurational isomers of both the cyclopentenyl/cyclohexenyl ring and the double bond in the straight chain were formed. The isomers were pre-fractionated by silver-ion HPLC on a column of Nucleosil 5SA (4.6 × 250 mm), converted to the silver-ion form as described by Juaneda *et al.* [283].

The presence of both alkyl and alkenyl ether groups in PtdIns preparations complicates the analysis of the fatty chain composition.

17.7.1.2 De-acylation

Milder chemical methods may be used for stepwise degradation of GPL: de-acylation, de-glyceration, and de-phosphorylation. Thus, in case of an unknown PtdIns, it may be started with a mild alkaline hydrolysis of [^{3}H]inositol-labeled PtdIns, purified by 2-D TLC [528]. Samples are suspended in 0.5 mL chloroform/methanol (1:4) and, after addition of 0.050 mL 1 *N* NaOH in methanol/water (1:1), the lipids are hydrolyzed at 37 °C for 10 min. Isobutyl alcohol is included in the subsequent partitioning in order to enhance the recovery of the lysoPtdIns in the organic phase. The resulting water-soluble products are chromatographed on Dowex columns, and the products are eluted stepwise with increasing concentrations of ammonium formate in formic acid. GroPIns is eluted with 60 m*M* ammonium formate/5 m*M* sodium borate. The glycerol derivatives of PtdInsP and PtdInsP_2, if any, are eluted with 12–14 mL of 0.4 *M* ammonium formate/0.1 *M* formic acid and 10–12 mL of 1 *M* ammonium formate/0.1 *M* formic acid, respectively. Alternatively, the total [^{3}H]-labeled lipids may be de-acylated with methanolic NaOH, as described by Downes and Michell [529], and the water-soluble products analyzed as above. De-acylated standard phosphatidyl-[2-^{3}H]-inositol 4,5-*bis*-phosphate was used for the calibration of the columns. The fatty acids were determined later by GLC, following preparation of methyl esters. De-acylation of PtdInsP serves two functions: It provides evidence of the composition and general structure of the phospholipid molecules and also provides derivatives that permit effective separation of isomeric PtdIns, including the novel 3-OH phosphates. According to Jones *et al.* [62], the de-acylation is best carried out when 500 μL of freshly prepared methylamine reagent (1-butanol/methanol/25% aq. methylamine, 11.5:45.7:42.8), containing 1 m*M* EDTA is added to the vials before heating them at 53 °C for 1 h. After cooling them to room temperature, they are transferred to Eppendorf tubes and vacuum-dried at room temperature. After resuspending the mixture in 500 μL of H_2O, FA and any unde-acylated lipids are removed by washing twice with 500 μL of a freshly prepared solvent mixture, consisting of 1-butanol/petroleum ether/ethyl formate (20:4:1). More than 95% of the radioactivity routinely partitions into the aqueous phase, indicating almost complete phospholipid de-acylation.

17.7.1.3 De-glyceration

In order to further characterize the GroPInsP, the glycerol moiety is removed by mild periodate oxidation. Both [^{32}P]-phosphate and [^{14}C]-inositol-labeled GroPInsPs can be used. Letcher *et al.* [67] used a modification of the method Brown and Stewart [530] to remove the glycerol moiety from the [5-^{32}P]-GroPIns(4,5)P_2. To the dry [5-^{32}P]-GroPIns(4,5)P_2 was added 1 mL of 50 m*M* sodium periodate. After mixing to dissolve the [5-^{32}P]-GroPIns(4,5)P_2, the tube was placed in the in the dark for 30 min at room temperature. If the oxidation is allowed to continue for longer, the periodate will start to oxidize the inositol ring. Ethylene glycol (150 μL of 10%) is added to stop the reaction

and, after 15 min, 0.5 mL of 1% 1,1-dimethylhydrazine in formic acid (pH 4) is added, and the mixture is allowed to stand for a further 60 min. The mixture is passed through 30 mL of a cation-exchange resin (BioRad 50WX8, 200–400 mesh, in acid form) in a 1.6-cm ID column. The column is eluted with 30 mL of H_2O, the pooled eluates are adjusted to pH 6 with dilute KOH, and evaporated to dryness in a rotary evaporator. The [^{32}P]-Ins(1,4,5)P_3 is now dissolved in a small volume of H_2O for HPLC. Letcher *et al.* [67] removed the glycerol moiety from the [^{14}C]-GroPIns(4)P exactly as described for[5-^{32}P]-GroPIns(4,5)P_2. After passing through a Dowex colum in acid form, and neutralizing with KOH, the [^{14}C]-Ins(1,4)P_2 is dissolved in 2 mL of H_2O for HPLC. The InsP are resolved by HPLC and identified as described below. Stephens [114] has described a scaled-down adaptation of the periodate oxidation method of Ballou and coworkers for the structure analysis of trace quantities of [^{3}H]-InsP with polyol dehydrogenase. The method involves the oxidation with periodate, reduction, and de-phosphorylation of the unknown inositol phosphate. The chirality of the final product, which was originally established by measuring the specific optical rotation, is now determined on the basis of the specificity of yeast polyol dehydrogenase.

17.7.1.4 De-phosphorylation

The chemical characterization of the InsPs derived by de-acylation and de-glyceration of PtdInsP may be continued to the formation of *myo*-inositol and its isomers, if present. This may be accomplished by chemical or by enzymatic de-phosphorylation (see below). The chemical de-phosphorylation of InsP is accomplished by alkaline hydrolysis, which leads to intermediate formation of a cyclic triester, provided a free hydroxyl group is available. The cyclic triester is then rapidly broken down to isomeric α- and β-phosphates. The isomerization of the phosphates can easily invalidate structure conclusions [114]. The confusion introduced by alkaline hydrolysis is eliminated by enzymatic hydrolysis of the InsP.

17.7.2 Enzymatic methods

17.7.2.1 Lipolysis

The positional distribution of the fatty acids in individual intact molecular species of the GPL may be determined by hydrolysis with PLA_2, which releases the FA from the sn-2-position. Alternatively, the FA in the sn-1-position can be specifically released by a fungal lipase, which specifically attacks the primary position of the acylglycerol molecule [532]. Because of the small amounts of mass likely to be involved, such analyses are not practical, unless radioactive markers are involved and carriers used. The fatty acid composition of the sn-1-position may be obtained in a variety of ways. It can be obtained by determining the FA recovered in the lysophospholipids, released by PLA_2, or by subtraction of the FA in the sn-2-position (released by PLA_2) from the total. The positional distribution of fatty acids may also be determined by lipase hydrolysis of the sn-1,2-diacylglycerol moieties, released from the GPL by nonspecific PLC or PtdIns-specific

PLC [108]. For this purpose, the bacterial enzymes (*e.g.* from *Crotalus adamanteus*) are utilized most commonly. These enzymes exhibit preference for GPL with choline and ethanolamine head-groups, and PtdIns is attacked at a slower rate. The substrate preference for FA chains in the sn-2-position of PtdCho is: 18:1 > 16:0 > 18:1 > 20:4. The latter type of positional analysis is best carried out following acetylation of the DAG, which prevents isomerization. The composition of the FA in the sn-1-position can be determined directly by hydrolysis of the phosphatide with the lipase from *Rhizopus arrhizus*, which specifically releases the fatty acid from the primary (sn-1-) position of the glycerol molecule [531]. This assay is best performed with the DAG moiety of the PdIns. The fatty acid composition of the sn-1-position may also be obtained by subtraction of the fatty acids in the sn-2-MAG from the total [531].

17.7.2.2 De-phosphorylation

Crude preparations of PLC from *Bacillus cereus* (such as Type III and some batches of Type V, Sigma) have activity against PtdCho, PtdEtn, and PtdSer, as well as PtdIns. Purer preparations (such as Type XIII, Sigma) have no PLC activity against PtdIns. PLC, specific for PtdIns and PtdInsP has been purified from several sources. These preparations yield DAG and inositol trisphosphate from $PtdInsP_2$, while PtdInsP yields DAG and $InsP_2$. Furthermore, $PtdInsP_2$ yields PtdInsP and inorganic phosphate, while the PtdInsP yields PtdIns and inorganic phosphate. Zhou *et al.* [532] have shown that the enzyme obtained from a recombinant strain of *B. subtilis*, after transfection with the *B. thuringiensis* PtdIns-PLC gene for overproduction of PtdIns-PLC enzyme, catalyzes the hydrolysis of PtdIns in discrete steps. First, an intramolecular phosphotransferase reaction yields Ins(1,2cyc)P, which is converted by a cyclic phosphodiesterase activity to Ins(1)P. The activity of the PtdIns-specific enzymes is limited to PtdIns and lyso-PtdIns. These enzymes do not attack PtdGro, SM, PtdCho, PtdEtn, or PtdSer. Bruzik *et al.* [533] have synthesized both 1-D- and 1-L-*chiro*-PtdIns in diastereomerically pure forms and have determined their substrate properties toward bacterial PtdIns-phospholipase C by ^{31}P-NMR. The results indicated that 1-L-chiro-PtdIns was accepted by PtdIns-PLC at a reduced rate, whereas the 1-D-diasteromer was not a substrate. The InsPs released by PLC may be identified by one of two basic methods: The first method determines directly any particular InsP isomer in an extract without initial chromatographic separation from other isomers, *e.g.*, radioligand competition assay with a highly specific high-affinity binding protein or enzymatic assays. In the second technique, InsP isomers are first separated chromatographically, most commonly by anion-exchange HPLC. Subsequently, their masses are determined by different methods, *e.g.*, phosphorus or inositol assays, which are laborious. Mayr [534,535] has developed an on-line post-column metal-dye detection system, in which a transition metal and a metal-specific dye act as reporter substances for InsP and other (oxy)anions, eluted from the HPLC column. InsP, ranging from InsP to Ins(1,3,4,5,6)P_5 and $InsP_6$, are separated by one of several elution procedures and quantified by the dye-metal binding method.

17.7.2.3 Phospholipase D

Phospholipase D catalyzes the hydrolysis of glycerophospholipids to produce PtdOH and their respective polar head-groups and is distinct from the PLD activity that hydrolyzes glycosyl PtdIns. At least two distinct isoenzymes of PLD are expressed in mammalian cells. Two major classes of mammalian PLD have been identified, a $PtdInsP_2$-dependent class (PLD 1 and PLD 2) and a FA (oleate)-sensitive form [536]. PtdIns-specific PLD liberates free Ins and PtdOH. PtdIns is also subject to transphosphatidylation by nonspecific PLD. Since inositol polyphosphatides are subject to hydrolysis by PLD, the phosphatidic acids generated by deliberate digestion or transphosphatidylation with PLD (methyl or ethyl phosphatides) or released during the course of a metabolic transformation, are amenable to analysis of molecular species by the methods described for the phosphatidic acids released from PtdIns. In view of the much smaller amounts of mass available for the analyses, the use of carriers would be necessary.

17.7.2.4 Other enzymes

Alkaline phosphatase preparations provide effective means of de-phosphorylation of PtdInsP as well as GroPInsP. Hydrolysis with alkaline phosphatase is critical for the de-phosporylation of InsPs without isomerization. Carter *et al.* [537] have provided a flow-chart of analysis of ^{32}P-labeled PtdIns(3,4,5)P_3, starting with the de-acylated and de-glycerated product and utilizing phosphomonoesterase and radiochromatography. In other instances, InsP and PtdInsP may be characterized by purified kinases and phosphatases of high specificity [538]. As already noted, an enzyme activity of great importance for the determination of the structure of the InsP is polyol dehydrogenase, which possesses total D *vs*. L selectivity [114]. Polyols recognized as substrates are oxidized to ketones in a β-NAD^+-linked reaction. The progress of the reaction is monitored in a spectrophotometer by measuring the accumulation of NADH or by detecting the formation of a ^{3}H-labeled ketone. A commercially available preparation of a yeast-derived L-iditol dehydrogenase (Sigma) oxidizes substrates with a specificity that is consistent with the rules set out by McCorkindale and Edson [114].

REFERENCES

1 A. Kuksis, in E. Heftmann (Ed.), *Chromatography*, 5th Edn. Elsevier, Amsterdam, 1992 p. B171.
2 G.A. Eiceman, H.H. Hill, Jr. and J. Gardea-Torresdey, *Anal. Chem.*, 72 (2000) 137R.
3 K. Eder, *J. Chromatogr. B*, 671 (1995) 113.
4 J.D. Morrow and L.J. Roberts, *Prog. Lipid Res.*, 36 (1997) 1.
5 V. Spitzer, *Prog. Lipid Res.*, 35 (1997) 387.
6 J.J. Myher and A. Kuksis, *J. Chromatogr. B*, 671 (1995) 3.
7 A. Kuksis and J.J. Myher, *J. Chromatogr. B*, 671 (1995) 35.
8 J. Sherma, *Anal. Chem.*, 72 (2000) 9R.

9 G.W. Somsen, W. Morden and I.D. Wilson, *J. Chromatogr. A*, 703 (1995) 613.
10 M. Niesen, *J. Chromatogr. A*, 856 (1999) 179.
11 R.C. Murphy, J. Fiedler and J. Hevko, *Chem. Rev.*, 101 (2001) 479.
12 A.I. Carapiso and C. Garcia, *Lipids*, 35 (2000) 1167.
13 C.J. Delong, P.R.S. Baker, M. Samuel, Z. Cui and M.J. Thomas, *J. Lipid Res.*, 42 (2001) 1959.
14 E.A. Podrez, E. Poliakov, Z. Shen, R. Zhang, Y. Deng, M. Sun, P.J. Finton, L. Shan, B. Gugiu, P.L. Fox, H.F. Hoff, R.G. Solomon and S.L. Hazen, *J. Biol. Chem.*, 277 (2002) 38503.
15 J. Folch, M. Lee and S.M. Sloane-Stanley, *J. Biol. Chem.*, 226 (1956) 497.
16 E.G. Bligh and W.J. Dyer, *Can. J. Biochem. Physiol.*, 37 (1959) 911.
17 R.E. Wuthier, *J. Lipid Res.*, 9 (1968) 68.
18 S.J. Iverson, S.L. Lang and M.H. Cooper, *Lipids*, 36 (2001) 1283.
19 J.E. Belle, N.G. Harris, S.R. Williams and K.K. Bhakoo, *NMR Biomed.*, 15 (2002) 37.
20 M.G. Low, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, p. 145.
21 K.R. Auger, L.A. Serunian and L.C. Cantley, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, p. 159.
22 J. van der Kaay, I.H. Batty, D.A. Cross, P.W. Watt and C.P. Downes, *J. Biol. Chem.*, 272 (1997) 5477.
23 A.K. Singh, *J. Chromatogr.*, 581 (1992) 1.
24 Y. Zeng, X. Han and R.W. Gross, *Biochem. J.*, 338 (1999) 651.
25 C. Costello, D.H. Beach and B.N. Singh, *Biol. Chem.*, 382 (2001) 275.
26 L.N.Y. Wu, B.R. Genge, M.W. Kang, A.L. Arsenault and R.E. Wuthier, *J. Biol. Chem.*, 277 (2002) 5126.
27 C. Nasuhoglu, S. Feng, J. Mao, M. Yamamoto, H.L. Yin, S. Earnest, B. Barylko, J.P. Albanese and D.W. Hilgemann, *Anal. Biochem.*, 301 (2002) 243.
28 K. Eder, A.M. Reichlmayr-Lais and M. Kirchgessner, *Clin. Chim. Acta*, 219 (1993) 93.
29 R.K. Richardson, *J. AOAC Int.*, 84 (2001) 1522.
30 E. Boselli, V. Velazco, M.F. Caboni and G. Lercker, *J. Chromatogr. A*, 917 (2001) 239.
31 T.W. Randolph, *Trends Biotechnol.*, 8 (1990) 78.
32 K.J. Choi, Z. Nakohost, V.J. Krukonis and M. Karel, *Food Biotechnol.*, 1 (1987) 263.
33 F.J. Eller and J.W. King, *J. Agric. Food Chem.*, 49 (2001) 4609.
34 C. Leray, T. Gric, G. Gutbier and M. Bnouham, *Analusis*, 23 (1995) 65.
35 H. Yoshida, Y. Hirakawa and S. Abe, *Eur. J. Lipid Sci. Technol.*, 103 (2001) 201.
36 J.R. Pare, G. Matni, J.M. Belanger, K. Li, C. Rule, B. Thibert, V. Yavlayan, Z. Liu, D. Mathe and P. Jacquault, *J. AOAC Int.*, 80 (1997) 928.
37 AOAC, *Official Methods and Recommended Practices, 1996–1997 Edn., Am 3-96*, Association of Official Analytical Chemists, Arlington, 1997.
38 S.N. Pedersen and C. Lindholst, *J. Chromatogr. A*, 864 (1999) 17.
39 L. Pensado, C. Casais, C. Mejuto and R. Cela, *J. Chromatogr. A*, 869 (2000) 505.
40 V.A. Yaylayan, J.R. Pare, G. Matni and J.M. Belanger, *Nat. Prod. Lett.*, 15 (2001) 187.
41 A.K. Menon, *Meth. Enzymol.*, 230 (1994) 418.
42 R.S. Naik, O.H. Branch, A.S. Woods, M. Vijaykumar, D.J. Perkins, B.L. Nahlen, A.A. Lal, R.J. Cotter, C.E. Costello, C.F. Ockenhouse, E.A. Davidson and D.C. Gowda, *J. Exp. Med.*, 192 (2000) 1563.
43 C. Santos de Macedo, P. Gerold, N. Jung, N. Azzouz, J. Kimmel and R.T. Schwarz, *Eur. J. Biochem.*, 268 (2001) 6221.

44 R.E. Wuthier, *J. Lipid Res.*, 9 (1968) 68.
45 C. Sevlever, D.R. Humphrey and T.L. Rosenberrry, *Eur. J. Biochem.*, 233 (1995) 384.
46 W.L. Roberts, S. Santikarn, V.N. Reinhold and T.L. Roseberry, *J. Biol. Chem.*, 263 (1988) 18776.
47 B.O. Keller and L. Li, *Anal. Chem.*, 73 (2001) 2929.
48 Z.E. Penton, *Adv. Chromatogr.*, 37 (1997) 205.
49 D.L. Mayer and J.S. Fritz, *J. Chromatogr. A*, 773 (1997) 189.
50 L. Pan and J. Pawliszyn, *Anal. Chem.*, 69 (1997) 196.
51 D.R. Janero and C. Burghardt, *J. Chromatogr.*, 526 (1990) 11.
52 J.J. Agren, A. Julkunen and J. Pentilla, *J. Lipid Res.*, 33 (1992) 1871.
53 S.T. Ingalls, M.S. Kriaris, Y. Xu, D.W. Dewulf, K.-Y. Tserng and C.L. Hoppel, *J. Chromatogr.*, 619 (1993) 9.
54 G.C. Burdge, P. Wright, A.E. Jones and S.A. Wootton, *Br. J. Nutr.*, 84 (2000) 781.
55 R.A. Hagerman, M.J. Anthony and R.A. Willis, *Anal. Biochem.*, 296 (2001) 141.
56 Z. Zhao, N.M. Hjelm, C.W. Lam and C.S. Ho, *Clin. Chem.*, 47 (2001) 1306.
57 R.E. Calaf, J. Pena, S. Paytubi, M. Carrascal, M. Posada, E. Gelpi and J. Abian, *J. Agric. Food Chem.*, 49 (2001) 5085.
58 T. Nakamura, Y. Hatori, K. Yamada, M. Ikeda and T. Yazuriha, *Anal. Biochem.*, 179 (1989) 127.
59 K. Yamada, S. Abe, K. Katayama and T. Sato, *J. Chromatogr.*, 424 (1988) 367.
60 T. Gunarsson, L. Ekblad, A. Karlsson, P.M. Michelsen, G. Odham and B. Jergil, *Anal. Biochem.*, 254 (1997) 293.
61 V. Vihma, H. Adlercreutz, A. Tiitinen, P. Kiuru, K. Wahala and M.J. Tikkanen, *Clin. Chem.*, 47 (2001) 1256.
62 D.R. Jones, A. Gonzalez-Garcia, E. Diez, C. Martinez-A, A.C. Carrera and I. Merida, *J. Biol. Chem.*, 274 (1999) 18407.
63 K. Anderson, J. Cummings and J.F. Smyth, *J. Chromatogr.*, 574 (1992) 150.
64 J.G. March, B.M. Simonet and F. Grases, *J. Chromatogr. B*, 757 (2001) 247.
65 J. Schacht, *Meth. Enzymol.*, 72 (1981) 626.
66 F.B. Palmer, *J. Lipid Res.*, 22 (1981) 1296.
67 A.J. Letcher, L.R. Stephens and R.F. Irvine, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, p. 31.
68 F. Irvine, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, p. 1.
69 T. Cronholm, M. Viesta Rains and J. Sjovall, *Biochem. J.*, 287 (1992) 925.
70 C.F. Poole and S.K. Poole, *Anal. Chem.*, 66 (1994) 27.
71 T. Holm, *J. Chromatogr. A*, 782 (1997) 81.
72 D.C. Gaudette, H.M. Aukema, C.A. Jolly, R.S. Chapkins and B.J. Holub, *J. Biol. Chem.*, 268 (1993) 13773.
73 F. Bressolle, M. Bromet-Petit and M. Audran, *J. Chromatogr. B*, 686 (1996) 3.
74 J. Abian, A.J. Oosterkamp and E. Gelpi, *J. Mass Spectrom.*, 34 (1999) 244.
75 V.P. Skipski, R.F. Peterson and M. Barclay, *Biochem. J.*, 90 (1964) 374.
76 J. Jolles, H. Zwiers, A. Dekker, K.W.A. Wirtz and W.H. Gispen, *Biochem. J.*, 194 (1981) 283.
77 G. Rouser, G. Kritchevsky, A. Yamamoto, G. Simon, C. Galli and A.J. Bauman, *Meth. Enzymol.*, 14 (1969) 272.
78 V.G. Mahadevappa and B.J. Holub, in A. Kuksis (Ed.), *Chromatography of Lipids in Biochemical Research and Clinical Diagnosis*, Elsevier, Amsterdam, 1987, p. 225.

79 G.Y. Sun and T.N. Lin, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, p. 153.
80 R. Wilson and J.R. Sargent, *J. Neurochem.*, 61 (1993) 290.
81 A.K. Singh and Y. Jiang, *J. Chromatogr. B*, 671 (1995) 255.
82 W. Zhang, B. Asztalos, P.S. Roheim and L. Wong, *J. Lipid Res.*, 39 (1998) 1601.
83 J.C. Touchstone, K.A. Snyder and S.S. Levin, *J. Lipid Chromatogr.*, 7 (1984) 2725.
84 B. Narasimhan, G. Plisa-Matysak, R. Kinnard, S. Carstense, M.A. Ritter, L. von Weyman and P.P.N. Murthy, *Plant Physiol.*, 113 (1997) 1385.
85 G. Carstensen, N. Pliska-Matyshak, K.M. Bhuvarahamurthy, P.P. Robbins and N. Murthy, *Lipids*, 34 (1999) 67.
86 J.E. Ferrell, Jr. and W.H. Huestis, *J. Cell Biol.*, 98 (1984) 1992.
87 R.A. Akhtar, C. Taft and A.A. Abdel-Latif, *J. Neurochem.*, 41 (1983) 1460.
88 H. Hegewald, *Anal. Biochem.*, 242 (1996) 152.
89 N.C. Shanta and G.E. Napolitano, *J. Chromatogr.*, 624 (1992) 37.
90 H. Tsukamoto, T. Hishinuma, T. Mikkaichi, H. Nakamura, T. Yamazaki, Y. Tomioka and M. Mizugaki, *J. Chromatogr. B*, 774 (2002) 205.
91 A. Kuksis, *Encyclopedia of Separation Science*, Academic Press, London, 2000, pp. 3219–3237.
92 C. Yang, C.C. Parish and R.J. Helleur, *J. Chromatogr. Sci.*, 34 (1996) 556.
93 E. Geeraert and P. Sandra, *J. Am. Oil Chem. Soc.*, 64 (1987) 100.
94 A. Kuksis, J.J. Myher and P. Sandra, *J. Chromatogr.*, 500 (1990) 427.
95 A. Kuksis, J.J. Myher and K. Geher, *J. Lipid Res.*, 34 (1993) 1029.
96 R.P. Evershed, *J. Am. Soc. Mass Spectrom.*, 7 (1996) 350.
97 R. Thompson, *J. Chromatogr. Sci.*, 35 (1997) 536 and 598.
98 R.L. Wolff and D. Precht, *Lipids*, 37 (2002) 627.
99 D. Precht, J. Molketin, M.A. McGuire and R.G. Jensen, *Lipids*, 36 (2001) 213.
100 R.J. Western, S.S.G. Lau, P.J. Marriott and P.D. Nichols, *Lipids*, 37 (2002) 715.
101 J.K.G. Kramer, C.B. Blackadar and J. Zhou, *Lipids*, 37 (2002) 823.
102 I. Rizov and A. Doulis, *J. Chromatogr. A*, 922 (2001) 347.
103 L.L. Dugan, P. Domediuk, C.I. Pendley and L.L. Horrocks, *J. Chromatogr.*, 378 (1986) 317.
104 P. Laakso and W.W. Christie, *Lipids*, 25 (1990) 349.
105 W.W. Christie, B. Nikolova-Damyanova, P. Laakso and B. Herslof, *J. Am. Oil Chem. Soc.*, 68 (1991) 595.
106 J. Agren and A. Kuksis, *Lipids*, 37 (2002) 613.
107 Y. Itabashi, J.J. Myher and A. Kuksis, *J. Chromatogr. A*, 893 (2000) 261.
108 A. Kuksis, J.J. Myher, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, pp. 187–216.
109 G. Dobson, Y. Itabashi, W.W. Christie and G.W. Robertson, *Chem. Phys. Lipids*, 97 (1998) 27.
110 G. Dobson and N. Deighton, *Chem. Phys. Lipids*, 111 (2001) 1.
111 S. Benard, J. Arnhold, M. Lehnert, J. Schiller and K. Arnold, *Chem. Phys. Lipids*, 100 (1999) 115.
112 T. Takagi, *Prog. Lipid Res.*, 29 (1990) 277.
113 A. Kuksis, in W.W. Christie (Ed.), *Advances in Lipid Methodology – Three*, The Oily Press, Ayr, 1996, pp. 1–36.
114 L.R. Stephens, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, p. 9.

115 C.E.L. Spencer, L.R. Stephens and R.F. Irvine, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, p. 39.
116 C.S. Kaiser, H. Rompp and P.C. Schmidt, *Pharmazie*, 56 (2001) 907.
117 C.M. Harris, *Anal. Chem.*, (2002) 87A.
118 E. Ibanez and F.J. Senorans, *J. Biochem. Biophys. Meth.*, 43 (2000) 25.
119 P.J. Sjoberg, L. Nyholm and K.E. Markides, *J. Mass Spectrom.*, 35 (2000) 330.
120 P.J. Sjoberg and K.E. Markides, *J. Chromatogr. A*, 855 (1999) 317.
121 T. Wang, M. Barber, I. Hardt and D.B. Kassel, *Rapid Commun. Mass Spectrom.*, 15 (2001) 2067.
122 M. Garzotti and M. Hamdan, *J. Chromatogr. B*, 770 (2002) 53.
123 J. Planeta, P. Rehulka and J. Chmelik, *Anal. Chem.*, 74 (2002) 39111.
124 T. Aro, C. Brede, P. Manninen and H. Kallio, *J. Agric. Food Chem.*, 50 (2002) 1970.
125 A. Kuksis, *Encyclopedia of Separation Science*, Academic Press, London, 2000, pp. 3237–3253.
126 Y. Itabashi, A. Kuksis, in *Abstracts, 24th World Congress and Exhibition, ISF*, Berlin, Germany, 2001, p. 57
127 Y. Itabashi, A. Kuksis, J.J. Myher, in *Abstracts*, 92nd AOCS Annual Meeting and Expo, Minneapolis, MN, 2001, p. S7
128 R.C. Murphy and K.A. Harrison, *Mass Spec. Rev.*, 13 (1994) 57.
129 K.A. Kayganich and R.C. Murphy, *Anal. Chem.*, 64 (1992) 2965.
130 P.G. Holbrook, L.K. Pannell, Y. Murata and J.W. Daly, *J. Biol. Chem.*, 267 (1992) 16834.
131 J. Jensen, K.B. Tomer and M.L. Gross, *Lipids*, 22 (1987) 480.
132 R. Sherman, K.E. Ackermann, R.H. Bateman, B.N. Green and I. Lewis, *Biomed. Mass Spectrom.*, 12 (1985) 409.
133 J.L. Kerwin and J.J. Torvik, *Anal. Biochem.*, 237 (1996) 56.
134 C.R. Cheng, M.L. Gross and E. Pittenauer, *Anal. Chem.*, 70 (1998) 4417.
135 F.F. Hsu and J. Turk, *J. Am. Soc. Mass Spectrom.*, 10 (1999) 600.
136 F.F. Hsu and J. Turk, *J. Am. Soc. Mass Spectrom.*, 10 (1999) 587.
137 X. Han and R.W. Gross, *Anal. Biochem.*, 295 (2001) 88.
138 X. Han and W.R. Gross, *Proc. Natl Acad. Sci. USA*, 91 (1994) 10635.
139 J.L. Kerwin, A.R. Tuininga and L.H. Ericsson, *J. Lipid Res.*, 35 (1994) 1102.
140 X. Han, R.A. Gubitosi-Klug, B.J. Collins and R.W. Gross, *Biochemistry*, 35 (1996) 5822.
141 S.D. Williams, F.F. Hsu and D.A. Ford, *J. Lipid Res.*, 41 (2000) 1585.
142 B. Brugger, G. Erben, R. Sandhoff, F.T. Wieland and W.D. Lehmann, *Cell Biol.*, 94 (1997) 2339.
143 S. Uran, A. Larse, P.B. Jacobsen and T. Skotland, *J. Chromatogr. B*, 758 (2001) 265.
144 A. Zacarias, D. Bolanowski and A. Bhatnagar, *Anal. Biochem.*, 308 (2002) 152.
145 E.K. Fridriksson, P.A. Shipkova, E.D. Sheets, D. Holowka, B. Baird and F.W. McLafferty, *Biochemistry*, 38 (1999) 8056.
146 P. Michelsen, B. Jergi and G. Odham, *Rapid Commun. Mass Spectrom.*, 9 (1995) 1109.
147 R. Schneiter, B. Brugger, R. Sandhoff, G. Zellnig, A. Leber, M. Lampl, K. Athenstaedt, C. Hrastnik, S. Eder, G. Daum, F. Paltauf, F. Wieland and S.D. Kohlwein, *J. Cell Biol.*, 146 (1999) 741.
148 S. Ramanadham, F.-F. Hsu, A. Bohrer, Z. Ma and J. Turk, *J. Biol. Chem.*, 274 (1999) 13915.
149 F.-F. Hsu, Z. Ma, M. Wohltmann, A. Bohrer, W. Nowatzke, S. Ramanadham and J. Turk, *J. Biol. Chem.*, 275 (2000) 16579.
150 W.C. Byrdwell and E.A. Emken, *Lipids*, 30 (1995) 173.
151 W.C. Byrdwell, *Lipids*, 36 (2001) 327.
152 W.C. Byrdwell, E.A. Emken, W.E. Neff and R.O. Adlof, *Lipids*, 31 (1996) 919.

153 H.R. Mottram and R.P. Evershed, *Tetrahedron Lett.*, 37 (1996) 8593.
154 P. Laakso and P. Voutillainen, *Lipids*, 31 (1996) 1311.
155 H. Mu, H. Sillen and C.E. Hoy, *J. Am. Oil Chem. Soc.*, 77 (2000) 1049.
156 H. Kallio and G. Currie, *Lipids*, 28 (1992) 207.
157 H. Kallio and G. Currie, in K.D. Moukherjee and N. Weber (Eds.), *Handbook of Chromatography*, CRC Press, Boca Raton, 1993, pp. 435–458.
158 P. Laakso and H. Kallio, *Lipids*, 31 (1996) 33.
159 D.J. Harvey, *J. Mass Spectrom.*, 30 (1995) 1333.
160 J.A. Marto, F.M. White, S. Seldomridge and A.G. Marshall, *Anal. Chem.*, 67 (1995) 379.
161 J. Schiller, J. Arnhold, J. Berard, S. Mueller, S. Reichl and K. Arnold, *Anal. Biochem.*, 267 (1999) 46.
162 J. Schiller and K. Arnold, in R.A. Myers (Ed.), *Encyclopedia of Analytical Chemistry*, Wiley, Chichester, 2000, pp. 559–585.
163 M. Petkovic, J. Schiller, M. Mueller, S. Benard, S. Reichl, K. Arnold and J. Arnhold, *Anal. Biochem.*, 268 (2000) 202.
164 M. Mueller, J. Schiller, M. Petkovic, W. Oehrl, R. Heinze, R. Wetzker, K. Arnold and J. Arnhold, *Chem. Phys. Lipids*, 110 (2001) 151.
165 G.W. Somsen, W. Morden and D. Wilson, *J. Chromatogr. A*, 703 (1995) 613.
166 J.T. Mehl, A.I. Gustav and D.M. Hercules, *Chromatographia*, 46 (1997) 358.
167 A.I. Gusev, O.J. Vasseur, A. Proctor, A.G. Sharkey and D.M. Hercules, *Anal. Chem.*, 67 (1995) 4565.
168 F. Couderc, *Lipids*, 30 (1995) 691.
169 P.E. Haroldsen and R.C. Murphy, *Biomed. Environ. Mass Spectrom.*, 14 (1987) 573.
170 F.A. Kuypers, P. Butikofer and C.H. Shackleton, *J. Chromatogr.*, 562 (1991) 191.
171 A. Ravandi, A. Kuksis, J.J. Myher and L. Marai, *J. Biochem. Biophys. Meth.*, 30 (1995) 271.
172 J. Becart, C. Chevalier and J.P. Riese, *J. High Resol. Chromatogr.*, 10 (1990) 126.
173 J.J. Myher, A. Kuksis, L. Marai and F. Manganaro, *J. Chromatogr.*, 283 (1983) 289.
174 S. Pind, A. Kuksis, J.J. Myher and L. Marai, *Can. J. Biochem. Cell Biol.*, 62 (1984) 352.
175 A. Kuksis, L. Marai and J.J. Myher, *J. Chromatogr.*, 588 (1991) 73.
176 L. Marai, A. Kuksis, J.J. Myher and Y. Itabashi, *Biol. Mass Spectrom.*, 21 (1992) 541.
177 A. Kuksis, J.J. Myher, L. Marai and K. Geher, in Y. Malkki (Ed.), *Proceedings, 17th Nordic Lipid Symposium*, A Lipidforum Publication, Imatra, Finland, 1993, pp. 230–238.
178 J.J. Myher and A. Kuksis, *INFORM*, 6 (1995) 1068.
179 R.D. Voyksner and T. Pack, *Rapid Commun. Mass Spectrom.*, 5 (1991) 263.
180 A. Kuksis, J.J. Myher, L.-Y. Yang and G. Steiner, in T. Matsuo, Y. Seyama, R.M. Caprioli and M.L. Gross (Eds.), *Biological Mass Spectrometry: Present and Future*, Wiley, Chichester, 1994, pp. 81–494.
181 K. Hartvigsen, A. Ravandi, K. Bukhave, G. Holmer and A. Kuksis, *J. Mass Spectrom.*, 36 (2001) 1116.
182 B. Malewicz and W.J. Baumann, *Lipids*, 31 (1996) 1189.
183 M.H. Nouri-Sorkhabi, L.C. Wright, D.R. Sullivan and P.W. Kuchel, *Lipids*, 31 (1996) 765.
184 M.H. Nouri-Sorkhabi, N.S. Agar, D.R. Sullivan, C. Gallagher and P.W. Kuchel, *Comp. Biochem. Physiol.*, (1996) 221.
185 G. Capuani, T. Aureli, A. Mccheli, M.E. Di Cocco, M.T. Ramacci and M. Delfini, *Lipids*, 27 (1992) 389.
186 J.B. Wissing and H. Behrbohm, *FEBS Lett.*, 315 (1993) 95.
187 L. Seijo, T.E. Merchant, L.T.M. van der Ven, B.D. Minsky and T. Glonek, *Lipids*, 29 (1994) 359.

188 J.M. Henderson, M. Petersheim, G.J. Templeman and B.J. Softly, *J. Agric. Food Chem.*, 42 (1994) 435, (1992).
189 P. Kalo, A. Kemppinen and I. Kilpelainen, *Lipids*, 31 (1996) 331.
190 P.M. Hansbro, S.J. Byard, R.J. Bushby, P.J.H. Turnbull, N. Boden, M.R. Saunders, R. Novelli and D.G. Reid, *Biochim. Biophys. Acta*, 1112 (1992) 187.
191 K.A. Lewis, V.R. Garigapati, C. Zhou and M.F. Roberts, *Biochemistry*, 32 (1993) 8836.
192 J.J. Volwerk, M.S. Shashidhar, A. Kuppe and O. Griffith, *Biochemistry*, 29 (1990) 8056.
193 C. Zhou, V. Garigapati and M.F. Roberts, *Biochemistry*, 36 (1997) 15925.
194 G. Leondoritis and D. Galanopoulou, *Lipids*, 35 (2000) 525.
195 A.R. Brash, W.E. Boeglin, J.H. Capdevila, Y. Suresh and I.A. Blair, *Arch. Biochem. Biophys.*, 321 (1995) 485.
196 E.H. Oliw, *Arch. Biochem. Biophys.*, 300 (1993) 434.
197 M. Hamberg, *Arch. Biochem. Biophys.*, 344 (1997) 194.
198 A.R. Brash, *Lipids*, 35 (2000) 947.
199 J. Nourooz-Zadeh, *Meth. Enzymol.*, (1999) 13.
200 J. Nourooz-Zadeh, N.K. Gopaul, S. Barrow, A.I. Mallet and E.E. Anggard, *J. Chromatogr. B*, 667 (1995) 199.
201 N.K. Goupal, E.E. Anggar, A.I. Mallet, D.J. Betteridge, S.P. Wolff and J. Nourooz-Zadeh, *FEBS Lett.*, 368 (1995) 225.
202 T. Hirano and K. Takahashi, *J. Chromatogr. Sci.*, 34 (1996) 341.
203 H. Yoshida, Y. Hirakawa, S. Abe and Y. Mizushina, *Eur. J. Lipid Sci. Technol.*, 104 (2002) 116.
204 J. Liu, T. Lee, E. Bobik, Jr., M. Guzman-Harty and C. Hastilow, *J. Am. Oil Chem. Soc.*, 70 (1993) 343.
205 H. Dreyfus, B. Guerold, L. Freysz and D. Hicks, *Anal. Biochem.*, 249 (1997) 67.
206 M.H.J. Bergqvist and B.G. Hersloff, *Chromatographia*, 40 (1995) 129.
207 J.B. Hauksson, M.H.J. Bergqvist and L. Rilfors, *Chem. Phys. Lipids*, 78 (1995) 97.
208 M.H.J. Bergqvist and P. Kaufmann, *J. Am. Oil Chem. Soc.*, 73 (1996) 211.
209 M. Hamberg, E. Liepinsh, G. Otting and W. Griffiths, *Lipids*, 33 (1998) 355.
210 C. Luquain, R. Dolmazon, J.M. Enderlin, C. Laugier, M. Lagarde and J.F. Pageaux, *Biochem. J.*, 351 (2000) 795.
211 M.S. Maier, A. Kuriss and A.M. Seldes, *Lipids*, 33 (1998) 825.
212 O. Sjovall, A. Kuksis, L. Marai and J.J. Myher, *Lipids*, 32 (1997) 1211.
213 O. Sjovall, A. Kuksis and H. Kallio, *J. Chromatogr.*, 905 (2001) 119.
214 O. Sjovall, A. Kuksis and H. Kallio, *Lipids*, 36 (2001) 1347.
215 O. Sjovall, A. Kuksis and H. Kallio, *Lipids*, 37 (2002) 81.
216 J.J. Myher, A. Kuksi, A. Ravandi and N. Cocks, *INFORM*, 4 (1994) 478.
217 V. Spitzer and R. Aichholz, *J. High Resol. Chromator.*, 19 (1996) 497.
218 J.-K. Limb, Y.H. Kim, S.-Y. Han and G.-J. Jhon, *J. Lipid Res.*, 40 (1999) 2169.
219 W.Q. Wang and A. Gustafson, *J. Chromatogr.*, 581 (1992) 139.
220 J.T. Lin, C.L. Woodruff, O.J. Lagouche, T.A. McKeon, A.E. Stafford, M. Goodrich-Tanrikulu, J.A. Singelton and C.A. Haney, *Lipids*, 33 (1998) 59.
221 G. Mallinger, J.K. Yao, A.S. Brown and C.S. Dippold, *J. Chromatogr.*, 614 (1993) 67.
222 G.G. Orgambide, Z.-H. Huang, D.A. Gage and F.B. Dazzo, *Lipids*, 28 (1993) 975.
223 F. Vitello and J.-P. Zaneta, *J. Chromatogr.*, 166 (1978) 637.
224 J.G. Parsons and S. Patton, *J. Lipid Res.*, 8 (1967) 696.
225 R. Gorges, G. Hofer, A. Somer, H. Stutz, H. Grillhofer, G.M. Kostner, F. Paltauf and A. Hermetter, *J. Lipid Res.*, 36 (1995) 251.
226 B. Engelmann, C. Kogl, R. Kulschar and B. Schaipp, *Biochem. J.*, 315 (1996) 781.

227 G.W. Small, J.C. Strum and L.W. Daniel, *Lipids*, 32 (1997) 715.
228 G. Rouser, S. Fleischer and A. Yamamoto, *Lipids*, 5 (1966) 494.
229 L.N.Y. Wu, T. Yoshimori, B.R. Genge, G.R. Sauer, T. Kirsch, Y. Ishikawa and R.E. Wuthier, *J. Biol. Chem.*, 268 (1993) 25084.
230 S.L. Abidi, *J. Chromatogr. B*, 717 (1998) 279.
231 M.F. Caboni, S. Menotta and G. Lercker, *J. Chromatogr. A*, 683 (1994) 59.
232 A. Ravandi, A. Kuksis, N. Shaik and G. Jackowski, *Lipids*, 32 (1997) 989.
233 W. Pruzanski, E. Stephanski, F.C. De Beer, M.C. De Beer, P. Vadas, A. Ravandi and A. Kuksis, *J. Lipid Res.*, 39 (1998) 2150.
234 W. Pruzanski, F.C. De Beer, M.C. De Beer, E. Stefanski, A. Ravandi and A. Kuksis, *J. Lipid Res.*, 41 (2000) 1035.
235 Z. Ahmed, A. Ravandi, G.F. Maguire, A. Emili, D. Draganov, B.N. La Du, A. Kuksis and P.W. Connelly, *J. Biol. Chem.*, 276 (2001) 24473.
236 Z. Ahmed, A. Ravandi, G.F. MaGuire, A. Emili, D. Draganov, B.N. La Du, A. Kuksis and P.W. Connelly, *Biochem. Biophys. Res. Commun.*, 290 (2002) 391.
237 M.G. Wiley, M. Przetakiewicz, M. Takahashi and J.M. Lowenstein, *Lipids*, 27 (1992) 295.
238 W.M.A. Hax and W.S.M.G. van Kessel, *J. Chromatogr.*, 142 (1977) 735.
239 L.M. Bonanno, B.A. Denizot, P.C. Tchoreloff, F. Puisieux and P.J. Cardot, *Anal. Chem.*, 64 (1992) 371.
240 Y. Bayon, M. Croset, V. Chirouze, J.L. Tayot and M. Lagarde, *Lipids*, 28 (1993) 631.
241 P. Juaneda, G. Rocquelin and P.O. Astorg, *Lipids*, 25 (1990) 756.
242 K.C. Arnoldsson and P. Kaufmann, *Chromatographia*, 38 (1994) 317.
243 W. Bernhard, M. Linck, H. Creutzburg, A.D. Posle, A. Arning, I. Martin-Carrera and K.F. Sewing, *Anal. Biochem.*, 220 (1994) 172.
244 L. Ingvardsen, S. Michaelse and H. Sorensen, *J. Am. Oil Chem. Soc.*, 71 (1994) 183.
245 K. Szucs, G.S. Verleysen, M.J.E. Duchateau, P. Sandra and B.G.M. Vandeginste, *J. Chromatogr. A*, 738 (1996) 25.
246 E.J. Schaefer, S.J. Robins, G.M. Patton, M.A. Sandberg, C.A. Weigel-DiFranco, B. Rosner and E.L. Berson, *J. Lipid Res.*, 36 (1995) 1427.
247 G.M. Patton and S.J. Robins, *Meth. Enzymol.*, 187 (1990) 195.
248 M.N. Vaghela and A. Kilara, *J. Am. Oil Chem. Soc.*, 72 (1995) 729.
249 A. Arduini, A. Peschechera, S. Dottori, A.F. Sciarroni, F. Sefarini and M. Calvan, *J. Lipid Res.*, 37 (1996) 684.
250 H.G. Rose and M. Oaklander, *J. Lipid Res.*, 6 (1965) 428.
251 W. Otten, P.A. Iaizzo and H.M. Eichinger, *J. Lipid Res.*, 38 (1997) 2023.
252 M. Seewald and H.M. Eichinger, *J. Chromatogr.*, 469 (1989) 271.
253 J.A. Singleton and L.F. Stikeleather, *J. Am. Oil Chem. Soc.*, 72 (1995) 485.
254 A.A. Karlsson, P. Michelsen, A. Larsen and G. Odham, *Rapid Commun. Mass Spectrom.*, 10 (1996) 775.
255 Y. Deng and R.G. Solomon, *J. Org. Chem.*, 65 (2000) 6660.
256 J.E. Ferrell, Jr. and W.H. Huestis, *J. Cell. Biol.*, 98 (1984) 1992.
257 N.G. Clarke and R.M.C. Dawson, *Biochem. J.*, 195 (1981) 301.
258 K.T. Michell, J.E. Ferrell, Jr. and W.H. Huestis, *Anal. Biochem.*, (1986) 158.
259 L. Vartikowski, D. Harrison-Findik, M. Keeler and M. Susa, *Biochim. Biophys. Acta*, 1226 (1994) 1.
260 A.E. Traynor-Kaplan, A.L. Harris, B.L. Thompson, P. Taylor and L.A. Sklar, *Nature*, 334 (1988) 353.
261 J.P. Walsh, K.K. Caldwell and P.W. Majerus, *Proc. Natl Acad. Sci. USA*, 88 (1991) 9184.
262 T. Okada, L. Sakuma, Y. Fukui, O. Hazeki and M. Ui, *J. Biol. Chem.*, 269 (1994) 3563.

263 A.K. Menon, *Meth. Enzymol.*, 230 (1994) 418.
264 H. Hegewald, E. Muller, R. Klinger, R. Wetzker and H. Frunder, *Biochem. J.*, 244 (1987) 183.
265 M. Kates, in R.H. Burdon and P.H. Van Knippenberg (Eds.), *Laboratory Techniques in Biochemistry and Molecular Biology*, Vol. 3, part 2, Elsevier, Amsterdam, 1986, 186–278.
266 J.M. Stein, G. Smith and J.P. Luzio, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, p. 167.
267 Y. Nagatsuka, T. Kasama, Y. Ohashi, J. Uzawa, Y. Ono, K. Shimizu and Y. Habayashi, *FEBS Lett.*, 497 (2001) 141.
268 D. Sevlever, S. Pickett, K.J. Mann, K. Sambamurti, M.E. Medof and T.L. Rosenberry, *Biochem. J.*, 343 (1999) 627.
269 R. Taguchi, N. Hamakawa, N. Maekawa and H. Ikezawa, *J. Biochem.*, 126 (1999) 421.
270 H. Natomi, T. Saitoh, K. Sugano, M. Iwamori, M. Fukayama and Y. Nagai, *Lipids*, 28 (1993) 737.
271 L.M. Ruwisch, B. Schafer-Korting and B. Kleuser, *Naunyn Schmiedebergs Arch. Pharmacol.*, 363 (2001) 358.
272 E. Yang-Boja, F. DeFelippes and H.M. Fales, *Anal. Biochem.*, 285 (2000) 205.
273 J.D. Craske, *J. Am. Oil Chem. Soc.*, 70 (1993) 325.
274 R.L. Wolff and C.C. Bayard, *J. Am. Oil Chem. Soc.*, 72 (1995) 1197.
275 W. Sattler, H. Reicher, P. Ramos, U. Panzenboeck, M. Hayn, H. Esterbauer, E. Malle and G.M. Kostner, *Lipids*, 31 (1996) 1303.
276 K. Ichihara, A. Shibahara, K. Yamamoto and T. Nakayama, *Lipids*, 31 (1996) 535.
277 C. Benistant, M.P. Dehouck, J.-C. Fruchart, R. Cecchelli and M. Lagarde, *J. Lipid Res.*, 36 (1995) 2311.
278 K.H. Weylandt, J.X. Kang and A. Leaf, *Lipids*, (1996) 977.
279 L.Y. Zang, H.L. Kalbach, B. Btrown, L.L. Jackson and J.G.M. Van Kuijk, *J. Chromatogr. B*, 677 (1996) 209.
280 O.-L. Brekke, E. Sagen and K.S. Bjerve, *J. Lipid Res.*, 38 (1997) 1913.
281 J.J. Agren, M.-L. Tormala, M.T. Nenonen and O.O. Hanninen, *Lipids*, 30 (1995) 365.
282 C. Borch-Jensen, M.P. Magnussen and J. Mollerup, *J. Am. Oil Chem. Soc.*, 74 (1997) 497.
283 P. Juaneda, J.L. Sebedio and W.W. Christie, *J. High Resol. Chromatogr.*, 17 (1994) 321.
284 R.O. Adlof, L.C. Copes and E.A. Emken, *J. Am. Oil Chem. Soc.*, 72 (1995) 571.
285 B. Nikolova-Damyanova, W.W. Christie and B. Herslof, *J. Chromatogr. A*, 693 (1995) 235.
286 S. Monchilova, B. Nikolova-Damyanova and W.W. Christie, *J. Chromatogr. A*, 793 (1998) 275.
287 E.D. George, *J. Am. Oil Chem. Soc.*, 71 (1994) 789.
288 C. Alasnier and G. Gandemer, *Meat Sci.*, 48 (1998) 225.
289 J.A. Zirolli and R.C. Murphy, *J. Am. Soc. Mass Spectrom.*, 4 (1993) 223.
290 J.-L. Le Quire, in W.W. Christie (Ed.), *Advances in Lipid Methodology – Two*, The Oily Press, Dundee, 1993, pp. 215–245.
291 W.W. Christie, in W.W. Christie (Ed.), *Advances in Lipid Methodology – Four*, The Oily Press, Dundee, 1997, pp. 119–169.
292 J.Y. Zhang, Q.T. Yu, B.N. Liu and Z.H. Huang, *Biomed. Environ. Mass Spectrom.*, 15 (1988) 33.
293 Y. Zhang, X.J. Yu, H.Y. Wang, B.N. Liu, Q.T. Yu and Z.H. Huang, *J. Am. Oil Chem. Soc.*, 66 (1989) 256.
294 G. Dobson, W.W. Christie, E.Y. Brechany, J.L. Sebedio and J.L. Le Quere, *Chem. Phys. Lipids*, 75 (1995) 171.
295 O. Berddeaux and R.L. Wolff, *J. Am. Oil Chem. Soc.*, 73 (1996) 1323.
296 W.W. Christie, *Lipids*, 33 (1998) 343.

297 T. Tanaka, K. Shibata, H. Hino, T. Murashita, M. Kayama and K. Satouchi, *J. Chromatogr. B*, 700 (1997) 1.
298 T. Tanaka, T. Hattori, M. Kouchi, K. Hirano and K. Satouchi, *Biochim. Biophys. Acta*, 1393 (1998) 299.
299 T. Tanaka, T. Takimoto, J. Morishige, Y. Kikuta, T. Sugiura and K. Satouchi, *Biochem. Biophys. Res. Commun.*, 264 (1999) 683.
300 C. Slater, H. Hardieck, T. Preston and T. Weaver, *J. Chromatogr. B*, 716 (1998) 1.
301 C. Li, A. McClory, E. Won and J.A. Yergey, *J. Mass Spectrom.*, 34 (1999) 521.
302 S.A. Lagerstedt, D.R. Hinrichs, S.M. Batt, M.J. Magera, P. Rinaldo and J.P. McConnell, *Mol. Genet. Metab.*, 73 (2001) 38.
303 F. Pragst, V. Auwaerter, F. Sporkert and K. Spiegel, *Forensic Sci. Int.*, 121 (2001) 76.
304 C.C. Metges, K. Kempe and G. Wolfram, *J. Mass Spectrom.*, (1994) 295.
305 A.F. Ruiter, M.T. Ackermans and E. Endert, *J. Chromatogr. B*, 759 (2001) 169.
306 R.O. Adlof, *Chem. Phys. Lipids*, 88 (1997) 107.
307 R.C. Murphy, J.A. Zirolli, R.C. Murphy and J.A. Zirrolli, in T. Matsuo, R.M. Caprioli, M.L. Gross and Y. Seyama (Eds.), *Biological Mass Spectrometry: Present and Future*, Wiley, Chichester, 1994, p. 464.
308 I. Knott, M. Raes, M. Dieu, G. Lenoi, M. Burton and J. Remacle, *Anal. Biochem.*, 210 (1993) 360.
309 H. Schweer, B. Watzer and H.W. Seyberth, *J. Chromatogr. B*, 652 (1994) 221.
310 A. Margalit, K.L. Duffin and P.C. Isakson, *Anal. Biochem.*, 235 (1996) 73.
311 A. Ferretti and V.P. Flanagan, *Chem. Phys. Lipids*, 74 (1994) 65.
312 A. Ferretti, V.P. Flanagan and J.M. Roman, *Organ. Mass Spectrom.*, 29 (1994) 360.
313 W.S. Powell, L. Wang, S.P. Khanapure, S. Manna and J. Rokach, *Anal. Biochem.*, 247 (1997) 17.
314 H. John and W. Schlegel, *J. Chromatogr. B*, 698 (1997) 9.
315 J. Sajiki and H. Kakimi, *J. Chromatogr. A*, 795 (1998) 227.
316 P. Wheelan and R.C. Murphy, *Anal. Biochem.*, 244 (1997) 110.
317 J. Rokach, S.P. Khanapure, S.-W. Hwang, M. Adiyaman, J.A. Lawson and G.A. FitzGerald, *Prostaglandins*, 54 (1997) 823.
318 J.A. Lawson, J. Rokach and G.A. FitzGerald, *J. Biol. Chem.*, 274 (1999) 24441.
319 J.D. Morrow, J.A. Awad, H.J. Boss, I.A. Blai and L.J. Roberts, II, *Proc. Natl Acad. Sci.*, 89 (1992) 10721.
320 R.J. Waugh and R.C. Murphy, *J. Am. Soc. Mass Spectrom.*, 7 (1996) 490.
321 J. Nourooz-Zadeh, B. Halliwell and E.E. Anggard, *Biochem. Biophys. Res. Commun.*, 236 (1997) 467.
322 J.D. Morrow, W.E. Zackert, J.P. Yang, E.H. Kuhrts, D. Callewaert, R. Dworski, K. Kanai, D. Taber, K. Moore, J.A. Oates and L.J. Roberts, *Anal. Biochem.*, 269 (1999) 326.
323 A. Burke, J.A. Lawson, E.A. Meagher, J. Rokach and G.A. FitzGerald, *J. Biol. Chem.*, 275 (2000) 2499.
324 M.F. Walter, J.B. Blumberg, C.G. Dolnikowski and G.J. Handelman, *Anal. Biochem*, 280 (2000) 73.
325 J. Bessard, J.L. Cracowski, F. Stanke-Labesque and G. Bessard, *J. Chromatogr. B*, 754 (2001) 333.
326 L.J. Roberts, II, T.J. Montine, W.R. Markesbery, A.R. Tapper, P. Hardy, S. Chemtob, W.D. Dettbarn and J.D. Morrow, *J. Biol. Chem.*, 273 (1998) 13605.
327 E.E. Reich, W.E. Zackert, C.J. Brame, Y. Chen, L.J. Roberts, D.L. Hachey, T.J. Montine and J.D. Morrow, *Biochemistry*, 39 (2000) 2376.

328 S.S. Fam, L.J. Murphey, E.S. Terry, W.E. Zacket, Y. Chen, L. Gao, S. Pandalai, G.L. Milne, L.J. Roberts, N.A. Porter, T.J. Montine and J.D. Morrow, *J. Biol. Chem.*, July (2002).
329 A.D. Jones, C.K. Winter, M.H. Buonarati and H.J. Segall, *Biol. Mass Spectrom.*, 22 (1993) 68.
330 D.L. Chance, K.O. Gerhardt and T.P. Mawhinney, *J. Chromatogr. A*, 79 (1998) 91.
331 W. Jira and G. Spiteller, *Chem. Phys. Lipids*, 79 (1996) 95.
332 W. Jira, G. Spiteller, W. Carso and A. Schramm, *Chem. Phys. Lipids*, 91 (1998) 1.
333 V.A. Folcik and M.K. Cathcart, *J. Lipid Res.*, 35 (1994) 1570.
334 M. Yamane, A. Abe and S. Yamane, *J. Chromatogr. B*, 652 (1994) 123.
335 M. Van Rollins and H.R. Knapp, *J. Lipid Res.*, 36 (1995) 952.
336 M.P. Yurawecz, J.K. Hood, M.M. Mossoba, J.A.G. Roach and Y. Ku, *Lipids*, 30 (1995) 595.
337 T. Hou, *J. Am. Oil Chem. Soc.*, 71 (1994) 975.
338 D. Laudert, P. Henning, B.A. Stelmach, A. Muller, L. Andert and E.W. Weiler, *Anal. Biochem.*, 246 (1997) 211.
339 C. Schneider, P. Schreier and M. Herderich, *Lipids*, 32 (1997) 331.
340 X.-H. Wang, T. Oshima, H. Ushino and C. Koizumi, *Lipids*, 34 (1999) 675.
341 T. Oshima, A. Hopia, J.B. German and E.N. Frankel, *Lipids*, 31 (1996) 1091.
342 P. Falardeau, M. Robillard and R. Hui, *Anal. Biochem.*, 208 (1993) 311.
343 P. Zollner, E. Lorbeer and G. Remberg, *Org. Mass Spectrom.*, 29 (1994) 253.
344 P. Zollner and E. Lorbeer, *J. Mass Spectrom.*, 30 (1995) 432.
345 P. Zollner and R. Schmid, *J. Mass Spectrom.*, 31 (1996) 411.
346 V.A. Liepkalns, J.J. Myherr, A. Kuksis, U. Leli, N. Freysz and G. Hauser, *Biochem. Cell Biol.*, 71 (1993) 141.
347 V.A. Liepkalns, J.J. Myherr, A. Kuksis, U. Leli, N. Freysz and G. Hauser, *J. Chromatogr. B*, 658 (1994) 223.
348 D.S. Woodard, B.L. Mealey, C.S. Lear, R.K. Satsangi, T.J. Prihoda, S.T. Weitraub, R.N. Pinchard and L.M. McManus, *Biochim. Biophys. Acta*, 1259 (1995) 137.
349 B.Y. Jeong, T. Oshima and C. Koizumi, *Lipids*, 31 (1996) 9.
350 W.C. Hubbard, T.R. Hundley, A. Oriente and D.W. MacGlashan, Jr., *Anal. Biochem.*, 236 (1996) 309.
351 E. Thyssen, J. Turk, A. Bohrer and W.F. Stenson, *Lipids*, 31 (1996) S-255.
352 S. Antonopoulou, N.K. Andrikopoulos and C.A. Demopoulos, *J. Liq. Chromatogr.*, (1994) 633.
353 J.T. Lin, C.L. Woodruff and T.A. McKeon, *J. Chromatogr. A*, 782 (1997) 41.
354 T. Takagi and Y. Ando, *J. Am. Oil Chem. Soc.*, 71 (1994) 459.
355 C. Lee, S.K. Fisher, B.W. Agronoff and A.K. Hajra, *J. Biol. Chem.*, 266 (1991) 22837.
356 P. Butikofer, M. Zollinger and U. Brodbeck, *Eur. J. Biochem.*, 208 (1992) 677.
357 P. Butikofer, F.A. Kuypers, C. Schackleton, U. Brodbeck and S. Stieger, *J. Biol. Chem.*, 265 (1990) 18983.
358 D. Brautigam, B. Engelmann, D. Reiss, U. Reinhardt, J. Thiery, W.O. Richter and T. Broshe, *Atherosclerosis*, 119 (1996) 77.
359 B. Engelmann, U.M. Schonthier, W.O. Richter and J. Duhm, *Biochim. Biophys. Acta*, 1165 (1992) 38.
360 T.W. Scott, J.R. Ashes, E. Fleck and S.K. Gulati, *J. Lipid Res.*, 34 (1993) 827.
361 K. Louie, R.D. Wiegand and R.E. Anderson, *Biochemistry*, 27 (1988) 9014.
362 H. Takamura, H. Narita, R. Urade and M. Kito, *Lipids*, 21 (1986) 356.
363 X. Zhu and J. Eichberg, *Biochim. Biophys. Acta*, 1168 (1993) 1.
364 M.L. Blank, M. Robinson, V. Fitzgerald and F. Snyder, *J. Chromatogr.*, 298 (1984) 473.
365 C. Lee and A.K. Hajra, *Meth. Neurosci.*, 18 (1993) 190.
366 W.E. Connor, D.S. Lin, G. Thomas, F. Ey, T. DeLoughery and N. Zhu, *J. Lipid Res.*, 38 (1997) 2516.

367 P.C. Schmid, I. Spimrova and H.H.O. Schmid, *Lipids*, 32 (1997) 1181.
368 P.C. Schmid, S.B. Johnson and H.H.O. Schymid, *J. Biol. Chem.*, 266 (1991) 13690.
369 P.C. Schmid, E. Deli and H.H.O. Schmid, *Arch. Biochem. Biophys.*, 319 (1995) 168.
370 P.C. Schmid, I. Spimrova and H.H.O. Schmid, *Arch. Biochem., Biophys.*, 322 (1995) 306.
371 B.J. McKeone, K. Osmundsen, D. Brauchi, Q. Pao, C. Payton-Ross, C. Klinc, F.A. Kummerow and H.J. Pawnall, *J. Lipid Res.*, 38 (1997) 429.
372 M.V. Bell and J.R. Dick, *Lipids*, 26 (1991) 565.
373 M.V. Bell, in W.W. Christie (Ed.), *Advances in Lipid Methodology – Four*, The Oily Press, Dundee, 1997, pp. 45–82.
374 Y. Bayon, M. Croset, D. Daveloose, F. Guerbette, V. Chirouze, J. Viret, J.-C. Kader and M. Lagarde, *J. Lipid Res.*, 36 (1995) 47.
375 Y. Xu and P.-A. Siegenthaler, *Lipids*, 31 (1996) 223.
376 C. Thevenon, S.E. Bawab, B. Chantegrel and M. Lagarde, *J. Chromatogr. B*, 708 (1998) 39.
377 F. Santinelli, P. Domiani and W.W. Christie, *J. Am. Oil Chem. Soc.*, 69 (1992) 552.
378 J.-H. Kim, H. Uzawa, Y. Nishida, H. Ohrui and H. Meguro, *J. Chromatogr. A*, 677 (1994) 35.
379 H.-Y. Kim, T.-Cl. Wang and Y.-C. Ma, *Anal. Chem.*, 66 (1994) 3977.
380 Y. Itabashi, A. Kuksis, L. Marai and T. Takagi, *J. Lipid Res.*, 31 (1990) 1711.
381 Y. Itabashi, A. Kuksis and J.J. Myher, *J. Lipid Res.*, 31 (1990) 2119.
382 Y. Itabashi, L. Marai and A. Kuksis, *Lipids*, 26 (1991) 951.
383 L. Marai, A. Kuksis, J.J. Myher and Y. Itabashi, *Biol. Mass Spectrom.*, 21 (1992) 541.
384 H.A. Norman, C.F. Mischke, B. Allen and J.S. Vincent, *J. Lipid Res.*, 37 (1996) 1372.
385 Y.H. Kim, J.S. Yoo and M.S. Kim, *J. Mass Spectrom.*, 32 (1997) 968.
386 Y.H. Kim, J.S. Yoo, C.H. Lee, Y.M. Goo and G.J. Jhon, *Rapid Commun. Mass Spectrom.*, 13 (1999) 481.
387 M. Yano, E. Kishida, Y. Muneyuki and Y. Musuzawa, *J. Lipid Res.*, 39 (1998) 2091.
388 L.H. Couch, M.I. Churchwell, D.R. Deerge, W.H. Tolleson and P.C. Howard, *Rapid Commun. Mass Spectrom.*, 11 (1997) 504.
389 M. Gu, J.L. Kerwin, J.D. Watts and R. Aebersold, *Anal. Biochem.*, 244 (1997) 347.
390 G. Liebisch, W. Drobnik, M. Reil, B. Trumbach, R. Arnecke, B. Olgemoller, A. Roscher and G. Schmitz, *J. Lipid Res.*, 40 (1999) 1539.
391 L.Y. Yang, A. Kuksis and J.J. Myher, *J. Lipid Res.*, 36 (1995) 1046.
392 J.J. Myher, A. Kuksis, K. Geher, P.W. Park and D.A. Diersen-Schade, *Lipids*, 31 (1996) 207.
393 J.J. Myher, A. Kuksis, C. Tilden and O.T. Oftedal, *Lipids*, 29 (1994) 411.
394 H. Oku, J. Shudo, J. Nagata and I. Chinen, *Biochim. Biophys. Acta*, 1300 (1996) 35.
395 A. Kawashima, Y. Shimada, T. Nagao, A. Ohara, T. Matsuhisa, A. Sugihara and Y. Tominaga, *J. Am. Oil Chem. Soc.*, 79 (2002) 871.
396 C.H. Winter, E.B. Howing and F.A.J. Muskiet, *J. Chromatogr.*, 616 (1993) 9.
397 A. Kempinen and P. Kalo, *J. Am. Oil Chem. Soc.*, 70 (1993) 1203.
398 V. Ruiz-Gutierrez, J.L. Prada and F. Perez-Jiminez, *J. Chromatogr.*, 622 (1993) 117.
399 M. Demirbuker, L.G. Blomberg, N.U. Olsson, M. Bergqvist, B.G. Herslof and F.A. Jacobs, *Lipids*, 27 (1992) 436.
400 P. Sundin, P. Larsson, C. Wesen and G. Odham, *Biol. Mass Spectrom.*, 21 (1992) 633.
401 R. Fabien, J.D. Craske and M. Wootton, *J. Am. Oil Chem. Soc.*, 70 (1993) 551.
402 M.H.J. Bergqvist and H.P. Kaufmann, *Lipids*, 28 (1993) 667.
403 M. Lamberto and M. Saitta, *J. Am. Oil Chem. Soc.*, 72 (1995) 867.
404 R. Irimescu, M. Yasui, Y. Iwasaki, N. Shimizu and T. Yamane, *J. Am. Oil Chem. Soc.*, 77 (2002) 501.
405 S. Kermasha, S. Kubow, M. Safari and A. Reid, *J. Am. Oil Chem. Soc.*, 70 (1993) 169.
406 Y. Itabashi, J.J. Myher and A. Kuksis, *J. Am. Oil Chem. Soc.*, 70 (1993) 1177.

407 C.C. Becker, A. Rosenquist and G. Holmer, *Lipids*, 28 (1993) 147.
408 D.C. Taylor, S.L. Acenzie, A.R. McCurdy, P.B.E. McVetty, E.M. Giblin, E.W. Pass, S.J. Stone, R. Scarth, S.R. Rimmer and M.D. Pickard, *J. Am. Oil Chem. Soc.*, 71 (1994) 163.
409 D.C. Taylor, E.M. Gibblin, D.W. Reed, L.R. Hogge, D.J. Olson and S.L. Mackenzie, *J. Am. Oil Chem. Soc.*, 72 (1995) 305.
410 M. Hori, Y. Sahashi, S. Koike, R.R. Yamaoka and M. Sago, *Anal. Sci.*, 10 (1994) 719.
411 C. Baiocchi, G. Saini, C. Cocito, D. Giacosa, M.A. Roggero, E. Merengo and M. Favale, *Chromatographia*, 37 (1993) 525.
412 D.G. Hayes and R. Keliman, *J. Am. Oil Chem. Soc.*, 73 (1996) 267.
413 P. Manninen, P. Laakso and H. Kallio, *Lipids*, 30 (1995) 665.
414 P. Manninen, P. Laakso and H. Kallio, *J. Am. Oil Chem. Soc.*, 72 (1995) 1001.
415 Y. Itabashi, L. Marai and A. Kuksis, *Lipids*, 26 (1991) 951.
416 P. Ruiz-Sala, M.T.G. Hierro, I. Martinez-Castro and G. Santa-Maria, *J. Am. Oil Chem. Soc.*, 73 (1996) 283.
417 J.J. Myher, A. Kuksis and L. Marai, *J. Am. Oil Chem. Soc.*, 70 (1993) 1183.
418 L. Marai, A. Kuksis and J.J. Myher, *J. Chromatogr. A*, 672 (1994) 87.
419 G.A. Spanos, S.J. Schwartz, R.B. van Breemen and C.H. Huang, *Lipids*, 30 (1995) 85.
420 W.E. Neff, E. Selke, T.L. Mounts, W. Rinsch, E.N. Frankel and M.A.M. Zeitoun, *J. Am. Oil Chem. Soc.*, 69 (1992) 111.
421 A. Kuksis, J.J. Myher, L. Marai and K. Geher, in Y. Malki (Ed.), *Proceedings, 17th Nordic Lipid Symposium, A Lipidforum Publication, Imatra, Finland*, 1993, pp. 230–238.
422 W.E. Neff and W.C. Byrdwell, *J. Chromatogr. A*, 818 (1998) 169.
423 W.C. Byrdwell and W.E. Neff, *Rapid Commun. Mass Spectrom.*, 16 (2002) 300.
424 E. Viinanen and A. Hopia, *J. Am. Oil Chem.*, 71 (1994) 537.
425 L. Steenhorst-Slikkerveer, A. Louter, H.-G. Janssen and C. Bauer-Plank, *J. Am. Oil Chem. Soc.*, 77 (2000) 837.
426 C. Bauer-Plank and L. Steenhorst-Slikkerveer, *J. Am. Oil Chem. Soc.*, 77 (2000) 477.
427 T. Kusaka, S. Ishihara, M. Sakaida, A. Mifune, Y. Nakano, K. Tsuda, M. Ikeda and H. Nakano, *J. Chromatogr. A*, 730 (1996) 1.
428 J.-P. Kurvinen, A. Kuksis, A. Ravandi, O. Sjovall and H. Kallio, *Lipids*, 34 (1999) 299.
429 A. Kuksis, *J. Chromatogr. A*, 935 (2001) 203.
430 L. Kritharides, W. Jessup, J. Gilford and R.T. Dean, *Anal. Biochem.*, 213 (1993) 79.
431 D.W. Johnson, H.J. ten Brink and C. Jacobs, *J. Lipid Res.*, 42 (2001) 1699.
432 W. Korytowski, P.G. Geiger and A.W. Girotti, *Meth. Enzymol.*, 300 (1999) 23.
433 J. Plat, H. Brzezinka, D. Lutjohann, R.P. Mensink and K. von Bergmann, *J. Lipid Res.*, 42 (2001) 2030.
434 G.M. Patton, J.M. Fasulo and S.J. Robins, *J. Lipid Res.*, 23 (1982) 190.
435 B.S. Beckman, C. Mallia and S. Clejan, *Biochem. J.*, 314 (1996) 861.
436 W. Bernhard, A.D. Postle, M. Linck and K.F. Sewing, *Biochim. Biophys. Acta*, 1255 (1995) 99.
437 J. McHowatt, J.H. Jones and M.H. Creer, *J. Lipid Res.*, 37 (1996) 2450.
438 H.Y. Kim and N. Salem, Jr., *Prog. Lipid Res.*, 32 (1993) 221.
439 S.L. Abidi and T.M. Mounts, *J. Liq. Chromatogr.*, 15 (1992) 2487.
440 S.L. Abidi and T.M. Mounts, *J. Liq. Chromatogr.*, 17 (1994) 105.
441 S.L. Abidi and T.M. Mounts, *J. Chromatogr. Sci.*, 31 (1993) 231.
442 S.L. Abidi and T.M. Mounts, *J. Chromatogr. A*, 694 (1995) 365.
443 J.F.H.M. Brouwers, B.M. Gadella, L.M.G. Van Golde and A.G.M. Tielens, *J. Lipid Res.*, 39 (1998) 344.
444 Y. Itabashi and A. Kuksis, *Analyt. Biochem.*, 254 (1997) 49.

445 L. Rizea-Savu, F. Silvestro, G. Sorgel, E. Montrucchio, G. Lupia and Camussi, *J. Chromatogr. B*, 682 (1996) 35.
446 S. Antonopoulou, C.A. Demopoulos, D. Argyropoulos, G. Baltas, H. Kotsifaki and A. Dimanti-Kipioti, *Biochem. J.*, 330 (1998) 791.
447 C. Li and J.A. Yergey, *J. Mass Spectrom.*, 32 (1997) 374.
448 C. Li, A. McClory, E. Wong and J.A. Yergey, *J. Mass Spectrom.*, 34 (1999) 521.
449 S. Ramanadham, F.-F. Hsu, S. Zhang, A. Bohrer, Z. Ma and J. Turk, *Biochim. Biophys. Acta*, 1484 (2000) 251.
450 L. Heikinheimo and P. Somerharju, *J. Biol. Chem.*, 273 (1998) 3327.
451 F. Hullin, H.Y. Kim and N. Salem, *J. Lipid Res.*, 30 (1989) 1963.
452 G.M. Patton, J.M. Fasulo and S.J. Robins, *J. Nutr. Biochem.*, 1 (1990) 549.
453 X. Han and R.W. Gross, *J. Am. Soc. Mass Spectrom.*, 6 (1995) 1202.
454 F.-F. Hsu, A. Bohrer and J. Turk, *J. Am. Soc. Mass Spectrom.*, 9 (1998) 516.
455 E. Hvattum, C. Rosjo, T. Gjoen, G. Rosenlund and B. Ruyter, *J. Chromatogr. B*, 748 (2000) 137.
456 J.-P. Kurvinen, A. Kuksis, A.J. Sinclair, L. Abedin and H. Kallio, *Lipids*, 35 (2000) 1001.
457 N. Khaselev and R.C. Murphy, *J. Am. Soc. Mass Spectrom.*, 11 (2000) 283.
458 R.C. Murphy, *Handbook of Lipid Research: Mass Spectrometry of Lipids*, Plenum Press, New York, 1993.
459 R. Taguchi, J. Hayakawa, Y. Takeuchi and M. Ishida, *J. Mass Spectrom.*, 35 (2000) 953.
460 D.L. Baker, D.M. Desiderio, D.D. Miller, B. Tolley and G.J. Tigyi, *Anal. Biochem.*, 292 (2001) 287.
461 S. Chen, *J. Chromatogr. B*, 661 (1994) 1.
462 M. Koivusalo, P. Haimi, L. Heikinheimo, R. Kostiainen and P. Somerharju, *J. Lipid Res.*, 42 (2001) 663.
463 Y. Endo, M. Tagiri-Endo, H.-S. Seo and K. Fujimoto, *J. Chromatogr. A*, 911 (2001) 39.
464 J.C. McPherson, J.G. Pavlovich and R.S. Jacobs, *Lipids*, 33 (1998) 931.
465 Y. Xiao, Y. Chen, A.W. Kennedy, J. Bekinson and Y. Xu, *Ann. N.Y. Acad. Sci.*, 905 (2000) 242.
466 T. Gunnarsson, A. Karlsson, P. Hansson, G. Johnson, C. Alling and G. Odham, *J. Chromatogr. B*, 705 (1998) 243.
467 K.R. Auger, L.A. Serunian, S.P. Soltoff, P. Libby and L.C. Cantley, *Cell*, 57 (1989) 167.
468 M. Whitman, C.P. Downes, M. Keele, T. Keller and L.C. Cantley, *Nature*, 332 (1988) 644.
469 A.-S. Anderson, R.A. Demel, L. Rilfors and G. Lindblom, *Biochim. Biophys. Acta*, 1369 (1998) 94.
470 A.-S. Anderson, L. Rilfors, R.N.A.H. Lewis, R.N. McElhaney and G. Lindblom, *Biochim. Biophys. Acta*, 1389 (1998) 43.
471 J.V. Zhang, B.J. Nobes, J. Wang and I.A. Blair, *Biol. Mass Spectrom.*, 123 (1994) 399.
472 J.-R. Zhang, A.R. Cazers, B.S. Lutzke and E.D. Hall, *Free Rad. Biol. Med.*, 18 (1995) 1.
473 H. Kamido, A. Kuksis, L. Marai and J.J. Myher, *J. Lipid Res.*, 36 (1995) 1876.
474 A. Ravandi and A. Kuksis, in R.A. Meyers (Ed.), *Encyclopedia of Analytical Chemistry*, Wiley, Chichester, 2000, p. 1.
475 C.M. Spickett, A.R. Pitt and A.J. Brown, *Free Rad. Biol. Med.*, 25 (1998) 613.
476 L.M. Hall and R.C. Murphy, *Anal. Biochem.*, 258 (1998) 184.
477 N. Baba, H. Daido, T. Kosug, M. Miyake and S. Nakajima, *Biosci. Biotechnol. Biochem.*, 52 (1998) 160.
478 S. Ponchaut, K. Veitch, R. Libert, F. Van Hoof, L. Hue and E. de Hoffman, *J. Am. Soc. Mass Spectrom.*, 7 (1996) 50.
479 L. Teesch and J. Adam, *Org. Mass Spectrom.*, 27 (1992) 931.

480 E. Bayer, P. Gefrorer and C. Rentel, *Angew. Chem. – Int. Ed.*, 38 (1999) 992.
481 C.M. Havrilla, D.L. Hachey and N.A. Porter, *J. Am. Chem. Soc.*, 122 (2000) 8042.
482 G.L. Milne and N.A. Porter, *Lipids*, 36 (2001) 1265.
483 M. Schlame, K. Beyer, M. Hayer-Hartl and M. Lingenberg, *Eur. J. Biochem.*, 199 (1991) 459.
484 B. Frey, R. Haupt, S. Alms, G. Holzmann, T. Konig, H. Kern, W. Kox, B. Rustow and M. Schlame, *J. Lipid Res.*, 41 (2000) 1145.
485 H. Kamido, H. Eguchi, H. Ikeda, T. Imaizumi, K. Yamana, K. Hartvigsen, A. Ravandi and A. Kuksis, *J. Lipid Res.*, 43 (2002) 158.
486 E.A. Podrez, E.E. Poliakov, Z. Shen, R. Zhang, Y. Deng, P.J. Finton, L. Shan, M. Febbraio, D.P. Hajjar, R.L. Silverstein, H.F. Hoff, R.G. Solomon and S.L. Hazen, *J. Biol. Chem.*, 277 (2002) 38517.
487 H. Kamido, A. Kuksis, L. Marai and J.J. Myher, *FEBS Lett.*, 304 (1992) 269.
488 H. Itabe, K. Suzuki, R. Hosoyya, M. Mori, Y. Ghigashi, Y. Fujimoto and T. Takano, *Anal. Biochem.*, 285 (2000) 151.
489 Z. Ou, A. Ogamo, L. Guo, Y. Konda, Y. Harigaya and Y. Nakagawa, *Anal. Biochem.*, 227 (1995) 289.
490 N.C. Shanta, *J. Chromatogr.*, 624 (1992) 21.
491 C.C. Parrish, C.H. McKenzie, B.A. MacDonald and E.A. Hatfield, *Mar. Ecol. Prog. Ser.*, 129 (1995) 151.
492 C.C. Parrish, G. Bodennec and P. Gentien, *J. Chromatogr. A*, 741 (1996) 91.
493 K. Okumura, K. Hayashi, I. Morishima, K. Murase, H. Matsui, Y. Toki and T. Ito, *Lipids*, 33 (1998) 529.
494 L. Striby, R. Lafont and M. Goutx, *J. Chromatogr. A*, 849 (1999) 371.
495 G. Peyrou, V. Rakotondrazafy, Z. Mouloungui and A. Gaset, *Lipids*, 31 (1996) 27.
496 E.D. Hudson, R.J. Helleur and C.C. Parrish, *J. Chromatogr. Sci.*, 39 (2001) 146.
497 A. Kuksis, in T. Shibamoto (Ed.), *Lipid Chromatographic Analysis*, Marcel Dekker, New York, NY, 1994, pp. 177–222.
498 H.-G. Janssen, W. Boers, H. Steenbergen, R. Horsten and E. Floter, *J. Chromatogr. A*, 1000 (2003) 385.
499 R.P. Evershed, C. Heron and L.J. Goad, *Analyst*, 115 (1990) 1339.
500 S. Charters, R.P. Evershed, L.J. Goad, P.W. Blinkhorn and V. Denham, *Archaeometry*, 35 (1993) 211.
501 R.P. Evershed, S.N. Dudd, M.S. Copley, R. Berstan, A.W. Stott, H. Mottram, S.A. Buckley and Z. Crossman, *Acc. Chem. Res.*, 35 (2002) 660.
502 L. Mondello, M. Catalfamo, G. Dugo and P. Dugo, *J. Chromatogr. Sci.*, 36 (1998) 201.
503 W.W. Christie and R.A. Urwin, *J. High Resol. Chromatogr.*, 18 (1995) 97.
504 B. Marcato and G. Cecchin, *J. Chromatogr. A*, 730 (1996) 83.
505 C. Silversand and C. Haux, *J. Chromatogr. B*, 703 (1997) 7.
506 J.-T. Liu, C.L. Woodruff and T.A. McKeon, *J. Chromatogr. A*, 782 (1997) 41.
507 E.J. Murphy, T.A. Rosenberger and L.A. Horrocks, *J. Chromatogr. B*, 685 (1996) 9.
508 Y.H. Kim, J.-S. Choi, J.S. Yoo, Y.-M. Park and M.S. Kim, *Anal. Biochem.*, 267 (1999) 260.
509 R. Welti, W. Li, M. Li, Y. Sang, H. Biesiada, H.-E. Zhou, C.B. Rajashekar, T.D. Williams and X. Wang, *J. Biol. Chem.*, 277 (2002) 31994.
510 K. Ekroos, I.V. Chernushevich, K. Simons and A. Shevchenko, *Anal. Chem.*, 74 (2002) 941.
511 J. Zhang, A. Okubo and S. Yamazaki, *Biosci. Biotechnol. Biochem.*, 65 (2001) 2573.
512 D.D. Wise, T.V. Barkhimer, P.A. Brault, J.R. Kirchhoff, W.S. Messer, Jr. and R.A. Hudson, *J. Chromatogr. B*, 775 (2002) 49.
513 N.U. Olsson, P. Kaufmann and S. Dzeletovic, *J. Chromatogr. B*, 698 (1997) 1.
514 J.T. Stull and J.E. Buss, *J. Biol. Chem.*, 252 (1977) 851.

515 P.A. Lanzetta, L.J. Alvarez, P.S. Rejnach and O.A. Candia, *Anal. Biochem.*, 100 (1979) 95.
516 B.P. Van Veldhoven, J.V. Swinnen, M. Esquenet and G. Verhoeven, *Lipids*, 32 (1997) 1297.
517 J.A. Maslanski and W.B. Bosa, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, pp. 113–126.
518 N. Ashizawa, M. Yoshida and T. Aotsuka, *J. Biochem. Biophys. Meth.*, 44 (2000) 89.
519 L.J. Rubin, F.F. Hsu and W.R. Sherman, in J.N. Fain (Ed.), *Lipid metabolism in Signaling Systems*, *Methods in Neurosciences*, Vol. 18, Academic Press, New York, 1993, pp. 201–212.
520 G.P. Heathers, T. Juehne, L.J. Rubin, P.B. Corr and A.S. Evers, *Anal. Biochem.*, 176 (1989) 109.
521 Y. Pak and J. Larner, *Biochem. Biophys. Res. Commun.*, 184 (1992) 1042.
522 R.E. Ostlund, Jr., J.B. McGill, I. Herskowitz, D. Kipnis, J.V. Santiago and W.R. Sherman, *Proc. Natl Acad. Sci. USA*, 90 (1993) 9988.
523 K.-S. Liu, *J. Am. Oil Chem. Soc.*, 71 (1994) 1179.
524 C. Major and B.A. Wolf, *J. Chromatogr. B*, 658 (1994) 233.
525 J.K.G. Kramer, V. Fellner, M.E.R. Dugan, F.D. Sauer, M.M. Mossoa and M.P. Yurawecz, *Lipids*, 32 (1997) 1219.
526 Y. Hallaq, T.C. Becker, C.S. Manno and M. Laposata, *Lipids*, 28 (1993) 355.
527 F. Rubino and L. Zecca, *J. Chromatogr.*, 579 (1992) 1.
528 J.C. Dittmer and M.A. Wells, *Meth. Enzymol.*, 14 (1969) 482.
529 C.P. Downes and R.H. Michell, *Biochem. J.*, 198 (1981) 133.
530 D.M. Brown and J.C. Stewart, *Biochim. Biophys. Acta*, 125 (1966) 413.
531 A.J. Slotboom, G.H. De Haas, P.P.M. Bonsen, G.J. Burbach-Westerhuis and L.L.M. Van Deenen, *Chem. Phys. Lipids*, 4 (1970) 15.
532 C. Zhou, Y. Wu and M.F. Roberts, *Biochemistry*, 36 (1997) 347.
533 S. Bruzik, A.A. Hakeem and M.-D. Tsai, *Biochemistry*, 33 (1994) 8367.
534 G.W. Mayr, *Biochem. J.*, 254 (1988) 585.
535 G.W. Mayr, in R.F. Irvine (Ed.), *Methods in Inositide Research*, Raven Press, New York, 1990, p. 83.
536 W.D. Singer, H.A. Brown and P.C. Sternweis, *Ann. Rev. Biochem.*, 66 (1997) 475.
537 A.N. Carter, R. Huang, A. Sorisky, C.P. Downes and E. Rittenhouse, *Biochem. J.*, 302 (1994) 415.
538 N. Divecha and R.F. Irvine, in R.F. Irvine (Ed.), *Methods in Inositide Reseach*, Raven Press, New York, 1990, pp. 179–185.

This page is intentionally left blank

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 18

Carbohydrates

SHIRLEY C. CHURMS

CONTENTS

18.1 INTRODUCTION

Since the publication of the previous edition of this textbook [1] there have been many further developments in the application of high-performance liquid chromatography (HPLC) to the chromatographic analysis of carbohydrates. The major area of growth has undoubtedly been in the use of what has become known as high-pH or high-performance anion-exchange chromatography (HPAEC), a form of ion chromatography developed specifically for analysis of sugars, which is now the method of choice in many laboratories worldwide. This technique, which has the signal advantage of requiring no derivatization of the sample prior to analysis, is applicable not only to monosaccharides but also to oligosaccharides over a wide range of degree of polymerization (DP), including the complex mixtures of oligosaccharides obtained in degradative studies of glycoconjugates. There has been continued interest in analytical methods of HPLC in other modes, as well as high-performance ion-exchange chromatography of the conventional type, but these techniques are increasingly being overshadowed because of the phenomenal growth of HPAEC. Size-exclusion chromatography (SEC) has, on the other hand, retained its importance in the carbohydrate field, due to the unique capacity this form of chromatography possesses of yielding valuable information on the molar mass distribution of polysaccharides. In recent years, the accuracy of this information has been much improved by the coupling of SEC systems to instruments capable of measuring molar mass by absolute methods, such as light scattering. Affinity chromatography, now increasingly possible in rapid, high-performance mode, has also remained a useful tool, particularly in studies of glycoconjugates.

During the past decade there have been significant advances in detection methods for HPLC analysis of carbohydrates. In particular, mass spectrometry (MS), interfaced with HPLC has become an important tool in carbohydrate studies, as in other fields. In this respect the development of HPLC analysis of carbohydrates has been similar to that of

gas/liquid chromatography (GLC), in which the coupling of MS to the chromatographic system is now considered essential. The use of GLC as an analytical method for carbohydrates has declined since the advent of HPAEC, but there have been some advances, notably in the resolution of enantiomeric pairs by the application of enantioselective stationary phases. There has been less interest in the preparation of chiral derivatives for this purpose, and indeed in derivatization in general, although there have been some improvements in the classical methodologies.

In planar chromatography, thin-layer chromatography (TLC) has now entirely superseded paper chromatography in carbohydrate analysis. The most important advance in this area has been in detection, with the use of overlay binding techniques in conjunction with methods, such as enzyme-linked immunosorbent assay (ELISA), greatly increasing the sensitivity of detection of complex carbohydrates. This has enhanced the usefulness of TLC in analyses involving such carbohydrates, particularly clinically important glycolipids and glycopeptides. The classical electrophoretic methods that were applied in the carbohydrate field have been superseded in recent years by the rapidly growing technique of high-performance capillary electrophoresis. This chapter will end with a brief overview of the current status of this important analytical method in carbohydrate chemistry.

18.2 LIQUID CHROMATOGRAPHY

The continued growth of HPLC as a major tool in carbohydrate analysis is reflected in the large number of reviews devoted to this topic that have appeared in the last decade [2–7] and the fact that a major reference book covering in detail many aspects of carbohydrate analysis by HPLC and capillary electrophoresis, which was first published in 1995 [8], has required extensive expansion and updating only seven years later [9].

18.2.1 Detection

From the outset, the universal refractive-index detector has been much used in HPLC analysis of carbohydrates without UV-absorbing chromophores. However, these detectors have the disadvantages of limited sensitivity and the effect of changes in solvent composition on their output, which precludes their use in gradient elution. Although the incorporation of lasers as light sources in refractive-index detectors has permitted detection of sugars at levels of 40–50 ng [10], or even lower with a *z*-configuration flow-cell and a position-sensitive detector included in the refractometer design [11], the evaporative light-scattering detector (ELSD) is now strongly recommended by some authors [12–15] as a better universal detector for carbohydrate analysis. The major advantage of the ELSD is its compatibility with gradient elution, and detection limits in the range of 1–10 ng have been claimed for analyses of sugars and alditols by this means [15]. In recent years the detector has been applied successfully in SEC of polysaccharides [16] (Sec. 18.2.8). The chiroptical properties of carbohydrates have also been applied in detection systems, although further developments in the technology involved are required before this type of detection can be regarded as an established technique in carbohydrate analysis [17]. Spectropolarimeters operating at multiple wavelengths [circular dichroism

(CD) detectors] are considered the best option, but they should be used in tandem with other detectors. An advantage of chiroptical detection is that it gives an indication of enantiomeric purity, but sensitivity is limited.

Because most carbohydrate species, including not only sugars but also alditols, sugar acids, amino sugars, and even oligo- and polysaccharides, are readily oxidized, they lend themselves to electrochemical detection without the necessity for derivatization. The use of such systems in HPLC of carbohydrates has been widely reported in the past decade. Two types of electrochemical detection have emerged, the best known being the pulsed-amperometric detector (PAD) that has become firmly established with its adoption for use in conjunction with HPAEC. However, constant-potential electrochemical detectors also have certain advantages [18]: first, the instrumentation required is simpler than that for the PAD and, secondly, the constant-potential mode should lower the detection limits because noise should be minimized when the applied potential is invariant. For underivatized carbohydrates, oxidation at a carbon electrode is kinetically unfavored and the required potentials are too high to be of practical use. Oxidation of carbohydrates for detection purposes has generally been carried out on electrodes composed of transition metals, either pure or in combination with other such metals [19]. For example, copper-wire electrodes were used [20] for the simultaneous detection of sugars, polyols, and carboxylic acids in HPLC, sugars and polyols being oxidized at $+0.5$ V in 100 m*M* NaOH and the acids (citric and acetic) being detected potentiometrically in Milli-Q water. Detection limits in the picomolar range were reported. The use of various alloys of copper and of nickel as electrode materials was investigated by Kuwana and co-workers [21,22] with the objective of improving the long-term stability of electrode response: Ni–Ti and Ni–Cr alloys were reported to give the best results. More recently, Morita *et al.* [23] have further optimized the composition of the Ni–Ti alloy (to 30 atom % of Ni) to permit highly sensitive detection of glucose (down to 50 fmol) in microbore HPLC. On its own, nickel functions as a nickel (III) oxide electrode, which in alkaline solution acts as a strong oxidant towards carbohydrates with electroactive hydroxyl groups [24]. Such an electrode has proved sufficiently sensitive for use in microbore HPLC of monosaccharides, alditols, and oligosaccharides to the trisaccharide level [25].

Chemically modified electrodes, containing one of the active transition metals in a specific molecular form, have also been successfully employed. For example, cobalt has been used as cobalt phthalocyanine [26,27], and nickel (II) nitrate, deposited on the surface of a glassy carbon electrode, has been reported [28] to give a chemically modified electrode, offering a useful compromise between background-current stability, catalytic activity, low detection limits, and reproducibility for the determination of carbohydrates in real samples, such as milk and fruit juice. A similar, cobalt-based, chemically modified electrode has also been described [29]. Another novel electrode, consisting of a glassy carbon electrode, coated with poly-1-naphthylamine, with copper (II) ions incorporated into the polymeric matrix, has been used in HPLC analysis of various carbohydrates [30]. The use of electrodes consisting of platinum [31] or gold [32] for HPLC applications requires pulsed-potential operation. This approach, which is the basis of the PAD [more recently also known as the pulsed-electrochemical detector (PED)], is necessary to avoid rapid deactivation of the electrode surface due to adsorption of the intermediates produced during carbohydrate oxidation. In the triple-pulse method originally described by Hughes

and Johnson [31,33], the potential necessary for oxidizing the carbohydrate analytes is applied to the working electrode for a short time only (100 msec or less), after which the potential is increased to oxidize fully any material adsorbed on the surface of the electrode. It is then reversed to a strongly reducing potential to convert the resulting oxide layer back to the metal. These detectors require highly alkaline conditions for their operation and are thus particularly suited to HPAEC of carbohydrates. Although satisfactory results have been obtained with both platinum and gold electrodes, it is the latter that have become standard in the commercial PAD, marketed by Dionex, mainly because of the higher signal/noise ratio, which permits lower detection limits [34]. The sensitivity of the detector can be varied over a wide range, with full-scale deflection at outputs ranging from 1 nA to 100 μA [32]. The pulse width is also variable. The ever-increasing role played by such detectors in HPLC of carbohydrates has been the subject of two comprehensive review chapters [35,36] as well as a recent book [37].

Conductivity detection is applicable only to carbohydrates carrying charged groups, and its use requires suppression of the conductivity of the eluent. A commercially available system (Dionex), in which an anion micromembrane suppressor is placed on-line between the separating column and the conductivity detector, has been successfully applied in, *e.g.*, anion-exchange chromatography of sugar phosphates [38], which can be detected in the 20–100 pmol range, and similar analysis of UDP-sugars and nucleotides [39]. This detection system, used in conjunction with ion-exchange or reversed-phase ion-pairing chromatography (the ion-pairing reagent being removed by the micromembrane suppressor prior to detection), also offers a sensitive method of analyzing oligosaccharides, produced on depolymerization of glycosaminoglycans, especially those having no UV-absorbing chromophore, such as the products of keratan hydrolase degradation of keratan sulfate [40]. Use of a conductivity detector, coupled on-line to a refractometer has proved valuable in SEC of pectins, since it affords a method of estimating the degree of esterification from the ratio between the areas of the peaks given by the two detectors [41]. The refractive index signal is proportional to the total mass of pectin injected, whereas the conductivity signal depends on the proportion of unesterified galacturonic acid residues.

Enzymes immobilized in hydrogels, conductive polymers and other materials have been used increasingly in detection systems for HPLC of carbohydrates, though not yet on a large, commercial scale. The enzymes applied in detection of sugars are generally oxidases, with determination of the hydrogen peroxide produced being carried out by post-column addition of chromogenic or chemiluminescent reagents, or amperometrically. In some cases, dehydrogenases are used; however, these require co-factors, such as nicotinamide adenine dinucleotide (NAD^+). Developments in this growing area have been summarized in a review [42] and, more recently, a chapter [43]. Detection by means of biospecific enzyme reactors is particularly useful in analyses of sugars in complex mixtures, such as spent sulfite liquors [44] or fermentation broths [45], where matrix components interfere with other detection methods. A very recent development in detection of the complex oligosaccharides released from glycoconjugates is the use of immobilized lectins in a detection method based on the phenomenon known as surface plasmon resonance (SPR). The SPR detector responds to changes in refractive index in the vicinity of the sensor surface. These changes are caused by an analyte binding to or dissociating from a surface-immobilized ligand. For example, Blikstad *et al.* [46] used

Sambucus nigra agglutinin to detect oligosaccharides containing terminal residues of sialic acid and *Ricinus communis* agglutinin to detect those with β-linked galactose end-groups. In this way, oligosaccharides having these features could be detected at femtomole levels after SEC separation. Monoclonal antibodies have also been used in SPR detection [47].

The ultraviolet (UV) detector, widely used in HPLC of other analytes, is not suitable for detection of neutral sugars or polyols without prior derivatization. Such compounds absorb only at wavelengths below 200 nm, a region in which there is generally interference from eluent constituents, such as the acetonitrile commonly used in partition chromatography on polar sorbents (Sec. 18.2.2). However, direct UV detection is possible in chromatography of carbohydrates containing chromophoric groups: These include alduronic and aldonic acids and their lactones, compounds containing 2-amino- or 2-acetamido-2-deoxyhexose or sialic acid residues, and unsaturated oligosaccharides, such as those produced by lyase degradation of polymers containing hexuronic acid residues. For example, UV detection at 245 nm has been reported [48] to afford a sensitive method for the determination of ascorbic acid in blood plasma by RPC. Compounds with any of the structural features mentioned may be detected at wavelengths in the range 200–254 nm, often at picomolar levels [49]. In some cases, multiple-wavelength UV detection can be advantageous, and for this purpose the photodiode-array (PDA) detector is employed. For example, although SEC of the glycosaminoglycan heparin, with UV detection at 206 nm [50], has long been used as a means of isolating the fractions of lower molar mass, which have the greatest anticoagulant activity, detection at a single wavelength gives no information on the purity of the isolated fractions. The introduction of PDA detection into this analysis [51] permits the use of techniques such as spectral overlay, in which the chromatogram is recorded at several characteristic wavelengths (206–280 nm). Computation of the absorbance ratios at different wavelengths over the peak profile reveals any discrepancies that may be due to impurities. More recently, the use of PDA detection has been recommended [52] for chromatographic identification of sugar acids in the presence of organic acids. It has also lowered the detection limit for HPLC analysis of gangliosides (to as low as 78 ng at 196 nm [53]), eliminating the necessity for precolumn derivatization (Sec. 18.2.1.1). For HPLC of analytes available only in very small quantities, radiochemical detection is often used. The complex oligosaccharides obtained on degradation of glycoconjugates may be detected at sub-nanomolar levels, if the oligosaccharides are reduced with sodium borotritiide before chromatography, permitting detection of the ^{3}H label by scintillation counting of the fractions emerging from the separation column [54,55]. Metabolic labeling, by exchange with D-mannose labeled with ^{3}H, or 2-amino-2-deoxy-D-glucose labeled with ^{14}C, is also used [56,57].

18.2.1.1 Derivatization

Although the possibility of analysis without prior derivatization has long been regarded as one of the advantages of HPLC over GLC, pre-column derivatization that introduces a chromophore or a fluorescent group can be beneficial in that it permits the use of sensitive UV or fluorimetric detection. During the last decade, there has been increased interest in

such derivatization methods in the carbohydrate field, possibly because of the rapid development of capillary electrophoresis and of HPLC/MS, in which techniques they are also used to advantage. The various methods employed in pre-column derivatization of carbohydrates have been discussed in depth in several recent reviews [58–60]. The reactions used include benzoylation, 4-nitrobenzoylation, fluorescent labeling by treatment with 5-dimethylaminonaphthalene-1-sulfonylhydrazine (dansyl hydrazine), and reductive amination with various reagents, such as 2-aminopyridine, 4-aminobenzoic acid ethyl ester (ABEE), 2-aminoacridine (AMAC), or 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS). Derivatization with anthranilic acid (2-aminobenzoic acid), which is highly fluorescent, has been recommended [61,62] for HPLC analysis of mono- and oligosaccharides produced in degradative studies of glycoconjugates. Labeling of oligosaccharides by reductive amination with *p*-nitrobenzylhydroxylamine (PNB) to form *N*-(*p*-nitrobenzyloxy)aminoalditol derivatives has proved effective in HPLC/MS of oligosaccharides [63]. Derivatization with 1-phenyl-3-methyl-5-pyrazolone (PMP) is also recommended [64] for this purpose as well as for sensitive detection of sialylated oligosaccharides in biological fluids [65].

The development of HPLC/MS and the necessity to recover derivatives after separation and analysis by HPLC for further structural investigation by various spectroscopic techniques, especially in studies of the complex oligosaccharides from glycoconjugates, have led to decreased use of post-column derivatization in chromatography of carbohydrates. Nevertheless, Honda [66] has stressed the advantages of such methods in some analyses. This author has listed the main reactions that have been used for the purpose, in automated systems with photometric, fluorimetric, or electrochemical detection. These are classified as follows, on the basis of the chemistry involved:

(a) Conversion to furfurals by treatment with strong acids, followed by condensation with chromogenic reagents, for photometric detection. Examples include the classical orcinol/sulfuric acid, anthrone, and cysteine methods, as well as those used in detecting specific classes of carbohydrates, such as the carbazole method for uronic acids and the thiobarbituric acid method for sialic acids. The necessity for corrosive acids in the analytical system is clearly a serious drawback of these methods.

(b) Use of the reducing power of sugars. A prime example is the copper bicinchoninate method developed by Mopper [67], in which the copper (II) ion included in the reagent is reduced to copper (I), which binds to 2,2′-bicinchoninate to form a chelate having an absorption maximum at 562 nm. The detection limit for monosaccharides is about 100 pmol. The reduction of anisyltetrazolium chloride (tetrazolium blue) to diformazan, with an absorption maximum at 520 nm, is another example of a post-column derivatization reaction of this type [68], but its use is restricted to eluents consisting largely of ethanol or acetonitrile, in which the product is freely soluble.

(c) Periodate oxidation, followed by Hantsch reaction to form pyridine derivatives, for photometric or fluorimetric detection. The formaldehyde produced on periodate oxidation of an alditol is allowed to react with 2,4-pentanedione in the presence of ammonia to give the pyridine derivative, with an absorption

maximum at 410–420 nm. Fluorimetric monitoring (ex 410 nm, em 503 nm) is more sensitive (alditol detection limit, 0.5 nmol [69]).

(d) Reactions with fluorogenic reagents, for fluorimetric or, in some cases, electrochemical detection. These reagents include ethylenediamine in a weakly alkaline medium, which gives products that can be detected fluorimetrically, with detection limits below 1 nmol [70], or electrochemically, with limits at picomolar level [71]. Ethanolamine in boric acid also gives fluorescent products that have proved useful in post-column derivatization for HPLC of sugars [72]. However, the most valuable of the reagents of this type has been 2-cyanoacetamide, which at pH 9–10 reacts with reducing sugars to give intensely fluorescent products (ex 330 nm, em 380 nm), as well as one containing a conjugated diene system that absorbs strongly at 270 nm, so that UV detection is also possible [73]. The UV-absorbing product is electrochemically oxidizable at a glassy carbon electrode, which affords yet another method of detection when this reagent is used [74]. Picomolar detection limits have been reported in analyses of neutral sugars by these methods.

(e) Condensation with hydrazino compounds, for photometric detection. Reagents used include 4-aminobenzoic acid hydrazide [75], which under alkaline conditions forms a yellow product with reducing carbohydrates, permitting their detection at levels of below 5 pmol. This is considered [76] to be one of the most sensitive of the post-column derivatization methods.

(f) Use of a standard amino acid analyzer. Amino sugars may be determined directly by use of the ninhydrin reagent, and this procedure may be applied to neutral reducing sugars if they are first converted to glycamines [77]. Greater sensitivity is possible if the standard reagent is replaced by 2-phthaldehyde, which permits fluorimetric detection [78].

18.2.1.2 Advances in high-performance liquid chromatography/mass spectrometry

Mass-spectrometric detection and characterization of oligosaccharides, particularly the complex mixtures encountered in degradative studies of glycoconjugates, has become increasingly important during the past decade, as the crucial role played by such compounds in biological processes and pathological conditions has been recognized. The problems arising from the availability of the oligosaccharides in very small quantities and the time-consuming purification processes necessary for their examination by classical techniques can be overcome by the use of mass spectrometry in conjunction with capillary HPLC, capillary electrophoresis or capillary electrochromatography. The ionization techniques used have been mainly electrospray ionization (ESI), matrix-assisted laser desorption ionization (MALDI) and fast atom bombardment (FAB). The development of the new generation of instruments, such as the time-of-flight (TOF) mass spectrometer generally employed with MALDI, has raised the upper limit of the mass range that can be analyzed, so that the oligosaccharides can be detected virtually intact, and further structural information can then be obtained by tandem mass spectrometry. These advances have revolutionized carbohydrate analysis in recent years. Detailed discussion of these valuable

techniques is beyond the scope of this chapter; interested readers are referred to an excellent comprehensive review chapter recently published by Mark Stahl *et al.* [79]. The special advantages of MALDI-TOF-MS in studies of intact glycopeptides and glycoproteins, as well as released oligosaccharides, have also been the subject of a review [80].

Some of the newer methods of pre-column derivatization mentioned in Sec. 18.2.1.1, such as reductive amination with 2-aminopyridine, 2-aminoacridine or PMP, have been used to advantage in analyses involving mass spectrometry coupled to reversed-phase HPLC [64,81]. It is this type of chromatography that has been most frequently used in conjunction with MS in the carbohydrate field. With partition chromatography on polar sorbents (Sec. 18.2.2) difficulties are posed by the acetonitrile-rich eluents frequently employed. However, a recent paper [82] describes the analysis of sulfated oligosaccharide-alditols from gastric mucins by ESI-MS, coupled on-line to such a system, which indicates that these problems are being overcome. The use of FAB-MS in detection of malto-oligosaccharides, as their 4-hexadecylaniline derivatives, after separation on porous silica with a chloroform/methanol/water gradient, has also been reported [83], and the possible application of this technique in studies of glycolipids has been considered. The potential of ESI-MS coupled on-line to HPAEC, with a suppressor to remove sodium hydroxide from the eluent, and preferably with another mass spectrometer in tandem (LC/MS/MS) to obtain clearer mass spectra for identification of the carbohydrate analytes, has been discussed in a review by Black and Fox [84], who see the technique as a viable alternative to GLC/MS for profiling the carbohydrates present in hydrolyzates from whole bacterial cells.

Direct interfacing of SEC with MS also presents some problems, unless the mobile phase contains only volatile salts. However, there has been some progress in this area too. For example, Lecchi and Abramson [85] have reported the characterization of various polymers, including heparin, by SEC with an ammonium acetate buffer as the eluent, the chromatographic system being interfaced with a chemical reaction chamber preceding an on-line mass spectrometer. Thus, with the problems that once beset the coupling of HPLC systems to MS being rapidly overcome, it is to be expected that applications of this powerful analytical method in the carbohydrate field will grow ever more rapidly in the future.

18.2.2 Hydrophilic-interaction chromatography on silica-based sorbents bearing bonded polar groups

During the last years of the 20th century there was a marked trend towards abandoning the term "normal-phase chromatography" in referring to the mode in which adsorption or partition chromatography is carried out on a polar sorbent, such as porous silica or a silica-based sorbent bearing bonded polar groups, with a less polar mobile phase. The reason for this is that, with the rapid growth of reversed-phase chromatography (RPC), especially in HPLC/MS analyses (Sec. 18.2.1.2), it became doubtful whether the adjective "normal" still applies to chromatography governed by polar forces, which is more restricted in its applications. Therefore, the name "hydrophilic interaction chromatography", with the acronym HILIC, was proposed in 1990 by Alpert [86]. This term will be used here in all discussion of chromatography on polar phases, whether based on silica or on polymeric

matrices (Sec. 18.2.4). The topic has been discussed in depth in a recent review chapter [87]. An overview of applications in the carbohydrate field of HILIC on silica-based sorbents is given below.

18.2.2.1 Adsorption and partition chromatography on unmodified silica

Unmodified silica sorbents find application mainly in HPLC of carbohydrate derivatives (Sec. 18.2.1.1). Benzoate and 4-nitrobenzoate derivatives have been widely used, but the production of multiple peaks due to resolution of anomers and of pyranose and furanose ring forms, though aiding identification of individual sugars, complicates the chromatograms given by mixtures [88,89]. As in GLC, this can be obviated by reduction of sugars to alditols prior to derivatization, and good resolution of a mixture of 6-deoxyhexoses, pentoses, and hexoses, as the derived alditol benzoates, on a column (150 × 4.5 mm ID) of 3-μm silica has been reported by Oshima and Kumanotani [90], who used isocratic elution with *n*-hexane/dioxane/dichloromethane (30:4:1). Amino- and acetamidodeoxyhexoses, similarly derivatized, can be resolved and distinguished from one another, well separated from the neutral sugar derivatives, on the same column with gradient elution (*n*-hexane/dioxane/dichloromethane, 22:2:1 to 4:2:1 in 80 min). This simultaneous analysis of neutral, aminodeoxy and acetamidodeoxy sugars, which can be used also for preparative purposes, is a major advantage over GLC as the alditol acetate derivatives, although resolution of the neutral sugar derivatives is inferior to that obtained by capillary GLC of alditol acetates (Sec. 18.3.1).

Single peaks are obtained also by conversion of sugars to 2,4-dinitrophenylhydrazones prior to chromatography. Karamanos *et al.* [91] applied this method to the analysis of the neutral sugar components of glycoproteins (fucose, xylose, mannose, galactose, and glucose). With a column (260 × 4.6 mm ID) of 5-μm silica, isocratic elution with chloroform, containing methanol (7.6%) and a trace (0.7%) of water, and detection at 352 nm, the five sugars of interest, converted to the 2,4-dinitrophenylhydrazones, were resolved as single peaks. The slight shoulders or minor peaks observed, which were believed to be due to the presence of geometric isomers of these derivatives, were sufficiently separated from the major peaks not to interfere with quantitation. Calibration plots were linear over the range 0.05–3.5 nmol for each sugar. The increased sensitivity possible when HPLC of sugars is preceded by derivatization with dansyl hydrazine, which permits the use of fluorimetric detection, has been mentioned (Sec. 18.2.1.1). For example, Takeda *et al.* [92] reported good resolution of a mixture of 6-deoxyhexoses, pentoses, hexoses, acetamidodeoxyhexoses, and the disaccharides maltose, cellobiose, gentiobiose, and lactose as their DNS-hydrazone derivatives on a column (250 × 4 mm ID) of LiChrosorb SI-100 in 25 min, by stepwise elution with eluents containing increasing proportions of ethanol (8–20%) in aq. chloroform (0.5–0.6% water). The use of a spectrofluorophotometer to monitor the effluent (ex 350 nm, em 500 nm) made possible detection and quantitation of sugars at levels down to 3–20 pmol. Only one peak was discernible for each sugar.

More recently, conversion of sugars to the pernaphthoates of the derived alditols before HPLC on a column, packed with 5-μm silica, has been reported [93] to give excellent resolution of a mixture of nine of the monosaccharides, including aminodeoxyhexoses,

that are commonly encountered in hydrolyzates of samples of biological origin. The separation was achieved by isocratic elution (15 min at a flow-rate of 1 ml/min) with dichloromethane, containing 2-propanol (0.025%) and acetonitrile (0.6%), followed by a gradient of acetonitrile (0.6% to 32% in 15 min) in the dichloromethane/2-propanol mixture. Separation of the strongly retained aminodeoxyhexose derivatives required isocratic elution with the final solvent mixture for a further 10 min. Of particular interest is the use by Oshima *et al.* [94] of derivatization of neutral aldoses by reductive amination with chiral L-(−)-α-methylbenzylamine, followed by acetylation, to resolve enantiomers by HPLC. On a column (150 × 4.6 mm ID) of 3-μm silica, eluted isocratically with *n*-hexane/ethanol (19:1) and monitored by UV (230 nm), the enantiomers of all the common neutral aldoses, except 2-deoxy sugars, were well resolved. The configuration at C-2 determines the elution order of the enantiomers of the diastereoisomeric 1-(*N*-acetyl-α-methylbenzylamino)-1-deoxyalditol acetates.

HILIC on silica has been much used in the separation and analysis of glycolipids. In the past, perbenzoylation prior to chromatography was generally employed to increase the sensitivity of detection of these biologically important compounds. With UV detection at 230 nm, perbenzoylated monosialogangliosides have been detected at levels of 50 pmol [95] and glycosphingolipids at 20 pmol [96]. Effective eluents for separation of these compounds have been linear gradients of dioxane in *n*-hexane, 7 to 23% dioxane in 18 min for the gangliosides [95] and 2.5 to 25% dioxane in 13 min for the glycosphingolipids [96]. More recently, the focus has shifted to the separation of glycolipids without pre-column derivatization, to yield samples more amenable to subsequent structural investigation by spectroscopic techniques. This approach has been facilitated by the use of detection methods such as PDA detection [53]. For the underivatized glycolipids, more polar solvent systems, such as mixtures of *n*-hexane with 2-propanol and aqueous ammonia in various proportions [97], are used as eluents. This indicates that partition chromatography is an important factor in the separation mechanism in these cases. The same applies to HILIC, without pre-column derivatization, of the series of oligosaccharides with 4,5-unsaturated residues at the non-reducing end that are produced by lyase degradation of glycosaminoglycans. The UV absorbance of these unsaturated oligosaccharides is sufficiently high to permit UV detection at nanomolar level, and therefore derivatization is unnecessary. For example, HPLC on 5-μm silica, with an eluent consisting of acetonitrile/methanol/0.5 *M* ammonium formate, pH 6.0 (10:6:3), has proved effective in the separation of the unsaturated oligosaccharides obtained on digestion of hyaluronic acid with *Streptomyces* hyaluronidase [98].

Until recently, these separations of glycolipids and unsaturated oligosaccharides were regarded as the only really important examples in the carbohydrate field of HILIC based mainly on partition chromatography on unmodified silica sorbents. Attempts to separate underivatized sugars and alditols under such conditions had achieved only limited success. For example, Iwata *et al.* [99] resolved D-glucose and its α-(1 → 4)-linked oligomers (malto-oligosaccharides) to DP 5, as well as the products (nigerosyl- and nigerotriosyler-ythritol) of Smith degradation of lichen polysaccharides, linked (1 → 3) and (1 → 4), on a column (250 × 4.6 mm ID) of 5-μm silica, eluted with ethyl acetate/methanol/water (7:3:2). Elution with the same solvents in proportions 12:3:1 resolved sucrose, maltose, and lactose. Some resolution of rhamnose, xylose, arabinose, mannose, and glucose was

achieved on the same column with ethyl formate/methanol/water (12:3:1). Nikolov and Reilly [100] measured the capacity factors for HPLC of digitoxose (2,6-di-deoxy-D-*ribo*-hexose), 2-deoxy-D-glucose (2-deoxy-D-*arabino*-hexose), D-glucose, sucrose, the 3 isomers of trehalose (αβ, αα, and ββ), and *myo*-inositol on a column (250 × 4.6 mm ID) of 5-μm silica, with acetonitrile/water eluents of varying acetonitrile content (60–90%). Comparison of these values with those obtained with a similar column, packed with amine-bonded silica (also of 5-μm particle diameter), showed that similar separations required a higher proportion of acetonitrile in the case of the unmodified silica. Correlation of capacity factors with both the proportion of water in the pores of the silica-based sorbents and the calculated hydration numbers of the carbohydrates indicated that partition, not adsorption, is the predominant mechanism governing HPLC of underivatized carbohydrates on both silica and amine-bonded silica.

In 1992 Herbreteau *et al.* [12] reported that unmodified silica sorbents show higher selectivities for sugars and alditols, if eluents consisting of dichloromethane/methanol mixtures, with only a trace of water, are used rather than the usual acetonitrile/water solvent systems. These authors achieved good resolution of the common monosaccharides, with reproducible retention times, on columns packed with 5-μm silica, eluted with dichloromethane/methanol (4:1) containing 0.2% of water, and when the proportion of methanol was 28%, a mixture of several disaccharides and raffinose was well separated. Fructose, glucose, sucrose, and raffinose could be completely separated within 15 min by use of a linear gradient of methanol (20 to 55%) in dichloromethane. Gradient elution (25 to 40% of methanol in 11 min) was also recommended for resolution of polyols. It is evident that the presence of water in only trace amounts in these solvent systems obviates the problems arising from variation in the degree of hydration of the silica with varying water content of the mobile phase. The high selectivities reported by Herbreteau *et al.* [12] for HILIC of sugars and polyols on unmodified silica under these conditions suggest that this could provide an attractive alternative to the use of aminopropyl silica sorbents (Sec. 18.2.2.3), which are less stable.

18.2.2.2 Partition chromatography on amine-modified silica

Partition chromatography of underivatized carbohydrates on silica can also be achieved by addition of a polyfunctional amine to the mobile phase, so that the silica is modified *in situ* to an amino-phase packing for normal partition chromatography. After the successful application of this method to a few sugars important in the food industry, its use was extended to a large number of sugars and alditols [101,102]. In these studies, a Radial-Pak cartridge (Waters), 100 × 8 mm ID, packed with 10-μm silica and operated under radial compression (Waters module RCM-100), was modified by equilibration with acetonitrile/water (7:3), containing 0.1% of tetraethylenepentamine (TEPA), and eluted with acetonitrile/water (81:19 [101] or 3:1 [102]), containing 0.02% of the polyamine. The authors claimed that operation under radial compression minimized the dissolution of the silica packing at the high pH (8.9–9.2) of the eluent, resulting in long column life; this claim was subsequently disputed [49]. Good resolution of many alditols, monosaccharides, and oligosaccharides up to tetrasaccharide level was obtained. TEPA has

generally been considered to be the best amine modifier for chromatography of these analytes, although Verzele *et al.* [103] found piperazine equally effective and expressed a preference for this compound as, being a solid, it was more easily added in the small proportions required.

For chromatography of higher oligosaccharides by this method White *et al.* [104], using stainless-steel columns of different dimensions, packed with 5-μm silica, tested various polyfunctional amine modifiers. Of these, TEPA, pentaethylenehexamine, commercial "polyamine modifier", and 1,4-diaminobutane gave the best resolution of the first 8 members of the malto-oligosaccharide series on a 100 × 5-mm ID column, eluted with acetonitrile/water (11:9), containing 0.01% of the amine, after previous equilibration with the same solvent system with 0.1% of the modifier. For resolution of the malto-oligosaccharides up to DP 20, on a 200 × 8-mm ID column, 1,4-diaminobutane proved to be the modifier of choice. Members of the (1 → 2)-linked D-fructo-oligosaccharide series (inulins) of DP 2-30 have been resolved by Praznik *et al.* [105], using a similar amine-modified silica system, in this case, with the column temperature raised to 35°C, which improved resolution, and with polyethylene glycol 35,000 (0.2%) added to both equilibrating and eluting solvent system to decrease interaction of the amino groups with the hydroxyl groups of the saccharides.

Advantages and disadvantages of the use of amine-modified silica, as opposed to amine-bonded silica (Sec. 18.2.2.3), have been detailed by several authors [49,102,103]. It is claimed [102] that amine-modified silica has longer life, since the amine adsorbed on the silica is continuously regenerated; the amine-modified silica system is fairly stable over a wide range of pH and solvent composition, has a high capacity for carbohydrate solutes, and is relatively inexpensive. Disadvantages include variations in retention times due to varying amine loading of the silica, fluctuating base lines due to variable delivery of amine into the mobile phase, and the difficulty of using UV detection, owing to the presence of amine in the eluent. Furthermore, it has been pointed out [49] that amine-modified silica shares with other silica-based sorbents the disadvantage of a tendency to gradual dissolution in water-rich eluents, a tendency likely to be enhanced in the presence of the amine. For these reasons, the use of amine-modified silica in HILIC of sugars and polyols has decreased considerably in recent years. This has been all the more noticeable since the feasibility of using unmodified silica sorbents for this purpose has been established [12]. Nevertheless, there have been two recent reports of the application of a "dynamically modified amino column" to the analysis of carbohydrates in foods and beverages [106,107]. In these studies, ethylenediamine, triethylamine, and ethanolamine were tested as amine modifiers, with acetonitrile/water eluents. It is claimed [107] that the low nucleophilicity of ethanolamine and triethylamine helps to decrease interactions between the amines and the sugars, thus improving the sensitivity and accuracy of the analysis. With evaporative light-scattering detection nanomolar sensitivity was achieved under these conditions.

18.2.2.3 Partition chromatography on aminopropyl silica

Aminopropyl silica sorbents were the first of the bonded-phase type to be used in HPLC of carbohydrates, and for about 20 years this was generally accepted as the standard

method for the analysis of neutral mono- and oligosaccharides, and even some higher oligosaccharides. The development of packings based on 3-μm silica permitted resolution of linear D-gluco-oligosaccharides to DP 30-35 on such sorbents. For example, Koizumi *et al.* [108], using a column (200 × 6 mm ID) designated ERC-NH-1171 (Erma Optical Works) and a differential refractometer of unusual sensitivity, reported resolution of the malto-oligosaccharide series to DP 30 within 35 min, and of the β-(1 → 2)-linked oligosaccharides of the sophoro series (from partial hydrolysis of cyclosophoraose) to DP 35 within 45 min, on isocratic elution with acetonitrile/water mixtures, containing 57–58% of acetonitrile. Oligosaccharides that were (1 → 6)-linked (α-or β-) were resolved to DP 26 within 40 min with 55–56% acetonitrile, but solubility considerations imposed upper limits of DP 18 and 10, respectively, for those linked β-(1 → 3) and the β-(1 → 4)-linked cellodextrin series. For cyclosophoraoses, the cyclic β-(1 → 2)-linked D-gluco-oligosaccharides produced by *Rhizobium* and *Agrobacterium* strains, the same group [109] achieved resolution of the oligomers of DP 17-40 within 50 min on the ERC-NH-1171 column, eluted with 57% acetonitrile. This performance was superior to that of an aminopropyl silica sorbent, based on 5-μm silica, which was found by Benincasa *et al.* [110] to resolve the same cyclic oligosaccharides over the range DP 17-33 (160 × 4.6-mm-ID column, eluted with 64% acetonitrile).

A recent report [111] has demonstrated the potential value, in HILIC of higher oligosaccharides, of new sorbents in which the aminopropyl phase is bonded to micropellicular silica. On a short column (100 × 4.6 mm ID), packed with this sorbent, in which the silica had an average particle diameter of 3 μm, isocratic elution with acetonitrile/water (4:1) gave excellent resolution of a mixture of fructo-oligosaccharides, produced from sucrose under the catalytic effect of immobilized β-fructofuranosidase. The slope of a logarithmic plot of retention factor against the number of fructosyl residues in the molecule was much steeper than that found on chromatography on an aminopropyl phase based on conventional microparticulate silica. It was concluded that the effect of steric hindrance was reduced to negligible proportion in the absence of microporous structure in the fluid-impervious micropellicular stationary phase, where most of the surface was available for binding of solutes. Such sorbents could find application in HILIC of oligosaccharides of very high DP, even those approaching macromolecular size. A study of the behavior of 65 neutral oligosaccharides, mainly of the type obtained in degradative studies of glycoproteins, by HPLC on a column (250 × 4 mm ID) of LiChrosorb-NH_2 (Merck; 5-μm particles), eluted isocratically for 30 min with a 4:1 mixture of acetonitrile and an aqueous 15 m*M* potassium phosphate buffer solution (pH 5.2), then with a linear gradient of increasing buffer content (0.5% per min), has enabled the effects of various structural features on retention time to be correlated. Blanken *et al.* [112] concluded that retention time is decreased by the presence of residues of L-fucose or 2-acetamido-2-deoxy-D-glucose, especially when the latter is at the reducing end, and increased for oligosaccharides having a 1 → 6 linkage. Five complex oligosaccharides of the *N*-acetyllactosamine type, linear, bi, tri-, and tetraantennary and containing 5–12 sugar residues, were resolved with a buffer gradient of 0.3% per min, starting with the 4:1 solvent mixture.

Dua *et al.* [113] have reported the separation of a series of reduced oligosaccharides, containing up to 7 residues (fucose, galactose, 2-acetamido-2-deoxyglucose, and

2-acetamido-2-deoxygalactitol), that are produced on alkaline borohydride degradation of mucin glycoproteins; columns of Alltech 605 NH and Micropak AX-5 (250 × 4.6 mm and 300 × 4 mm ID, respectively, both 5-μm) were eluted with 3:2 acetonitrile/water or 1 m*M* KH_2PO_4 buffer solution (pH 5.4). Both analytical and preparative separations of lacto-*N*-hexaose and mono-and difucosylated derivatives, isolated from milk, have been achieved [114] on the Micropak AX-5 column, eluted with 1:1 acetonitrile/buffer. The resolution of oligosaccharides of this type by normal-phase partition chromatography is vastly superior to that given by HPLC on C_{18}-silica packings with water (Sec. 18.2.3.1). Brain gangliosides, including highly sialylated members of the series, are well resolved by HPLC on 5-μm aminopropyl silica by gradient elution with phosphate buffers (pH 5.5–5.6) in acetonitrile [53,115,116]. The longer retention times and higher proportion of phosphate required for elution as the degree of sialylation increases indicate that ion exchange plays a major role in the chromatographic mechanism. Although aminopropyl silica sorbents have been successfully applied in separations of higher oligosaccharides, a serious disadvantage of their use in HILIC of reducing sugars is the formation of glycosylamines by interaction between the sugars and the amino groups. This reaction, which results in both loss of the sugar analytes and de-activation of the column, is the main reason for the widespread trend away from the use of these sorbents that has been noticeable in recent years. It has been claimed, however, that losses of reducing sugars under these conditions can be minimized by replacing the water in the eluent by a phosphate buffer [117]. The presence of such a buffer is indeed essential, to prevent irreversible adsorption, where the analytes include acidic substances, such as ascorbic acid [118] or hexuronic acids [119]. It has been suggested [119] that the role of the $H_2PO_4^-$ anion in the eluent may be not only to adjust the pH of the mobile phase but also to form a complex with the amino phase.

18.2.2.4 Hydrophilic-interaction chromatography on other polar phases bonded to silica

In addition to their studies on the 3-μm aminopropyl silica sorbent, Koizumi *et al.* [120] tested two different sorbents with respect to their resolving power for oligosaccharides. These were YMC Pack PA-03 (Yamamura Chemical Company), in which a polyamine resin is bonded to silica, and TSK gel Amide-80 (Toyo Soda), which has carbamoyl amide groups instead of amino groups as the bonded phase. However, their resolving power for malto-oligosaccharides was inferior to that of ERC-NH-1171, the upper limit of resolution being 25 when columns (250 × 4.6 mm ID) packed with these sorbents were eluted with acetonitrile/water mixtures containing 50–53% of acetonitrile. In the same study, these columns and ERC-NH-1171 were applied to HPLC analysis of α-, β-, and γ-cyclodextrins and series in which these cyclodextrins carried branches. Here, the best resolution was obtained with YMC Pack PA-03, eluted with 55% acetonitrile, although the amide column, which is stable at higher temperatures (up to 80°C) than the other sorbents tested gave improved resolution at 50–70°C, with eluents containing 60% of acetonitrile.

The sorbent with bonded polyamine has proved to be considerably less stable than that carrying amide groups, and therefore has found little further application in HILIC of carbohydrates. Nevertheless, it has been reported [121] to give rapid and sharp resolution

of simple mixtures of mono- and disaccharides, such as D-fructose, D-glucose, sucrose, and maltose, and with acetonitrile/water (3:1) as the eluent it has been applied in monitoring the nutrient content of fermentation broths. More recently, a newer form of this sorbent, YMC Polyamine II, has proved useful in evaluating the purity of cyclodextrins to be used for pharmaceutical purposes [122]. Baseline resolution of α-, β-, and γ-cyclodextrins, well separated from D-glucose and the linear malto-oligosaccharides to DP 6, was obtained by HILIC on a column (250 × 4.6 mm ID), packed with this sorbent, with 64% acetonitrile as the eluent.

The sorbent with bonded amide groups has found application mainly in the separation of oligosaccharides. For example, the α-linked disaccharides of D-glucose, and also their alditols, are well resolved on a column (250 × 4.6 mm ID), packed with TSK gel Amide-80, with 70% acetonitrile as the eluent [123]. Akiyama *et al.* [124] applied such a column in the determination of the glycosaminoglycans hyaluronic acid, chondroitin sulfate and dermatan sulfate, as the dansyl derivatives of the unsaturated oligosaccharides produced on lyase digestion. This analysis was performed at a column temperature of 50°C, with a 4:1 mixture of acetonitrile and a 0.15 *M* acetate buffer (pH 5.0) as the mobile phase. The amide column has also proved effective in separations of oligosaccharides of the type released in degradative studies of glycoconjugates, and it is now used in preference to aminopropyl silica, in conjunction with a reversed-phase column, in two-dimensional mapping of such oligosaccharides (Sec. 18.2.5).

An amino-cyano phase, bonded to silica, Partisil-PAC (Whatman), was one of the first to be introduced with the objective of overcoming the problem of the instability of the alkylamino-bonded phase. This sorbent, too, can be used at slightly elevated temperatures. For example, in another study aimed at monitoring the purity of cyclodextrins, a column (250 × 4.6 mm ID) of Partisil-5 PAC (based on 5-μm silica) was operated at 45°C, with 73% acetonitrile as the eluent at a flow-rate of 1 mL/min, which within 25 min gave baseline resolution of α-, β-, and γ-cyclodextrins, D-glucose and the linear malto-oligosaccharides to DP 7 [125]. The use of the higher temperature prevents peak broadening due to anomeric separation, as the rate of mutarotation is increased. The amino-cyano phase has also proved effective in resolution of all the unsaturated disaccharides derived from the chondroitin sulfates, dermatan sulfate and hyaluronic acid [126], and can thus be applied in determination of these glycosaminoglycans. In this case, the recommended mobile phase is a mixture (24:7:19) of acetonitrile, methanol, and an aq. 0.5 *M* Tris–HCl buffer(pH 8.0), to which 0.1 *M* boric acid and 23.4 m*M* sulfuric acid have been added. The presence of borate prevents peak broadening, and the addition of sulfuric acid in low concentration optimizes differences in retention of these sulfated oligosaccharides.

Another sorbent introduced as a possible alternative to aminopropyl silica for HILIC of sugars and polyols is LiChrosorb (or LiChrospher) DIOL (Merck). Brons and Olieman [127] found that this hydroxylic phase behaved similarly to the amino phase in HPLC of monosaccharides, simple oligosaccharides, and alditols, but a higher proportion of acetonitrile (85%) was required in the eluent. To avoid peak-broadening due to anomeric separation, it was necessary to add a small proportion (0.1%) of diisopropylethylamine, but the presence of this base was detrimental to the stability of the silica support. Far better results were obtained with the dichloromethane/methanol eluents recommended by

Herbreteau *et al.* [13]. The selectivity shown by LiChrospher DIOL for monosaccharides and alditols with dichloromethane/methanol (84:16) as the eluent was much higher than that with acetonitrile/water (85:15), and anomeric separation was apparent only for D-galactose. A higher proportion of methanol (22–25%) was required for the separation of di- and trisaccharides. Gradient elution (7 to 20% of methanol in 5 min, 20 to 30% in 3 min, then isocratic elution for 10 min) at a flow-rate of 1 mL/min permitted sharp resolution, within 20 min, of a mixture of monosaccharides, the disaccharides sucrose and melibiose, and the trisaccharide raffinose. The use of the ELSD with this chromatographic system allowed detection of the sugars in nanogram quantities.

The stationary phases in which α- or β-cyclodextrin is bonded to 5-μm silica (Cyclobond; Advanced Separation Technologies) have proved to be the most versatile of the newer sorbents for HPLC of carbohydrates. Armstrong and Jin [128] published retention times for a wide variety of carbohydrates, including alditols, monosaccharides from triose to heptose, deoxy sugars, di-, tri-, and tetrasaccharides, and cyclodextrins on columns (250 × 4.6 mm ID), containing these sorbents, with eluents consisting of aq. acetonitrile (80–85%) or acetone (85–90%), which indicated the feasibility of separating many of these compounds that are not easily resolved by other HPLC methods. Some remarkable separations of complex mixtures, by isocratic or gradient elution, were achieved. More recently, Simms *et al.* have demonstrated the high selectivity and efficiency of Cyclobond I (with β-cyclodextrin as the bonded phase) in separating homologous series of neutral oligosaccharides [129] as well as oligogalacturonic acids [130]. For example, the malto-oligosaccharides can be resolved to DP 25 on the standard Cyclobond I column, with acetonitrile/water (65:35) as the eluent [129]. In the case of the acidic oligosaccharides, the presence of a buffer in the eluent is necessary to prevent irreversible adsorption. Because the higher members of the oligogalacturonic acid series are insoluble in the presence of acetonitrile, the organic solvent was omitted here, and an aqueous buffer (150 m*M* sodium phosphate, pH 5.0) was used as the mobile phase. This permitted resolution of the normal oligogalacturonic acid series to DP 17 and of the unsaturated oligomers to DP 10 [130]. With a sodium acetate gradient in the mobile phase the members of the normal oligogalacturonic acid series could be resolved to DP 24. In the absence of the organic modifier, the cyclodextrin-bonded silica was evidently functioning in anion-exchange mode, although it contains no ionizable groups. It has been postulated [130] that this might be due to the inclusion within the cyclodextrin cavity of a cationic component from the mobile phase, forming a "dynamic" anion-exchange site. Further separations of oligosaccharides [131] and of phosphorylated carbohydrates [132], in the latter case with the chromatographic system coupled on-line to ESI-MS, continue to afford evidence of the remarkable versatility and efficiency of this sorbent. The performance of this and some of the other silica-based sorbents in the HILIC of sugars and polyols is compared in Table 18.1

18.2.3 Reversed-phase and hydrophobic-interaction chromatography

RPC is now extensively applied in carbohydrate analysis, mainly because of the increased use of precolumn derivatization (Sec. 18.2.1.1), and the relative ease with which such chromatographic systems can be coupled on-line to MS (Sec. 18.2.1.2).

TABLE 18.1

CAPACITY FACTORS FOR HILIC OF SUGARS AND ALDITOLS ON SILICA-BASED SORBENTS

Compound	Capacity factor (k')			
	P1	P2	P3	P4
Sugars				
Erythrose				0.48
Rhamnose	1.8	1.3	1.78	0.73
Ribose	1.6	1.1	1.35	0.78
Xylose	2.2	1.5	2.22	0.96
Arabinose	2.2	1.8	2.04	1.05
Mannose	2.9	2.8	3.30	1.34
Glucose	4.0	3.3	3.78	1.55
Galactose	3.5/3.9*	3.5	3.21/3.71*	1.65
Fructose	2.8	2.3	2.43	1.25
Sorbose	3.0	2.3	3.13	1.29
Tagatose				1.16
Sucrose				2.71
Cellobiose				3.30
Maltose				3.33
Lactose				3.84
Maltotriose				6.83
Raffinose				7.19
Stachyose				17.57
Alditols				
m-Erythritol	2.5	1.2	1.78	0.94
Ribitol				1.25
Xylitol	4.8	2.0	2.22	1.33
Mannitol	5.7	3.1	4.00	1.76
Glucitol				1.75
Galactitol				1.80

P1 = Unmodified silica, 5 μm, Zorbax-Sil (DuPont), 250 × 4.6 mm ID column; eluent dichloromethane/methanol/water (80:19.8:0.2), flow-rate 1 mL/min [12].

P2 = Aminopropyl silica, Waters Carbohydrate Analysis Column, 300 × 3.9 mm ID; eluent acetonitrile/water (4:1), flow-rate 1 mL/min [12].

P3 = Bonded hydroxylic phase, LiChrospher DIOL (Merck), 250 × 4 mm ID column; eluent dichloromethane/methanol (84:16), flow-rate 1 mL/min [13].

P4 = Bonded β-cyclodextrin phase on 5-μm silica (Cyclobond I), 250 × 4.6 mm ID column; eluent acetonitrile/water (85:15), flow-rate 1.5 mL/min [128].

* Double peak due to resolution of anomers.

The technique has been used in the separation of both large and small carbohydrate molecules. Hydrophobic-interaction chromatography (HIC) was developed primarily for the separation of proteins, but some coverage of its use in studies of glycoproteins seems appropriate here. The applications of these two modes of chromatography in analyses of carbohydrates and glycoconjugates have been the subject of comprehensive reviews [133,134]. The use of sorbents consisting of graphitized carbon in RPC of carbohydrates is a relatively new technique that may be regarded as the modern equivalent of the classical method in which columns packed with charcoal were used in preparative separations of oligosaccharides. Developments in the application of this form of chromatography to the carbohydrate field, which were recently reviewed by Koizumi [135], will also be briefly surveyed.

18.2.3.1 Reversed-phase chromatography on octadecylsilica sorbents

Packings in which a long alkyl chain, usually C_{18}, is bonded to silica, can operate in different modes when applied to HPLC of carbohydrates. The reversed-phase partition mechanism, for which they are primarily designed, applies only after introduction of non-polar groups by derivatization of the carbohydrate solutes, a procedure that is frequently employed to increase sensitivity of detection by conferring chromophoric or fluorescent properties upon the molecules. For example, sugars have been analyzed on C_{18}-silica columns as dansylhydrazones [136] and pyridylamino derivatives [137], which permit sensitive fluorimetric detection. The latter method has the advantage that neutral and amino sugars can be analyzed simultaneously. For example, Takemoto *et al.* [137], using two columns, each 250 × 4.6 mm, connected in series, were able to resolve the common neutral sugars, 2-acetamido-2-deoxymannose, -glucose, and -galactose, and *N*-acetylneuraminic acid as their pyridylamino derivatives on 5-μm C_{18}-silica, eluted with 0.25 *M* sodium citrate buffer (pH 4.0), containing 1% of acetonitrile. Fluorimetric detection (ex 320 nm, em 400 nm) allowed the determination of 0.01–10 nmol of each sugar. Hydrolyzates from glycoconjugates (glycoproteins and gangliosides) could be analyzed by this method in samples of 100–200 pmol. The technique has also been successfully applied in analyses of the complex oligosaccharides released from glycoproteins [138], as well as neutral oligosaccharides, such as xyloglucans [139]. Gradient elution, *e.g.*, linear gradients of acetonitrile (0–40%) in water, is required. More recently, some of the newer methods of precolumn derivatization (Sec. 18.2.1.1) have been favored over reductive pyridylamination in preparing carbohydrates for RPC. These include derivatization with ABEE, which affords a very sensitive method for analyzing the monosaccharide components of glycoproteins [140] and anthranilic acid [141] or PMP [65] for the oligosaccharides from glycoproteins.

Perbenzoylation, followed by reversed-phase HPLC, has been proposed as an alternative to GLC of the trimethylsilylated product (Sec. 18.3) for the analysis of methanolysates of glycoproteins, the necessity for the use of empirically determined molar response factors, as in GLC, being obviated by the direct relationship between UV absorbance and the number of benzoyl groups in each derivative. Jentoft [142] reported good resolution of the benzoylated methyl glycosides of all the sugar constituents

(neutral and amino sugars) of glycoproteins on a column (150 × 4.6 mm ID) of 3-μm C_{18}-silica within 45 min, using stepwise elution with 50% and then 60% acetonitrile. Multiple peaks were obtained by this method. The limit of detection under the conditions used by Jentoft was of the order of l nmol, but with microbore columns, analysis of benzoylated methyl glycosides at picomolar levels is possible [143]. Gradient elution (acetonitrile in water) produces the best resolution in such analyses [144]. Benzoylation prior to RPC has also proved useful in analyses of the monosaccharide constituents of glycosaminoglycans, released after carbodiimide reduction of the acidic residues and acid hydrolysis [145]. There have been several reports of RPC of acetylated carbohydrates. For example, Velasco *et al.* [146] investigated the possibility of using this method as an alternative to GLC in the analysis of sugars as the peracetylated aldononitrile or ketoxime derivatives and, with methanol/water, acetonitrile/water, or water/tetrahydrofuran/methanol (65:10:25) as eluents, satisfactory resolution of such derivatives was obtained. Attempts to use HPLC on C_{18}-silica in analyses of partially methylated sugars without prior reduction have met with only limited success because of the difficulties arising from the resolution of anomers, which give double peaks for most methylated sugars. For HPLC of the mixtures of partially methylated sugars, obtained in methylation analysis of polysaccharides, reduction to alditols is necessary to eliminate the doublets. With eluents consisting of acetonitrile (1–5% or a gradient) in water, the partially methylated alditols are eluted in the order of increasing degree of methylation [147]. However, separation of isomers having the same degree of methyl substitution is difficult. In contrast, RPC has proved useful in the characterization of permethylated oligosaccharides, especially when used in conjunction with ESI-MS [148].

Underivatized carbohydrates have also been subjected to RPC, usually with plain water as the mobile phase. However, the chromatograms of sugars [149] and methyl glycosides [150] analyzed in this way are complicated by the formation of double peaks due to anomer separation. Procedures that have been adopted in attempts to overcome this problem include reduction to the alditol, increasing the column temperature, and inclusion of a detergent (such as Triton X-100) or base in the eluent [133,134]. The method has proved more successful in the chromatography of oligosaccharides. Separation of underivatized oligosaccharides on C_{18}-silica, eluted with water or aqueous solutions, takes place by a mechanism that is not really reversed-phase partition but probably a form of HIC, involving interaction of the Van der Waals type between the oligosaccharide chains and the C_{18} on the stationary phase. Not only chain-length, but also molecular structure affects the degree of interaction [134]. For example, in a separation of the fructo-oligosaccharides from red squill by RPC with pure water as the eluent, there was good resolution of two trisaccharides, 1-kestose and neokestose, which differ only in the position of the sugar residues [151]. Cyclic oligosaccharides, which contain no reducing sugars and therefore no anomers, are more easily resolved by this method. Koizumi *et al.* [109] fractionated the cyclosophoraoses of DP 17-33 on a column (250 × 4 mm ID) of 5-μm C_{18}-silica, eluted with water, containing 5.5% methanol, but the resolution obtained was inferior to that produced by HILIC on 3-μm aminopropyl silica (Sec. 18.2.2.3). In contrast, the Koizumi group considers C_{18}-silica columns superior to amino-phase columns for analyses of cyclodextrins and branched cyclodextrins, having obtained satisfactory resolution of these cyclic oligosaccharides with several C_{18} columns, eluted

with water, containing 3–7% methanol [152]. The method has proved useful in the isolation and purification of branched cyclodextrins [153]. Application of RPC in pure water to separations of the complex oligosaccharides of human milk and those obtained in degradative studies of glycoproteins has met with only limited success, where the presence of anomeric centers gives rise to double peaks. Nevertheless, several oligosaccharides have been successfully isolated in this way, including the fucose-containing pentasaccharides of goat's milk [154] and the *O*-linked neutral oligosaccharides from human meconium glycoproteins [155]. Reduced oligosaccharides, produced by treatment of glycoproteins with alkaline borohydride, give single peaks in HPLC on C_{18}-silica. The method has been useful in, *e.g.*, the isolation of the carbohydrate chains of mucus glycoproteins [156].

Another mode of chromatography in which C_{18}-silica columns have been used to fractionate carbohydrates is ion-pair chromatography. As an alternative to ion-exchange chromatography (Sec. 18.2.6), ionic carbohydrates can be separated on C_{18}-silica columns with eluents containing an ion-pairing reagent. For anionic solutes, the tetrabutylammonium cation is particularly effective in conferring sufficient hydrophobic character on the ion-pair complex to permit resolution on a reversed-phase column. Alkyltrimethylammonium salts have also been used. For example, the normal and unsaturated oligogalacturonic acid series, produced by degradation of pectic acid with endo-polygalacturonase and endo-pectic acid lyase, respectively, have been resolved (the normal series to DP 4, the unsaturated acids to DP 7) on a column (250 × 4.6 mm ID) of 10-μm C_{18}-silica at 40°C with eluents containing 0.05 *M* phosphate buffer (pH 7.0), methanol (10% for normal, 30% for unsaturated acids), and 25 m*M* tetrabutylammonium bromide [157]. The technique has also been successfully applied to the determination of hyaluronic acid in biological tissues and fluids, by quantitative HPLC of the unsaturated tetra- and hexasaccharides produced on degradation of the hyaluronic acid with *Streptomyces* hyaluronidase. These oligosaccharides were well resolved on a column (250 × 4.6 mm ID) of 5-μm C_{18}-silica by use of an acetonitrile gradient (20 → 22%) in 8 m*M* H_3PO_4, containing 10 m*M* tetrabutylammonium hydroxide [158]. Analyses of glycosaminoglycans have been among the most important applications of ion-pair RPC [159,160]. The technique has also proved valuable in studies of sugar phosphates [161] and UDP-sugars [162].

18.2.3.2 Reversed-phase chromatography on graphitized carbon

An important development in carbohydrate chromatography during the last decade has been the use of graphitized carbon as sorbent in the HPLC of underivatized carbohydrates. Preliminary studies by Koizumi *et al.* [163] on the behavior of mono- and oligosaccharides on a column (100 × 4.6 mm ID), packed with Hypercarb (Shandon), showed that, as in the case of the classical charcoal column, monosaccharides were weakly retained and were eluted with water in less than 4 min at a flow-rate of 1 mL/min and a column temperature of 30°C. Disaccharides required the addition of methanol (15%) or acetonitrile (4%) for their elution. Double peaks due to anomeric resolution were observed in all cases, unless 1 m*M* NaOH was added to the eluent. Good resolution of a mixture of nine

gluco-oligosaccharides was achieved by gradient elution (1.5 to 5.0% acetonitrile in 1 m*M* NaOH over 15 min at a flow-rate of 1 mL/min). The column effluent was made more alkaline to allow the use of the sensitive pulsed amperometric detector. Chromatography of cyclodextrins and their 6-*O*-α-D-glucopyranosyl derivatives on this column required a higher percentage of organic modifier (13–15% of acetonitrile) and a higher column temperature (50°C) than was the case for linear oligosaccharides. It was subsequently reported by the same group [164] that positional isomers of trimaltosyl-β-cyclodextrin, which are difficult to resolve on other columns, were well separated on the graphitized carbon column.

The potential value of this sorbent in HPLC analysis of the oligosaccharide-alditols and glycopeptides, encountered in degradative studies of glycoproteins, was investigated by Davies *et al.* [165]. They found that the reduced oligosaccharides were strongly retained in water, but could be eluted by use of a gradient of acetonitrile (0–25% in 40 min, at a flow-rate of 0.75 mL/min) in 0.05% aq. trifluoroacetic acid. Mono- to hexasaccharides could be separated, elution patterns depending not only on chain-length but also on the nature of the glycosidic linkages and the presence or absence of charged groups. Isomeric oligosaccharide-alditols, differing only in linkage type, could be separated in some cases. For example, an isomer having a (1 → 3) linkage was eluted before one having a (1 → 4) linkage. The presence of a (1 → 6) linkage increased the retention time, as did sialic acid residues, with a (2 → 3)-linked residue resulting in a longer retention time than one linked 2 → 6. These findings were subsequently applied by the same group [166] in the HPLC analysis of the oligosaccharides and reduced oligosaccharides released from glycoproteins by enzymatic degradation or alkaline borohydride treatment. Advantages of the method included the fact that peptides, glycopeptides, reduced oligosaccharides, and sialylated oligosaccharides could be separated under the same conditions, with volatile eluents, which facilitated the recovery of the products from the column effluent for further structural studies.

Fan *et al.* [167] demonstrated that a further advantage of the use of graphitized carbon columns in the chromatography of oligosaccharides from glycoproteins and of glycopeptides with few amino acids was that those not retained on octadecylsilica columns, even at low pH or in the presence of electrolyte in high concentration, can be effectively retained and separated by gradient elution when graphitized carbon is used as the sorbent. This applies also to chito-oligosaccharides, the homologous series of β-(1 → 4)-linked oligomers of 2-acetamido-2-deoxy-D-glucose that are released on hydrolysis of chitin. These oligosaccharides could be resolved to DP 9 on a Hypercarb column (100 × 4.6 mm ID) by elution with a gradient of acetonitrile (0–25% in 40 min, at flow-rate 1 mL/min) in 10 m*M* aq. ammonia, at a column temperature of 50°C [167]. According to Koizumi [135,168], the unique ability of graphitized carbon columns to resolve isomeric or closely related compounds is related to the flat surface of the sorbent. Retention is mainly by an adsorption mechanism, planar molecules being in general more retained than non-planar molecules, although hydrophobic interaction is also involved. The exceptional physical and chemical stability of the highly structured graphite surface permits operation at elevated temperatures and with eluents of high or low pH. The conditions used in chromatography on this sorbent are conducive to coupling the system to a pulsed amperometric detector [169] or ESI-MS [170].

18.2.3.3 Hydrophobic-interaction chromatography of glycoproteins

In HIC the stationary phase is less hydrophobic than in RPC. The surface of the rigid support is usually covered with a hydrophilic coating, to which the hydrophobic ligands, generally short alkyl or aryl groups, are attached. The mobile phase initially contains a high concentration of salt, which promotes hydrophobic interaction of proteins, and the adsorbed solutes are subsequently eluted by stepwise or gradient elution with a mobile phase of decreasing salt concentration. In surveying the application of HIC to the purification of glycoproteins, El Rassi [133,134] has pointed out that, unlike RPC, HIC has little denaturing effect, because no organic modifier is required for elution. This permits recovery of a protein in its native form. In the case of lipoprotein constituents, *e.g.*, β-2-glycoprotein I, the lipid content is preserved under these conditions [171]. HIC is generally used in combination with other chromatographic techniques, such as ion-exchange chromatography or SEC in purification schemes. It is often more specific in indicating micro-heterogeneity in glycoproteins than other methods [172], as HIC can detect differences in degree of glycosylation [173] or hydrophobicity [171].

18.2.4 Hydrophilic-interaction and reversed-phase chromatography on polymer-based sorbents

Polymeric sorbents that are rigid enough for use in HPLC include highly cross-linked polystyrenesulfonate ion-exchange resins, which function as supports for partition chromatography with aq. organic solvents as eluents. In this case, the separation mechanism depends on the hydrophilicity of the anionic groups on the resin and their counter-ions. Ion exchange is not a factor under these conditions. A resin that has proved useful for HILIC of carbohydrates is Shodex RSPak DC-613 (Showa Denko), which is very highly cross-linked (55% divinylbenzene) and therefore sufficiently rigid to serve as a sorbent for HPLC. In its sodium form, with an eluent consisting of acetonitrile, methanol, and 0.8 *M* ammonium formate, pH 4.5 (13:3:4) and a column temperature of 70°C, the resin has been successfully applied to the analysis of the unsaturated oligosaccharides produced by lyase degradation of glycosaminoglycans [174]. The use of this resin, in the hydrogen form, for separating the monomers and lower oligomers obtained on hydrolysis of sialic acid polymers has also been reported [175]. At a column temperature of 35°C, isocratic elution with 67–75% acetonitrile in a 20–25 m*M* sodium phosphate buffer (pH 7.4) allowed separation of the monomers, dimers, trimers, and tetramers of the two sialic acids, *N*-acetyl- and *N*-glycolylneuraminic acid, and of deaminoneuraminic acid. Polymers developed specifically for HPLC include the macroporous cross-linked vinylpyridinium type, used by Sugii and Harada [176] in HILIC of sugars with aq. acetonitrile. Since these polymers are stable at elevated temperature, columns have been used at 70°C; with the resins in the phosphate or sulfate forms. Elution with acetonitrile/water (4:1) gave baseline resolution of a mixture of the common monosaccharides and some disaccharides, such as maltose and lactose.

Sorbents in which a polymer replaces silica as the support for the aminopropyl bonded phase, much used in HILIC of carbohydrates, have also proved useful. For example,

Akiyama [177] reported good resolution of a number of mono- and disaccharides, including 2-acetamido-2-deoxy-D-glucose and its β-(1 → 4)-linked dimer, chitobiose, after pre-column derivatization with ABEE (Sec. 18.2.1.1), on a column (250 × 4.5 mm ID), packed with an amine-bonded vinyl alcohol copolymer, Asahipak NH_2P-50 (Asahi Kasei). With acetonitrile/water (85:15) as the eluent, resolution of the derivatized sugars was better than that of the free sugars. The lower members (to DP 5 or 6) of the malto-, cello-, and isomalto-oligosaccharide series, similarly derivatized, were also well resolved on this column, when the acetonitrile content of the eluent was reduced to 70%. The upper limit can be extended (to DP 13 for the isomalto-oligosaccharides) by further decrease in the acetonitrile content, to 65%. Sorbents carrying C_{18} chains, bonded to a vinyl alcohol copolymer (Asahipak ODP-50), have been used in RPC of carbohydrates. Koizumi and Utamura [178] achieved excellent resolution of the linear D-gluco-oligosaccahrides on this stationary phase, the malto-oligosaccharides being resolved to DP 23, the β-(1 → 2)-linked sophoro series to DP 12, and the isomalto series to DP 11. Elution with water resulted in the formation of double peaks, but this could be prevented by using a basic mobile phase (aq. NaOH, pH 11), which is not possible with silica-based sorbents. This stability at high pH is a major advantage of polymeric sorbents.

18.2.5 Two-dimensional high-performance liquid chromatography

It should be evident from Secs. 18.2.2 and 18.2.3 that HILIC and RPC are complementary, especially when applied to oligosaccharides. This led to a technique for the identification of glycoprotein-derived oligosaccharides by reference to a two-dimensional "map", based upon the retention times of standard oligosaccharides on chromatography by each of these two modes [179,180]. In both cases, the oligosaccharides, produced by sequential digestion of glycoproteins with pepsin and *N*-oligosaccharide glycopeptidase [179], or hydrazinolysis followed by *N*-acetylation [180], were derivatized by reductive amination with 2-aminopyridine, allowing fluorimetric detection down to 10 pmol. Tomiya *et al.* [179] used an amide column (Sec. 18.2.2.4), Hase *et al.* [180] an aminopropyl silica column to fractionate the oligosaccharides mainly according to size, whereas several structural factors governed their retention on the C_{18}-silica columns. The retention times of a large number of oligosaccharides, representing the different types (*N*-acetyllactosamine, oligomannoside, hybrid and xylose-containing) isolated from glycoproteins, were compared by Tomiya *et al.* [179] with those of the α-(1 → 6)-linked isomalto series, similarly derivatized, on the two columns and plotted as equivalent numbers of glucose units. Those of a group of 45 oligosaccharides [180] were related to the number of D-mannosyl residues present. These seminal papers have provided an extensive database for the identification of unknown oligosaccharides from HPLC data. This two-dimensional mapping technique, with the amide column eventually replacing the aminopropyl silica column in all cases, has been applied to the identification of many of the oligosaccharides released by glycoproteins [181–184] or glycosphingolipids [185]. The ABEE derivatives have also been used for mapping of oligosaccharides from these glycoconjugates [186,187]. The applications of oligosaccharide mapping by two-dimensional HPLC in determining the structures of oligosaccharides released from glycoconjugates have been reviewed by Takahashi [188,189].

18.2.6 Ion-exchange chromatography and ion-moderated partitioning

Ion-moderated partitioning (IMP) has been discussed in the previous edition of this textbook [1]. Generally, micro-particulate cation-exchange resins are used for HPLC in the analysis of sugars and polyols on cross-linked polystyrenesulfonate resins, loaded with various counter-ions, usually Ca^{2+}, Pb^{2+} or Ag^{+}, and water is used as the eluent, at elevated column temperatures. Homologous series of oligosaccharides, both neutral and charged, are separated on such resins in the hydrogen form, with dilute acid (*e.g.*, 5 m*M* H_2SO_4) as the eluent. Separations of the former type are believed to involve ligand exchange as the predominating mechanism, while in the latter type ion- and size-exclusion are the most important factors. More recently, the role in carbohydrate analysis of ligand-exchange chromatography at alkaline pH, on resins loaded with lanthanide ions or Fe^{3+}, has been reviewed [190]. Chromatographic separations based on all of these principles are included in a current review [191] covering HPLC of carbohydrates on cation- and anion-exchangers in general.

During the past decade the use of chromatographic methods based on IMP has declined dramatically, probably because of the now widespread preference for high-performance anion-exchange chromatography (HPAEC) (Sec. 18.2.7). Therefore, applications of IMP to carbohydrate analysis will not be considered further, and the present discussion will be confined to developments in ion-exchange chromatography (IEC) *per se*. In adaptations of ion-exchange chromatography of carbohydrates to HPLC, both silica-based packings with bonded anion exchangers and highly efficient, micro-particulate ion-exchange resins have been used. The silica-based anion exchangers, which bear quaternary ammonium (strongly basic) or primary amino groups (weakly basic), tend to deteriorate rapidly, even with guard columns, and therefore their lifetime is much shorter than that of the resins. The latter are relatively stable, if protected by suitable guard columns of similar resin and if excess pressure and high temperatures are avoided. However, it is often necessary to operate resin columns at elevated temperature to maximize efficiency, because of the slow diffusion processes involved [49], and this reduces their lifetime.

18.2.6.1 Ion-exchange chromatography on silica-based anion exchangers

Sorbents of this type that have been used in carbohydrate analysis include both those with strong-base anion-exchange groups, such as quaternary ammonium groups, and those with weakly basic groups. Examples of the former are Nucleosil 10 SB (Macherey-Nagel), Zorbax SAX (DuPont), and Partisil SAX (Whatman). With regard to the latter type, those that have found application have generally been aminopropyl silica sorbents, which function as ion-exchangers with eluents consisting mainly or entirely of an aqueous buffer. The use of IEC under these conditions is obviously limited to analytes having charged groups, such as acidic carbohydrates. The hexuronic acids themselves are not well resolved by HPLC on strongly basic anion-exchangers bonded to silica, as peaks due to co-existing lactones can cause problems in analysis [192]. Applications of IEC in carbohydrate analysis have been confined mainly to separations involving acidic oligosaccharides. For example, Voragen *et al.* [157] resolved the normal oligogalacturonic

acids from pectic acid to DP 4, and the unsaturated series to DP 6, by HPLC on silica-based anion exchangers (Nucleosil 10 SB or Zorbax SAX), eluted at 40°C with 0.3 *M* sodium acetate buffer (pH 5.4). Use of 0.4 *M* buffer effected resolution of the unsaturated acids to DP 8 within 15 min, whereas ion-pair reversed-phase chromatography (Sec. 18.2.3.1) achieved resolution to DP 7 in about the same time.

Strongly basic anion exchangers have also been used in the analysis of the sulfated oligosaccharides, produced from glycosaminoglycans by nitrous acid deamination [193] or enzymatic degradation [194]. Stepwise or gradient elution with phosphate buffers permits resolution of oligosaccharides differing in DP or degree of sulfation. As the strong-base anion-exchangers, supported on silica, tend to deteriorate very rapidly, it is the weak-base type that has been more widely used in IEC of carbohydrates, mainly the oligosaccharides, produced by degradation of glycosaminoglycans and glycoproteins. For example, Nebinger *et al.* [195] reported complete separation of all the saturated oligosaccharides, even and odd-numbered, derived from hyaluronic acid. In this case, HPLC on Ultrasil-NH_2 (Beckman), eluted isocratically with 100 m*M* KH_2PO_4 (pH 4.75), resolved all members of the series, containing 2–8 sugar residues, within 30 min. HPLC on similar weakly basic anion exchangers, eluted with 0.3 *M* ammonium formate (pH 5.5) containing 4% of methanol [196], or 10 m*M* sodium sulfate containing 1 m*M* acetic acid [197], achieves good resolution of the unsaturated disaccharides, produced on lyase digestion of chondroitin 4- and 6-sulfates and of dermatan sulfate. The unsaturated tetra- and hexasaccharides generated by digestion of hyaluronic acid with *Streptomyces* hyaluronidase are also quantitatively resolved, within 12 min, by this method [196]. More recently, even better resolution of the unsaturated disaccharides from the chondroitin sulfates was obtained on a sorbent of this type with a combination of the eluents used earlier: Imanari *et al.* [198] recommended a mobile phase consisting of a 20 m*M* ammonium formate buffer (pH 5.0), containing 10 m*M* sodium sulfate and 4% acetonitrile. Karamanos *et al.* [199] were able to distinguish the various glycosaminoglycan-derived oligosaccharides with respect to their degree of sulfation by stepwise elution with 5 m*M* NaH_2PO_4 (pH 2.55), 50 m*M* NaH_2PO_4 (pH 2.50), and 50 m*M* sodium sulfate, containing sodium acetate, buffered to pH 5.0 with acetic acid. Optimal conditions for resolution of neutral and sialylated oligosaccharides from glycoproteins by chromatography on a weak-base anion-exchanger, bonded to silica, have been given in a review by Baenziger [200]. The method has also proved effective in the separation of gangliosides. A highly sensitive analytical procedure, involving the use of a micro-bore HPLC column (250 × 1 mm ID), packed with the weak-base anion-exchanger Spherisorb-NH_2 (Phase Separations) and eluted with a phosphate gradient in acetonitrile, has been described by Wagener *et al.* [201]. The gangliosides are eluted according to their degree of sialylation with increasing phosphate concentration. There is also some separation of gangliosides having an equal number of sialic acid residues, due to differences in polarity.

18.2.6.2 Ion-exchange chromatography on micro-particulate resins and gels

Chromatography of sugars as anionic borate complexes, once an important analytical method, may now be regarded as obsolete, even though the introduction of more efficient

resin columns and post-column derivatization with 2-cyanoacetamide by Honda *et al.* [202] reduced analysis time and greatly improved sensitivity. Ion-exchange chromatography of neutral sugars is now performed almost exclusively by HPAEC (Sec. 18.2.7). IEC on microparticulate resins has afforded a rapid method of analyzing sugar acids, such as the aldonic acids present in fermentation broths. These are well resolved on the strongly basic anion-exchange resins Aminex A27 (13–17 μm) or A29 (5–8 μm) (BioRad) at a column temperature of 45°C, with 0.2 *M* ammonium formate (pH 3.2) or 0.2 *M* KH_2PO_4 (pH 3.35) as eluents [203]. Aminex A-29, packed into a short column (40 × 4.6 mm ID) has also proved effective in analyses of mixtures of the sialic acids *N*-acetyl- and *N*-glycolylneuraminic acid, mono-, di-, and triacetylated derivatives, methyl glycosides, and some sialyloligosaccharides [204]. These were sharply resolved in 15 min at room temperature by elution, at 0.5 mL/min, with 0.75 m*M* sodium sulfate or, in some cases, 40 m*M* sodium acetate buffer (pH 5.5). The same method can be used to resolve 3-deoxy-D-*manno*-octulosonic acid (KDO) and its methyl glycosides, but for KDO disaccharides a higher ionic strength (10 m*M* sodium sulfate) is necessary to shorten retention times [204].

In recent years, some newer resins have found application in IEC of anionic carbohydrates, especially sialylated oligosaccharides released from glycoproteins. As weakly basic anion exchangers, supported on silica, proved effective in such separations, so have weak-anion-exchange resins. For example, Guile *et al.* [205] reported in 1994 on the usefulness of the weakly basic anion-exchange resin Vydac 301VHP575 (Hichrom) in both analytical and preparative HPLC of the *N*- and *O*-linked oligosaccharides from fetuin. A short column (50 × 7 mm ID), packed with this resin, and gradient elution with ammonium formate buffers gave good resolution of these oligosaccharides, and also of some of the sulfated oligosaccharides from glycosaminoglycans. In preparative chromatography, use of the resin resulted in purer products, without the contamination from the exchanger matrix that is a disadvantage of silica-based sorbents in such applications. Currently, applications are being tested of novel anion-exchange resins, in which the cross-linked polystyrene matrix carries tertiary amino groups, with one of the substituents on the nitrogen atom a methylene or oxyethylene chain bearing a terminal dimethylamino group [206,207]. With 100 m*M* NaOH as the eluent, these resins can separate a number of mono- and disaccharides. Use of a linear gradient of sodium acetate in 100 m*M* NaOH permits resolution of the malto-oligosaccharides to DP7 [206].

As successors to the soft dextran and cellulose gels carrying anionic – usually diethylaminoethyl (DEAE) – groups that have been in use for many years in preparative chromatography of anionic oligo- and polysaccharides [1], more rigid sorbents, bearing the same groups, have been developed so that preparative chromatography may be carried out more efficiently by HPLC techniques. For example, Maness and Mort [208] achieved separations of oligomers of the normal oligogalacturonic acid series over the DP range 3–25 by HPLC of these oligosaccharides, as their pyridylaminated derivatives, on a column (250 × 4.6 mm ID), packed with Spherogel TSK DEAE-2SW (Beckman). With a column temperature of 35°C and a sodium acetate gradient (77 m*M* to 406 m*M* in 63 min, at a flow-rate of 1 mL/min and pH 5.2) the separation was complete within 80 min. More recently, Müthing and Unland [209] reported high resolution of mixtures of gangliosides on Fractogel (Merck) carrying trimethylaminoethyl (TMAE) groups. With the anion exchanger in the acetate form, the gangliosides were eluted with a gradient of ammonium

acetate in methanol (0 to 0.1 *M* ammonium acetate in 20 min, rising to 1 *M* in 220 min, then isocratic elution for 60 min) at a flow-rate of 0.5 mL/min. While the degree of sialylation was the dominant factor in the separation, there was also some resolution of isomeric compounds differing only in the position of sialylation. Gangliosides that were identical in all respects in their sialic acid substitution, but differed in their respective neutral sugar components, could also be separated on TMAE-Fractogel.

18.2.7 High-performance anion-exchange chromatography

A search of the current literature reveals that there are far more references to HPAEC than to any other chromatographic technique of carbohydrate analysis. The method was formerly termed "high-pH anion-exchange chromatography", because, as developed originally, it relied on the utilization of high-pH conditions, but in recent years the acronym HPAEC has been accepted as standing for "high-performance anion-exchange chromatography". Non-alkaline separation conditions have also been explored. A very comprehensive review on the current status of HPAEC was recently published [210] and additional information on this now-essential method can be found in some earlier reviews [211–214].

18.2.7.1 Principles

It was in a seminal paper, published in l983 by Rocklin and Pohl [215], that the principles of HPAEC were first stated. When chromatography is performed at high pH (≥ 12), most carbohydrates, having pK_a values in the range 12–14, become anionic and can be sorbed on a strongly basic anion exchanger in the hydroxide form. However, carbohydrates are liable to undergo base-catalyzed reactions – such as isomerization of reducing sugars by the Lobry de Bruyn–van Ekenstein transformation or degradation of oligosaccharides by β-elimination – on prolonged exposure to strong alkali. For this reason, and because of the obvious desirability of rapid analysis, sorbents capable of much faster interaction than the usual exchangers, based on porous resins or silica, are essential. High efficiency is also essential, as the carbohydrate anions are very weakly retained, compared to common inorganic anions. Dionex has developed special pellicular packings, with a thin film of strongly basic anion exchanger, coated on nonporous latex beads, of 5- or 10-μm particle diameter, for this particular application. It was the first of these, designated HPIC-AS6 (10-μm beads), that was introduced by Rocklin and Pohl [215]. It was the precursor of the CarboPac series, now marketed by Dionex. Another innovation at the time was the use, in conjunction with the HPIC-AS6 column, of the highly sensitive pulsed-amperometric detector (Sec. 18.2.1), which allowed detection of carbohydrates at picomolar level without prior derivatization. With this column (250 × 4 mm ID), eluted at 1 mL/min with 150 m*M* sodium hydroxide, a range of alditols, monosaccharides, and simple oligosaccharides, including lactose, sucrose, maltose, raffinose, and stachyose, could be separated within 15 min at 36°C. Retention factors are reduced in the presence of acetate ions, and this variable is used to improve resolution and to promote elution of higher oligosaccharides. Initially, the malto-oligosaccharides were resolved to DP 10

within 10 min at 34°C by isocratic elution with a solution in which both sodium hydroxide and sodium acetate were 200 m*M*. The potential of gradient elution for resolution of oligosaccharides to much higher DP was later demonstrated by Olechno *et al.* [216], who used a linear gradient (0–600 m*M* sodium acetate in 100 m*M* NaOH over 30 min, at a flow rate of 1 mL/min), followed by isocratic elution at the final concentration for 5 min, to achieve excellent resolution of the malto-oligosaccharides, to DP as high as 43, at ambient temperature.

The prototype column used in this pioneering work on HPAEC of carbohydrates was later marketed by Dionex as CarboPac PA-1. This is still the most widely used column, but in recent years more specialized sorbents have been introduced. The CarboPac PA-10 column is optimized for monosaccharide analysis, giving more evenly distributed retention times for these sugars and also for alditols [217] and sialic acids [218]. The CarboPac PA-100 column has been reported to give better performance in the separation of *N*-glycans from glycoproteins than does CarboPac PA-1 [219]. Both of these newer sorbents are more tolerant of traces of organic solvent than CarboPac PA-1 [210]. There is also a sorbent based on a macro-porous substrate which, although the surfaces of the resin pores are coated with a layer of ion-exchange groups, so that it is still regarded as a pellicular resin, has very different properties from the CarboPac PA series, having higher anion-exchange capacity and requiring operation at a lower flow-rate (0.4 mL/min) [220]. The use of this sorbent, designated CarboPac MA-1, is mostly limited to analysis of reduced oligosaccharides from glycoproteins [210]. Even more specialized HPAEC systems are available. Sugar phosphates have been analyzed on a Dionex column (IonPac AS4), equipped with an anion micro-membrane suppressor for conductivity detection of analytes carrying charged groups [38]. More recently, this was done on the newer IonPac AS11, to which they are less strongly bound and thus are better separated [221]. The same is true of uronic acids [222]. The NucleoPac (now called DNAPac) PA-100 column, though not originally intended for carbohydrate analysis, has been used occasionally for the isolation of glycopeptides [223] and polysialic acids [224]. Guard or trap columns to remove traces of amino acids or borate are available for all these columns [210].

The PAD has remained the most frequently used detector for HPAEC, although other methods are occasionally employed. In the analysis of complicated biological samples, such as cell lysates, on-line radioactivity detection can be advantageous. For example, simultaneous usage of the PAD and this type of detection allows labeled components to be compared with chromatographic standards [225]. Compounds having UV-absorbing chromophores can be analyzed by HPAEC, coupled to UV detection, and those that have been appropriately derivatized, by connecting the system to a fluorimetric detector [210]. Coupling of HPAEC to MS is used increasingly, following improvements in desalting of the column effluent by use of micro-membrane suppressors. Sodium acetate is added to the eluent for HPAEC of higher oligosaccharides, because the acetate ion has a slightly higher affinity than OH^- for the strongly basic anion-exchange groups, and therefore, closely bound solutes, such as oligosaccharides of high DP or acidic saccharides, are more easily displaced in the presence of acetate, which has become known in this context as the "pushing agent". Wong and Jane [226] examined the effects of other pushing agents, anions that are more strongly bound to the anion-exchanger than acetate, and concluded

that a nitrate gradient could give better resolution of higher oligosaccharides than the acetate gradient generally employed. One problem is that detection of the highest members of a homologous series requires greater sensitivity than that afforded by the PAD, but this can been achieved by means of an on-line enzyme reactor, which causes hydrolysis of the oligosaccharides in the column effluent to the monomer before they reach the PAD. Thus, by a combination of sodium nitrate elution and the enzyme reactor, coupled to the PAD, the members of the malto-oligosaccharide series may now be separated and quantitatively determined up to DP 80 [227]. Superior resolution of polysialic acids (to DP 90) on CarboPac PA-100 has also been achieved by use of a nitrate gradient [228]. An added advantage of the presence of nitrate in the eluent is that a lower concentration of NaOH may be used, and this reduces the chances of degradation when the oligosaccharides contain alkali-sensitive terminal sugars.

For specific purposes, other anions have sometimes been introduced into the eluent. The presence of oxalate at pH 6.0 has been recommended [229] as a means of improving the separation of oligogalacturonic acids by HPAEC, because it prevents precipitation of these solutes from the alkaline eluents. However, oxalate is not easily removed from the eluted products, and therefore Spiro *et al.* [230] used a potassium acetate gradient at pH 8.0 for the preparative purification of biologically active oligogalacturonic acids, which are more soluble under these conditions than in sodium acetate solutions. Gradient elution with sodium phosphate buffer (pH 6.3) has been found effective in separating the oligosaccharides from hyaluronic acid on a CarboPac PA-100 column [231], while isolation of glycopeptides on a NucleoPac PA-100 column was achieved with Tris–HCl-buffered sodium chloride (pH 8.0) as the eluent [223]. Since neither phosphate nor chloride is compatible with the PAD, UV detection was used in these cases. The introduction of alkaline-earth cations in small proportion (1–2 m*M*) into dilute alkaline eluents (5–20 m*M* NaOH) has recently been recommended for peak sharpening and greater reproducibility of retention times in HPAEC of monosaccharides and alditols on CarboPac PA-1 columns [232,233]. For example, with an eluent consisting of 5 m*M* NaOH containing 1 m*M* barium acetate, a mixture of alditols and monosaccharides, including the amino- and acetamidodeoxyhexoses, was separated on this column within 15 min at a flow-rate of 1 mL/min [233]. With a higher concentration of alkali (20 m*M* NaOH) and the same concentration of barium acetate, the amino sugars and the neutral sugars (fucose, galactose, glucose, and mannose) that are common constituents of glycoconjugates can be separated in a very short time (11 min). The presence of Ba^{2+} or Sr^{2+} ions (but not Ca^{2+}) in the eluent has been observed to increase PAD response [234]. This applies also to acidic sugars: for example, a threefold increase in PAD response to galacturonic acid was observed when the alkaline eluent was "spiked" with 2 m*M* barium nitrate. Carbohydrates having anionic groups in the molecule can be separated by HPAEC under neutral or even mildly acidic conditions. Such analytes include uronic acids, sialic acids, sugar nucleotides, and phosphorylated or sulfated saccharides [210]. HPAEC/PAD under these conditions usually requires post-column addition of NaOH for detector response, although direct detection by PAD at pH 8 has been used in preparative-scale separations of oligogalacturonic acids [230].

18.2.7.2 Applications

One of the early successes of HPAEC was base-line resolution within 15 min of all the neutral and amino sugars commonly found in hydrolysates from glycoconjugates. Using one of the first CarboPac PA-1 columns, Hardy *et al.* [235] achieved this by isocratic elution with 22 m*M* NaOH at a flow-rate of 1 mL/min. The recommended concentration of the alkaline eluent was later reduced to 16 m*M* [210]. However, it was found that xylose, which occurs in glycoconjugates of plant origin, was not separated from mannose under these conditions. For the analysis of hydrolysates from these sources, Lee [211] recommended elution with 1 m*M* NaOH, containing 0.03 m*M* sodium acetate, which required post-column addition of NaOH for effective functioning of the PAD. Subsequent studies have been aimed, *inter alia*, at finding the optimal conditions for separating sugars from their alditols [236], a problem that has recently been solved by the inclusion of barium or strontium ions in the alkaline eluent. Cataldi *et al.* [232,237] attribute the effect of the alkaline-earth cations to two factors: the formation of soluble complexes with the sugars and alditols, and the removal of the carbonate formed by dissolution of atmospheric carbon dioxide in the alkaline eluent. The importance of this new approach in the determination of sugars and alditols in complex matrices, such as foods and vegetable tissues has been highlighted in a recent review [238]. The method has also found application in the simultaneous analysis of sugars, alditols, and glycerol in cell cultures and fermentation broths [239].

The separation of mixtures containing acidic monosaccharides presents its own problems. In an early investigation, aimed at the determination of hexuronic acids as well as neutral and amino sugars in biological materials, it was found necessary to use different elution conditions for chromatographing these three different types of monosaccharide [240]. However, complete resolution of 19 monosaccharides, 9 of which were neutral sugars, 3 aminodeoxy sugars and 7 acidic sugars (including *N*-acetylneuraminic acid, muramic acid, and KDO), that are frequently found in bacterial polysaccharides, was achieved in a single run by Clarke *et al.* [241]. The CarboPac PA-1 column was eluted with 15 m*M* NaOH at 1 mL/min for 15 min, and then a linear gradient of 0–100% 100 m*M* NaOH, containing 150 m*M* sodium acetate, was applied over 40 min at the same flow-rate, resulting in the elution of all the neutral sugars, including the rare hexoses altrose and talose, and of the acidic sugars in sharp peaks. The retention times for acidic sugars were later reduced by using eluents spiked with Ba^{2+} or Sr^{2+} ions [234].

The application of HPAEC to the separation of sialic acids, a diverse group of carboxylated 2-ketononuloses which differ mainly in their hydroxyl substitution, has been widely investigated [242]. In an early study, Manzi *et al.* [243] pointed out that, while the parent compounds, *N*-acetyl- and *N*-glycolyl-neuraminic acid, can safely be separated on a CarboPac PA-1 column by elution with 100 m*M* NaOH, containing 150 m*M* sodium acetate, alkaline eluents must be avoided for the *O*-acetyl esters, as saponification would be likely to occur during chromatography. For this purpose these authors recommended isocratic elution with 5 m*M* sodium acetate for 5 min, followed by a 30-min linear gradient of acetic acid (0–2.5 m*M*) in 5 m*M* sodium acetate, at a flow-rate of 1 ml/min. This resulted in separation of mono- and diacetylated species from the parent acids,

although there was overlap between some of the acetylated compounds. The use of a mildly acidic eluent, containing acetate at pH 5.0, has been shown [244] to decrease the difference in retention time between *N*-acetyl- and *N*-glycosylneuraminic acid, which is large under alkaline conditions and results in a considerable increase in analysis time if both are present. The distance between the two peaks is shorter on the Carbo Pac PA-10 column than on PA-1 under the same elution conditions [218].

The monophosphates of D-glucose have been well resolved on the IonPac AS6 column by isocratic elution with 50 m*M* NaOH, containing 300 m*M* sodium acetate [245]. HPAEC has become a recommended method for sensitive analysis of sugar phosphates, removed from biological samples by solid-phase extraction [246]. To preserve the stability of the nucleotide sugars ADP- and UDP-glucose, the sugar phosphates should be eluted at a low temperature and a relatively low concentration of alkali (<4 m*M*) [247,248]. Koizumi *et al.* [245] examined the possibility of using HPAEC on CarboPac PA-1 for separating the mono-*O*-methyl ethers of D-glucose. Under the conditions used (isocratic elution with 50 m*M* NaOH), differences in retention were small, with much overlap between the 4- and 6-*O*-methyl isomers and between the 2- and 3-*O*-methyl ethers. Better results were achieved by using a CarboPac PA-1 column eluted with 20 m*M* NaOH to resolve all the *O*-methyl ethers released on hydrolysis of permethylated (1 → 4)-linked glucans, and this method was applied to the determination of the substitution patterns of amyloses [249]. HPAEC has also been used in determining the distribution of substituents in carboxymethylcelluloses [250]. The mixture of carboxymethylated glucoses, obtained on perchloric acid hydrolysis of the polymer, was separated on CarboPac PA-1 by application of an acetate gradient (0–1.0 *M* in 15 min, at a flow-rate of 4 mL/min) in 100 m*M* NaOH.

One of the most important advantages of HPAEC is the high resolving power of this chromatographic method for homologous series of oligosaccharides. Koizumi *et al.* [251] performed a systematic study of the application of HPAEC to the analysis of several series of D-gluco-oligosaccharides: linked β-(1 → 2), α- and β-(1 → 3), α- and β-(1 → 4) and α- and β-(1 → 6). Using the HPIC-AS6 column and isocratic elution with 150 m*M* NaOH, containing 100 m*M* sodium acetate, these workers first investigated the relationship between log k' and DP for each of the homologous series. This was found to be linear in all cases, the slope being steepest for the strongly retained (1 → 3)-linked oligosaccharides and lowest for the α-(1 → 6)-linked isomalto series. Effective separations of the individual members of these oligosaccharide series were achieved with different gradients of sodium acetate in 150 m*M* NaOH, at a flow-rate of 1 mL/min. Under these conditions, the (1 → 2)- and (1 → 3)-linked series were resolved to DP 30, the β-(1 → 4)-linked series to DP 20 (this lower limit was set mainly by solubility considerations), and those of the β- and α-(1 → 6)-linked series to DP 45 and 50, respectively, in all cases within 45 min. The α-(1 → 4)-linked oligosaccharides of the malto series were also resolved to DP 50 under the conditions used, and there were indications that, if the sensitivity of the PAD could be further enhanced, even higher oligomers would be discernible (this was later achieved by use of an enzyme reactor on line with the PAD [227]). HPAEC was also applied to an estimation of the chain-length distribution of the linear α-(1 → 4)-linked segments in amylopectins, the branched components of starch [252]. Based on the results just discussed, a comparison of the performance of the HPAEC system with that of other HPLC systems that have been applied to the resolution of malto-oligosaccharides is presented in Table 18.2.

TABLE 18.2

RETENTION DATA FOR HPLC OF MALTO-OLIGOSACCHARIDES

DP	Capacity factor (k′)				
	P1	P2	P3	P4	P5
2	0.67	0.43	0.48	0.56	0.20
3	0.80	0.57	0.64	0.92	0.40
4	1.00	0.71	0.80	1.28	0.75
5	1.20	0.86	0.96	1.66	1.00
6	1.43	0.98	1.30	2.16	1.50
7	1.67	1.08	1.70	3.00	2.00
8	1.93	1.18	2.08	3.66	2.45
9	2.46	1.28	2.50	4.33	2.80
10	2.60	1.40	2.94	5.00	3.00
11	3.00	1.55	3.44	5.67	3.55
12	3.40	1.71	3.94	6.34	4.00
13	3.86	1.86	4.48	7.00	4.60
14	4.33	2.06	5.16	7.67	5.00
15	4.86	2.28	5.86	8.67	5.60
20	8.73	3.72	11.5	17.0	9.0
25		5.57	22.8	31.0	10.2
30		8.26			11.0
35					11.8
40					13.0
45					14.2
50					15.0

P1 = 5-μm silica equilibrated with 1,4-diaminobutane by passage of solution (300 ml) of this base (0.1%) in acetonitrile/water (1:1) through column (200 × 8 mm ID); eluent acetonitrile/water (1:1) containing 1,4-diaminobutane (0.01%), flow-rate 1 mL/min [104].

P2 = ERC-NH-1171 (aminopropyl phase on 3-μm silica), 200 × 6 mm ID column; eluent acetonitrile/water (57:43), flow-rate 1 mL/min [108].

P3 = YMC-Pack PA-03 (polyamine phase on 5-μm silica), 250 × 4.6 mm ID column; eluent acetonitrile/water (1:1), flow-rate 1 mL/min [120].

P4 = TSK gel Amide 80 (amide phase on 5-μm silica), 250 × 4.6 mm ID column; eluent acetonitrile/water (53:47), flow-rate 1 mL/min [120].

P5 = Dionex HPIC-AS6, 250 × 4 mm ID column; eluent 150 mm NaOH with sodium acetate gradient (200–300 m*M* in 5 min, 300–350 m*M* in 15 min, 350–400 m*M* in 25 min, then 400–450 m*M* in 25 min), flow-rate 1 mL/min [251].

Koizumi and coworkers [253] also applied HPAEC on HPIC AS-6 to the analysis of cyclic glucans, including the cyclodextrins and their branched derivatives, which were well resolved by isocratic elution with 150 m*M* NaOH, containing 200 m*M* sodium acetate. The cyclosophoroaoses in the DP range of 17–25 were resolved with 140 m*M* sodium acetate in the same alkaline eluent. Cyclic glucans have shorter retention times than linear glucans with the same DP (because of the absence of two hydroxyl groups, including the anomeric group, in the cyclic form [211]), unless the retention is affected by conformational differences [210]. Other neutral oligosaccharides that have been successfully analyzed by HPAEC include fructans, which are of importance in food and agricultural science. Since 1994, when a report appeared describing the efficacy of HPAEC on CarboPac PA-1, with a gradient of sodium acetate (50–225 m*M* in 15 min, at a flow-rate of 1 mL/min) in 150 m*M* NaOH, in resolving the mixtures of glucose, fructose, sucrose, isomeric fructosyl trisaccharides and fructans (to DP 7) that occur in cereal grasses [254], there has been interest in the use of the method for this purpose. Since EU regulations now require the characterization and quantitative analysis of fructans in stewed fruit products, the HPAEC procedure for fructan analysis is being accepted as a standard method in Europe [255]. It has also proved useful in the separation and identification of the fructose oligosaccharides, produced as intermediates in the enzymatic hydrolysis of sucrose [256].

HPAEC has also found application in studies of acidic oligosaccharides, such as pectins and polygalacturonic acids. The technique has proved valuable in investigation of esterification patterns, the distribution of neutral sugars, and the mode of action of enzymes used in degradation. Oligogalacturonic acids afford an example of analytes that do not require alkaline eluents in HPAEC analysis. In early work in this field, Hotchkiss and Hicks [229] separated the oligogalacturonic acids of the normal series to DP 50 by use of a potassium oxalate gradient, and subsequently they applied this method to an analysis of the unsaturated oligogalacturonic acids generated by pectate lyase degradation, which were found to be more strongly retained than their counterparts of like size in the normal series [257]. This was observed also by Lieker *et al.* [258], who used an alkaline eluent, containing the usual acetate ions, as the "pushing agent".

Mort and coworkers [259] were the first to demonstrate the usefulness of HPAEC in the determination of the patterns of methyl esterification in pectins. The products of sodium borohydride reduction of the esterified galacturonic acid residues, followed by HF solvolysis of the glycosidic linkages involving the galactose residues thereby produced, were analyzed on a CarboPac PA-1 column. Application of a sodium acetate gradient (30–200 m*M* over 27 min, to 500 m*M* over 25 min, then after a 5-min hold to 750 m*M* over 5 min), at a flow-rate of 1 mL/min, resolved the mixtures of oligogalacturonic acids generated from the pectins by the reactions described. Quantification of these oligomers then revealed the lengths and distribution of the stretches of contiguous nonesterified galacturonic acid residues in the original pectin. The isolation of oligogalacturonic acids having DP up to 20 by HPAEC on a preparative-scale Carbo-Pac PA-1 column (21 mm ID), with a non-linear gradient of potassium acetate (pH 7.5), has recently been reported [260]. The high resolution and sensitivity of HPAEC/PAD is proving valuable in analyses of sialic acid

oligo/polymers. As has been mentioned, the use of a nitrate gradient in HPAEC on the CarboPac PA-100 column produced exceptionally sharp separations of polysialic acids [228]. Oligomers of the α-(2 → 8)-linked series with *N*-acetyl- or *N*-glycolyl-neuraminic acid or KDN (keto-3-deoxynonulosonic acid) monomers were resolved to DP 80–90. Further work by Lin *et al.* [261], who studied five different homologous series of polysialic acids, with different glycosidic linkages, and with and without sulfation, has demonstrated similar resolution by HPAEC on CarboPac PA-100 under these conditions. The aim was to explore the use of highly sensitive analytical methods in the identification of diverse polysialic acids in studies of biological phenomena associated with the expression of polysialic acid chains with different DP. In this application the authors considered HPAEC to be superior to other modern techniques, such as capillary electrophoresis.

HPAEC has been applied to analyses of sulfated oligosaccharides, such as those obtained on enzymatic degradation of glycosaminoglycans. However, the presence of the sulfate groups tends to suppress PAD responses, and therefore other methods of detection are often used. For this purpose pre-column derivatization is sometimes employed: for example, the keratan sulfate oligosaccharides have been resolved as the anthranilic acid derivatives on the IonPac AS4A column [262]. Oligosaccharides from chondroitin and dermatan sulfates [263] and heparin [264] have been profiled by HPAEC on CarboPac PA-1, with UV detection at 232 nm. In the case of the heparin oligosaccharides a semi-preparative column was used, the oligosaccharides being eluted with a sodium chloride gradient in neutral medium, where they are stable. Subsequent desalting permitted isolation of the oligosaccharides in a pure state. This work is particularly interesting in that it affords an example of the use of HPAEC as part of a chromatographic system in which the depolymerized heparin is first fractionated by SEC and fractions of uniform size are then separated on the basis of charge by HPAEC [264].

18.2.7.3 Glycoconjugate analysis

Separation of the complex oligosaccharides released in degradative studies of glycoconjugates has been a major application of HPAEC. The potential of the technique in this regard was first recognized in 1988, when publication of a seminal paper by Hardy and Townsend [265] drew attention to its capacity for separating neutral oligosaccharides of this type according to molecular size, sugar composition, and the glycosidic linkages between the monosaccharide units. These studies were subsequently extended by the same researchers to sialylated oligosaccharides [266], which were found to be separated not only on the basis of degree of sialylation but also according to the nature of the sialic acid residues and the position of the glycosidic linkages between these residues and the neutral sugar components. Fucosylated oligosaccharides, too, were clearly distinguished from one another and from their non-fucosylated counterparts [267]. In all cases, chromatography was carried out on the CarboPac PA-1 column, generally with an acetate gradient in an alkaline eluent, although it was found that some of the monosialylated oligosaccharides were best separated at lower pH (sodium acetate gradient at pH 4.65) [266].

The pioneering work of this research group was soon summarized in comprehensive review articles [212,268]. There was also some interest at this early stage in the use of the method for separating the various oligosaccharides present in human milk; most of these were resolved by isocratic elution with 30 m*M* NaOH, containing 25 m*M* sodium acetate [269].

The extension of oligosaccharide profiling by HPAEC to those with even more complex structures, such as the di-, tri,- and tetraantennary oligosaccharides and the high-mannose and hybrid types [270,271], and to *O*-linked [272,273] as well as *N*-linked glycans, firmly established the technique as an indispensable method in glycobiology. This was all the more so when the development of desalting by the use of an on-line anionic micro-membrane suppressor facilitated isolation of the separated oligosaccharides for characterization by spectroscopic methods, such as NMR and FAB-MS [270,273]. Hermentin *et al.* [274] established a database for mapping of *N*-glycans, into which were entered the retention data for over 100 different oligosaccharides of known structure, analyzed by HPAEC on the CarboPac PA-100 column with two different linear gradients of acetate in 100 m*M*NaOH, one considered to be optimal for resolution of sialylated oligosaccharides, the other for asialo structures. Several reviews on the analysis of glycoconjugates by this technique are available [210,213,275].

Not only oligosaccharides as such but also asparagine-linked glycopeptides, produced on trypsin digestion of glycoproteins, have been successfully resolved by HPAEC. Rohrer [276] has compiled a list of empirical relationships between molecular structure and retention time for these glycopeptides, to facilitate interpretation of the chromatographic data in their analysis. There has also been some accumulation of data on structural features governing the HPAEC retention of the neutral and acidic lactose-based oligosaccharides of human milk [277]. The CarboPac PA-1 column with an acetate gradient (0–250 m*M* in 30 min, at a flow-rate of 1 mL/min) in 100 m*M* NaOH provides good resolution of positional and structural isomers, so that the effects of increasing chain-length and additional fucosylation or sialylation can be easily determined. Molar response factors for use in quantitative analysis have been calculated for 28 of these oligosaccharides.

In a recent study [278] the effects of temperature on the behavior of different types of *N*-linked oligosaccharides from glycoproteins, as well as the neutral and sialylated oligosaccharides of human milk, have been investigated. By performing HPAEC analysis at different ambient temperatures, over the range 13–30°C, it has been shown that the retention times of all these oligosaccharides decrease with increasing temperature, even relatively small temperature differences (about 5°C) resulting in appreciable changes in retention times. Furthermore, the relative changes in retention time have been found to vary from one oligosaccharide to another, so that reversals in elution order with change in temperature may be observed. It can be inferred from these results that the use of retention times, even relative to an internal standard, for identification of oligosaccharides by HPAEC analysis is invalid, unless the temperature is carefully controlled. Change in temperature can, however, afford an effective means of achieving optimal separations in HPAEC of oligosaccharides of these types.

18.2.7.4 Coupling with mass spectrometry

There have been significant recent advances in the coupling of HPAEC systems to ESI-MS, mainly due to improvements in on-line desalting of the column effluent, which now permits the use of coupled MS with NaOH/sodium acetate gradients up to a total Na^+concentration of 0.6 *M*. Under these conditions, it has been found possible to determine the molar mass of glucose oligomers of the α-(1 → 4)-linked malto series to DP 7, with a detection limit of 20 ng for each compound [279]. Galacturonic acid oligomers could be detected to DP 4, at a concentration of 70 ng each. Further improvements have been achieved by the use of position- and time-resolved ion counting (PATRIC) array detection, with a magnetic-sector mass spectrometer, equipped with an ion-spray interface [280]. PATRIC array detection is used in static or scanning modes for molecular mass determination and structure characterization, respectively. Use of this system in static mode has lowered the detection limits for the malto-oligosaccharides to 700 pg and, with PATRIC in scanning mode, structural information has been obtained for galacturonic acid oligomers and the components of a digest from an exocellular bacterial polysaccharide. Inclusion of on-line micro-dialysis in the HPAEC/MS system, in addition to the micro-membrane desalting device, has further improved sensitivity, with disaccharides such as sucrose, maltose, and trehalose becoming detectable at levels as low as 3 pg [281].

18.2.8 Size-exclusion chromatography

During the past decade the more rigid sorbents developed for SEC under HPLC conditions (HPSEC) have almost entirely superseded the older gels, especially as increased efficiency of these sorbents has improved resolution. Extensive reviews on the topic will be found in chapters included in two recent books [282,283]. The coupling of HPSEC systems to detectors other than the conventional differential refractometer or UV photometer has proved very advantageous in increasing the sensitivity and scope of the method. Enzymatic methods can be applied to increase the sensitivity of detection of polysaccharides that are readily degraded by specific enzymes. For example, a technique has been developed for the continuous determination of the α-glucan components of entire or debranched starch by SEC, followed by treatment of the column effluent, first, with dilute acid to neutralize the alkaline eluent used, and then with amyloglucosidase to hydrolyze the α-(1 → 4)-linked glucans [284]. The glucose produced is analyzed by on-line spectrophotometric determination of the product of glucose oxidase conversion of the sugar to hydrogen peroxide and treatment of this with peroxidase and a chromogenic reagent. In some cases, direct spectrophotometric detection in the visible range is possible, if the analyte forms a colored complex on post-column addition of a particular reagent. A prime example is the use of iodine in detection of starch and starch components, emerging from a polymethacrylate SEC column [285]. The specific interaction of β-glucans with Calcofluor dye has been utilized in the SEC of the (1 → 3) (1 → 4)-linked β-D-glucans that are the major soluble components of the cell walls of barley and oats [286]. Both, this reagent and iodine, are compatible with the alkaline eluents used in HPSEC of glucans on polymeric sorbents.

The need for a highly sensitive detector in SEC of polymers producing viscous solutions, *i.e.*, measuring sample concentrations of 1 mg/mL or less, as well as the problem of finding suitable calibrants for determination of molar-mass distributions of polymers by SEC, have led to the introduction of an on-line light-scattering photometer into SEC systems. Since the output of a LSD is proportional to both the molar mass and the concentration of the sample, lower concentrations may be used in SEC as molar mass increases. The intensity of the scattered light is a function of the cosine of the scattering angle, and is therefore highest with scattering angles close to 0°. This is the principle behind the operation of the instrument most commonly employed in SEC, the low-angle laser light-scattering (LALLS) photometer. The use of this instrument permits sensitive detection at low sample concentrations of high-polymers that form viscous solutions or gels at concentrations above a certain limit. It also makes possible simultaneous determination of the molar-mass distribution and average molar mass (M_w) by an absolute method that does not require the use of standards for calibration. Coupling of a LALLS detector, usually in tandem with a differential refractometer, to the SEC system is thus a fundamentally sound approach to the determination of molar masses of polymers. Use of SEC/LALLS in the carbohydrate field is growing, especially in the characterization of polysaccharides having functionalities that are dependent on molar mass. The technique has been applied to many of the commercially important polysaccharides, such as starch and its components [287,288], pectins [289], carrageenans [290,291], alginates [292], agarose [293], and microbial polysaccharides, like xanthan [294]. SEC/LALLS is also finding application in the characterization of clinically valuable biopolymers, especially heparin [295,296], since its anticoagulant activity is restricted to fractions of lower molar mass.

A more recent advance in the use of the LSD in SEC has been the introduction of the multi-angle laser light-scattering (MALLS) instrument, in which the cell assembly through which the laser beam passes is surrounded by 18 collimated detectors at different fixed angles. This permits intensities of scattered light to be measured simultaneously at many angles and enables the determination not only of molar mass but also of the radius of gyration of a polymer molecule, from which information on molecular conformation can be deduced. The MALLS detector was first used in conjunction with HPSEC of a polysaccharide by Beri *et al.* [297], who applied the technique to the characterization of samples of the cationic biopolymer chitosan (produced by de-*N*-acetylation of chitin). They succeeded in establishing structure/function relationships for polymer fractions having M_w above 100,000, but results obtained with samples of lower average molar mass were less accurate. In recent years, the coupling of MALLS to HPSEC has been increasingly recognized as an important technique for the characterization of carbohydrate polymers, as it affords information on the degree of branching and molecular shape, as well as molecular size. The method is now strongly recommended as a complement to LALLS in the characterization of industrially important polysaccharides [298–302]. Its potential in the assessment of biological samples, such as gastric mucins, with respect to both molecular size and purity has also been explored [303,304]. The importance of both LALLS and MALLS in SEC analysis of complex polysaccharides and glycoconjugates has been emphasised in a recent review by Jumel [305].

Another detector that has facilitated the characterization of polysaccharides when used in conjunction with SEC is the viscosity detector. In the SEC analysis of pectins, Fishman *et al.* [306,307] have made extensive use of a viscosity detector, on-line with the column system, and a differential refractometer, so that the intrinsic viscosity of each pectin component could be determined as it was eluted from the columns. Because of the dependence of intrinsic viscosity on molar mass, these data could be used to estimate molar masses. The method was subsequently applied to other polysaccharides of industrial importance, such as alginates, carboxymethylcellulose, locust bean gum, and gum arabic [308]. The data obtained from the viscosity detector have been found to complement those given by MALLS in SEC characterization of the molecular size and shape of polysaccharides [309]. As with HPLC in other modes, there is currently much interest in the use of mass spectrometry in conjunction with the chromatographic system. As has been mentioned (Sec. 18.2.1.2), direct interfacing of SEC with MS poses some problems, but these are being overcome by innovative methods [85]. The use of the modern desalting techniques that are now proving useful in HPAEC/MS could also be advantageous here. In the carbohydrate field, research on SEC/MS has been directed mainly at the characterization of polymers, such as the glycosaminoglycans, for which there are no suitable calibration standards to permit molar-mass determination by SEC alone. Yeung and Marecak [310] have reported the use of MALDI-MS to determine the molar masses of microfractions, collected across peaks emerging during SEC of enzymatically degraded hyaluronic acid. The data thus obtained could be used subsequently in establishing a valid calibration, so that molar masses of other samples of hyaluronic acid could be estimated from SEC retention data alone.

It has long been accepted that the problem of obtaining suitable calibration standards for the determination of molar masses of polysaccharide fractions by SEC can be overcome by application of the "universal calibration", in which V_e or K_{av} is plotted against log $[\eta].M_w$, where $[\eta]$ is the intrinsic viscosity (Chap. 5). The product $[\eta].M_w$ is directly related to the hydrodynamic volume of the polymer molecule in solution. A calibration of this type, obtained with well-characterized dextran standards, has been found to hold satisfactorily for pullulans, amyloses, and amylopectins on both silica-based [311] and polymeric sorbents [312]. Linear correlations of SEC elution parameters for polysaccharide fractions with the function log $[\eta].M_w$, determined by viscosimetry and light-scattering measurements of each individual fraction, have been reported for other, more diverse polysaccharides, including carrageenans [313], pectins [306,307], and chitosan [314]. Where such narrow fractions of the polymer being examined are not available, the dextran fractions marketed by Pharmacia have often been used as standards in establishing the universal calibration plot. However, a dextran calibration is not valid for glycosaminoglycans, and charged polymers must be used for this purpose. Sodium polystyrene sulfonate was suggested as a calibrant for SEC of hyaluronic acid [315], but this procedure has not yielded satisfactory results and its validity has been questioned [316]. More recently, fractions of the polymer to be analyzed, obtained by random depolymerization, preparative chromatography, and characterization of the products by absolute methods, have been routinely used as calibrants in molar-mass determination of glycosaminoglycans, especially heparin [317–319]. Another failure of the universal calibration was reported [320,321], following attempts to apply a calibration established

with polysaccharides having flexible chains (pullulans) to those having rigid, rod-like molecules, such as the (1 → 3)-linked β-D-glucan schizophyllan. Thus, the general validity of the so-called "universal calibration" in the carbohydrate field is doubtful, and the application of absolute methods of molar-mass determination, especially MS and the light-scattering techniques, is now considered essential for accurate determination of molar masses of polymers analyzed by SEC. Although the range of sorbents available does include some with smaller average pore diameter, which are capable of fractionating oligosaccharides, the use of SEC for this purpose has now been largely superseded by HPAEC. It is in the fractionation and molar-mass distribution analysis of polymeric analytes, namely polysaccharides, glycoconjugates, and their degradation products of larger molecular size, that SEC has retained its value. A few examples of the chromatographic systems that have been successfully applied in this field are given in Table 18.3.

18.2.9 Affinity chromatography

Affinity chromatography (Chap. 3) on immobilized lectins was originally introduced mainly for the recognition or isolation of polysaccharides and glycoproteins having specific structural features, and in this regard it has assumed great importance in biochemistry and clinical chemistry. The technique has proved invaluable, *e.g.*, in procedures designed to distinguish between normal and malignant cells [323]. More recently, the focus has shifted towards the use of affinity chromatography in the separation of the glycopeptides and oligosaccharides produced in degradative studies of glycoconjugates. For this purpose, a series of different sorbents, bearing lectins with binding capacities that are specific for each of the various major structural features of these glycopeptides and oligosaccharides, is generally employed. The applications in glycobiology of this technique, which is known as serial lectin affinity chromatography, have been discussed in several reviews [324–328]. The strength of binding between a lectin and an oligosaccharide is determined by the presence or absence of a particular sequence of sugar residues in the oligosaccharide chain. Some examples of lectins that are much used in serial lectin affinity chromatography, and the sequences for which strong affinity is shown have been listed by Endo [327,328].

Quantification of the carbohydrate-binding specificities of various immobilized lectins in columns is best performed by frontal analysis [328]. In this procedure, a large volume of the analyte solution is continuously applied to the column until no more of the analyte can be retained. The volume of the elution front is then measured. Disadvantages of this method are that it is time-consuming and that a large sample of analyte is required. Recently, these disadvantages have been overcome by the use of miniature columns (10 × 4 mm ID), which are coupled to an electrospray mass spectrometer [329]. The amount of analyte may also be reduced by pyridylamination of the sample, which is then monitored by fluorimetry [330]. Under these conditions, the amount of sample required is decreased to only about 20 nanomoles, and the analysis time with the miniature column is only 12 min per cycle. The speed of affinity chromatography has been increased by the use of sorbents in which the lectins that have proved most useful in conventional affinity chromatography have been coupled to LiChrosorb DIOL [331] or unmodified

TABLE 18.3

EXAMPLES OF SEC SYSTEMS AND CONDITIONS USED IN FRACTIONATION OF CARBOHYDRATE POLYMERS

Polymer	Sorbent	Eluent	Conditions	Detector	Reference
Starch	Ultrahydrogel 250 and 2000 (polymethacrylate), 2 columns (300 × 7.8 mm ID) coupled in series	50 m*M* NaOH	0.5 mL/min, RT	RI and spectrophotometric monitoring of starch/I_2 complex at 546 and 658 nm	[285]
Cellulose, chitin	TSK gel GM HXL (styrene-DVB copolymer), column 300 × 7.8 mm ID	*N*,*N*-dimethylacetamide solution of LiCl (5%, w/w)	0.1 mL/min, RT	RI	[322]
Agarose	Shodex OHpak B804 and/or B805 (polymethacylate), single or coupled columns (each 500 × 8 mm ID); choice depends on molecular-size range	0.1 *M* $NaNO_3$	1 mL/min, 45 °C	RI and LALLS	[293]
Carrageenans	TSK G6000 PW and G5000 PW (polymethacrylate), 2 columns (600 × 7.5 mm ID) coupled in series	0.1 *M* LiCl	1 mL/min, 60 °C	RI and LALLS	[290]
Chitosan	Bio-Gel SEC-60 (hydroxylated polyether) column coupled to two Bio-Gel SEC-50 columns, each 300 × 7.5 mm ID	Buffer, 0.33 *M* acetic acid/0.1 *M* sodium acetate, pH 4.2	0.6 mL/min, RT	RI and MALLS	[297]

(*continued on next page*)

TABLE 18.3 (continued)

Polymer	Sorbent	Eluent	Conditions	Detector	Reference
Pectins	TSK G6000 PW and G4000 PW, 2 columns (each 600 × 7.5 mm ID) coupled in series	0.1 *M* Na NO_3	1 mL/min, 30 °C	RI and LALLS	[289]
	μ-Bondagel E-High and E-1000 (polyether bonded to silica), 2 columns (each 300 × 3.9 mm ID) coupled to SynChropak GPC-100 (glycerylpropyl silica) column, 250 × 4.6 mm ID	50 m*M* $NaNO_3$	0.5 mL/min, 35 °C	RI and viscosity detector	[307]
Heparin	TSK G2000 SW and/or G3000 SW (silica-based), each column 300 × 7.5 mm ID	0.1 *M* NaCl	0.5 mL/min, RT	UV (diode-array detection over range 206-280 nm)	[51]
	TSK G2000 SW column coupled to TSK G3000 SW, each 300 × 7.5 mm ID	0.2 *M* Na_2SO_4, containing Tris–HCl buffer (50 m*M*), pH 7.0	0.6 mL/min, 30 °C	RI and UV (235 nm)	[296]
	TSK G3000 PW (polymethacrylate)	Buffered Na_2SO_4, as above	0.6 mL/min, 30 °C	RI and LALLS	[296]

RT = room temperature, RI = refractive-index detector.

micro-particulate silica. In the latter case, the coupling is performed through an amino group on the lectin and an aldehyde group formed on the silica gel support through periodate oxidation of vicinal hydroxyl groups [332]. The amino group of the lectin is coupled to the aldehyde group on the support by reductive amination in the presence of sodium cyanoborohydride. Honda [332] emphasizes that the amino group utilized for coupling must be located at a position remote from the binding site, so that the binding ability of the lectin is not affected, and that the silica should have an average pore size of not more than 60 angstroms. Silica gel with larger pore sizes will have a higher capacity for the lectins, but carbohydrate peaks will be broadened due to delayed interaction inside the pores.

The broad peaks, sometimes obtained in affinity chromatography on sorbents in which lectins are coupled to silica, have been ascribed to slow dissociation of the carbohydrate from its complex with the immobilized lectin. After a detailed thermodynamic and kinetic study of affinity chromatography on silica-based concanavalin A, Muller and Carr [333] reported that dissociation constants for glycosides in such complexes are about ten times smaller than those of the complexes in solution, and those for complexes involving glycoproteins are even smaller. The effect of temperature on the resolution of glycoproteins on concanavalin A, immobilized on micro-particulate silica, was investigated [334], and it was found that resolution was much improved, due to the production of narrower peaks, by temperature-programmed elution (30°C to 70–80°C at 1.5°C/min). However, while this may be acceptable for analytical purposes, the possibility of denaturing glycoproteins on exposure to such elevated temperatures, even for a short time, makes this technique inapplicable in preparative chromatography.

A more feasible alternative, which can be used for preparative purposes, is the substitution of weakly interactive ligands, instead of lectins, as the active agents in high-performance affinity chromatography (HPAC) on silica. Some success has been achieved by use of monoclonal antibodies for this purpose [335,336]. Coupling of antibody 39.5 (IgG2b) to silica, and elution with 0.2 *M* NaCl in 0.02 *M* sodium phosphate buffer (pH 7.5) from a column (100 × 5 mm ID), containing this sorbent, gave sharp peaks over the temperature range 37–50°C for the reduced oligosaccharide Glcα(1 → 6)Glcα(1 → 4) Glcα(1 → 4)Glc-ol. The dissociation constants involved were such that the column efficiency approached that generally associated with HPLC [335]. The efficacy of IgM monoclonal antibody bound to silica through concanavalin A, immobilized on the surface of the support, has also been investigated [336]. The active oligosaccharides of blood group A (penta-, hexa-, and heptasaccharides) were well resolved by isocratic elution with phosphate-buffered saline, by stepwise temperature programming from 35°C to 50°C. In more recent studies [337], a number of carbohydrate antigens, including some that are similar in structure, have been separated under isocratic conditions according to their weak interactions with various monoclonal antibodies, immobilized on silica. The separations could be modified by variation in temperature or pH. A generally recognized advantage of using weakly interacting ligands in PPAC is that a true chromatographic process is involved in the separation, as compared with traditional lectin affinity, which is more of a discontinuous "on/off" process.

18.3 GAS/LIQUID CHROMATOGRAPHY

Although gas/liquid chromatography (GLC) remains an important technique in carbohydrate analysis, few reviews dealing with GLC of carbohydrates have been published during the past decade, owing to the rapidly growing interest in HPLC methods. What reviews have appeared have been devoted specifically to certain areas. Fox [338,339] has emphasised the advantages of GLC, coupled to tandem mass spectrometry, in the profiling of carbohydrates, as alditol acetate derivatives, in complex matrices, where the sugar components may only be present at trace levels. The alditol acetate method is widely recognized as the standard technique for GLC and GLC/MS analysis of neutral and amino sugars, but where acidic sugars are present, other derivatization methods have to be employed. Molnár-Perl [340] has demonstrated that the resolution and high sensitivity of GLC of the trimethylsilylated (TMS) oxime derivatives are superior to HPLC methods in the simultaneous analysis of sugars, polyols, uronic acids, and other carboxylic acids in natural matrices, such as fruit and vegetables, and in fermentation broths. The value of this method in the food industry, where mixtures of sugars, carboxylic acids, and amino acids are often analyzed, has been shown by the same author [341] in a recent comprehensive review of the chromatographic methods currently in use for such analyses. The advantages of methanolysis, followed by GLC analysis of the TMS derivatives of the resulting methyl glycosides, in the simultaneous characterization of the neutral sugar and uronic acid components of acidic polysaccharides, such as plant gum exudates, have also been the subject of two recent reviews [342,343]. The many alternative methods of derivatization that were explored during the 1980 s for possible use in GLC and GLC/MS analysis of carbohydrates [1] have not, in general, been followed up. Therefore, this section will be confined mainly to the three procedures just mentioned, together with some promising new techniques. Other aspects of GLC analysis that have remained current in the carbohydrate field are the enantioselective resolution of chiral compounds and, of course, applications of mass spectrometry coupled to GLC systems.

18.3.1 Derivatization

Conversion of sugars and methylated sugars to their alditol acetate derivatives for GLC analysis is still a universally accepted method; this applies also to GLC/MS, since a wealth of mass spectral data for alditol acetates has been accumulated However, this method of derivatization does have the disadvantage of being time-consuming, despite the modifications introduced during the 1980s [1]. Recent progress in solving this problem is the development of a fully automated system for the preparation of alditol acetate derivatives [339,344]. This is a computer-controlled instrument that enables the multi-stage process to be performed sequentially, without manual intervention, and several samples to be processed simultaneously. In this system, a series of electrically driven solenoid valves are connected on-line to a 21-sample manifold of glass/PTFE reaction chambers. The manifold is seated in a movable heating block. A set of valves controls the input of solvent and/or nitrogen to each sample chamber, and a set of gas valves controls connections to the atmosphere or vacuum, while closure of all valves allows the samples to

be sealed in closed chambers. The temperature is also adjusted automatically. Solvent exchange is achieved by automated evaporation of the first solvent, followed by input of the new solvent under nitrogen pressure. After reduction of the samples in sodium borohydride or borodeuteride solution at room temperature for 2 h, methanol/acetic acid (200:1) is added. The program then sets the heating block to 60°C, and evaporation under nitrogen occurs for 30 min. This step is automatically repeated several times to ensure removal of borate as volatile tetramethyl borate. After the last evaporation, the system is evacuated, and the samples are dried at room temperature. Acetic anhydride (300 μl) is then added, and the samples are acetylated at 100°C for 13 h, with the sodium acetate remaining after evaporation serving as the catalyst. Only the final post-derivatization clean-up (which requires only 30 min) has to be performed manually; after the excess acetic anhydride has been decomposed by addition of water, the samples are extracted with chloroform for GLC. The advent of this innovative system, which removes the tedium from derivatization, may well lead to a resurgence of interest in GLC analysis [338].

Derivatization of sugars to oximes prior to acetylation, trimethylsilylation, or trifluoroacetylation for GLC analysis has the advantage that it is applicable to ketoses as well as aldoses, and to acidic sugars, such as aldonic and alduronic acids, as well as neutral and amino sugars. Oximation involves reaction with hydroxylamine hydrochloride (or *O*-methylhydroxylamine hydrochloride) in pyridine for only a short time (generally, 30 min or less). A disadvantage, however, is that double peaks, corresponding to geometrical isomers, are produced for each sugar on GLC of oxime derivatives. While acetylation of oximes and aldononitriles of sugars has been widely used as a means of derivatization for GLC analysis, the TMS oximes have generally been preferred in recent years. The use of oxime derivatives in GLC has the advantage that aldoses can be distinguished from alditols existing as such in complex mixtures from natural sources. It is for this reason that there has been renewed interest in GLC analysis of the derived TMS oximes for the simultaneous analysis of neutral sugars, polyols, sugar acids, and components, such as carboxylic acids and amino acids in fruit and other natural matrices [340,345]. A GLC/MS database has been accumulated for this purpose [345,346].

TMS oximes are rather unstable derivatives, which cannot be stored, and the trifluoroacetylated oximes even more so, as they are unstable in the presence of moisture, which complicates post-derivatization clean-up [339]. There has recently been renewed interest in GLC of carbohydrates as the acetylated *O*-pentafluorobenzyl oximes, a method first introduced in 1987 by Biondi *et al.* [347]. These derivatives are more stable, and their use, like that of the trifluoroacetyl derivatives, allows coupling of the gas chromatograph to the sensitive electron-capture detector. The derivatization procedure involves oximation by reaction with *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride, followed by acetylation with acetic anhydride/pyridine. Good separations of amino sugars from neutral sugars within a short time are achieved by GLC using these derivatives, which give highly characteristic mass spectra [348]. GLC/MS by this method has proved useful in detection and quantitation of traces of amino sugars in hydrolyzates from glycoproteins and bacterial cell walls. For example, Kozar and Fox [349] recently described the application of the technique in analysis of muramic acid (3-*O*-lactylglucosamine), an amino sugar present in the cell wall of the peptidoglycan of *Eubacteria*, which is used as a

chemical marker for the detection of traces of bacteria or bacterial debris in complex matrices. The acetylated pentafluorobenzyl oxime (PFBO) derivatives are particularly suited to MS in negative-ion chemical ionization mode.

Capillary GLC of the trimethylsilylated derivatives of the methyl glycosides produced on methanolysis of the carbohydrate components of glycoproteins is a highly sensitive method, allowing the determination of individual components at levels down to 100 pmol, and analysis of glycoprotein samples of 0.5–10 μg. Excellent resolution of all the monosaccharide components of glycoproteins is achieved by this method, the derivatives from 2-acetamido-2-deoxy-D-glucose and -galactose, and from *N*-acetylneuraminic acid, being well separated from the neutral sugar derivatives but still emerging within a short time (about 20 min). The TMS methyl ester methyl glycosides, derived from D-glucuronic and D-galacturonic acid, are also well resolved and may be analyzed by capillary GLC together with the TMS glycosides from neutral sugars in methanolysates of acidic polysaccharides. The method has therefore been exploited to advantage in the analysis of acidic gums of industrial importance, such as gums arabic, karaya, and tragacanth [350]. The characteristic pattern of multiple peaks given by each sugar helps identification, which in recent years has been greatly facilitated by the accumulation of a large body of EI-MS data for TMS methyl glycosides [342,343,351]. GLC/MS data have been compiled for several different classes of monosaccharides, including all the aldopentoses and -hexoses, some deoxy sugars and ketoses, and the common hexuronic acids. An interesting application has been the use of GLC/MS of the TMS methyl glycosides from methanolysates as a means of characterizing the plant gums and other substances of vegetable origin present in manuscript inks and objects of archaeological interest [343].

A novel method for rapid, sensitive analysis of the monosaccharide constituents of glycoconjugates, by GLC of the heptafluorobutyrate derivatives of the methyl glycosides produced on methanolysis, was recently described by Zanetta *et al.* [352]. The rationale behind this technique is that acylation with heptafluorobutyric anhydride (HFBAA) binds in a single step not only hydroxyl groups but also amino groups, forming products that do not interact strongly with the methyl siloxane phases commonly used in GLC analysis of apolar derivatives. Thus, all constituents of glycoconjugates, including the amino sugars, can be analyzed in a single run within a relatively short time. This applies also to acidic sugars [353]: the method has proved successful in GLC analysis of methanolysates from glycosaminoglycans, most of which contain hexuronic acids, such as glucuronic and iduronic acids, in addition to aminodeoxy sugars and, in some cases, neutral sugars from the proteoglycan linkage region. These are sulfated polysaccharides, and therefore methanolysis (0.5 *M* methanolic HCl, 80°C, 20 h) was performed in the presence of barium acetate to minimize degradation due to release of sulfuric acid [353]. In the derivatization procedure recommended by Zanetta and coworkers, the dried methanolysate is dissolved in acetonitrile, and HFBAA is added (25 μl for every 200 μl of acetonitrile). The reaction mixture is then heated at 150°C for 15 min and, after evaporation under nitrogen, the residue is redissolved in acetonitrile for GLC analysis.

The heptafluorobutyrate method of derivatization has also been applied in examination of the diversity of the sialic acid components occurring in glycoconjugates [354]. In this case, the sialic acids, after liberation by mild acid hydrolysis, are methyl-esterified by

treatment with diazomethane/methanol, and the esters produced are then derivatized with HFBAA. GLC/MS analysis of these derivatives allows the separation and identification of a variety of sialic acids, including *N*-acetyl- and *N*-glycolylneuraminic acids, KDN, and the acetylated derivatives of all three, as well as 8-*O*-methylated and 8-*O*-sulfated types. The sialic acid compounds can be identified at picogram level by this method.

18.3.2 Enantiomer resolution

In the previous edition of this textbook there was some discussion of the preparation of chiral derivatives of sugars, with the objective of achieving resolution of enantiomers by GLC [1] However, with the exception of a brief review describing the technique of determining the absolute configuration of sugars by GLC of the acetylated (+)-2-octyl glycosides [355], the use of such derivatives for this purpose was not followed up during the 1990s. In recent years the emphasis has shifted entirely towards the use of enantioselective stationary phases for GLC resolution of chiral compounds, as these phases have become increasingly more effective. König and coworkers [356] have produced their most successful stationary phases for this purpose by modifying cyclodextrins through the introduction of hydrophobic groups. The objective was both to lower their melting points from those of the free cyclodextrins, to permit the use of the modified cyclodextrins as liquid phases in GLC, and to increase thermal stability. In the first phase of this type, all the free hydroxyls in α-cyclodextrin (cyclomaltohexaose) were pentylated by reaction of the cyclodextrin with 1-bromopentane and NaOH in dimethyl sulfoxide, followed by further pentylation in the presence of NaH in tetrahydrofuran. This pentylated cyclodextrin was found to exhibit a high degree of enantioselectivity towards trifluoroacetylated aldoses, methyl glycosides, and alditols, including the 1,4- and 1,5-anhydroalditols, which have assumed importance in studies of polysaccharides by the reductive cleavage method. Base-line resolution of enantiomers was achieved within 5–10 min on glass capillary columns (20 or 40 m), coated with this phase, GLC being performed isothermally at temperatures varying from 80°C to 120°C [357,358]. The thermal stability of the pentylated cyclodextrin was such that no deterioration of column performance was observed, even after operation at 200°C [357]. Since trifluoroacetylated carbohydrate enantiomers, but not peralkylated or TMS derivatives were resolved, it was postulated that chiral recognition probably depended on specific dipole/dipole interactions between the trifluoroacetyl groups and the asymmetric centers in the pentylated cyclodextrin, rather than inclusion phenomena, involving the cavities in the cyclodextrin matrix [358]. However, it was subsequently recognized that enantioselective recognition was dependent on both the size and the shape of the molecule, the latter being influenced by the nature of the substituent groups introduced by derivatization [359,360].

The pentylated α-cyclodextrin [hexakis (2,3,6-tri *O*-pentyl)cyclomaltohexaose] was given the trade name Lipodex A, and capillary columns coated with this phase were marketed by Macherey-Nagel. Another enantioselective phase developed by the König group, by pentylation of β-cyclodextrin [the systematic name of this derivative is heptakis(2,3,6-tri-*O*-pentyl)cyclomaltoheptaose], which was named Lipodex C, has also proved to be successful in resolution of the D- and L-enantiomers of trifluoroacetylated

carbohydrates, among other compounds [359]. More recently, the same group has reported the use of other modified cyclodextrins in enantioselective GLC of permethylated methyl glycosides and anhydroalditols [361]. Capillary GLC on 25-m columns, coated with heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)- cyclomaltoheptaose (20%, dissolved in polysiloxane; Column A) or octakis(2,6-di-*O*-methyl-3-*O*-pentyl)cyclomaltooctaose (50%, dissolved in polysiloxane; Column B), at temperatures ranging from 85°C to 110°C, produced resolution of enantiomeric pairs in the per-*O*-methylated methyl glycosides of all the common aldopentoses and -hexoses, and the deoxyhexoses. There was also clear differentiation between α- and β-anomers, and between pyranoside and furanoside ring forms. Permethylated anhydroalditols, which were produced by reduction of the permethylated methyl glycosides by the reductive cleavage method, were also examined by GLC on Columns A and B; in most cases even better separations of enantiomeric pairs were observed with these compounds. The technique was successfully applied in simultaneous determinations of linkage position and absolute configuration of the galactose residues in a snail galactan. Reductive cleavage of the permethylated polysaccharide, followed by acetylation of the hydroxyl groups exposed by the cleavage, gave a mixture of *O*-acetyl-1,5-anhydro-*O*-methylgalactitols, which were well resolved by temperature-programmed capillary chromatography on Column A. In this way, the occurrence of terminal residues of L-galactose in the snail galactan, the remainder having the more usual D-configuration, was clearly established. This is a striking example of the power of enantioselective GLC in structural studies of polysaccharides.

18.3.3 Mass spectrometry

The use of a mass spectrometer, usually in EI mode, on-line with the gas chromatograph is considered essential for the identification of components separated by GLC, especially in the analysis of complex mixtures of closely related compounds, such as the partially methylated sugars in hydrolysates of methylated polysaccharides. The sensitivity of GLC analysis can be increased considerably if the usual FID detector is replaced by the mass spectrometer, and the intensities of certain selected, diagnostic ions are used to monitor the emergence from the column of specific components. During the 1990s, tandem mass spectrometry became available as an adjunct to GLC, allowing detection limits to be even further lowered. GLC/MS/MS is now an important technique in analyses at trace levels, for example, in the identification of trace carbohydrates in complex matrices, such as bacterial cell walls [338,339], where optimal specificity and sensitivity are required. The use of a mass spectrometer in selected ion monitoring (SIM) mode increases selectivity and decreases background noise. However, extraneous background peaks are still found, and for enhanced specificity, Fox [338] strongly advocates having a tandem mass spectrometer in mutiple reaction monitoring (MRM) mode. In the first use of GLC/MS/MS by Fox *et al*. [362], a triple quadrupole instrument was employed, but later successful deployment of the less expensive ion-trap MS/MS for the same purpose [363] brought the technique more within the scope and budget of analytical laboratories. Although quantitation is more accurate with the triple quadrupole mass

spectrometer, sensitivity is higher with the ion-trap instrument, and the reproducibility obtained is sufficient for most clinical and environmental applications. The sensitivity of GLC/MS/MS for trace analysis of sugars in complex matrices is presently unmatched by that of LC/MS/MS [364,365]. The current instruments can be run in EI or CI mode, with positive- or negative-ionization capability, but most GLC/MS and GLC/MS/MS analyses of sugars are still performed with EI-MS in positive-ion detection mode [339].

GLC/MS analysis of carbohydrates has been greatly facilitated by the availability of databases containing m/z values and relative intensities of selected ions among those produced by the various carbohydrate compounds or their methyl ethers, appropriately derivatized. The use of alditol acetate derivatives in GLC/MS of carbohydrates is now a mature technique, for which a vast body of data has been collected over the years. There is also now a comprehensive library of GLC/MS data for the TMS derivatives of the methyl glycosides of neutral sugars and hexuronic acids [343] and, as has been mentioned (Sec. 18.3.1), diagnostic MS data for use where sugars and sugar acids are analyzed as their TMS oximes in the presence of other acids is accumulating [345,346].

The power of GLC/MS in structural studies of intact oligosaccharides has been clearly demonstrated by a recent study [366] in which the technique was applied to the determination of both the position and the anomeric configuration of the linkage in disaccharides. A series of eight reducing disaccharides of D-glucose were analyzed, as the permethylated and the peracetylated oligosaccharide-alditols, and the GLC retention times, together with the mass numbers and relative intensities of the major ions in the EI-MS spectra, were subjected to pattern recognition studies with multivariate data analysis. The relative intensities were especially interesting, as the data obtained indicated that, at least for the disaccharides investigated, an axial glycosidic bond is more easily cleaved in the ionization process than an equatorial one. Anomeric configuration could be assigned on this basis. The technique is potentially valuable for structural elucidation of more complex oligosaccharides.

18.4 PLANAR CHROMATOGRAPHY

While commercial aluminum- or plastic-backed plates, coated with Silica Gel 60, are widely used in TLC of carbohydrates, HPTLC plates are generally recommended for applications, such as the resolution of homologous series of oligosaccharides [367] and of gangliosides [368,369]. The latter application, which has been extended to glycosphingolipids in general [370], is of importance in medical science, where rapid analysis of very small samples is often required. The role of TLC in this field is highlighted in Sec. 18.4.2. Analysis of glycosphingolipids and other glycoconjugates, which are usually available in only minute quantities, has necessitated the development of some new techniques, involving immunochemistry or affinity binding, for their detection on TLC plates, as the usual spray reagents lack the required sensitivity. Mass spectrometry is also being used to an increasing extent as an adjunct to TLC.

18.4.1 Methods

18.4.1.1 Overlay binding

The use of detection methods that depend on interaction of carbohydrates on TLC plates with antibodies, lectins, and other binding proteins, even viruses and bacteria, through overlay of the developed plate with a solution containing the binding agent serves not only to detect the analytes but also to yield some information on their molecular structures. Such methods were developed mainly for detection in TLC of glycosphingolipids, and they have been described in some detail in the two comprehensive reviews by Müthing [369,370]. However, they are applicable also to other glycoconjugates having the specific structural features targeted by the binding agents. The various overlay procedures share some common steps. Müthing [369,370] recommends coating the plate, after development and drying, with a thin film of plastic, usually polyisobutyl methacrylate (PIBM), by dipping it for 30 sec into a dilute solution (0.2% or less) of PIBM in *n*-hexane. The purpose of this is to prevent the silica gel from flaking off the plate during the subsequent incubation and washing steps, and also to reduce nonspecific binding [371]. In the next step, the plate is overlaid with the primary binding agent (such as an antibody) and incubated. Then, after washing with an appropriate buffer, it is sometimes overlaid again with a secondary agent (for example, an enzyme-labelled secondary antibody). After a second washing, the plate is dried prior to the actual detection procedure, which may involve X-ray autoradiography or color development. A companion plate, run in the same TLC chamber under identical conditions, is subjected to chemical detection with a general visualization reagent for carbohydrates, such as the orcinol/sulfuric acid spray, to show the location of the carbohydrate-containing spots. The exact conditions used in overlay precedures obviously depend on the nature of the analyte and the binding agent. Some examples, drawn mainly from the two reviews by Müthing and that by Schnaar and Needham [369–371], follow.

Sensitive *immunoassay* is particularly useful in the detection of picomolar quantities of glycosphingolipids on TLC plates. This is important in clinical analysis, as the distribution patterns of these glycolipids, especially gangliosides, in brain and neural tissues are diagnostic of certain pathological conditions. Two methods have been developed for immunoassay of glycosphingolipids (or other oligosaccharides having the required structural features). In radioimmunoassay, the plate, after being coated with PIBM, is sprayed with a buffer [usually phosphate-buffered saline (PBS), pH 7.4], containing bovine serum albumin (1%), as a further precaution against nonspecific binding. It is then overlaid with a solution containing an appropriate monoclonal antibody (2–5 μg/mL) in the same buffer and incubated for 3 h at 4°C. After being washed in cold PBS, the plate is overlaid again with a solution containing a secondary antibody (about 0.01 μg/mL), such as $F(ab')_2$ rabbit anti-mouse IgG, labelled with ^{125}I (about 40 μc/μg). After 1 h at ambient temperature, the plate is again washed in PBS, dried, and placed on X-ray film for autoradiography. This requires an exposure time of at least 16 h, sometimes longer, even several days.

In order to accelerate the detection procedure, and avoid the use of radioactive isotopes, the alternative method of enzyme-linked immunosorbent assay (ELISA) has been introduced. In this case, the secondary antibodies are linked to peroxidase or alkaline phosphatase and, after overlaying the plate with these enzyme-linked immunosorbents, it is immersed in a solution, containing a substrate for the enzyme together with a chromogenic reagent capable of sensitive detection of unreacted enzyme. This permits densitometric scanning of the colored spots or bands produced. For example, Schnaar and Needham [371] described the use of peroxidase-conjugated goat anti-mouse IgM as the secondary antibody, following overlay of glycosphingolipids on a TLC plate with monoclonal antibody. After incubating and washing the plate, it was immersed in a freshly made solution, containing 0.01% hydrogen peroxide and 0.5 mg/mL diaminobenzidine tetrahydrochloride in PBS. The chromatographic zones, where the glycosphingolipids are located, became clearly visible as brown spots or bands against a light background. For alkaline phosphatase-linked secondary antibodies, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in a 0.1 *M* glycine buffer (pH 10.4), containing $ZnCl_2$ and $MgCl_2$, each 1 m*M*, is an effective chromogenic reagent [372].

The sensitivity of immunoassays is sometimes increased if the overlay of the plate with the primary antibody is preceded by an overlay with an enzyme that attacks glycosidic linkages involving specific structural features in the analyte molecules. This applies especially to analyses of gangliosides, which have one or more sialic acid residues in the oligosaccharide chain. Techniques have been described [369–371] that involve overlay of the TLC plate with a buffer solution containing a neuraminidase that removes both external and internal sialic acid residues from the oligosaccharide core. The resulting asialogangliosides are then incubated on the plate with anti-gangliotetraosylceramide ($GgOse_4Cer$) and/or anti-gangliotriosylceramide ($GgOse_3Cer$) antiserum. Subsequent detection is possible by radioimmunoassay, after incubation with ^{125}I-labelled staphylococcal Protein A [371], or by ELISA. An example of the latter is a method described by Müthing and Neumann [373] for the selective detection of gangliosides of the sialylated neolacto series, which involves treatment of the gangliosides on the TLC plate with neuraminidase, incubation of the plate with chicken anti-$nLcOse_4Cer$ specific antibodies, and then overlaying with alkaline phosphatase-conjugated secondary antibodies (rabbit anti-chicken IgG antiserum). After removal of phosphate by washing with 0.1 *M* glycine buffer (pH 10.4), containing $ZnCl_2$ and $MgCl_2$ as described above, the bound primary antibodies are visualized by generation of a blue dye with BCIP in the same buffer. This method distinguishes α-(2 → 3)-sialylated gangliosides from α-(2 → 6)-, as the latter can be detected without prior treatment with neuraminidase. Sialylation of galactose in the 6-position does not prevent binding of the anti-$nLcOse_4Cer$ antibody to the Galβ(1 → 4)GlcNAc sequence, whereas steric hindrance results from sialylation at the 3-position. This highly specific and sensitive technique allows detection of the gangliosides to levels of about 10 ng.

The specific carbohydrate-binding properties of *lectins*, which have found wide application in affinity chromatography (Chap. 3) (Sec. 18.2.9), can also be utilized in the detection of glycolipids, glycopeptides, and oligosaccharides from glycoproteins on TLC plates. If labelled with a radioisotope, usually ^{125}I, the bound lectin can be located by autoradiography. Alternatively, the ELISA technique can be employed: The plate is

overlaid with a lectin/peroxidase conjugate in PBS, and a solution containing diaminobenzidine tetrahydrochloride is used as a chromogenic reagent. A recently published procedure is based on the use of digoxigenin-labelled lectins and their detection with anti-digoxigenin antibody, conjugated with horseradish peroxidase [374]. Lectins from *Maackia amurensis* and *Sambucus nigra*, respectively, were used for specific detection of α-(2 → 3)- and α-(2 → 6)-sialylated neolacto series gangliosides. Studies of the binding of lectins to glycolipids, or oligosaccharides from glycoproteins, immobilized on TLC plates, afford a rapid method of determining recognition specificities. Furthermore, by serial use of different lectins, information can be obtained on the structural features of unknown oligosaccharides.

The binding of *toxins*, *viruses*, and *bacteria* can be similarly exploited. The cholera toxin, a globular protein consisting of two subunits, A and the pentameric B_5, binds specifically to the monosialylated ganglioside G_{M1} or structurally related compounds substituted with *N*-acetyl- or *N*-glycolylneuraminic acid residues. The mechanism of binding to animal cells has been found to involve specific affinity of the B_5 subunit (choleragenoid) for the ganglioside receptors on the cell surface, followed by dissociation between the subunits and penetration of the smaller subunit A through the membrane [369]. This binding phenomenon can be used for analytical purposes. Gangliosides that bind cholera toxin can be detected on TLC plates by overlaying with ^{125}I-labelled toxin, followed by autoradiography; a detection limit of 0.1 ng (65 fmol) of G_{M1} has been reported [369,370]. Alternatively, ELISA can be applied in detection. In this case, native cholera toxin is used for binding, which is followed by incubation with a specific antiserum to the toxin and a species-specific antiserum, coupled to horseradish peroxidase. This is a more sensitive method, with detection limits as low as 0.01 ng (6.5 fmol) of G_{M1}. The presence of other members of the gangliotetraose family (G_{D1a}, G_{D1b}, G_{T1b}, and G_{Q1b}) can be revealed by this method, if the plate is first overlaid with *Vibrio cholerae* neuraminidase, an enzyme which does not cleave the internal α-(2 → 3)-linkage of the *N*-acetylneuraminic acid residue in the G_{M1} structure, but does attack these residues in the terminal positions they occupy in the other gangliosides of this family. For example, Davidsson *et al.* [372] have used the technique, in conjunction with ELISA (with alkaline phosphatase-linked secondary antibodies and BCIP as the chromogenic reagent), in TLC assays of all the gangliotetraose gangliosides in human cerebrospinal fluid. TLC, combined with neuraminidase treatment and overlaying with choleragenoid, has been widely applied in the detection and determination of these gangliosides in various clinical investigations [369,370].

Gangliosides with sialic acid residues in terminal positions are important binding sites for viruses, such as the influenza C virus, which can bind a wide spectrum of sialoglycoconjugates containing 9-*O*-acetylated *N*-acetylneuraminic acid. Specific detection of such gangliosides on TLC plates can be achieved by exploiting the binding capacity of the virus [375]. The plate is overlaid with a suspension of the virus in PBS (pH 7.2), containing bovine serum albumin (0.5%), and this is followed by incubation at 4°C for 1 h. The acetylesterase activity of the bound virus is the basis for its subsequent visualization by incubation of the plate at ambient temperature with 1 m*M* 2-naphthyl acetate in the same buffer; the solution also contains 0.1% of 2-amino-5-chlorotoluene 0.5[$ZnCl_4$] (Fast Red TR-salt), which forms a red dye with the 2-naphthol liberated from

the ester. This method has a detection limit as low as 65 fmol. The technique is potentially useful in clinical investigations, as *O*-acetylation of neuraminic acid residues is known to be altered in cells undergoing malignant transformation. During the last decade there has been increasing interest in the binding of intact bacteria to glycosphingolipids on TLC plates. The bacteria are labelled externally with ^{125}I, or metabolically with other isotopes, and detected by autoradiography. These methods have much potential for use in the detection and characterization of carbohydrate receptors for microbes in the infection process [369,370].

18.4.1.2 Mass spectrometry

Although the use of overlay binding techniques in TLC has the power to generate information on the structural features of glycosphingolipids and complex oligosaccharides, such methods have their limitations, especially with regard to assessing purity The production by an analyte of a single spot or band on TLC, even in several different solvent systems, does not necessarily indicate homogeneity, as some analytes of this type are difficult to resolve by TLC, despite differences in monosaccharide composition and/or ceramide moieties. Furthermore, caution should be exercised in making structural assignments purely on the basis of immunochemical methods, in case underlying minor components in crude or insufficiently purified fractions are responsible for the observed binding. Even in methods involving modification of the analytes with enzymes, results should be interpreted with care, as other structural features may hinder enzymatic attack on an otherwise susceptible linkage. Therefore, these techniques should be used in conjunction with other analytical methods for unambiguous interpretation of TLC data. For this purpose the use of high-resolution mass spectrometry is attracting growing interest [376].

Initially, the combination of MS with TLC was indirect, necessitating time-consuming extraction of the separated analytes from the TLC plate before MS analysis, which was generally by EI-MS of appropriate derivatives. The development of soft ionization methods, especially liquid secondary-ion mass spectrometry (LSI-MS), ESI-MS and FAB-MS, and of high-field instruments brought progress in that direct analysis on TLC plates became possible. After separation of the analytes on a flexible aluminum- or plastic-backed plate and nondestructive localization, the segments containing the analytes are excised and mounted inside the ion source or, in the case of FAB-MS, attached to the probe tip, together with the matrix liquid. Use of a blotting technique, in which the separated analytes are transferred from the plate to a polyvinylidene difluoride membrane, from which the zones of interest are excised for attachment to the mass spectrometer probe, has been recommended for TLC/MS of glycosphingolipids [377]. In continuation of this approach, direct MALDI-TOF-MS of glycosphingolipids, separated on TLC plates, has been achieved after heat transfer of the analytes to polymer membranes of various types [378]. There was a gain in sensitivity compared with that possible in TLC/LSI-MS, the detection limits being lowered to 5–50 pmol, as opposed to 1–10 nmol. It was found that glycosphingolipids, bound to antibodies in an overlay assay on the TLC plate, could be transferred to the membranes and analyzed by MALDI-TOF-MS without interference

from the antibody or the buffers used during the binding process. In all cases, the mass spectra obtained in TLC/MS are comparable with those obtained by conventional methods [369,370]. LSI-MS, used in conjunction with TLC, has also proved effective in the sequencing of oligosaccharide-alditols, derived from glycoproteins. The techniques used in analysis of glycolipids are applicable, if the reduced oligosaccharides are converted to neoglycolipids by coupling with dipalmitoylglycerophosphoethanolamine [379]. These derivatized oligosaccharides are located by spraying the plates with the fluorogenic reagent primuline (specific for lipids), which does not interfere in LSI-MS.

18.4.2 Clinical analysis of glycoconjugates

TLC can be a valuable aid in the diagnosis of disorders of carbohydrate metabolism. The existence of such conditions generally results in excretion of characteristic oligosaccharides or glycopeptides in the urine, and TLC affords a rapid, sensitive method for the detection of these markers. For example, Schindler *et al.* [380] described a procedure for the simultaneous detection of abnormal urinary oligosaccharides and glycopeptides that are diagnostic of lysosomal storage diseases, such as α-*N*-acetylgalactosaminidase deficiency. This involved TLC of urine samples on duplicate plates, which were developed simultaneously in the same tank (two-fold development with 1-butanol/acetic acid/water, 22:9:9). One plate was sprayed with Bial's reagent (40 m*M* orcinol and 33 m*M* $FeCl_3$ in acidified ethanol) to visualize the oligosaccharides, and the other with the ninhydrin/Cu(II) reagent to detect glycopeptides.

The efficacy of TLC in the analysis of small samples of glycosphingolipids, especially gangliosides, has been discussed [371]. The solvent systems most used in clinical laboratories concerned with TLC of glycosphingolipids are mixtures of chloroform and methanol with water or an aqueous electrolyte solution. The proportions depend on the polarity of the analytes, ranging from 66:30:4 for those of relatively low polarity to 50:40:10 for polar analytes. The presence of electrolyte in the solvent system improves resolution of gangliosides and other anionic glycosphingolipids. Müthing [381] has used automated multiple development to enhance separations of gangliosides in HPTLC, with a solvent system consisting of chloroform/methanol/20 m*M* aqueous $CaCl_2$, in proportions of the order of 52:38:10. Decreasing the proportion of the aqueous component resulted in better resolution of the complex mixtures of mono- and disialogangliosides isolated, *e.g.*, from human granulocytes, while a solvent system containing slightly more of the aqueous component was necessary for an effective separation of polysialogangliosides. The TLC plates were automatically cycled through a preset number of developments, usually at least three. Multiple development by this method in chloroform/methanol/20 m*M*$CaCl_2$ (55:40:5) resulted in the separation of complex gangliosides of the G_{M1b} type, substituted with *N*-glycolyl- as well as the usual *N*-acetylneuraminic acid residues, and with both C_{24} and C_{16} fatty acids, which are characteristic of murine tumor cell lines [382]. This could not be achieved by standard methods. The importance of TLC in the separation and identification of glycosphingolipids in tumors, bacterial and viral infections (including HIV), and other pathological states is discussed in the recent review chapter by Müthing [370].

18.5 CAPILLARY ELECTROPHORESIS

During the decade that has elapsed since applications of electrophoresis to carbohydrates were briefly reviewed in the previous edition of this textbook [1] the field has been revolutionized by the rapid development of high-performance capillary electrophoresis (HPCE) (Chap. 9). This technique is such an attractive alternative to conventional planar electrophoresis because of its high speed and efficiency, small sample requirements, and precise instrumentation, adapted from that used in HPLC, that the older methods have been largely superseded by HPCE in recent years. There is some continued interest in slab gel electrophoresis in the form of fluorophore-assisted carbohydrate electrophoresis (FACE), in which the carbohydrate analytes are labelled by reductive amination with reagents such as ANTS or AMAC (Sec. 18.2.1.1), in order to replace the older staining procedures by sensitive fluorimetric detection [383,384]. However, the superior resolution and other advantages of capillary electrophoresis, which can be regarded as being complementary to HPLC, have made this the method of choice in most analytical laboratories, especially those concerned with separations of complex mixtures of oligosaccharides and glycopeptides. The development of HPCE as an important technique in carbohydrate analysis has been documented in three reviews by El Rassi [385–387]. Specific aspects of HPCE, as applied to carbohydrates have been the focus of several other reviews [388–390].

Honda [332,391] has demonstrated the usefulness of HPCE in studying carbohydrate/protein interactions in solution, which permits rapid estimation of binding constants. Much of the recent work on HPCE of carbohydrate analytes has been concerned with its application to the analysis of glycoproteins, glycopeptides, and oligosaccharides released from glycoconjugates [392,393], as well as glycosaminoglycans and proteoglycans, both intact and de-polymerized [394]. There is special interest in the use of HPCE, sometimes in combination with HPLC, in multi-dimensional techniques for oligosaccharide mapping [393,395], and even mapping of glycopeptides as such [393]. The development of electrophoretic conditions for the characterization of glycoforms of glycoproteins by HPCE/ESI-MS has also been the topic of a review [396]. More recent work has been devoted to the application of HPCE/MS to the identification of heparin oligosaccharides [397], as well as those from glycoproteins [398]. Pre-column derivatization with reagents such as ANTS [399] and AMAC [400] has proved advantageous here. The use of an on-line electrospray ion-trap mass spectrometer in profiling of both ANTS-derivatized and underivatized glycans from glycoproteins has been recommended [401]. There is an increasing tendency to use both HPLC and HPCE, as complementary methods, in applications such as the separation and purification of glycopeptides [402].

REFERENCES

1 S.C. Churms, in E. Heftmann (Ed.), *Chromatography*, 5th Edn. Elsevier, Amsterdam, 1982, pp. 229–292.
2 B. Herbreteau, *Analusis*, 20 (1992) 355.
3 S.C. Churms, *J. Chromatogr. A*, 720 (1996) 75.

4 Z. El Rassi, *J. Chromatogr. A*, 720 (1996) 93.
5 Y.C. Lee, *J. Chromatogr. A*, 720 (1996) 137.
6 S. Kishino and K. Miyazaki, *J. Chromatogr. B*, 699 (1997) 371.
7 I. Molnār-Perl, *J. Chromatogr. A*, 891 (2000) 1.
8 Z. El-Rassi (Ed.), *Carbohydrate Analysis: High Performance Liquid Chromatography and Capillary Electrophoresis*, Elsevier, Amsterdam, 1995.
9 Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002.
10 D.J. Bornhop, T.G. Nolan and N.J. Dovichi, *J. Chromatogr.*, 384 (1987) 181.
11 D.O. Hancock and R.E. Synovec, *J. Chromatogr.*, 464 (1989) 83.
12 B. Herbreteau, M. Lafosse, L. Morin-Allory and M. Dreux, *Anal. Chim. Acta*, 267 (1992) 147.
13 B. Herbreteau, M. Lafosse, L. Morin-Allory and M. Dreux, *Chromatographia*, 33 (1992) 325.
14 A. Clement, D. Jong and C. Brechet, *J. Liq. Chromatogr.*, 15 (1992) 221.
15 M. Lafosse and B. Herbreteau, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 1101–1134.
16 L. Bento and S.D. Susana, *Carbohydr. Polym.*, 37 (1998) 257.
17 N. Purdie, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 1135–1149.
18 R.P. Baldwin, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 947–959.
19 R.P. Baldwin, *J. Pharm. Biomed. Anal.*, 19 (1999) 69.
20 Z.-L. Chen and D.B. Hibbert, *J. Chromatogr. A*, 766 (1997) 27.
21 P.F. Luo and T. Kuwana, *Anal. Chem.*, 66 (1994) 2775.
22 P.F. Luo, T. Kuwana, D.K. Paul and P.M.A. Sherwood, *Anal. Chem.*, 68 (1996) 1330.
23 M. Monta, O. Niwa, S. Tou and N. Watanabe, *J. Chromatogr. A*, 837 (1999) 17.
24 R.E. Reim and R.M. van Effen, *Anal. Chem.*, 58 (1986) 3203.
25 M. Goto, H. Miyahara and D. Ishii, *J. Chromatogr.*, 515 (1990) 213.
26 L.M. Santos and R.P. Baldwin, *Anal. Chim. Acta*, 206 (1988) 85.
27 J. Zhou and S.M. Lunte, *Anal. Chem.*, 67 (1995) 13.
28 I.G. Casella, E. Desimoni and T.R.I. Cataldi, *Anal. Chim. Acta*, 248 (1991) 117.
29 T.R.I. Cataldi, I.G. Casella, E. Desimoni and T. Rotunno, *Anal. Chim. Acta*, 270 (1992) 161.
30 F. D'Eramo, J.M. Marioli, A.A. Arevalo and L.E. Sereno, *Electroanalysis*, 11 (1999) 481.
31 S. Hughes and D.C. Johnson, *Anal. Chim. Acta*, 132 (1981) 11.
32 P. Edwards and K.K. Haak, *Am. Lab*, 4 (1983) 78.
33 S. Hughes and D.C. Johnson, *Anal. Chim. Acta*, 149 (1983) 1.
34 G.G. Neuberger and D.C. Johnson, *Anal. Chem.*, 59 (1987) 204.
35 D.C. Johnson and W.R. LaCourse, in Z. El Rassi (Ed.), *Carbohydrate Analysis: High Performance Liquid Chromatography and Capillary Electrophoresis*, Elsevier, Amsterdam, 1995, pp. 391–429.
36 W.R. LaCourse, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 905–946.
37 W.R. LaCourse, *Pulsed Electrochemical Detection in High Performance Liquid Chromatography*, Wiley, New York, 1997.
38 R.E. Smith, S. Howell, D. Yourtee, N. Premkumar, T. Pond, G.Y. Sun and R.A. MacQuarrie, *J. Chromatogr.*, 439 (1988) 83.
39 S.R. Hull and R. Montgomery, *Anal. Biochem.*, 222 (1994) 49.

40 R.J. Linhardt, K.N. Gu, D. Loganathan and S.R. Carter, *Anal. Biochem.*, 181 (1989) 288.
41 A. Plöger, *J. Food Sci.*, 57 (1992) 1185.
42 J. Emnēus and G. Marko-Varga, *J. Chromatogr. A*, 703 (1995) 191.
43 L.J. Nagels, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 1071–1100.
44 G. Marko-Varga, E. Dominguez, B. Hahn-Hägerdal and L. Gorton, *J. Chromatogr.*, 506 (1990) 423.
45 G. Marko-Varga, E. Dominguez, B. Hahn-Hägerdal, L. Gorton, H. Irth, G.J. de Jong, R.W. Frei and U.A.Th. Brinkman, *J. Chromatogr.*, 523 (1990) 173.
46 I. Blikstad, L.G. Fagerstam, R. Bhikhabhai and H. Lindblom, *Anal. Biochem.*, 233 (1996) 42.
47 C. Jungar, M. Strandh, S. Ohlson and C.-F. Mandenius, *Anal. Biochem.*, 281 (2000) 151.
48 L.S. Liau, B.L. Lee, A.L. New and C.N. Ong, *J. Chromatogr.*, 612 (1993) 63.
49 K.B. Hicks, *Adv. Carbohydr. Chem. Biochem.*, 46 (1988) 17.
50 J. Harenberg and J.X. de Vries, *J. Chromatogr.*, 261 (1983) 287.
51 J.X. de Vries, *J. Chromatogr.*, 465 (1989) 297.
52 P. Lehtonen and H. Vuorela, *J. Liq. Chromatogr.*, 17 (1994) 1245.
53 M. Previti, F. Dotta, G.M. Pontieri, U. di Mario and L. Lenti, *J. Chromatogr.*, 605 (1992) 221.
54 S.J. Mellis and J.U. Baenziger, *Anal. Biochem.*, 114 (1981) 276.
55 K. Yamashita, T. Mizuochi and A. Kobata, *Methods Enzymol.*, 83 (1982) 105.
56 L.S. Lohmander, *Anal. Biochem.*, 154 (1986) 75.
57 J.P. Fuhrer, *J. Chromatogr.*, 528 (1990) 65.
58 S. Hase, *J. Chromatogr. A*, 720 (1996) 173.
59 S. Hase, in Z. El Rassi (Ed.), *Carbohydrate Analysis: High Performance Liquid Chromatography and Capillary Electrophoresis*, Elsevier, Amsterdam, 1995, pp. 555–575.
60 S. Hase, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 1043–1069.
61 K.R. Anumula, *Anal. Biochem.*, 220 (1994) 275.
62 K.R. Anumula and S.T. Dhume, *Glycobiology*, 8 (1998) 685.
63 M. Pauly, W.S. York, R. Guillen, P. Albersheim and A.G. Darvill, *Carbohydr. Res.*, 282 (1996) 1.
64 X. Shen and H. Perreault, *J. Chromatogr. A*, 811 (1998) 47.
65 D. Fu and D. Zopf, *Anal. Biochem.*, 269 (1999) 113.
66 S. Honda, *J. Chromatogr. A*, 720 (1996) 183.
67 K. Mopper, *Anal. Biochem.*, 85 (1978) 528.
68 K. Mopper and E.T. Degens, *Anal. Biochem.*, 45 (1972) 147.
69 S. Honda, M. Takahashi, S. Shimada, K. Kakehi and S. Ganno, *Anal. Biochem.*, 128 (1983) 429.
70 K. Mopper, R. Dawson, G. Liebezeit and H.-P. Hansen, *Anal. Chem.*, 52 (1980) 2018.
71 S. Honda, K. Enami, T. Konishi, S. Suzuki and K. Kakehi, *J. Chromatogr.*, 361 (1986) 321.
72 T. Kato and T. Kinoshita, *Anal. Biochem.*, 106 (1980) 238.
73 S. Honda, K. Kakehi, K. Fujikawa, Y. Oka and M. Takahashi, *Carbohydr. Res.*, 183 (1988) 59.
74 S. Honda, T. Konishi and S. Suzuki, *J. Chromatogr.*, 299 (1984) 245.
75 A. Vrātny, U.A.Th. Brinkman and R.W. Frei, *Anal. Chem.*, 57 (1985) 224.
76 M. Kramer and H. Engelhardt, *J. High Resolut. Chromatogr.*, 15 (1992) 24.
77 S. Hara, H. Ikegami, A. Shono, T. Mega, T. Ikenaka and Y. Matsushima, *Anal. Biochem.*, 97 (1979) 166.

78 F. Perini and B.P. Peters, *Anal. Biochem.*, 123 (1982) 357.
79 M. Stahl, A. von Brocke and E. Bayer, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 961–1042.
80 D.J. Harvey, *J. Chromatogr. A*, 720 (1996) 429.
81 R. Grimm, H. Birrell, G. Ross, J. Charlwood and P. Camilleri, *Anal. Chem.*, 70 (1998) 3840.
82 K.A. Thomsson, N.G. Karlsson and G.C. Hansson, *J. Chromatogr. A*, 854 (1999) 131.
83 L. Johansson, H. Karlsson and K.-A. Karlsson, *J. Chromatogr. A*, 712 (1995) 149.
84 G.E. Black and A. Fox, *ACS Symp. Ser.*, 619 (1996) 81.
85 P. Lecchi and F.P. Abramson, *J. Chromatogr. A*, 828 (1998) 509.
86 A.J. Alpert, *J. Chromatogr.*, 499 (1990) 177.
87 S.C. Churms, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 121–163.
88 C.A. White, J.F. Kennedy and B.T. Golding, *Carbohydr. Res.*, 76 (1979) 1.
89 M. Petchey and M.J.C. Crabbe, *J. Chromatogr.*, 307 (1984) 180.
90 R. Oshima and J. Kumanotani, *J. Chromatogr.*, 265 (1983) 335.
91 N.K. Karamanos, T. Tsegenidis and C.A. Antonopoulos, *J. Chromatogr.*, 405 (1987) 221.
92 M. Takeda, M. Maeda and A. Tsuji, *J. Chromatogr.*, 244 (1982) 347.
93 N. Ikemoto, L.-C. Lo and K. Nakanishi, *Angew. Chem. Int. Ed. Engl.*, 31 (1992) 890.
94 R. Oshima, Y. Yamauchi and J. Kumanotani, *Carbohydr. Res.*, 107 (1982) 169.
95 E.G. Bremer, S.K. Gross and R.H. McLuer, *J. Lipid Res.*, 20 (1979) 1028.
96 S.K. Gross and R.H. McLuer, *Anal. Biochem.*, 102 (1980) 429.
97 M. Suzuki, T. Yamakawa and A. Suzuki, *J. Biochem. (Tokyo)*, 109 (1991) 503.
98 I. Takazono and Y. Tanaka, *J. Chromatogr.*, 288 (1984) 167.
99 S. Iwata, T. Narui, K. Takahashi and S. Shibata, *Carbohydr. Res.*, 133 (1984) 157.
100 Z.L. Nikolov and P.J. Reilly, *J. Chromatogr.*, 325 (1985) 287.
101 D.L. Hendrix, R.E. Lee, Jr., J.G. Baust and H. James, *J. Chromatogr.*, 210 (1981) 45.
102 J.G. Baust, R.E. Lee, Jr., R.R. Rojas, D.L. Hendrix, D. Friday and H. James, *J. Chromatogr.*, 261 (1983) 65.
103 M. Verzele, G. Simeons and F. van Damme, *Chromatographia*, 23 (1987) 292.
104 C.A. White, P.H. Corran and J.F. Kennedy, *Carbohydr. Res.*, 87 (1980) 165.
105 W. Praznik, R.H.F. Beck and E. Nitsch, *J. Chromatogr.*, 303 (1984) 417.
106 Y. Wei and M.-Y. Ding, *J. Chromatogr. A*, 904 (2000) 113.
107 Y. Wei and M.-Y. Ding, *J. Liq. Chromatogr.*, 25 (2002) 1769.
108 K. Koizumi, T. Utamura and Y. Okada, *J. Chromatogr.*, 321 (1985) 145.
109 K. Koizumi, Y. Okada, T. Utamura, M. Hisamatsu and A. Amemura, *J. Chromatogr.*, 299 (1984) 215.
110 M. Benincasa, G.P. Cartoni, F. Coccioli, R. Rizzo and L.P.T.M. Zevenhuizen, *J. Chromatogr.*, 393 (1987) 263.
111 S.-C. Lin and W.-C. Lee, *J. Chromatogr. A*, 803 (1998) 302.
112 W.M. Blanken, M.L.E. Bergh, P.L. Koppen and D.H. van den Eijnden, *Anal. Biochem.*, 145 (1985) 322.
113 V.K. Dua, V.E. Dube and C.A. Bush, *Biochim. Biophys. Acta*, 802 (1984) 29.
114 V.K. Dua, K. Goso, V.E. Dube and C.A. Bush, *J. Chromatogr.*, 328 (1985) 259.
115 G. Gazzotti, S. Sonnino and R. Ghidoni, *J. Chromatogr.*, 348 (1985) 371.
116 S. Ando, H. Waki and K. Kon, *J. Chromatogr.*, 408 (1987) 285.
117 B. Porsch, *J. Chromatogr.*, 253 (1982) 49.

118 G.M. Sapers, F.W. Douglas, Jr., M.A. Ziolkowski, R.L. Miller and K.B. Hicks, *J. Chromatogr.*, 503 (1990) 431.
119 Y.-A. Wei and J.-N. Fang, *J. Chromatogr.*, 513 (1990) 227.
120 K. Koizumi, T. Utamura, Y. Kubota and S. Hizukuri, *J. Chromatogr.*, 409 (1987) 396.
121 R.G. Bell and K.L. Norman, *J. Chromatogr.*, 632 (1993) 87.
122 D.J. Platzer, K.A. Mills, E.L. Ciolkowski and T. Ramstad, *J. Chromatogr. A*, 793 (1998) 57.
123 M. Shiota and S. Kobayashi, *Carbohydr. Res.*, 215 (1991) 209.
124 H. Akiyama, S. Shidawara, A. Mada, H. Toyoda, T. Toida and T. Imanari, *J. Chromatogr.*, 579 (1992) 203.
125 G. White, T. Katona, J.P. Zodda and M.N. Eakins, *J. Chromatogr.*, 625 (1992) 157.
126 M. Zebrower, F.J. Kieras and J. Heaney-Kieras, *Glycobiology*, 1 (1991) 271.
127 C. Brons and C. Olieman, *J. Chromatogr.*, 259 (1983) 79.
128 D.W. Armstrong and H.L. Jin, *J. Chromatogr.*, 462 (1989) 219.
129 P.J. Simms, R.M. Haines and K.B. Hicks, *J. Chromatogr.*, 648 (1993) 131.
130 P.J. Simms, A.T. Hotchkiss, Jr., P.L. Irwin and K.B. Hicks, *Carbohydr. Res.*, 278 (1995) 1.
131 A. Berthod, S.S.C. Chang, J.P.S. Kullman and D.W. Armstrong, *Talanta*, 47 (1998) 1001.
132 J. Feurle, H. Jomaa, M. Wilhelm, B. Gutsche and M. Herderich, *J. Chromatogr. A*, 803 (1998) 111.
133 Z. El Rassi, *J. Chromatogr. A*, 720 (1996) 93.
134 Z. El Rassi, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 41–102.
135 K. Koizumi, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 103–119.
136 K. Mopper and L. Johnson, *J. Chromatogr.*, 256 (1983) 27.
137 H. Takemoto, S. Hase and T. Ikenaka, *Anal. Biochem.*, 145 (1985) 245.
138 Y.C. Lee, B.I. Lee, N. Tomiya and N. Takahashi, *Anal. Biochem.*, 188 (1990) 259.
139 Z. El Rassi, D. Tedford, J. An and A. Mort, *Carbohydr. Res.*, 215 (1991) 25.
140 H. Kown and J. Kim, *Anal. Biochem.*, 215 (1993) 243.
141 K.R. Anumula and P. Du, *Anal. Biochem.*, 277 (1999) 236.
142 N. Jentoft, *Anal. Biochem.*, 148 (1985) 424.
143 D.J. Gisch and J.D. Pearson, *J. Chromatogr.*, 443 (1988) 299.
144 E.Y.J. Kang, R.D. Coleman, H.J. Pownall, A.M. Gotto, Jr. and C.Y. Yang, *J. Protein Chem.*, 9 (1990) 31.
145 M. Formato, A. Senes, F. Soccolini, R. Coinu and G.M. Cherchi, *Carbohydr. Res.*, 255 (1994) 27.
146 D. Velasco, J. Castells and F. Lopez-Calahorra, *J. Chromatogr.*, 519 (1990) 228.
147 S. Saadat and C.E. Ballou, *Carbohydr. Res.*, 119 (1983) 248.
148 N. Viseux, E. de Hoffmann and B. Domon, *Anal. Chem.*, 69 (1997) 3193.
149 A. Heyraud and M. Rinaudo, *J. Liq. Chromatogr.*, 3 (1980) 721.
150 N.W.H. Cheetham and P. Sirimanne, *J. Chromatogr.*, 208 (1981) 100.
151 W. Praznik and T. Spies, *Carbohydr. Res.*, 243 (1993) 91.
152 K. Koizumi, Y. Kubota, Y. Okada, T. Utamura, S. Hizukuri and J.-I. Abe, *J. Chromatogr.*, 437 (1988) 47.
153 S. Kobayashi, K. Nakashima and M. Arahira, *Carbohydr. Res.*, 192 (1989) 223.
154 P. Chaturvedi and C.B. Sharma, *Carbohydr. Res.*, 203 (1990) 91.
155 C. Capon, P. Cache, Y. Leroy, G. Strecker, J. Montreuil and B. Fournet, *J. Chromatogr.*, 425 (1988) 35.
156 A. Klein, C. Carnoy, G. Lamblin, P. Roussel, J.A. van Kuik, P. de Waard and J.F.G. Vliegenthart, *Eur. J. Biochem.*, 198 (1991) 151.

157 A.G.J. Voragen, H.A. Schols, J.A. de Vries and W. Pilnik, *J. Chromatogr.*, 244 (1982) 327.
158 L.E. Chun, T.J. Koob and D.R. Eyre, *Anal. Biochem.*, 171 (1988) 197.
159 N.K. Karamanos, P. Vanky, A. Syrokou and A. Hjerpe, *Anal. Biochem.*, 225 (1995) 220.
160 I. Koshiishi, M. Takenouchi, T. Hasegawa and T. Imanari, *Anal. Biochem.*, 265 (1998) 49.
161 S.K. Henderson and D.E. Henderson, *J. Chromatogr. Sci.*, 24 (1986) 198.
162 R. Lagunas and J.C. Diez-Masa, *Anal. Biochem.*, 216 (1994) 188.
163 K. Koizumi, Y. Okada and M. Fukuda, *Carbohydr. Res.*, 215 (1991) 67.
164 Y. Okada, K. Koizumi and S. Kitahata, *Carbohydr. Res.*, 254 (1994) 1.
165 M.J. Davies, K.D. Smith, A.-M. Harbin and E.F. Hounsell, *J. Chromatogr.*, 609 (1992) 125.
166 M.J. Davies, K.D. Smith, P.A. Carruthers, W. Chai, A.M. Lawson and E.F. Hounsell, *J. Chromatogr.*, 646 (1993) 317.
167 J.-Q. Fan, A. Kondo, I. Kato and Y.C. Lee, *Anal. Biochem.*, 219 (1994) 224.
168 K. Koizumi, *J. Chromatogr. A*, 720 (1996) 119.
169 J. Wang, P. Sporns and N.H. Low, *J. Agric. Food Chem.*, 47 (1999) 1549.
170 K.A. Thomsson, N.G. Karlsson and G.C. Hansson, *J. Chromatogr. A*, 854 (1999) 131.
171 N. Takahashi and F.W. Putnam, in K.M. Gooding and F.E. Regnier (Eds.), *HPLC of Biological Macromolecules*, Dekker, New York, 1990.
172 R.A. Wallace, O. Carnevali and T.G. Hollinger, *J. Chromatogr.*, 519 (1990) 75.
173 R.C. Grebenau, D.M. Golgenberg, C.-H. Chang, G.A. Koch, D.V. Gold, A. Kunz and H.J. Hansen, *Mol. Immun.*, 29 (1992) 751.
174 K. Murata and Y. Yokoyama, *J. Chromatogr.*, 415 (1987) 231.
175 S. Kitazume, K. Kitajima, S. Inoue and Y. Inoue, *Anal. Biochem.*, 202 (1992) 25.
176 A. Sugii and K. Harada, *J. Chromatogr.*, 544 (1991) 219.
177 T. Akiyama, *J. Chromatogr.*, 588 (1991) 53.
178 K. Koizumi and T. Utamura, *J. Chromatogr.*, 436 (1988) 328.
179 N. Tomiya, J. Awaya, M. Kurono, S. Endo, Y. Arata and N. Takahashi, *Anal. Biochem.*, 171 (1988) 73.
180 S. Hase, K. Ikenaka, K. Mikoshiba and T. Ikenaka, *J. Chromatogr.*, 434 (1988) 51.
181 H. Oku, S. Hase and T. Ikenaka, *Anal. Biochem.*, 185 (1990) 331.
182 N. Tomiya, Y.C. Lee, T. Yoshida, Y. Wada, J. Awaya, M. Kurono and N. Takahashi, *Anal. Biochem.*, 193 (1991) 90.
183 N. Takahashi, Y. Wada, J. Awaya, M. Kurono and N. Tomiya, *Anal. Biochem.*, 208 (1993) 96.
184 N. Kuraya and S. Hase, *Anal. Biochem.*, 233 (1996) 205.
185 K. Ohara, M. Sano, A. Kondo and I. Kato, *J. Chromatogr.*, 586 (1991) 35.
186 F. Matsuura, M. Ohta, K. Murakami, K. Hirano and C.C. Sweeley, *Biomed. Chromatogr.*, 6 (1992) 77.
187 H. Higashi, M. Ito, N. Fukaya, S. Yamagata and T. Yamagata, *Anal. Biochem.*, 186 (1990) 355.
188 N. Takahashi, *J. Chromatogr. A*, 720 (1996) 217.
189 N. Takahashi, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 347–385.
190 M. Stefansson and D. Westerlund, *J. Chromatogr. A*, 720 (1996) 127.
191 C.W. Huck, C.G. Huber and G.K. Bonn, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 165–205.
192 P. Gacesa, A. Squire and P.J. Winterburn, *Carbohydr. Res.*, 118 (1983) 1.
193 S.R. Delaney, M. Leger and H.E. Conrad, *Anal. Biochem.*, 106 (1980) 253.
194 S.R. Delaney, H.E. Conrad and J.H. Glaser, *Anal. Biochem.*, 108 (1980) 25.
195 P. Nebinger, M. Koel, A. Franz and E. Werries, *J. Chromatogr.*, 265 (1983) 19.

196 T. Gherezghiher, M.C. Kuss, R.E. Nordquist and C.P. Wilkinson, *J. Chromatogr.*, 413 (1987) 9.
197 J. Macek, J. Krajickova and M. Adam, *J. Chromatogr.*, 414 (1987) 156.
198 T. Imanari, H. Toyoda, S. Yamanashi, K. Shinomiya, I. Koshiishi and T. Oguma, *J. Chromatogr.*, 574 (1992) 142.
199 N.K. Karamanos, A. Syrokou, P. Vanky, M. Nurminen and A. Hjerpe, *Anal. Biochem.*, 221 (1994) 189.
200 J.U. Baenziger, *Methods Enzymol.*, 230 (1994) 237.
201 R. Wagener, B. Kobbe and W. Stoffel, *J. Lipid Res.*, 37 (1996) 1823.
202 S. Honda, M. Takahashi, K. Kakehi and S. Ganno, *Anal. Biochem.*, 113 (1981) 130.
203 R.A. Lazarus and J.L. Seymour, *Anal. Biochem.*, 157 (1986) 360.
204 A.K. Shukla, R. Schauer, F.M. Unger, U. Zahringer, E.T. Rietschel and H. Brade, *Carbohydr. Res.*, 140 (1985) 1.
205 G.R. Guile, S.Y.C. Wong and R.A. Dwek, *Anal. Biochem.*, 222 (1994) 231.
206 T. Masuda, Y. Nishimura, M. Tonegawa, K. Kitahara, S. Arai, J. Yamashita and N. Takai, *J. Chromatogr. A*, 845 (1999) 401.
207 T. Masuda, K. Kitahara, Y. Aikawa and S. Arai, *J. Chromatogr. A*, 961 (2002) 89.
208 N.O. Maness and A. Mort, *Anal. Biochem.*, 178 (1989) 248.
209 J. Müthing and F. Unland, *J. Chromatogr. B*, 658 (1994) 39.
210 Y. Zhang and Y.C. Lee, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 207–250.
211 Y.C. Lee, *Anal. Biochem.*, 189 (1990) 151.
212 R.R. Townsend and M.R. Hardy, *Glycobiology*, 1 (1991) 139.
213 R.R. Townsend, in Z. El Rassi (Ed.), *Carbohydrate Analysis: High Performance Chromatography and Capillary Electrophoresis*, Elsevier, Amsterdam, 1995, pp. 181–209.
214 Y.C. Lee, *J. Chromatogr. A*, 720 (1996) 137.
215 R.D. Rocklin and C.A. Pohl, *J. Liq. Chromatogr.*, 6 (1983) 1577.
216 J.D. Olechno, S.R. Carter, W.T. Edwards and D.S. Gillen, *Am. Biotechnol. Lab.*, 5(5) (1987) 38.
217 M.L. Salto, C. Gallo-Rodriguez, C. Lima and R.M. de Lederkremer, *Anal. Biochem.*, 279 (2000) 79.
218 J.S. Rohrer, J. Thayer, M. Weitzhandler and N. Avdalovic, *Glycobiology*, 8 (1998) 35.
219 G.A. Cooper and J.S. Rohrer, *Anal. Biochem.*, 226 (1995) 182.
220 R. Andersen and A. Sorensen, *J. Chromatogr. A*, 897 (2000) 195.
221 S. Ricci, A. Bardotti, S. D'Ascenzi and N. Ravenscroft, *Vaccine*, 19 (2001) 1989.
222 D.J. Lefeber, G.R. Gutierrez, C.H. Grun, D. Proietti, S. D'Ascenzi, P. Constantino, J.P. Kamerling and J.F. Vliegenthart, *Carbohydr. Res.*, 337 (2002) 819.
223 J.S. Rohrer, *J. Chromatogr.*, 667 (1994) 75.
224 S. Inoue, S.L. Lin, Y.C. Lee and Y. Inoue, *Glycobiology*, 11 (2001) 819.
225 S. Degroote, M.P. Ducourouble, P. Roussel and G. Lamblin, *Glycobiology*, 9 (1999) 1199.
226 K.-S. Wong and J.-L. Jane, *J. Liq. Chromatogr.*, 18 (1995) 63.
227 K.-S. Wong and J.-L. Jane, *J. Liq. Chromatogr. Related Technol.*, 20 (1997) 297.
228 Y. Zhang, Y. Inoue, S. Inoue and Y.C. Lee, *Anal. Biochem.*, 250 (1997) 245.
229 A.T. Hotchkiss, Jr. and K.B. Hicks, *Anal. Biochem.*, 184 (1990) 200.
230 M.D. Spiro, K.A. Kates, A.L. Koller, M.A. O'Neill, P. Albersheim and A.G. Darvill, *Carbohydr. Res.*, 247 (1993) 9.
231 S. Holmbeck and L. Lerner, *Carbohydr. Res.*, 239 (1993) 239.
232 T.R.I. Cataldi, C. Campa, G. Margiotta and S.A. Bufo, *Anal. Chem.*, 70 (1998) 3940.
233 T.R.I. Cataldi, C. Campa, M. Angelotti and S.A. Bufo, *J. Chromatogr. A*, 855 (1999) 539.

234 T.R.I. Cataldi, C. Campa and I.G. Casella, *J. Chromatogr. A*, 848 (1999) 71.
235 M.R. Hardy, R.R. Townsend and Y.C. Lee, *Anal. Biochem.*, 170 (1988) 54.
236 T.J. Pakasch, H.-P. Lieker, P.J. Reilly and K. Thielecke, *Carbohydr. Res.*, 215 (1991) 1.
237 T.R.I. Cataldi, G. Margiotta and C.G. Zambonin, *Food Chem.*, 62 (1998) 109.
238 T.R.I. Cataldi, C. Campa and G. de Benedetto, *Fresenius J. Anal. Chem.*, 368 (2000) 39.
239 V.P. Hanko and J.S. Rohrer, *Anal. Biochem.*, 283 (2000) 192.
240 D.A. Martens and W.T. Frankenberger, Jr., *J. Chromatogr.*, 546 (1991) 297.
241 A.J. Clarke, V. Sarabia, W. Keenleyside, P.R. MacLachlan and C. Whitfield, *Anal. Biochem.*, 199 (1991) 68.
242 J.S. Rohrer, *Anal. Biochem.*, 283 (2000) 3.
243 A.E. Manzi, S. Diaz and A. Varki, *Anal. Biochem.*, 188 (1990) 20.
244 T.J. Monica, S.B. Williams, C.F. Goochee and B.L. Maiorella, *Glycobiology*, 5 (1995) 175.
245 K. Koizumi, Y. Kubota, H. Ozaki, K. Shigenobu, M. Fukuda and T. Tanimoto, *J. Chromatogr.*, 595 (1992) 340.
246 H.P. Smits, A. Cohen, T. Buttler, J. Nielsen and L. Olsson, *Anal. Biochem.*, 261 (1998) 36.
247 S.M. de Bruijn, R.G.F. Visser and D. Vreugdenkil, *Phytochem. Anal.*, 10 (1999) 107.
248 N. Tomiya, E. Ailor, S.M. Lawrence, M.J. Betenbaugh and Y.C. Lee, *Anal. Biochem.*, 293 (2001) 129.
249 J. Heinrich and P. Mischnick, *J. Chromatogr. A*, 749 (1996) 41.
250 E.A. Kragten, J.P. Kamerling and J.F.G. Vliegenthart, *J. Chromatogr.*, 623 (1992) 49.
251 K. Koizumi, Y. Kubota, T. Tanimoto and Y. Okada, *J. Chromatogr.*, 464 (1989) 365.
252 K. Koizumi, M. Fukuda and S. Hizukuri, *J. Chromatogr.*, 585 (1991) 233.
253 K. Koizumi, Y. Kubota, T. Tanimoto and Y. Okada, *J. Chromatogr.*, 454 (1988) 303
254 L.H. Slaughter and D.P. Livingston, III, *Carbohydr. Res.*, 253 (1994) 287.
255 C. L'Homme, J.L. Peschet, A. Puigserver and A. Biagini, *J. Chromatogr. A*, 920 (2001) 291.
256 S. Farine, C. Versluis, P.J. Bonnici, A. Heck, J.L. Peschet, A. Puigserver and A. Biagini, *J. Chromatogr. A*, 920 (2001) 299.
257 A.T. Hotchkiss, Jr. and K.B. Hicks, *Carbohydr. Res.*, 247 (1993) 1.
258 H.-P. Lieker, K. Thielecke, K. Buchholz and P.J. Reilly, *Carbohydr. Res.*, 238 (1993) 307.
259 A.J. Mort, F. Qiu and N.O. Maness, *Carbohydr. Res.*, 247 (1993) 21.
260 A.T. Hotchkiss, S.L. Lecrinier and K.B. Hicks, *Carbohydr. Res.*, 334 (2001) 135.
261 S.L. Lin, Y. Inoue and S. Inoue, *Glycobiology*, 9 (1999) 807.
262 K.M. Whitham, J.L. Hadley, H.G. Morris, S.M. Andrew, I.A. Nieduszynski and G.M. Brown, *Glycobiology*, 9 (1999) 285.
263 R.M. Lauder, T.N. Huckerby and I.A. Nieduszynski, *Glycobiology*, 10 (2000) 393.
264 W.L. Chuang, H. McAllister and L. Rabenstein, *J. Chromatogr. A*, 932 (2001) 65.
265 M.R. Hardy and R.R. Townsend, *Proc. Natl Acad. Sci. USA*, 85 (1988) 3289.
266 R.R. Townsend, M.R. Hardy, O. Hindsgaul and Y.C. Lee, *Anal. Biochem.*, 174 (1988) 459.
267 M.R. Hardy and R.R. Townsend, *Carbohydr. Res.*, 188 (1989) 1.
268 R.R. Townsend, M.R. Hardy and Y.C. Lee, *Methods Enzymol.*, 179 (1989) 65.
269 W.T. Wang and D. Zopf, *Carbohydr. Res.*, 189 (1989) 1.
270 L.J. Basa and M.W. Spellman, *J. Chromatogr.*, 499 (1990) 205.
271 R.R. Townsend, P.H. Atkinson and R.B. Trimble, *Carbohydr. Res.*, 215 (1991) 211.
272 S. Honda, S. Suzuki, S. Zaiki and K. Kakehi, *J. Chromatogr.*, 523 (1990) 189.
273 G.P. Reddy and C.A. Bush, *Anal. Biochem.*, 198 (1991) 278.
274 P. Hermentin, R. Witzel, J.F.G. Vliegenthart, J.P. Kamerling, M. Nimtz and H.S. Conrad, *Anal. Biochem.*, 203 (1992) 281.
275 M.R. Hardy and R.R. Townsend, *Methods Enzymol.*, 230 (1994) 208.

276 J.S. Rohrer, *Glycobiology*, 5 (1995) 359.
277 C. Kunz, S. Rudloff, A. Hintelmann, G. Pohlentz and H. Egge, *J. Chromatogr. B*, 685 (1996) 211.
278 E. Landberg, A. Lundblad and P. Pahlsson, *J. Chromatogr. A*, 814 (1998) 97.
279 R.A.M. van der Hoeven, A.J.P. Hofte, U.R. Tjaden, J. van der Greef, N. Torto, L. Gorton, G. Marko-Varga and C. Bruggink, *Rapid Commun. Mass Spectrom.*, 12 (1998) 69.
280 R.A.M. van der Hoeven, U.R. Tjaden, J. van der Greef, W.H.M. van Casteren, H.A. Schols, A.G.J. Voragen and C. Bruggink, *J. Mass Spectrom.*, 33 (1998) 377.
281 N. Torto, A. Hofte, R. van der Hoeven, U. Tjaden, L. Gorton, G. Marko-Varga, C. Bruggink and J. van der Greef, *J. Mass Spectrom.*, 33 (1998) 334.
282 A. Hüber and W. Praznik, in C. Wu (Ed.), *Column Handbook for Size Exclusion Chromatography*, Academic Press, San Diego, 1999, pp. 459–497.
283 S.C. Churms, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 267–303.
284 J.F. Karkalas and R.F. Tester, *J. Cereal Sci.*, 15 (1992) 175.
285 T. Suortti and E. Pessa, *J. Chromatogr.*, 536 (1991) 251.
286 T. Suortti, *J. Chromatogr.*, 632 (1993) 105.
287 A. Hüber, *Biochem. Soc. Trans.*, 19 (1991) 505.
288 M.H. Ong, K. Jumel, P.F. Tokarczuk, J.M.V. Blanshard and S.E. Harding, *Carbohydr. Res.*, 260 (1994) 99.
289 G. Brigand, A. Denis, M. Grall and D. Lecacheux, *Carbohydr. Polym.*, 12 (1990) 61.
290 D. Lecacheux, R. Panaras, G. Brigand and G. Martin, *Carbohydr. Polym.*, 5 (1985) 423.
291 D. Slootmaekers, J.A.P.P. van Dijk, F.A. Varkevisser, C.J. Bloys van Treslong and H. Reynaers, *Biophys. Chem.*, 41 (1991) 51.
292 A. Martinsen, G. Skjak-Braek and O. Smidsrod, *Carbohydr. Polym.*, 15 (1991) 171.
293 C. Rochas and M. Lahaye, *Carbohydr. Polym.*, 10 (1989) 289.
294 D. Lecacheux, Y. Mustiere, R. Panaras and G. Brigand, *Carbohydr. Polym.*, 6 (1986) 477.
295 K. Jumel, P. Browne and J.F. Kennedy, *Biochem. Soc. Trans.*, 19 (1991) 486.
296 H. Komatsu, K. Yoshii, S. Ishimitsu, S. Okada and T. Takahata, *J. Chromatogr.*, 644 (1993) 17.
297 R.G. Beri, J. Walker, E.T. Reese and J. Rollings, *Carbohydr. Res.*, 238 (1993) 11.
298 W.S. Bahary, M.P. Hogan, M. Jilani and M.P. Aronson, *Adv. Chem.*, 247 (1995) 151.
299 W.S. Bahary and M.P. Hogan, *Int. J. Polym. Anal. Charact.*, 2 (1996) 121.
300 S. You, M. Fiedorowicz and S.-T. Lim, *Cereal Chem.*, 76 (1999) 116.
301 D.R. White, Jr., *ACS Symp. Ser.*, 73 (1999) 299.
302 G.A. Morris, T.J. Foster and S.E. Harding, *Food Hydrocoll.*, 14 (2000) 227.
303 K. Jumel, I. Fiebrig and S.E. Harding, *Int. J. Biol. Macromol.*, 18 (1996) 133.
304 K. Jumel, F.J.J. Fogg, D.A. Hutton, J.P. Pearson, A. Allen and S.E. Harding, *Eur. Biophys. J.*, 25 (1997) 477.
305 K. Jumel, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 305–345.
306 M.L. Fishman, D.T. Gillespie, S.M. Sondey and R.A. Barford, *J. Agric. Food Chem.*, 37 (1989) 584.
307 M.L. Fishman, D.T. Gillespie, S.M. Sondey and Y.S. El-Atawy, *Carbohydr. Res.*, 215 (1991) 91.
308 P.D. Hoagland, M.L. Fishman, C. Konya and E. Clauss, *J. Agric. Food Chem.*, 41 (1993) 1274.
309 A. Müller, H.A. Pretus, R.B. McNamee, E.L. Jones, I.W. Browder and D.L. Williams, *J. Chromatogr. B*, 666 (1995) 283.
310 B. Yeung and D. Marecak, *J. Chromatogr. A*, 852 (1999) 573.

311 T. Kuge, K. Kobayashi, H. Tanahashi, T. Igushi and S. Kitamura, *Agric. Biol. Chem.*, 48 (1984) 2375.
312 M.L. Fishman, W. Damert, J.C. Phillips and R.A. Barford, *Carbohydr. Res.*, 160 (1987) 215.
313 G. Sworn, W.M. Marrs and R.J. Hart, *J. Chromatogr.*, 403 (1987) 307.
314 C. Yomota, T. Miyazaki and S. Okada, *Colloid Polym. Sci.*, 271 (1993) 76.
315 M. Terbojevich, A. Cosani, M. Palumbo and F. Pregnolatao, *Carbohydr. Res.*, 157 (1986) 269.
316 T. Malfait, D. Slootmaekers and F. van Cauwelaert, *J. Appl. Polym. Sci.*, 39 (1990) 571.
317 E. Ovrisky, L. Soltes, P. Chabrecek, I. Novak, V. Kery, M. Stancikova and I. Vins, *J. Liq. Chromatogr.*, 15 (1992) 3203.
318 H. Komatsu, T. Takahata, M. Tanaka, S. Ishimitsu and S. Okada, *Biol. Pharm. Bull.*, 16 (1993) 1189.
319 B. Mulloy, C. Gee, S.F. Wheeler, R. Wait, E. Gray and T.W. Barrowcliffe, *Thromb. Haemostasis*, 77 (1997) 668.
320 P.L. Dubin and J.M. Principi, *Macromolecules*, 22 (1989) 1891.
321 P.L. Dubin, J.I. Kaplan, B.-S. Tian and M. Mehta, *J. Chromatogr.*, 515 (1990) 37.
322 M. Hasegawa, A. Isogai and F. Onabe, *J. Chromatogr.*, 635 (1993) 334.
323 H.-J. Gabius, *Angew. Chem. Int. Ed. Engl.*, 27 (1988) 1267.
324 T. Osawa, *Pure Appl. Chem.*, 61 (1989) 1283.
325 A. Kobata and T. Endo, *J. Chromatogr.*, 597 (1992) 111.
326 R.D. Cummings, *Methods Enzymol.*, 230 (1994) 66.
327 T. Endo, *J. Chromatogr. A*, 720 (1996) 251.
328 T. Endo, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 251–265.
329 D.C. Schriemer, D.R. Bundle, L. Li and O. Hindsgaul, *Angew. Chem. Int. Ed. Engl.*, 37 (1998) 3383.
330 H. Hirabayashi, Y. Arata and K. Kasai, *J. Chromatogr. A*, 890 (2000) 261.
331 S. Honda, S. Suzuki, T. Nitta and K. Kakehi, *J. Chromatogr.*, 438 (1988) 73.
332 S. Honda, in Z. El Rassi (Ed.), *Carbohydrate Analysis: High Performance Liquid Chromatography and Capillary Electrophoresis*, Elsevier, Amsterdam, 1995, pp. 211–231.
333 A.J. Muller and P.W. Carr, *J. Chromatogr.*, 357 (1986) 11.
334 A.F. Bergold and P.W. Carr, *Anal. Chem.*, 61 (1989) 1117.
335 S. Ohlson, A. Lundblad and D. Zopf, *Anal. Biochem.*, 169 (1988) 204.
336 D. Zopf, S. Ohlson, J. Dakour, W. Wang and A. Lundblad, *Methods Enzymol.*, 179 (1989) 55.
337 M. Bergstrom, A. Lundblad, P. Pahlsson and S. Ohlson, *J. Mol. Recognit.*, 11 (1998) 110.
338 A. Fox, *J. Chromatogr. A*, 843 (1999) 287.
339 A. Fox, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 829–843.
340 I. Molnār-Perl, *J. Chromatogr. A*, 845 (1999) 181.
341 I. Molnār-Perl, *J. Chromatogr. A*, 891 (2000) 1.
342 J. Bleton, P. Mejanelle, J. Sansoulet, S. Goursaud and A. Tchapla, *J. Chromatogr. A*, 720 (1996) 27.
343 P. Mejanelle, J. Bleton, A. Tchapla and S. Goursaud, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 845–902.
344 P. Steinberg and A. Fox, *Anal. Chem.*, 71 (1999) 1914.
345 Z.F. Katona, P. Sass and I. Molnār-Perl, *J. Chromatogr. A*, 847 (1999) 91.
346 I. Molnār-Perl and K. Horvath, *Chromatographia*, 45 (1997) 321.
347 P.A. Biondi, F. Manca, A. Negri, C. Secchi and M. Montana, *J. Chromatogr.*, 411 (1987) 275.

348 P.A. Biondi, L.M. Chesa, C. Mariani and P. Renon, *J. Chromatogr. A*, 726 (1996) 246.
349 M.P. Kozar and A. Fox, *J. Chromatogr. A*, 946 (2002) 229.
350 Y.W. Ha and R.L. Thomas, *J. Food Sci.*, 53 (1988) 574.
351 T. Doco, M.A. O'Neill and P. Pellerin, *Carbohydr. Polym.*, 46 (2001) 249.
352 J.-P. Zanetta, P. Timmerman and Y. Leroy, *Glycobiology*, 9 (1999) 255.
353 J.-P. Zanetta, P. Timmerman and Y. Leroy, *Glycoconjugate J.*, 16 (1999) 617.
354 J.-P. Zanetta, A. Pons, M. Iwersen, C. Mariller, Y. Leroy, P. Timmerman and R. Schauer, *Glycobiology*, 11 (2001) 663.
355 K. Leontein and J. Lönngren, in J.N. BeMiller, R.L. Whistler and D.H. Shaw (Eds.), *Methods in Carbohydrate Chemistry*, Vol. 9, Wiley-Interscience, New York, 1993, pp. 87–89.
356 W.A. König, S. Lutz and G. Wenz, *Angew. Chem. Int. Ed. Engl.*, 27 (1988) 979.
357 W.A. König, S. Lutz, P. Mischnick-Lübbecke, B. Brassat and G. Wenz, *J. Chromatogr.*, 447 (1988) 193.
358 W.A. König, P. Mischnick-Lübbecke, B. Brassat, S. Lutz and G. Wenz, *Carbohydr. Res.*, 183 (1988) 11.
359 W.A. König, *Carbohydr. Res.*, 192 (1989) 51.
360 D.W. Armstrong, W. Li, A.M. Stalcup, H.V. Secor, R.R. Izac and J.I. Seeman, *Anal. Chim. Acta*, 234 (1990) 365.
361 J. Heinrich, W.A. König, H. Bretting and P. Mischnick, *Carbohydr. Res.*, 299 (1997) 1.
362 A. Fox, L. Wright and K. Fox, *J. Microbiol. Methods*, 22 (1995) 11.
363 A. Fox, M. Krahmer and D. Harrison, *J. Microbiol. Methods*, 27 (1996) 129.
364 G.E. Black and A. Fox, *J. Chromatogr. A*, 720 (1996) 51.
365 M. Shahgholi, S. Ohorodnik, J. Callahan and A. Fox, *Anal. Chem.*, 69 (1997) 1956.
366 I. Faengmark, A. Jansson and B. Nilsson, *Anal. Chem.*, 71 (1999) 1105.
367 L.W. Doner, *Methods Enzymol.*, 160 (1988) 176.
368 S. Ando, H. Waki and K. Kon, *J. Chromatogr.*, 405 (1987) 125.
369 J. Müthing, *J. Chromatogr. A*, 720 (1996) 3.
370 J. Müthing, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 423–482.
371 R.L. Schnaar and L.K. Needham, *Methods Enzymol.*, 230 (1994) 371.
372 P. Davidsson, P. Fredman, J.E. Mansson and L. Svennerholm, *Clin. Chim. Acta*, 197 (1991) 105.
373 J. Müthing and U. Neumann, *Biomed. Chromatogr.*, 7 (1993) 158.
374 L. Johansson, P. Johansson and H. Miller-Podraza, *Anal. Biochem.*, 267 (1999) 239.
375 G. Zimmer, G. Reuter and R. Schauer, *Eur. J. Biochem.*, 204 (1992) 209.
376 G.W. Somsen, W. Morden and I.D. Wilson, *J. Chromatogr.*, 703 (1995) 613.
377 T. Taki, D. Ishikawa, S. Handa and T. Kasama, *Anal. Biochem.*, 225 (1995) 24.
378 J. Guittard, X.L. Hronowski and C.E. Costello, *Rapid Commun. Mass Spectrom.*, 13 (1999) 1838.
379 A.M. Lawson, E.F. Hounsell, M.S. Stoll, J. Feeny, W. Chai, J.R. Rosankiewicz and T. Feizi, *Carbohydr. Res.*, 221 (1991) 191.
380 D. Schindler, T. Kanzaki and R.J. Desnick, *Clin. Chim. Acta*, 190 (1990) 81.
381 J. Müthing, *J. Chromatogr. B*, 657 (1994) 75.
382 J. Müthing and H. Ziehr, *J. Chromatogr. B*, 687 (1996) 357.
383 C.M. Starr, I. Masada, C. Hague, E. Skop and J.C. Klock, *J. Chromatogr. A*, 720 (1996) 295.
384 C. Hague, I. Masada and B.K. Brandley, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 787–798.
385 Z. El Rassi, *Adv. Chromatogr.*, 34 (1994) 177.

386 Z. El Rassi and W. Nashabeh, in Z. El Rassi (Ed.), *Carbohydrate Analysis: High Performance Liquid Chromatography and Capillary Electrophoresis*, Elsevier, Amsterdam, 1995, pp. 267–360.
387 Z. El Rassi, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 597–676.
388 A. Paulus and A. Klockow, *J. Chromatogr. A*, 720 (1996) 353.
389 R.J. Linhardt and A. Pervin, *J. Chromatogr. A*, 720 (1996) 323.
390 S. Honda, *J. Chromatogr. A*, 720 (1996) 337.
391 S. Honda and A. Taga, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 677–690.
392 K. Kakehi and S. Honda, *J. Chromatogr. A*, 720 (1996) 377.
393 N.T. Tran, M. Cabanes-Macheteau and M. Taverna, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 691–785.
394 N.K. Karamanos and A. Hjerpe, in Z. El Rassi (Ed.), *Carbohydrate Analysis by Modern Chromatography and Electrophoresis*, Elsevier, Amsterdam, 2002, pp. 799–826.
395 L.R. Zieske, F. Daotian, S.H. Khan and R.A. O'Neill, *J. Chromatogr. A*, 720 (1996) 395.
396 J.F. Kelley, S.J. Locke, L. Ramaley and P. Thibault, *J. Chromatogr. A*, 720 (1996) 409.
397 S. Duteil, P. Gareil, S. Girault, A. Mallet, C. Feve and L. Siret, *Rapid Commun. Mass Spectrom.*, 13 (1999) 1898.
398 A.D. Hooker and D.C. James, *Mol. Biotechnol.*, 14 (2000) 241.
399 M. Larsson, R. Sundberg and S. Folestad, *J. Chromatogr. A*, 934 (2001) 75.
400 M. Militsopoulou, F.N. Lamari, A. Hjerpe and N.K. Karamanos, *Electrophoresis*, 23 (2002) 1104.
401 L.A. Gennaro, J. Delaney, P. Vouros, D.J. Harvey and B. Domon, *Rapid Commun. Mass Spectrom.*, 16 (2002) 192.
402 S.L. Wu, *Anal. Biochem.*, 253 (1997) 85.

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 19

Nucleic acids and their constituents

YOSHINOBU BABA and LIHUA ZHANG

CONTENTS

19.1 INTRODUCTION

Nucleic acids provide essential functions in all living organisms. The most vital functions are the storage and transmission of genetic information and the use of this information in the synthesis of proteins. Considerable advances in nucleic acid technology have generated an enormous amount of genetic information [1]. One of the most exciting research projects is the almost completed sequencing of human genome in early 2001 [2,3]. The importance of nucleic acids in all areas of biological sciences calls for the rapid development of effective techniques for their isolation, separation, quantitation, and structural analysis [4]. In this chapter, based on a brief introduction on the structure of nucleic acids and how to prepare nucleic acid samples, we will focus on the subsequent analysis of nucleic acids and their constituents by appropriate chromatographic and electrophoretic methods, which have greatly contributed to the tremendous achievements obtained in the Human Genomic Project (HGP).

19.2 STRUCTURE OF NUCLEIC ACIDS

Nucleic acids are generally long-chain polymers, consisting of phosphoric acid, a pentose, and purine and pyrimidine bases. There are two kinds of nucleic acids, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). The main sugar component of DNA is β-D-2-deoxyribose in the D-furanose form. In RNA this sugar is replaced by β-D-ribose (Fig. 19.1). This apparently small difference between the two types of nucleic acids

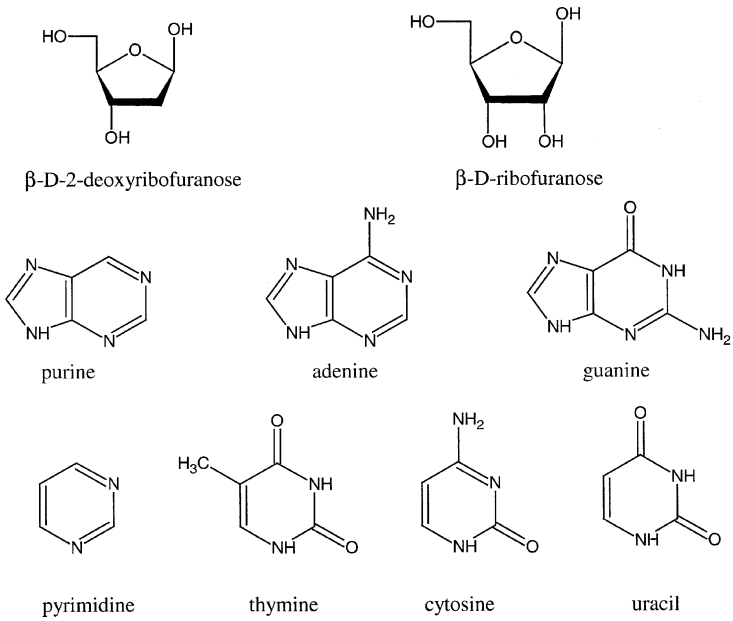
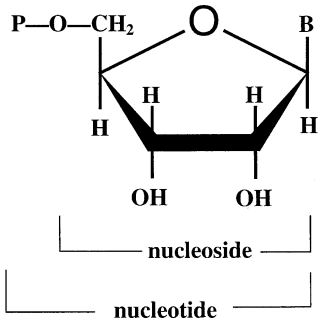


Fig. 19.1. Structure of pentose and bases in DNA and RNA.

has wide-ranging effects on both their chemistry and structure, since the presence of the bulky hydroxyl group on the 2′-position of the sugar not only limits the range of possible secondary structures available to the RNA molecule, but also makes it more susceptible to chemical and enzymatic degradation. Another difference in the composition between DNA and RNA is that DNA has two purines, adenine (A) and guanine (G), and two pyrimidines, cytosine (C) and thymine (T), whereas RNA contains the same bases except that uracil (U) replaces thymine (Fig. 19.1).

The monomeric unit of nucleic acids is called nucleotide (Fig. 19.2). In each unit, the pentose is joined to the base by a β-*N*-glycosyl bond between carbon atom 1′ of the pentose and nitrogen atom 9 of the purine base, or nitrogen atom 1 of the pyrimidine base. The phosphate group is in ester linkage with carbon atom 5′ of the sugar, forming a 5′-monophosphate, 5′-diphosphate, or 5′-triphosphate. When the phosphate group is completely removed from the nucleotide by hydrolysis, the structure remaining is called a nucleoside (Fig. 19.2). The connection between successive monomer units in nucleic acids is through a phosphate residue, attached to the hydroxyl on the 5′ carbon of one unit and the 3′ hydroxyl of the next one; thus, a phosphodiester link is formed between the two sugar residues which make up the backbone of the molecule (Fig. 19.3).



B: purine or pyrimidine base; P: 1,2 or 3 phosphate groups

Fig. 19.2. Structure of the nucleoside and nucleotide.

Both DNA and RNA can form a secondary structure through the interaction of hydrogen bonds. In 1953, Watson and Crick advanced the view that the DNA molecule is double-stranded and in the form of a right-handed helix [5,6]. In double-stranded DNA, a double-helical structure secludes the hydrophobic bases inside the double helix, whereas the two helical sugar-phosphate chains spiral down the outside of the double-stranded structure, presenting a polyanionic and hydrophilic structure to the solvent. The DNA double helix is stabilized through specific complementary pairs of nucleotides, where A and T are bridged by two hydrogen bonds and G and C by three. Although RNA molecules do not possess the regular inter-strand hydrogen-bond structure characteristic of DNA, they have the capacity to form double-helix regions between two separate RNA chains, but more frequently between two segments of the same chain, folded back on itself. The helical regions formed in this manner do not have entirely complementary sequences, so non-bonded residues loop out of the structure. For nucleic acid research, the studies of

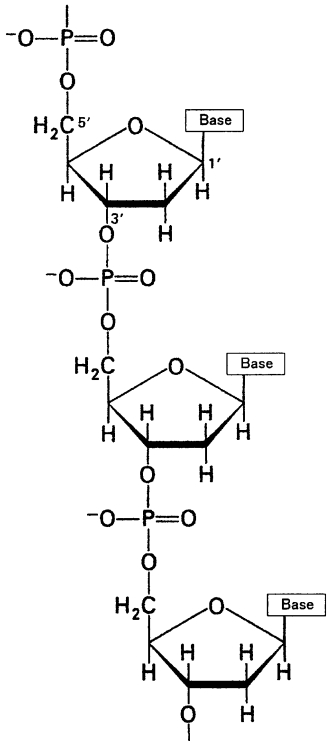


Fig. 19.3. Structure of part of a DNA chain.

bases, nucleosides, nucleotides, and polynucleotides by appropriate methods are all very important. However, before each analysis, sample preparation is an indispensable procedure to eliminate interfering compounds without greatly diluting the sample or altering the components of analytical interest.

19.3 SAMPLE PREPARATION

Methods of sample preparation of nucleic acids may be classified into two groups, purification of crude synthetic oligonucleotides, especially DNA oligonucleotides, and purification of nucleic acids from biological materials.

19.3.1 Purification of crude synthetic oligonucleotides

Most modern nucleic acid technologies, such as DNA sequencing, quantification, clinical analysis, and DNA genotyping, are based on the polymerase chain reaction (PCR), which was first described by Panet and Khorana in 1974 [7], and owes its name to Mullis and co-workers [8]. PCR uses short, single-stranded DNA oligonucleotides (primers) to amplify an interrogated region of double-stranded DNA. Accordingly, the purity of the

primers has great effects on the amplification efficiency and selectivity. With the introduction of DNA synthesizers, the synthesis of DNA primers now is an inexpensive and routine operation. Although oligonucleotide synthesis is automated and relatively fast, the purification generally requires multiple steps. The commonly used methods are high-performance liquid chromatography (HPLC), slab gel electrophoresis, and solid-phase extraction (SPE). The former two methods will be further discussed in Secs. 19.4 and 19.5. After purification, the oligonucleotides, purified by liquid chromatography, are recovered from the eluent manually or automatically. The extraction of nucleic acid fragments from gels is more complex [9]. SPE, making use of the adsorption/distribution of analytes between a mobile and a stationary phase, has gained wide acceptance in sample preparation, due to the ease of automation, high analyte recovery, extraction reproducibility, ability to increase the analyte concentration selectively, and the commercial availability of SPE devices and sorbents (Chap. 12) [10]. It can be successfully used in the "trityl-on" method of oligonucleotide purification. "Trityl-on" purification takes advantage of the hydrophobicity of the dimethoxytrityl (DMT) protecting group from the DNA synthesis. When in the last step of DNA synthesis the DMT is not cleaved from the full-length oligonucleotide, this leads to greater retention of the target product on reversed-phase sorbents. Lo *et al*. [11] have utilized this method for the selective extraction of target oligonucleotides on SPE cartridges. Recently, Gilar *et al*. [12] have developed high-throughput purification methods for synthetic oligonucleotides, using 96-well Oasis HLP extraction plates, which combine excellent pH stability with a high load capacity, thus allowing automatic single-step purification in a 0.2 μmol/L-scale synthesis. The purity of target products is typically *ca*. 90% or higher.

19.3.2 Purification of nucleic acids from biological materials

Preparation of biological samples for nucleic acid analysis is relatively complex. Examples of specimen types processed include: whole blood, serum, saliva, urine, stool, cells, spinal fluid, and tissues from biopsies. Although each specimen type has different sample preparation requirements, the purification procedure generally includes the following steps: cell separation/concentration, cell disruption, extraction and purification of nucleic acids, and amplification.

19.3.2.1 Cell separation/concentration

Some applications of sample preparation will require cell separation to reduce the complexity of the original sample. Microfiltration is one technique that exploits the size differences among cell types. Using conventional computer-controlled machining, different designs and sizes of filters have been fabricated, mainly for the isolation of white cells from whole blood [13,14]. Recently, Yuen *et al.* [15] demonstrated that a weir-type filter with a 3.5-μm gap could isolate white cells with $>99.9\%$ elimination of red blood cells. Di-electrophoresis (DEP), which utilizes the interactions between the intrinsic dielectric properties of cells and the applied AC electric field, is an active separation approach, since different types of cells have different responses to the applied electric

field. Therefore, by selecting the appropriate frequency of electric field, different types of cells can be separated [16–19]. In addition, hydrodynamic flow [20] and electro-osmotic flow [21] have also been used to transport and sort cells on microchips. Laser capture micro-dissection (LCM) is a new technique for obtaining pure populations of cells from heterogeneous tissues [22]. This technique has been extensively used in combination with mutation detection studies and analyses of gene expression at the messenger RNA (mRNA) level [23].

When the sample is complex and the target copy number is low, enrichment and sometimes purification of the target cells are necessary. Fluorescence-activated cell sorting (FACS) [24] and magnetic-activated cell sorting (MACS) [25] have been employed for the enrichment of rare fetal cells in blood. Other, less complex specimen types may require only a concentration step. Urine, saliva, serum, or plasma generally fall into this category when one is assaying for the presence of micro-organisms, or for the low levels of DNA or cells that have been reported to be present in these fluids [26]. The concentration required varies with specimen type and target species. The isolation and analysis of tumor DNA in plasma and serum has been reviewed by Jen *et al.* [27].

19.3.2.2 Cell disruption

After isolating the desired cells from the raw samples, the cells must be lysed or disrupted to release the nucleic acids. Many mechanical and non-mechanical methods have been developed for cell disruption. In mechanical disruption, the cell is physically broken and releases all intracellular components. Several kinds of equipment for this purpose are commercially available, such as high-pressure homogenizers, bead mills, and high-frequency ultrasonication devices. Mechanical disruption methods have several deficiencies [28]. Because cells are broken completely, all intracellular materials are released, and this makes the separation of DNA from a complex mixture of proteins and membrane fragments difficult.

Nonmechanical lysis methods include chemical, enzymatic, osmotic, thermal, and electric methods. Chemical methods extract intracellular components from micro-organisms by making the outer cell wall permeable with organic solvents that create channels through the cell membrane. A variety of enzymes specific for cell-wall components have often been used in the lysis procedure, which is generally followed by addition of detergent and then heating [29]. The osmotic method for cell disruption is based on the rapid changes in external osmolarity of cells. Repeated freezing and thawing or elevated temperature can also lyse mammalian cells [30]. Furthermore, cells can also be lysed electrically on microelectrode arrays after pulsed high-electric-field treatment [31].

19.3.2.3 Purification of nucleic acids

A variety of approaches have been adopted for the purification of nucleic acids after lysis. Traditionally, DNA and RNA are purified by digestion of proteins with proteinase K in the presence of detergents, extraction with phenol/chloroform, and concentration by precipitation with alcohol in the presence of salts [32]. In addition, organic solvents,

inorganic salts, or strong acids have also been used to precipitate proteins [33,34]. DNA and RNA have often been purified with chaotropic reagents, such as guanidinium hydrochloride or guanidinium thiocyanate. This purification procedure takes advantages of the observation that in the presence of chaotropic reagents, DNA and RNA bind to silica. Non-nucleic acid components of the cell lysates can be washed away, and the purified nucleic acid can be eluted later [35]. In addition, SPE has also been used in the isolation of nucleic acids from biological materials [36–38]. Besides the traditional reversed-phase, ion-exchange, and mixed-mode sorbent, selective sequence-specific hybridization of DNA strains has been used for the construction of affinity-like DNA clean-up and enrichment devices. Immobilized DNA probes [39,40], morpholino-backbone-modified oligonucleotides [41], and peptide nucleic acid oligonucleotides [42–44] have been utilized for the capture of sequence-specific DNA or RNA strands.

19.3.2.4 Amplification

The last step of sample preparation is the amplification of low copy numbers of target nucleic acids, so that they can subsequently be analyzed or detected. The most widely used amplification method is PCR, which is capable of amplifying as few as 20 copies of a target to over hundreds of millions of copies through a temperature-variant procedure. Ligase chain reaction (LCR) is another amplification method that requires a similar thermal cycling procedure [45]. Furthermore, some isothermal amplification methods have also been proposed, *e.g.*, the strand displacement amplification (SDA) [46], the transcription-mediated assays [47], and loop-mediated isothermal amplification [48].

19.3.2.5 Integrated systems

Whether in laboratory manipulation or in clinical diagnostics, it is most useful to integrate the above-mentioned steps in one instrument, not only to avoid contamination and loss of target molecules, but also to shorten the time spent on the whole procedure. In the past decade, the micro-electromechanical system (MEMS) has proved to be a promising approach for the development of automated sample preparation, with the principal advantages of

(a) small instrument footprint,
(b) multiplexing so that several analyses can be completed simultaneously,
(c) microfluidics for fluid manipulation, lessening the need for traditional macro-pumps,
(d) potential for integrating various functional components in the device,
(e) reduced volume requirements for sample and reagents, and
(f) unsupervised automation [28].

Up until now, several reviews on microfabricated devices (Chap. 11) for genetic diagnostics have been published to record the advances in this field [28,49–51]. Some of the proposed designs could be regarded as masterpieces, and the design shown in Fig. 19.4 is one example [52]. Such a disposable disk consists of chambers and channels, which form the sample and reagent storage reservoirs, fluid-controlling valves, fluid-mixing

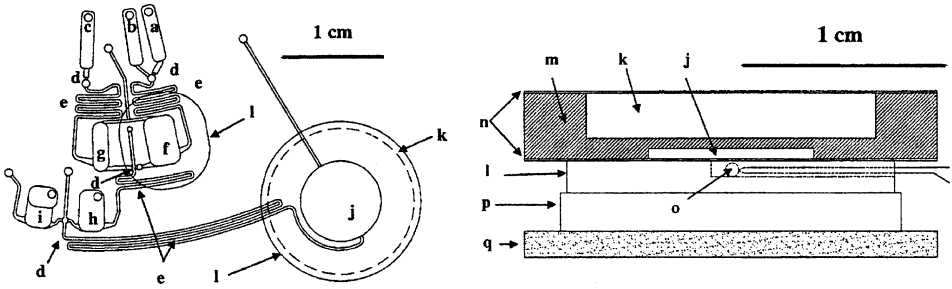


Fig. 19.4. LabCD™ for PCR (Tecan). Left: Top view of one area of the LabCD. The center of the disc would be in the upper left and the outer edge at the bottom. The elements are: (a) sample, (b) NaOH for lysing cells, (c) Tris–HCl for neutralizing the lysate, (d) capillary valves, (e) mixing channels, (f) lysis chamber, (g) Tris–HCl holding chamber, (h) neutralized-lysate holding chamber, (i) PCR reagents, (j) thermal-cycling chamber, and (k) air gap. Fluids loaded in (a), (b), and (c) are driven at a first RPM into reservoirs (g) and (f), at which time (g) is heated to 95 °C. The RPM is increased and the fluids are driven into (h). The RPM is increased and fluids in (h) and (i) flow into (j). Right: The cross-section shows the disc body (m), air gap (k), sealing layers (n), heat sink (l), thermoelectric (p), PC-board (q), and thermistor (o). (Reproduced from Ref. 52 with permission.)

chambers, and heating chambers. By rotating the disk at different speeds, cells and reagents can be sequentially moved to different compartments to accomplish most sample preparation steps from cell lysis to DNA purification and amplification. In addition, the throughput may be augmented by placing multiple structures of the design on one disk. Some manufactures, such as Nanogen and Cepheid, have even worked on developing new MEMS for the clinical laboratory or bedside setting.

Under some circumstances, desalting of samples is necessary prior to analysis, especially mass-spectrometric analysis. Up until now, several methods have been reported, including dialysis, ultrafiltration, size exclusion, affinity purification, and SPE [10,53]. The nucleic acids obtained by the above-mentioned procedures can be analyzed by chromatography or electrophoresis, two methods that have been used not only in genomics, but also for the determination of free nucleotides, nucleosides, and their bases [54].

19.4 CHROMATOGRAPHIC METHODS

By virtue of its high resolving capability, short analysis time, advanced instrumentation, and preparative separation capacity, chromatography has proved to be an excellent technique for the separation and quantitation of nucleic acids. Initial work on the chromatographic separation of nucleic acids and their constituents is credited to Cohn [55]. He used ion-exchange chromatography, and with this technology he separated nucleotides quickly and efficiently. In the middle of the 1960s, Horváth *et al.* [56,57] reported the analysis of nucleotides, nucleosides, and their bases by HPLC, and Brown *et al.* [58,59] adapted these analyses for use in biological matrices. Subsequently, with the significant improvements in column packings [60], instrument design, and the number and

kind of detectors commercially available, HPLC has become an important method for nucleic acid research.

Up until now, six major chromatographic modes have been utilized: ion-exchange HPLC, reversed-phase HPLC, ion-pair reversed-phase HPLC, mixed-mode HPLC, size-exclusion chromatography, and affinity chromatography. The choice of the appropriate mode depends on the properties of samples. In dealing with nucleic acid and their constituents, a rule-of-thumb is that, if the compounds are ionic (nucleic acids, oligonucleotides, nucleotides), ion exchange is generally used. However, the reversed-phase mode can also be adopted for ionic compounds, if techniques, such as ion pairing or ion suppression are employed. With non-ionic compounds, such as purines, pyrimidines, and their nucleosides, the reversed-phase mode gives the best separations. Size-exclusion chromatography is generally used to separate compounds by class, if they differ greatly in molecular weight (nucleic acids, oligonucleotides, nucleotides), or in the separation of various types of nucleic acids differing in size. Affinity chromatography is utilized for the separation of specific nucleic acids with specifically designed stationary phases. In this section, our focus will be on the first three chromatographic modes.

19.4.1 Ion-exchange chromatography

Ion-exchange chromatography was the earliest chromatographic methods used for the analysis of nucleic acids and their constituents [55]. Although both anion and cation exchange were used to separated the purines and pyrimidines or individual members of the nucleotide group [61–63], anion-exchange chromatography is now most commonly used to analyze nucleic acids and their constituents with anionic sites. Virtually all anion-exchange functional groups on the stationary phase are based on amino groups, where the degree of alkylation of the nitrogen determines the pH range over which the functional group is positively charged. Secondary and tertiary amino groups, diethylaminoethyl (DEAE) groups, *e.g.*, serve as weak anion-exchange functionalities with a practical operating pH range between 6 and 9. Quaternary ammonium groups, such as trimethlyammonium (TMA), are another kind of functionality that ensures a constant charge density, independent of the eluent pH. In anion-exchange chromatography of nucleic acids, the mobile phase is a buffer solution, containing a strong electrolyte as eluting salt, and a linear gradient of increasing salt concentration is usually applied for elution.

The predominant attractive force in anion-exchange chromatography is the electrostatic interaction between two charged surfaces in contact with a buffered salt solution [64]. Accordingly, the retention behavior of nucleotides is predicable, the monophosphate nucleotides being eluted first, followed by the diphosphate, and then triphosphate nucleotides (Fig. 19.5) [63]. Nucleotide separations within each group, *e.g.*, the mono-, di- or triphosphate nucleotides, are dependent on the base. The base order for each group is: cytidine, uridine, thymine, adenosine, and guanosine. As far as the separation of nucleic acids is concerned, since the charge densities of DNA and RNA are constant, anion-exchange separation is primarily based on chain-length (Fig. 19.6) [65]. However, under some circumstances, the separation of double-stranded DNA fragments shows deviations or inversions in retention time as a function of chain-length [66–69], which may be

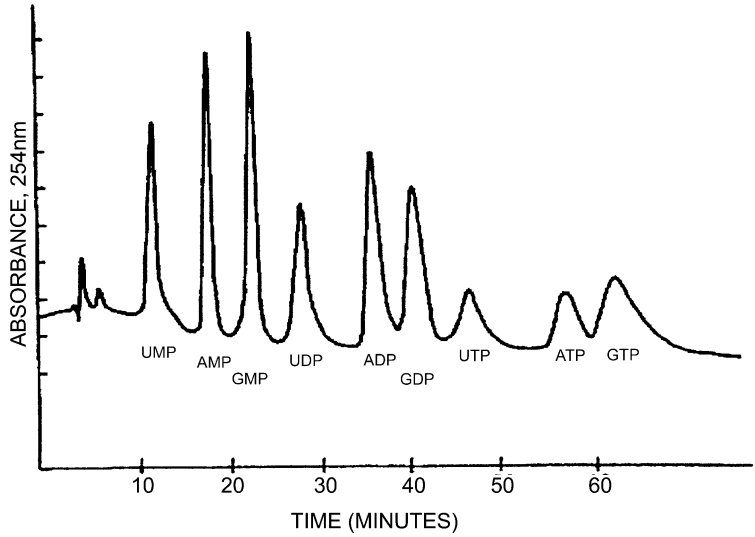


Fig. 19.5. Separation of mono-, di- and triphosphate nucleotides of adenine, guanine, and uracil by ion-exchange HPLC. Conditions: Partisil-10 SAX column; ambient temperature; detector sensitivity, 0.08 AUFS; eluents, (low) 0.007 mol/L KH_2PO_4 (pH 4.0), (high) 0.25 *M* KH_2PO_4, 0.50 mol/L KCl (pH 4.5); linear gradient program, rate 1.5 mL/min. (From Ref. 63 with permission.)

attributed to the high percentage of A and T in a fragment [66] or a curvature in the geometry of the molecule [68,69]. As suggested by Bloch [70], a more length-relevant separation of DNA fragments is feasible when a gradient of tetraethylammonium chloride instead of sodium chloride is applied. The efficiency of anion-exchange chromatography

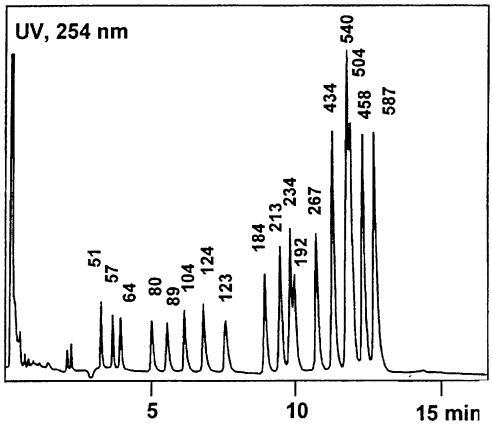


Fig. 19.6. Separation of pBR322 DNA-*Hae* III digest by anion-exchange chromatography. Sample: 4.8 μg of pBR322 DNA-*Hae* III digest. Conditions: column, TSKgel DEAE-NPR (2.5 nm, 35 × 4.6 mm ID); mobile phase, (A) 20 m*M* Tris–HCl (pH 9.0), (B) 1 *M* sodium chloride in 20 m*M* Tris–HCl (pH 9.0); linear gradient, 25–45% B in 0.1 min, 45–50% B in 2.9 min, 50–100% B in 57 min; flow-rate, 1.5 mL/min; temperature, 25 °C. (Reproduced from Ref. 65 with permission.)

in nucleotides and nucleic acids analyses is affected by several operational variables. A micropellicular stationary phase with 1- to 3-μm particle diameter has proven superior for nucleic acid analysis, having the advantages of fast mass transfer, high column efficiency, high speed, and fast column equilibration [60,65,71]. The most widely employed buffer is tris(hydroxymethyl)aminomethane (Tris). Alternative buffer compounds used include phosphate, acetate, ethanolamine, and piperazine. Mobile-phase parameters of polarity, pH, ion types, and concentration have been shown to affect the separation [65,66,72–75]. The flow-rate and gradient mode of the mobile phase also exert a significant impact on resolution (Fig. 19.7) [76]. For DNA analysis, with fragments shorter than 3,000 bp, the

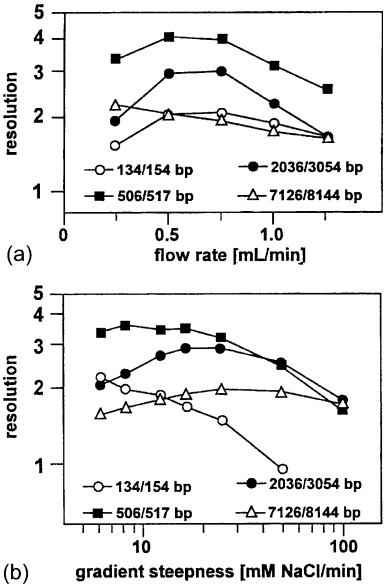


Fig. 19.7. Dependence of resolution on flow-rate (a) and gradient steepness (b) in anion-exchange HPLC on a TSKgel DNA-NPR column. (Reproduced from Ref. 76 with permission.)

highest resolution was observed at a flow-rate of 0.5–0.75 mL/min. Large DNA fragments were better resolved at flow-rates less than 0.5 mL/min [76]. Although several authors have reported that there is little effect of temperature on the quality of DNA separation in anion-exchange chromatography, Mueller [66] found that an increase in temperature from ambient to 60 °C improved the resolution of DNA fragments with simultaneous increase in retention times. All of the above-mentioned parameters need to be carefully considered in order to obtain optimal separations of charged nucleic acids and their constituents.

Aside from the routine analysis of nucleotides and nucleic acids, anion-exchange chromatography has also been employed for sample preparation. The charged compounds, such as nucleotides and nucleic acids, can be selectively retained, while the uncharged compounds, such as nucleosides and bases are eluted. Their preparative fractionation requires elution with gradients of increasing salt concentration. Therefore, removal of the salt from the collected nucleic acid fractions by precipitation, dialysis, ultrafiltration, size-exclusion chromatography, or SPE is required before subsequent analyses.

19.4.2 Reversed-phase liquid chromatography

It was not until the development of reversed-phase liquid chromatography (RP-LC) that nucleosides and bases could be separated in one run, since these compounds are not usually ionized at the pH of the mobile phase [77]. By now, RP-LC has become the method of choice for nucleosides and/or bases for all types of analyses, from a single nucleoside to the complete assay of all the nucleosides and bases in a physiological fluid, such as serum or plasma (Fig. 19.8) [59]. Reversed-phase bonded silica is the most popular column packing material used in modern HPLC. Typically, these materials have C_8 or C_{18} ligands, bonded *via* siloxane linkages to a silica support. For RPLC, the mobile phase generally contains an organic solvent, such as methanol or acetonitrile. The separation mechanism of nucleosides and bases by RPLC is primarily based on solvophobic interaction between the hydrophobic analytes and the non-polar surface of the stationary phase. Compounds with highly polar substituents interact more strongly with the polar hydro-organic mobile phase; thus, there is a decrease in the retention compared to similar solutes having no polar moiety. However, the retention order of nucleic acid compounds has been found to be unpredictable. For example, ribonucleosides, while much more soluble in aqueous solvents than their corresponding bases, have longer retention times. On the basis of solubility and the presence of more hydroxyl groups, it could be expected

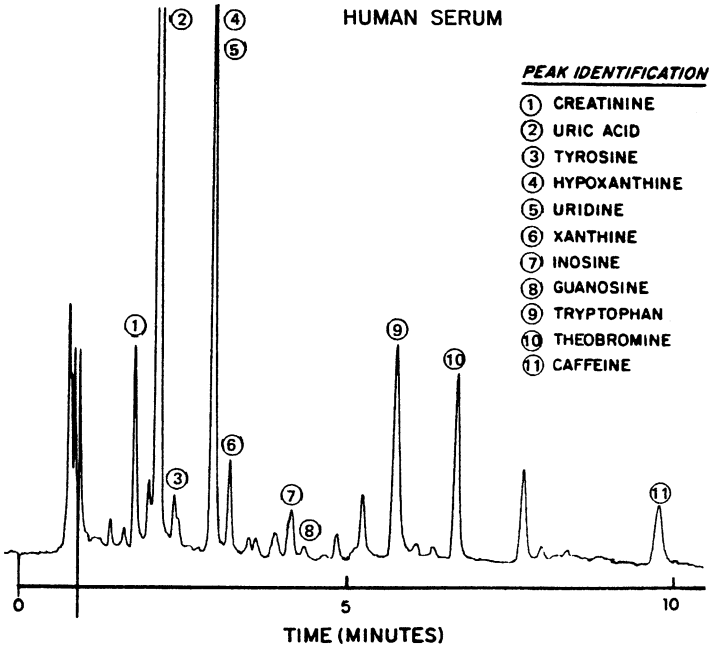


Fig. 19.8. Separation of nucleosides and bases in serum by reversed-phase liquid chromatography Conditions: column, PE HS-5 C18; elution mode, 0.5% to 40% A in 7 min; eluents, A, 60% MeOH/H_2O, B, 0.02 mol/L NaH_2PO_4 (pH 5.6); flow-rate, 2.0 mL/min; detector: PE LC-8J at 254 nm. (Reproduced from Ref. 59 with permission.)

that the ribonucleosides have greater attraction to the polar mobile phase than to the non-polar stationary phase. Accordingly, they should be eluted first. However, a different retention order was found experimentally; the bases were eluted before their corresponding nucleosides. Moreover, although substituent groups on the bases are known to affect retention behavior, 1-methyladenine was eluted before adenine but 7-methyladenine had an elution time almost twice that of adenine. Further investigation indicated that this phenomenon should be attributed to the "vertical base stacking" of the nucleic acid constituents [78]. Assenza and Brown [79] have further investigated the quantitative structure/retention relationship, which is helpful for postulating structures of unknown purine and pyrimidine derivatives on the basis of retention order.

Not only the classical columns, but also microbore columns with reversed-phase stationary phases have been enlisted for analyses of nucleosides and bases. They have the proven advantages of high efficiency and high speed. Hartwick *et al*. [59] have demonstrated the separation of the four major ribonucleosides C, U, G, A in 24 sec on a short, 1-mm-ID column, packed with C_{18} stationary phase. A flow-rate of 0.50 mL/min was used, which is equivalent to 10.5 mL/min in a 4.6-mm-ID column, In addition, short columns (5–10 cm), filled with 5-μm packing materials can also give faster separation. Such high-speed separations should have a major impact on clinical and routine biochemical separations. In the RPLC separation of nucleosides and bases, ionic strength has little influence on the retention of analytes, whereas pH affects only those components that have a pKa in the range of the pH values of the eluents used [77]. On the other hand, an increase in the organic solvent concentration of the mobile phase will obviously decrease the retention of these compounds (Fig. 19.9) [80]. RPLC has been developed as a powerful and practical tool for the determination of nucleosides, bases, and their metabolites in body fluids. Comprehensive studies of nucleosides and bases in serum or plasma were first performed by Brown and her collaborators [81,82]. Some methods for the selective analysis of nucleosides through the treatments of serum or plasma prior to HPLC analysis have also been reported [83]. Additionally, analyses of endogenous compounds in saliva [84] and urine [85] have been carried out. There has been much research activity in this field [54,86–88].

19.4.3 Ion-pair reversed-phase chromatography

Ion-pair reversed-phase (IPRP) chromatography is a well-established technique for separating nucleic acids and their constituents in both the ionized and non-ionized forms simultaneously [60]. A variety of silica- and polymer-based supporting materials are commercially available for IPRP chromatography. Octadecylated poly(styrene/divinylbenzene) (PS/DVB) is one of the commonly used types. In addition, a stationary phase formed by *in situ* co-polymerization of styrene and divinylbenzene has been proposed [89]. Separation of nucleic acids and their constituents is achieved by means of a hydro-organic eluent, containing an amphiphilic ion [*e.g*., triethylammonium (TEA) ion], and a small, hydrophilic counter-ion (*e.g*., acetate). As in anion-exchange chromatography, the gradient elution mode is widely employed. However, in IPRP chromatography the gradient is not affected by an increase in ionic strength, but by an increase in

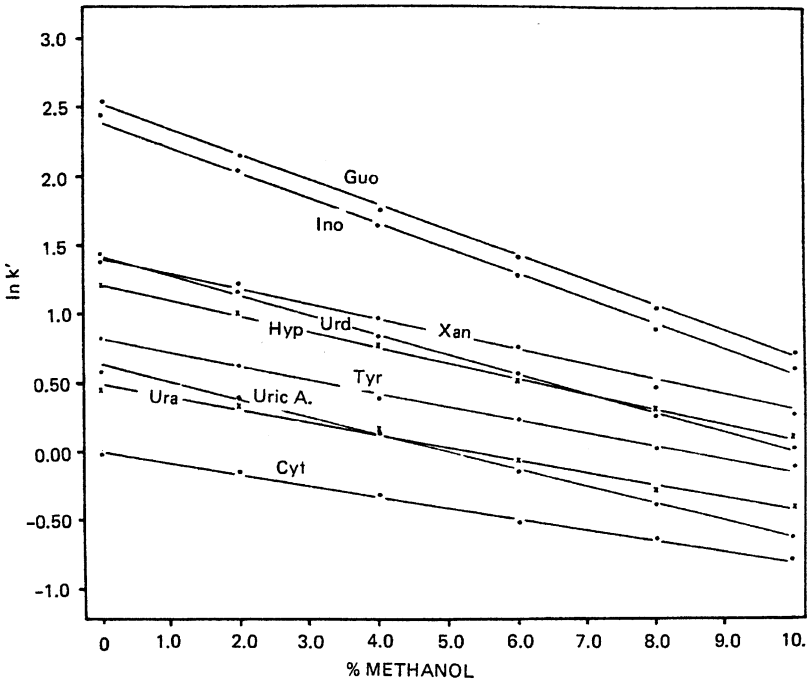


Fig. 19.9. Effect of percentage of methanol in the mobile phase on retention factors (k) of eight nucleosides and their bases and the amino acid tyrosine in a reversed-phase separation Conditions: column, Whatman Partisil-5, C8; particle size, 5 μm; mobile phase, 0.005 mol/L NaH_2PO_4 (pH 5.0); flow-rate, 1.0 mL/min; temperature, 25 °C. (Reproduced from Ref. 80 with permission.)

the concentration of organic solvent in the mobile phase, resulting in the desorption of amphiphilic ions from the hydrophobic surface of the stationary phase.

For the analysis of neutral nucleosides, the retention mechanism is similar to that in reversed-phase chromatography, depending on solvophobic interaction. However, the retention of charged nucleic acids and nucleotides by IPRP chromatography is very similar to that in anion-exchange chromatography. Briefly, the positively charged TEA ions are adsorbed at the interface between the nonpolar stationary phase and the hydro-organic mobile phase, resulting in the formation of a positive surface, which is determined by several factors, including the hydrophobicity of the column packing, charge, hydrophobicity, concentration, and ionic strength of the pairing ion, by the temperature, and by the dielectric constant of the organic solvent. Retention of analytes is governed mainly by electrostatic interactions between the positive surface potential generated by the TEA ions adsorbed on the stationary phase and the negative surface potential generated by the dissociated phosphate group. Therefore, double-stranded DNA molecules are retained according to chain-length. IPRP chromatography has been widely used in analyses of nucleic acids and their constituents. [60,89–94]. Fig. 19.10 is a typical chromatogram for the analysis of restricted DNA fragments [94]. Sufficient equilibration of a newly packed PSDVB C_{18} column is an important determinant for obtaining satisfactory

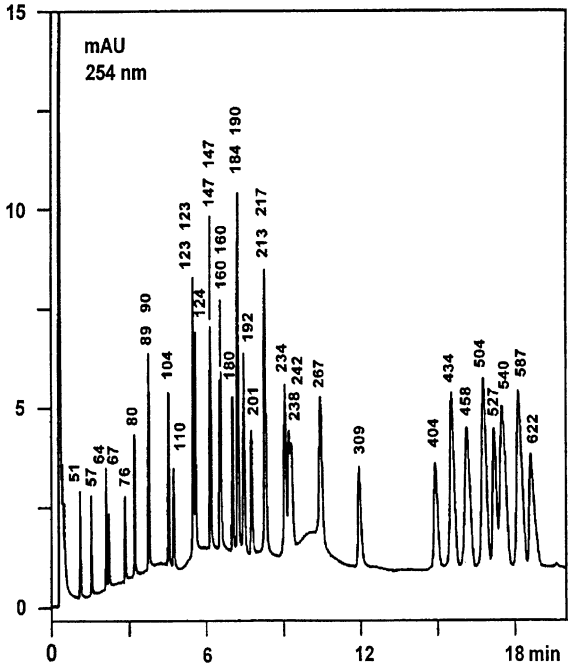


Fig. 19.10. High-resolution separation of double-stranded DNA fragments by IP-RP chromatography. Sample: 0.75 μg pBR322 DNA-*Hae* III digest and 0.4 μg pBR322 *Msp* I digest. Conditions: column, PS-DVB-C_{18} (2.1 μm, 50 × 4.6-mm ID); mobile phase, (A) 0.1 *M* TEAA (pH 7.0), (B) 0.1 *M* TEAA, 25% acetonitrile (pH 7.0); linear gradient, 37–55% B in 6 min 55–65% B in 14 min; flow-rate, 1 mL/min; temperature, 50 °C. (Reproduced from Ref. 94 with permission.)

high-performance separations. TEA acetate (TEAA) is the ion-pair reagent of choice for IPRP chromatography. Its concentration in the mobile phase has a major effect on the retention and resolution of analytes. In addition, pH, type and concentration of counter-ion, concentration of organic solvent, flow-rate and gradient slope of the mobile phase, all influence the separation of nucleic acids and their constituents [60,92].

It should be pointed out that the column temperature has a special effect on the separation of DNA. Generally, retention times and resolution increase with increasing temperature. However, it has been observed that at a temperature over 50 °C, DNA heteroduplices are less stable than perfectly matched duplices under IPRP chromatographic conditions, and even partial denaturation of DNA causes a noticeable shift in retention. These phenomena have given rise to denaturing high-performance liquid chromatography (DHPLC) [95]. In DHPLC analysis, PCR products of DNA clones with one single base difference are generally mixed at equimolar ratio, denatured, renatured, and analyzed at increasing column temperature. At a suitable temperature, four species, two heteroduplices and two homoduplices, may be separated, the former two migrating faster than the latter. The order of elution can be dictated by the degree of destablization, which is influenced by both, neighboring stacking interaction [96] and the ability of certain bases to form atypical Watson-Crick base-pairs [97]. Since different mismatches display

different degrees of destabilization at a given temperature, different chromatographic profiles for different matches may be obtained. Interestingly, mutations located at the same nucleotide position always yield a different chromatographic profile, while mutations located within the same melting domain tend to show very similar profiles [95]. In DHPLC, the optimum temperature for detection should be determined by the degree of sensitivity desired and the extent of lower-melting domains. This maybe arrived at either empirically, or by means of computation, if the sequence of interest is known. In addition, the TEAA concentration in the mobile phase may also affect the optimum temperature. Huber *et al*. [98] have found that for DNA fragments, a 50-mmol/L increase in TEAA concentration corresponds to a 2 °C increase in melting temperature.

The past decade has seen great improvements in the development of new kinds of stationary phases for DHPLC. The classical phase is generally made of alkylated, non-porous PS/DVB particles, 2–3 μm in diameter [99]. Now, a 3.5-μm wide-pore alkylated silica matrix and a polymer-coated 3-μm wide-pore alkylated silica-based stationary phase, having an improved column lifetime, are available from Agilent and Varian, respectively (Chap. 12). More recently, Huber *et al.* [100] introduced monolithic capillary columns that had been prepared by *in situ* co-polymerization of styrene and divinylbenzene inside a 200-μm-ID fused-silica capillary. Compared with the conventional packings, 40% improved column performance was obtained in the separation of DNA fragments. DHPLC has played an important role in mutational analysis of genes. The predominant application of DHPLC is the mutation analysis of *BRCA1* and *BRCA2*. Cross *et al*. [101] have detected all the 432 heterozygotes in *BRCA1* and 136 in *BRCA2* studied, with only one false positive. Spiegelman *et al*. [102] have amplified 476 fragments from two *Arabidopsis thaliana* ecotypes, covering various regions on three chromosomes, without prior knowledge of the presence or absence of polymorphisms. Parallel analysis by DHPLC and sequencing demonstrated that DHPLC can detect 165 out of 166 polymorphic fragments, with only four false positives. All of these results demonstrate the excellent sensitivity and specificity of DHPLC in the detection of mutations and polymorphisms. The throughput of DHPLC can be increased by labeling different fragments that share a common melting temperature with different fluorophores [95]. In addition, DHPLC can be employed for the quantitative analysis of gene expression [103], identification of biallelic polymorphism on the Y-chromosome [104], and gene mapping [105]. In some instances, it is not enough just to distinguish heterozygotes from homozygotes. Therefore, DHPLC under partially denaturing conditions cannot be employed for genotyping, unless homozygotes are mixed with reference material of known genotype prior to DHPLC analysis [106]. DHPLC under completely denaturing conditions may prove a worthwhile alternative, based on its ability to resolve single-stranded nucleic acids as a function of base composition rather than size (Fig. 19.11) [107]. In summary, completely denaturing HPLC enables direct genotyping of biallelic sites contained in short amplicons, as well as the analysis of primer extension products. In addition to the above-mentioned three modes of chromatography, mixed-mode [108–110], size-exclusion [111–113], and affinity chromatography [114–116] have also been employed for the purification and separation of nucleic acids and their constituents.

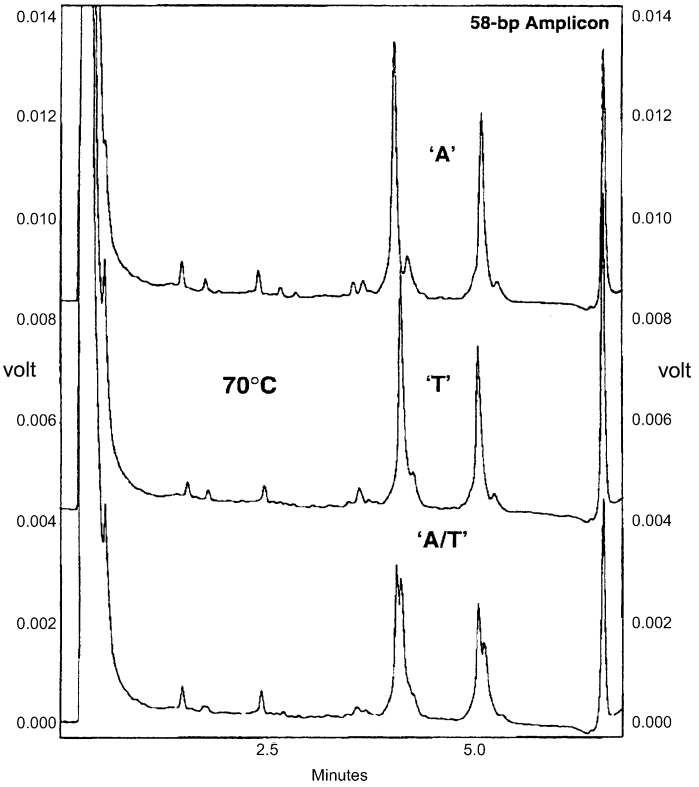


Fig. 19.11. Allelic discrimination of an A → T transversion by DHPLC under completely denaturing conditions Conditions: column, DNASep™, 50 × 4.6 mm ID; mobile phase, 0.1 *M* TEAA (pH7.0); linear gradient, 7–10% acetonitrile in 5.5 min; flow-rate, 0.9 mL/min; temperature, 70 °C; detection, UV at 254 nm. (Reproduced from Ref. 107 with permission.)

19.5 ELECTROPHORETIC METHODS IN ANALYSES OF NUCLEIC ACIDS AND THEIR CONSTITUENTS

Not only chromatography, but also a variety of electrophoretic methods, such as gel electrophoresis, capillary electrophoresis (CE), and microchip electrophoresis have been used extensively in analyses of nucleic acids and their constituents.

19.5.1 Gel electrophoresis

The term gel electrophoresis encompasses a whole series of techniques for separating charged molecules in an electrical field and a gel matrix, exploiting differences in net electrical charge, shape, and size of the molecules [117]. Gel electrophoresis has been successfully applied to the purification and separation of nucleic acids on the basis of their migration velocities in the electric field, with gels either in tubes or slabs [118]. DNA and

RNA molecules are highly charged near pH 7, because the phosphate group in each nucleotide contributes one negative charge. As a result, DNA and RNA molecules move toward the positive electrode during gel electrophoresis. Smaller molecules move through the gel matrix more readily than larger molecules, so that molecules of different length, such as restriction fragments, are separated. Because the gel matrix restricts random diffusion of the molecules, molecules of different length separate into "bands", having the same width as that of the well into which the original mixture was placed. The resolving power of gel electrophoresis is so great that single-stranded DNA molecules up to about 500 nucleotides long can be separated if they differ in length by as little as 1 nucleotide. This high resolution is critical to the DNA-sequencing procedures. DNA molecules composed of up to *ca.* 2000 nucleotides are usually separated electrophoretically on polyacrylamide gels, and molecules from 500 nucleotides to 20 kilobase (kb) on agarose gels. For RNA, both polyacrylamide and agarose gels have been employed. Although the distance traversed by a charged nucleic acid molecule is inversely related to its $\log_{10}$ molecular weight, its base composition, secondary structure, and other factors can affect this relationship. In addition, gel electrophoresis has also been employed to study nucleic acid/protein interactions, based on the retardation assay [118,119]. After migration, the DNA or RNA bands on polyacrylamide or agarose gels can be detected by a variety of very sensitive staining procedures or autoradiography of the gel, before or after it is dried. The accuracy of analyses by gel electrophoresis depends to a large extend on the quality of the imaging system. Recently, Edwards *et al*. [120] have published a review that offers some guidance in the selection of digital imaging systems for gel-electrophoretic analysis.

The gel-electrophoretic techniques described so far can resolve DNA fragments up to *ca*. 20 kb in length. Larger DNAs, ranging from 2×10^4 to 10^7 bp [20 kb to 10 megabases (mb)] in length, can be separated according to size by pulsed-field gel electrophoresis [118, 121–123]. This technique depends on the unique behavior of large DNAs in an electric field that is turned on and off (pulsed) at short intervals. When an electric field is applied to large DNA molecules in a gel, the molecules not only migrate in the direction of the field but also stretch out lengthwise. If the current then is stopped, the molecules begin to "relax" into random coils. The time required for relaxation is directly proportional to the length of a molecule. The electric field is then reapplied at 90° or 180° to the first direction. Longer molecules relax less than shorter ones during the time the current is turned off. Since the molecules must relax into a random coil before moving off in a new direction, longer molecules start moving in the direction imposed by the new field more slowly than shorter ones. Repeated alternation of the field direction gradually forces large DNA molecules of different size farther and farther apart. Pulsed-field gel electrophoresis is very important for purifying long DNA molecules up to *ca.* 10^7 bp in length. The technique is required for analyzing cellular chromosomes, which range in size from about 5×10^5 bp (smallest yeast chromosomes) to $2–3 \times 10^8$ bp (animal and plant chromosomes). Very large chromosomes must be split into fragments of 10^7 bp or less before they can be analyzed.

Temperature-gradient gel electrophoresis (TGGE) is another variant of traditional gel electrophoresis, which can be employed for the detection of genetic mutants, exploiting two physical properties of DNA, the effect of base-pair sequence on the temperature, including unwinding or melting transition, and the influence of partial denaturation on electrophoretic mobility [124,125]. In a typical experiment, a DNA fragment migrates into

a gel with a superimposed temperature gradient until it reaches a temperature that induces partial strand unwinding. As a consequence of being partially denatured, the DNA undergoes a large decrease in mobility. Homologous DNAs, differing in a single-base pair in their least stable region, migrate to different depths before undergoing abrupt changes in mobility. TGGE experiments can be carried out with the migration of DNA bands perpendicular or parallel to the temperature gradient. Fig. 19.12 illustrates the adoption of the two formats [125]. The parallel TGGE format shown in Fig. 19.12a is generally used to screen for base-pair changes in multiple DNA samples, while the perpendicular TGGE format shown in Fig. 19.12b provides a better approach for analyzing the unwinding behavior of a given DNA.

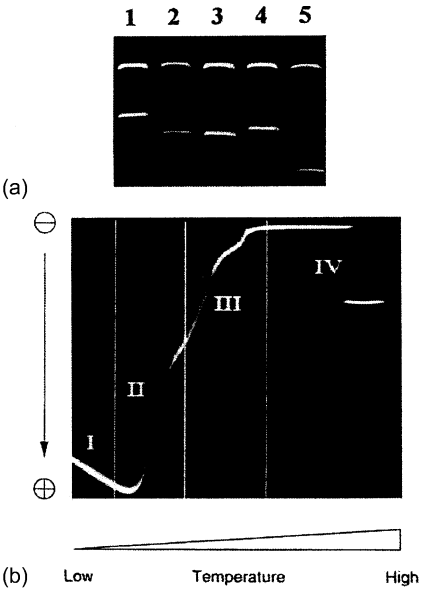


Fig. 19.12. (a) Parallel TGGE experiment. The temperature gradient was 4 °C parallel to the direction of electrophoresis, 29 to 33 °C. The experiment was run at a constant voltage of 80 V for 14.5 h. The samples were DNA plasmids treated with RsaI/EcoR I enzymes. For each lane, the top band is the 676-bp DNA and the bottom one is the 373-bp DNA. Lanes and plasmids: (1) pUC8-5, (2) pUC8-36, (3) pUC8 + 3, (4) pUC8-15, (5) pUC8-31, the wild type. The plasmids in Lanes 1 to 4 differ from pUC8-31 by single base-pair change (G → A) in the first melting domain of the 373-bp DNA. The locations of mutations for pUC8-5, pUC8-36, pUC8 + 3 and pUC8-15 are 48, 17, 55 and 38 base-pairs away from the duplex end with the EcoR I site. (b) Mobility curve of the wild type 373 bp DNA with perpendicular TGGE. The temperature gradient was from 15 to 60 °C across the gel, perpendicular to the direction of electrophoresis; run for 12 h at 120 V. Four distinct melting regions are shown. In Region I the DNA is not yet melted, and its mobility increases slightly with temperature. Region II contains the first transition step, which is correlated with the first melting step in the melting map. Region III shows two further melting steps. Region IV includes the later stages of the transition. Both partial duplex and the faster moving band assigned to single-stranded DNA can be observed in a 1.5 °C-wide temperature zone. (Reproduced from Ref. 125 with permission.)

Denaturing gradient-gel electrophoresis (DGGE) is also applicable to the separation of DNA fragments that differ at a single base pair site. It depends on the fact that different double-stranded DNA molecules "melt" or are denatured to form single strands at different temperatures or under different chemical conditions, depending on their specific sequences [126,127]. For example, one can construct electrophoresis gels that contain a gradient of increasing concentrations of denaturants, such as urea or formamide, and if a DNA molecule is made to migrate through such a gel, it will stop at the position at which it is denatured. If two DNA fragments of identical length differ in their sequences by as little as one base pair, the concentration of denaturant at which the two fragments melt will be slightly different. Thus, electrophoresis of these two DNA fragments through a gradient of denaturant will distinguish them by the positions at which the two fragments stop migrating. One could begin with fragments isolated by polymerase chain reaction (PCR), making this a convenient way to screen for the presence of common mutations, using only a small amount of sample.

In order to improve the separating ability of complex nucleic acids, two-dimensional gel electrophoresis has also been developed for nucleic acids analysis. The major advantage of two-dimensional electrophoresis is that it provides a high-resolution system for screening multiple fragments under the same condition. For RNA analysis, there are mainly three types of two-dimensional systems [118]. In the "urea shift" method, the gel for the first and second dimension has the same concentration and both are run in essentially neutral medium. However, the gel for either the first or the second dimension contains a high concentration of urea; the other one is used under non-denaturing conditions. For the dimension in the absence of urea, fragments containing hidden breaks migrate as base-paired complexes. In the urea-containing gel the complex will dissociate into its constituents, which run considerably faster. In the "concentration shift" method, the only difference between the gels for the first and second dimension resides in a change in polyacrylamide concentration. In this case, nothing is done to change the RNA conformation between these two dimensions, but the differential effect of a tighter gel matrix on RNA molecules with different conformation is exploited to change their order of mobility. The third type of two-dimensional gel separation combines a pH shift with a urea and a polyacrylamide concentration shift. This method was originally designed for separating complex mixtures of RNA fragments obtained when large RNA molecules are partially digested with RNase in preparation for sequence analysis. However, its main area of application later developed into the fingerprinting of RNase T_1 digests, mainly of large viral RNAs. The electrophoresis of DNA continues to be practiced mostly through the use of one-dimensional separation methods. However, for a number of applications, particularly those dealing with the comparative analysis of large DNA fragments or of entire genomes that do not involve the use of a sequence-specific probe, various two-dimensional approaches have been developed [128,129]. The simplest procedure for two-dimensional DNA electrophoresis involves the use of separate gels for the first-dimensional separation according to size, and for the subsequent separation of the same fragments by DGGE on the basis of their melting temperature. In practice, by using a two-dimensional system, it is possible to visualize every fragment corresponding to an entire gene in a particular DNA sample and to recognize each exon and the variants therein immediately. This has been demonstrated for several large genes for human disease, *e.g.*, the *CFTR*

(cystic fibrosis transmembrane regulator) gene [130], the *RB1* (retinoblastoma tumor suppressor) gene [131], the *MLH1* (MutL protein homolog 1) [132], and the TP53 (p53 tumor suppressor) gene [133]. These results suggest that two-dimensional electrophoresis can provide a generally applicable, practical, and fast way to diagnose with high accuracy large genes for a broad spectrum of possible disease-related mutations.

The emerging need for large-scale, high-resolution analysis of nucleic acids, such as DNA sequencing, PCR product sizing, and single-nucleotide polymorphism (SNP) hunting, led to the development of automated and high-throughput gel electrophoresis methods, providing rapid, high-performance separations over a wide molecular-weight range. Ultrathin-layer gel electrophoresis is a possible solution, which has been reviewed by Guttman *et al*. [134] (Sec. 11.3.1). It has a number of advantages, such as high efficiency, short analysis time, reagent and sample saving, and low cost (Table 19.1) [135]. In order to accomplish high-throughput analysis, microplate-array diagonal gel electrophoresis (MADGE) has also been developed. It combines direct compatibility with microplates, convenient polyacrylamide gel electrophoresis, and economy of time and reagents, at minimal capital cost, and enables one user to run up to several thousand gel lanes per day for direct assays of single-base variants [136].

TABLE 19.1

COMPARISON OF SEPARATION PERFORMANCE AND SENSITIVITY OF AUTOMATED ULTRATHIN-LAYER AGAROSE GEL ELECTROPHORESIS AND MANUAL SUBMARINE GEL SLAB ELECTROPHORESIS SYSTEMS

Feature	dsDNA fragment analysis	
	Automated	Manual
Gel preparation and loading	10 min	35 min
Separation	25 min	60 min
Staining/destaining	None	30 min
Imaging/evaluation	None	15 min
Total time	40 min	155 min
Number of lanes	32	14
Time per sample	1.09 min	10 min
Sensitivity (ethidium bromide)	0.04 ng/band	0.2 ng/band*
Separation range (single gel)	20–25 000 bp	50–500, 500–5k, 5k–50k
Resolution	4 bp at 240 bp in 12 min	4 bp at 240 bp in 5 h
Required buffer volume	25 mL	250 mL
Required gel volume	2 mL	20 mL

* With UV trans-illumination and charge-coupled device camera detection imaging. (Reproduced from Ref. 135 with permission.)

19.5.2 Capillary electrophoresis

During the last decade, CE has developed into an eminently useful analytical technique, which played an important role in the HGP and the post-genomic-sequencing era for nucleic acid analyses. An advantage of CE is that one can apply a higher voltage than in gel slab electrophoresis, and thus one can analyze nucleic acids at high resolution and high speed. Several books [137–139] and reviews [140–144] have been published on this subject. Since RNA and DNA molecules have a constant charge-to-size ratio and therefore identical electrophoretic mobility, they can generally not be separated in free solution, except when some special modifications are adopted to break the charge-to-size symmetry. Accordingly, CE separation of nucleic acid species requires addition of a sieving matrix to the background electrolyte to retard the analytes in proportion to their size. This has been achieved either by casting a cross-linked gel in the capillary, or by incorporating a linear gel polymer in the running buffer. The selection of an appropriate sieving matrix will depend upon the size of the molecules that are to be separated, the resolution required, and the capabilities of the analytical instrument.

Much of the earlier DNA separations in CE were performed in gel-filled capillaries, hence the name capillary gel electrophoresis (CGE) [145]. The mostly commonly used gel matrix is polyacrylamide, which can be prepared by *in situ* polymerizing acrylamide monomers in the presence of a cross-linker to form a three-dimensional network [146]. The pore size of the gel is dependent on both the monomer and the cross-linker concentration. The choice of the gel concentration is determined by the competition between the maximum inter-band spacing and the range of fragment sizes to be separated. For small fragments, such as oligonucleotides, a better separation can be obtained with higher gel concentration, giving a smaller pore size. However, the resolution is drastically reduced for the separation of large molecules (over 200 bases). Dilute gels can separate much larger molecules, but at low resolution. Therefore, a compromise must be found for a particular separation problem. CGE has played an important role in DNA sequencing [147], restriction-fragment-length polymorphism (RFLP) analysis [148], and PCR product analysis [149].

More recently, the separation of nucleic acids with linear-polymer sieving matrices has attracted much attention, since it can offer many advantages over conventional cross-linked matrices, including ease of operation, a homogeneous sieving structure, and low susceptibility to damage due to bubble formation. A number of polymers used so far, including agarose and its different modifications, various cellulose derivatives, such as methylcellulose (MC), hydroxyethylcellulose (HEC), hydroxypropylcellulose (HPC) and hydroxypropylmethylcellulose (HPMC), and synthetic polymers, including polyethylene glycol (PEG) and polyethylene oxide (PEO), have been well reviewed in Ref. 141. Co-polymer solutions have also been employed as separation media. The separation matrices can be easily prepared by dissolving the polymer in the buffer at the desired concentration. Under these conditions, the chains of the polymer will become entangled, forming a transient network for the sieving of nucleic acid molecules.

For the DNA separation mechanism we can give just a basic introduction here; detailed information may be found in the relevant reviews [137,140]. On the basis of the Ogston model, a separation medium, either gel or polymer, is treated as a random network of fibers

with limited length. A sample molecule migrates as a random coil adopting a spherical shape with a radius of gyration, *Rg*, in the direction of the electric field, diffusing laterally until it encounters a pore large enough to allow its passage. When the average pore size of the sieving network is larger than *Rg*, at low field intensities the DNA fragment migrates through the gel. The net mobility must then be related to the tortuosity of the path as well as to the DNA/gel fiber interaction. In the Ogston region, there is a linear relationship between the logarithm of mobility and DNA size. However, this model is only suitable for small DNA fragments and at low electric fields. For DNA with *Rg* larger than the average pore size of the matrix, the reptation theory has been established to explain its movement. According to that theory, a polymer chain moves through a network, not as an undeformable particle, but rather snake-like through "tubes" in the sieving network. When the electric field is low enough, the DNA molecule can be considered as a random-coil polymer with some degree of flexibility. Theoretically, its mobility under these conditions has a linear relationship to its size, which is named reptation-without-stretching regime. However, when the electric field is strong, DNA is no longer considered to move as a random-coil polymer, but moves with extended rod-like conformation. If DNA is completely extended, it moves straight in the electric field direction, and in this situation, its mobility no longer depends on its molecular size. This regime is defined as reptation-with-stretching. The electrophoretic behavior of DNA under the above-mentioned three regimes is shown in Fig. 19.13.

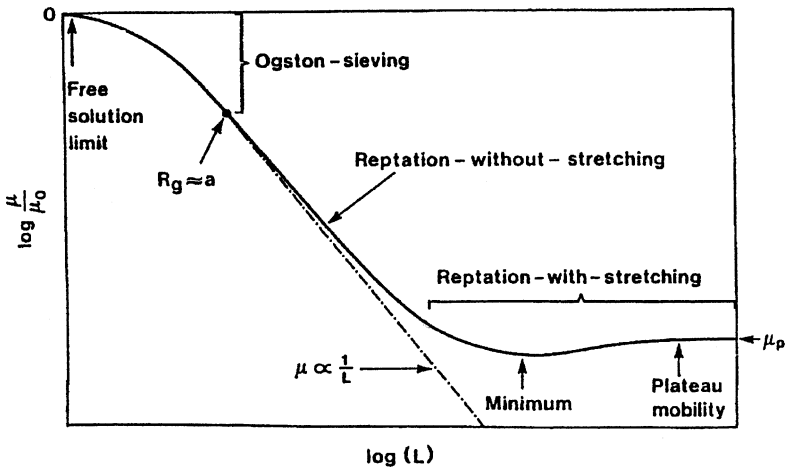


Fig. 19.13. Electrophoretic behavior of DNA in capillary electrophoresis.

Based on this separation mechanism, it is evident that in the sieving system there are several parameters that can affect the migration time, the resolution, and the speed of analysis of DNA fragments. The choice of a suitable polymer is very important, since, depending on their physical properties, different polymers have a different intrinsic viscosity and also form networks with different pore sizes. The concentration of the polymer may also affect the pore size of the sieving matrix [150]. Polymers with self-coating ability are preferred. Otherwise, a coated capillary should be employed to inhibit

electro-osmotic flow (EOF). Once the polymer is chosen, other operational parameters will become the main factors that affect the separation [151]. Electric field strength is an important one. Although increasing the applied voltage will result in faster migration and shorten the overall run time of the experiment, the Joule heat will also increase, and the resolution of large DNA fragments will decrease when the electric field is too strong. Therefore, it is necessary to optimize the electric field strength to strike a balance between analysis time and resolution. The concentration of the buffer as well as the temperature affect the separation.

CE is widely used in nucleic acid analyses. It has contributed greatly to the completion of the draft sequence of the human genome. The use of CE for DNA sequencing was first demonstrated in the early 1990s [152,153]. More recently, the development of capillary-array electrophoresis (CAE), with 96- or 384-capillary arrays, has greatly improved the throughput of DNA sequencing [154,155]. The two most powerful commercially available capillary array sequencers are the ABI PRISM 3700 DNA sequencer from PE Biosystems (Applied Biosystems) and the MegaBACE™ 1000 from Molecular Dynamics (Amersham-Pharmacia Biotech). Most of the human genome was sequenced with these two systems. In DNA sequencing, the read length depends on various factors, such as sample preparation, sequencing software, and resolution [156]. Resolution is the most important parameter, since it is a quantitative measure of the discriminatory power of the electrophoretic separation system for DNA sequencing fragments. Because it strongly depends on the characteristics of the separation media, there is great interest in improved separation matrices. Barron *et al.* [141] have reviewed the wide range of polymeric materials that have been employed for DNA sequencing by CE, including linear polyacrylamide (LPA), poly-*N,N*-dimethylacrylamide (PDMA), and polyethylene oxide (PEO). Since the read length for each capillary of the commercial instrument is limited to less than 400–500 bases with $>99\%$ accuracy, long-read sequencing of more than 1,000 bases with high accuracy is required to achieve a higher throughput for DNA sequencing. Other improvements, such as elevating the capillary temperature [157], changing the denaturing agent [158], and using a gradient of electric field strength [159], have also been proposed. The instrumentation detector systems are a cornerstone of the successful implementation of any DNA sequencing device. The two most successful detector designs in this respect are the sheath-flow detector and the scanning confocal detector (Sec. 19.6.2) [156]. More recently, Yeung *et al.* [160] have developed a two-color excitation system for two-dimensional step scanning across a 96-capillary array for DNA sequencing.

The detection of DNA mutations and natural variants has become central to the characterization and diagnosis of human congenital diseases [161]. Several recent reviews provide detailed descriptions of CE applications, developed for the detection of point mutations [161–165]. CE-based single-strand conformation polymorphism (SSCP) and heteroduplex analysis (HA) are two commonly used methods, based on the differences in electrophoretic mobilities of wild-type and mutant DNA species [164–167]. SSCP involves dissociation of the double-stranded DNA fragment, after which each of the two single-stranded fragments assumes a folded conformation, determined by the specific nucleotide sequence, which leads to a different mobility. The sample preparation of HA assay is similar to that shown in Sec. 19.4.3. The mismatches within the heteroduplexes result in conformational changes, which retard their electrophoretic mobilities relative to

the homoduplex. PCR-based technologies have been playing an important role in the highly sensitive and selective detection of mutations or polymorphisms of human genes. Previously, slab gel electrophoresis was commonly used for the analysis of the PCR products, but the technique is rather time-consuming, labor-intensive, and not quantitative. Now, CE is rapidly becoming an important tool for the analysis of PCR products [168–170] and the restriction fragments of PCR products for disease diagnosis [171,172]. In addition, capillary affinity gel electrophoresis (CAGE), which combines the high specificity of affinity ligands with the high resolving power of CGE, has been developed for the recognition of specific DNA bases or sequences [173–175]. Furthermore, CE-based short-tandem-repeat (STR) length polymorphism is also used to identify mutations [176–178].

CE has also been applied to various analyses of nucleic acids and their constituents. In order to obtain highly specific amplification products in PCR, careful control of the quality of deoxynucleoside triphosphate (dNTP) and oligonucleotide primers is required. Pearce *et al*. [179] have established a CE-based method that can be used for both of them. Chemically modified, synthetic, single-stranded oligonucleotides have been analyzed by CE, offering important information for the development of drugs [180]. The interaction of DNA with proteins is a central theme of molecular biology, which can also be studied by CE mobility-shift assay [181]. In this method, protein and DNA are mixed in the reaction buffer and then the mixture is injected. Based on the charge-to-mass ratio, free DNA can be quickly separated from the DNA/protein complex during electrophoresis. Changing the DNA or protein concentration usually does not change the electrophoretic mobilities, but the peak areas for both free DNA and DNA/protein complex. The dissociation constant can be then calculated by measuring both peak areas. It should be emphasized that the commonly used modes of CE at constant electric field strength is not suitable for the separation of large DNAs. Pulsed-field CE (Sec. 19.5.1) should be employed [182]. CE has also been successfully applied to the analysis of nucleosides and RNA. Xu *et al*. [183] have used micellar electrokinetic capillary chromatography (MEKC) to analyze the free normal and modified ribonucleosides in urine (Fig. 19.14). The levels of some of them were later correlated to those of conventional tumor markers [184]. Although the detection sensitivity of CE is relatively low because of the short UV-light path length, the resolution of some compounds is better and less sample is required than in the traditional methods for nucleosides by RP-HPLC. Analyses of transfer RNA, 5S ribosomal RNA, and the fingerprinting of low-molecular-mass RNA have also been performed by CGE and CE with linear-polymer solutions [185,186]. Separations are influenced by differences in three-dimensional structure, in charge, in conformation during electrophoresis, and interactions with the separation matrix.

Recently, a new kind of separation mode, capillary electrochromatography (CEC), which could be regarded as a combination of HPLC and CE, has been proposed (Chap. 7). In CEC, the stationary phase is either coated on or bonded to the inner wall of a capillary, or either packed into or *in situ* polymerized in the capillary, and the mobile phase and samples are driven through the capillary with EOF as the propelling force. Accordingly, both neutral and ionic compounds can be separated by CEC, based on differences in their partition between the mobile and stationary phases, as well as their electric mobility in an electrical field. Several papers have been published on the analysis of monomeric

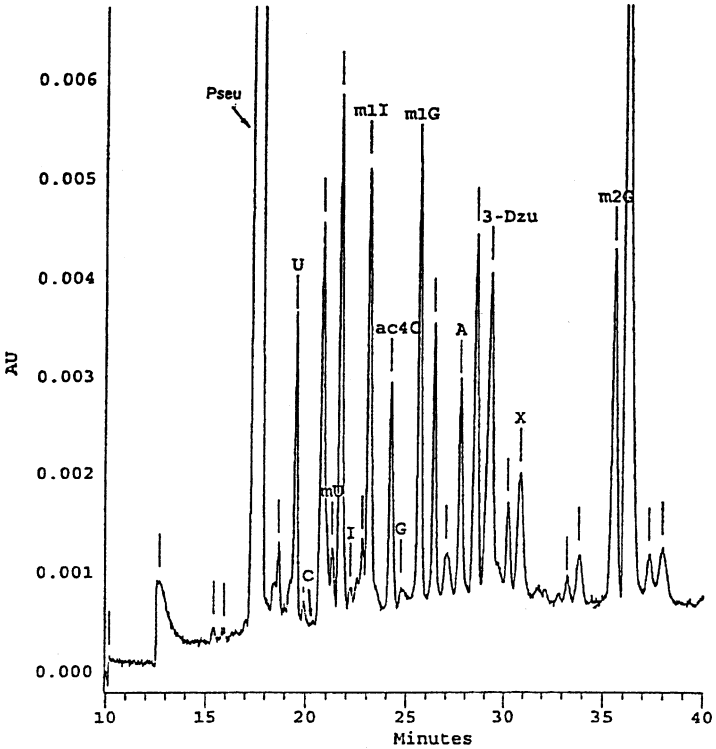


Fig. 19.14. Typical electropherogram of normal and modified nucleosides, extracted from urine Sample: nucleosides extracted from urine. Conditions: capillary: 500 mm × 50 μm ID (437.5 mm to window); buffer, 300 m*M* SDS-25 m*M* borate-50 m*M* phosphate (pH 6.7); voltage, 7.0 kV with 49 μA of current; detection wavelength, 260 nm; peak identifications, Pseu = pseudouridine, Dhu = dihydrouridine, U = uridine, C = cytidine, mU = 3-methyluridine + 5-methyluridine, I = inosine, m1I = 1-methylinosine, ac4C = N4-acetylcytidine, G = guanosine, m1G = 1-methylguanosine, A = adenosine, 3-Dzu = 3-deazauridine, X = xanthosine, m2G = 2-methylguanosine, m6A = N6-methyladenosine. (Reproduced from Ref. 183 with permission.)

nucleosides and nucleotides by CEC [187,188]. A typical application is the separation of oligonucleotides, carried out by Bayer *et al.* [189]. In their study, they used the term pressurized gradient electro-HPLC (electro-HPLC), which is a variation of pressurized CEC (PEC). A HPLC system was employed with a voltage applied across a capillary, packed with 5-μm C_{18} RP silica gel. Compared to HPLC and MEKC, the analysis time was shorter and the efficiency was dramatically increased with PEC. Electric field strength, voltage gradient, pH, applied external pressure, eluent composition, and overall mobile phase gradient, are the most important parameters that can be varied to optimize the performance of PEC. Compared with MEKC, without any packing material or external pressurized flow, even under isocratic conditions, PEC offers everything that MEKC can provide in terms of analyte selectivity and resolution, as well as excellent peak shape and

efficiency, but in much less time. Accordingly, PEC should be a useful alternative in oligonucleotides analysis.

19.5.3 Microchip electrophoresis

Miniaturization of analytical instruments has developed rapidly in the passed ten years. Earlier, different kinds of micro total analysis systems (μTAS) had been devised and applied to practical analyses. Microchip electrophoresis is now emerging as a new technology that promises to lead the next revolution in chemical analysis [190]. Since the first demonstrations in 90s by Manz *et al.* [191], microchip electrophoresis has kept on developing. At present, many important reviews on microchip electrophoresis have been published (Chaps. 9 and 11) [191–196]. Although micro-machining is applicable to silicon as a substrate, its semiconductor properties are not compatible with the high voltage in microchip electrophoresis, and glass is the preferred substrate. In most situations, the channels of a microchip are fabricated by standard photo-lithographic and chemical wet-etching techniques, and then a second glass plate is thermally bonded to the top of the etched surface. Polymers, such as poly(dimethylsiloxane) (PDMS), poly(methylmethacrylate) (PMMA), polyacrylate, and polycarbonate are of increasing interest now [197,198].

In microchip electrophoresis, migration is controlled by the electric field. For the sample loading procedure, generally, small pinching voltages are applied to the buffer reservoirs to avoid the leakage of samples into the separation channel. Both the injector geometry and the electric field distribution are of paramount importance to the best trade-off between separation efficiency and detection sensitivity [199,200]. As far as the separation is concerned, many parameters, such as the column geometry [201,202], sieving matrix [141], temperature, and electric field strength [203], have major effects on analysis speed and resolution. Although single-channel microchips have enabled high-speed separation, to achieve high-throughput analysis, parallel analyses with multiple channels on one microchip are indispensable. Capillary-array electrophoresis (CAE) on microchips has developed very quickly with respect to both chip design and detection systems [204,205]. Mathies *et al*. have made major contribution to the development of CAE on microchips, increasing the analysis rate by advancing from 12 channels [206] to 384-channel chips [207].

The application of microchip electrophoresis to DNA analysis has undergone fast development in DNA sizing, genetic analysis, and DNA sequencing. The first DNA separations on a microchip were reported by Effenhauser *et al*. in 1994 [208]. A mixture of DNA oligomers, from 10 to 15 bases long, was separated with high efficiency. Subsequently, many fundamental studies of microchip electrophoresis have been carried out with DNA ladders as examples [209]. Ueda *et al.* [210] have proposed field-inversion electrophoresis on a microchip and demonstrated its potentialities in the fast separation of short DNA fragments. DNA digestion fragments have also been analyzed by microchip electrophoresis, either with a single channel [211] or multiple channels [212]. Channels of limited length not only reduce analysis time, but also provide high resolution. Zhang *et al*. [213] have recently proposed a stepwise gradient of linear polymer concentration in the microchannel. Different pore sizes in the sieving matrix obviously improve the resolution

of DNA fragments. Microchip electrophoresis with associated integrated processes has played an important role in genetic analysis [214–216]. Fast analyses of the candidate gene for hereditary hemochromatosis [206], alleles of the D1S80 locus [217], multiplexed short tandem repeats [218], and specific PCR products of hepatitis C virus [219] have been published. Compared to the traditional methods of CGE and CE, the analytical efficiency of microchip electrophoresis constitutes a 10- to 100-fold improvement, and this is quite meaningful for clinical diagnosis. Rapid detection of deletion, insertion, and substitution mutations by combining allele-specific DNA amplification with heteroduplex analysis by microchip electrophoresis has been accomplished within 130 sec [220,221]. Array electrophoresis on microchips further increases the analysis speed. With multi-channel microchips, 96 allele-specific amplification products related to the *HFE* gene could be analyzed within 100 sec [222,223]. In addition, fast detections of SNP and gene mutations by enzymatic mutation detection [224], and SSCP [225] and RFLP analysis [226] by microchip electrophoresis have been reported. CAE has contributed greatly to the HGP since it has accelerated DNA sequencing. However, technological improvements are still needed for mapping the genomes of individuals. DNA sequencing on a microchip was first demonstrated in 1995 by Mathies *et al*. [227]. At that time, single-base resolution to *ca*. 150 bases was achieved in 540 sec with 97% accuracy. Energized by the HGP, DNA sequencing on microchips has experienced fast improvement [228–235]. In Mathies's recent work [235], DNA sequencing data from 95 successful lanes (out of 96 lanes run in parallel) were batch-processed, producing an average read length of 430 bp at a rate of 1.7 kb/min. This system permits lower reagent volumes and lower sample concentrations, and it also offers numerous possibilities for integrated sample preparation and handing. Recent progresses in microfabrication and nanofabrication technologies, based on computer-chip technology, have created a new research area for integrated microchip and nanochip technology [236]. In the near future, so-called exa-bioinformatics, which integrates both ultra-fast information technology and exa-sequencing technology based on microchips and nanochips, will be launched and enable huge amounts of information to be obtained for personalized medicine.

19.6 DETECTION

For practical analysis, high-efficiency and high-resolution separations of analytes are not sufficient. A well-designed detector with high sensitivity is indispensable. For nucleic acids a variety of detection methods have been developed, such as ultraviolet (UV), laser-induced fluorescence (LIF), mass spectrometry (MS), nuclear magnetic resonance (NMR), Fourier transform infrared (FT-IR), electrochemical detection, and microscopy.

19.6.1 UV detection

Purine and pyrimidine derivatives show very strong absorption in the ultraviolet region, from 250 to 280 nm. The high absorbance of these compounds, combined with the low noise and large linear dynamic range of the absorbance detector, make it possible to measure both trace and major components of a mixture in a single separation. The relative

TABLE 19.2

FLUORESCENT DYES USEFUL FOR LABELING DNA FRAGMENTS

Fluorophore*	Ex**	Em***	$(\epsilon \times 10^{-3})$****	QY*****
Propidium iodide	536	617	5.8	–
Ethidium bromide	510	595	5.5	–
Ethidium homodimer-1	528	617	7.4	0.1
Acridine orange	502	526	62	–
TO	509	525	54	0.2
YO-PRO-1	491	509	52	0.44
TO-PRO-1	515	531	62	0.25
YO-YO-1	491	509	84	0.52
TO-TO-1	514	533	112	0.34
YOYO-3	612	631	167	0.15
POPO-3	534	570	146	0.46
SYBR Green I	494	521	–	–

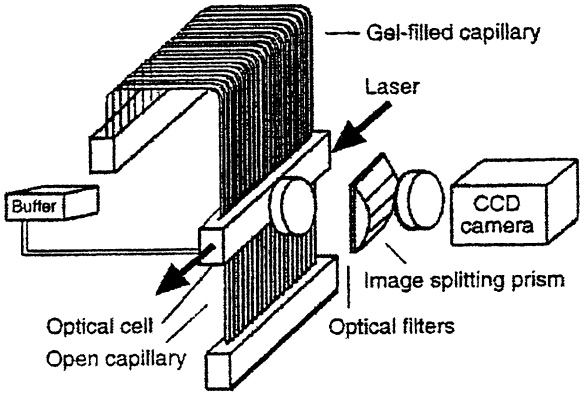
* YO represents oxazole yellow (benzoxazolium-4-quinolinium dye); TO represents thiazole orange (benzothiazolium-4-quinolinium dye); PO represents benzoxazolium-4-pyridiniumdye.
** Ex, wavelength (nm) at the excitation of absorption maximum.
*** Em, wavelength (nm) at the emission maximum.
**** $(\epsilon \times 10^{-3})$, extinction coefficient ($cm^{-1}\ M^{-1}$) multiplied by 1000.
***** QY, quantum yield. (Reproduced from Ref. 163 with permission.)

insensitivity of the absorbance detector to changes in temperature, flow-rate, and mobile-phase composition contributes to its wide-spread use. Accordingly, the UV detector has been a basic tool in the analysis of nucleic acid and their constituents by both chromatographic and electrophoretic methods.

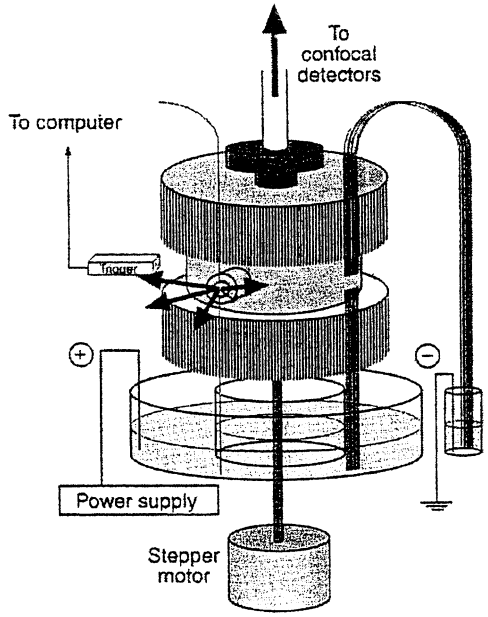
19.6.2 Laser-induced fluorescence detection

LIF detection, which can offer excellent sensitivity (up to 10^{-12} mol/L), has proven to be extremely well suited to the analysis of DNA by CE and microchip electrophoresis. Although the autofluorescence of DNA is very low, it can be derivatized prior to analysis with a fluorescence label. The intercalating dyes listed in Table 19.2 are commonly used for the LIF detection of DNA fragments. Other LIF detection schemes for DNA involve directly labeling of the analyte with a suitable fluorophore. Fluorescently labeled probes and primers are used in many molecular biology application, involving hybridization, PCR, DNA sequencing, and multicolor detection for accurate SSCP analysis. The most frequently used fluorescent labeling agents are so-called ABI dyes, including FAM, JOE, TAMRA, and ROX. Recently, new types of labeling reagents, called energy-transfer (ET) primers have gained wide acceptance for sequencing, owing to significant advantages over traditional four-color automated DNA sequencing [237,238].

LIF has been used not only for detection in single-channel separation, it has also been successfully coupled with CAE and CAE on microchips to meet the needs of high-throughput analysis. A schematic diagram of the multiple-sheath flow system developed by Kambara *et al*. is shown in Fig. 19.15a [239]. The electrophoretic detection cell consists of a gel-filled capillary array, a gel-free optical cell, and a gel-free open capillary array. The analytes from the separation capillaries are eluted into the sheath flow, and then flow out through the open capillaries. This results in higher sensitivity and an



(a)



(b)

Fig. 19.15. Commonly used detector of capillary array electrophoresis (a). Multiple-sheath flow detection (Reproduced from Ref. 239 with permission); (b) Rotary confocal scanning detection. (Reproduced from Ref. 240 with permission.)

excellent signal-to-noise ratio, because the sample is irradiated in a gel-free optical cell. In addition, a high-throughput rotary-scanner detection device, coupled with CAE, has been reported by Sherer *et al*. [240], as shown in Fig. 19.15b. This device has been designed to analyze over 1,000 capillaries in parallel. In this system, a microscope objective and a mirror assembly revolve inside a ring of capillaries, exciting fluorophores and collecting fluorescence from each capillary. For CAE on microchips, similar designs have been employed [204,205].

19.6.3 Mass spectrometry

MS not only provides highly sensitive detection, but also offers information on molecular mass and structure of analytes. Accordingly, its coupling with HPLC and CE has played an important role in the analysis of nucleic acids [241]. Among the various chromatographic modes, RP-LC and affinity chromatography are particularly compatible with MS (Chap. 10). Electrospray ionization (ESI) is one of the commonly used interfaces for nucleic acid analysis. For on-line HPLC/ESI-MS, the optimization of chromatographic and mass-spectrometric experimental conditions is indispensable in achieving highly sensitive detection. Among all the parameters, the eluent composition and the concentration of organic solvent in the electrosprayed solution are the most important ones to consider. HPLC/ESI-MS has been successfully applied to analyses of synthetic oligodeoxynucleotides [242], sequence determinations [241], studies of antisense oligonucleotides and oligonucleotide metabolism [243], identification of DNA adducts [244], ribonucleic acids [241], products of PCR [245], double-stranded DNA restriction fragments [241], and the detection of mutations [246]. Moreover, since the range of the flow-rates from CE or CEC columns is compatible with that of micro- and nano-electrospray, femtomole amounts of samples or samples with a wide dynamic range in concentration can be measured. The application of CE/MS and CEC/MS in the analysis of DNA adducts has been reported by Vouros *et al.* [188,247].

In addition to the above-mentioned detectors, NMR [248], FTIR [249], and the electrochemical detector [250] have been put to good use for the on-line detection of nucleic acids in chromatographic or electrophoretic methods.

19.7 PROSPECT

Because sequencing of the human genome is almost completed [2,3], the HGP will soon enter the post-genome-sequencing era, including SNP analysis, functional genomics, mutation analysis, and metabolome analysis, directed towards personalized medicine [251–253]. In this era, further development of the analytical technology for DNA, mRNA, proteins, and metabolites is urgently required in high-throughput analysis, a stimulus to further advancements of chromatographic and electrophoretic methods.

ACKNOWLEDGMENTS

The present work is partially supported under the CREST program of the (Japan Science and Technology Corporation (JST), a Grant from the New Energy and Industrial Technology Development Organization (NEDO) of the Ministry of Economy, Trade and Industry, Japan, a Grant-in-Aid for Scientific Research from the Ministry of Health and Welfare, Japan, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Technology, Japan.

REFERENCES

1 A.J.F. Griffiths, J.H. Miller and D.T. Suzuki, *An Introduction to Genetic Analysis*, W.H. Freeman, New York, 1996.
2 J.C. Venter et al. *Science*, 291 (2001) 1304.
3 International Human Genome Sequencing Consortium, *Nature*, 409 (2001) 860.
4 C.A. Dangler, *Nucleic Acid Analysis: Principles and Bioapplications*, Wiley, New York, 1996.
5 J.D. Watson and F.H.C. Crick, *Nature*, 171 (1953) 737.
6 J.D. Watson, *The Double Helix*, Atheneum, New York, 1968.
7 A. Panet and H.G. Khorana, *J. Biol. Chem.*, 249 (1974) 5213.
8 P.K. Saiki, S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erilich and N. Arnheim, *Science*, 230 (1985) 1350.
9 B.T. Kurien and R.H. Scofield, *Anal. Biochem.*, 302 (2002) 1.
10 M. Gilar, E.S.P. Bouvier and B.J. Compton, *J. Chromatogr. A*, 909 (2001) 111.
11 K.M. Lo, S.S. Jones, N.R. Hackett and H.G. Khorana, *Proc. Natl Acad. Sci. USA*, 82 (1984) 2285.
12 M. Gilar and E.S.P. Bouvier, *J. Chromatogr. A*, 890 (2000) 167.
13 P. Wilding, J. Pfahler, H.H. Bau, J.N. Zemel and L.J. Kricka, *Clin. Chem.*, 40 (1994) 43.
14 J.P. Cheng, S. Fortina, L.J. Sorrey and P. Wilding, *Mol. Diag.*, 1 (1996) 183.
15 P.K. Yuen, L.J. Kricka, P. Fortina, N.J. Panaro, T. Sakazume and P. Wilding, *Genome Res.*, 11 (2001) 405.
16 X.B. Wang, Y. Huang, R. Hoelzel, J.P.H. Burt and P. Pethig, *J. Phys. D: Appl. Phys.*, 26 (1993) 1278.
17 Y. Huang, K.L. Ewalt, M. Tirado, R. Haigis, A. Forster, D. Ackley, M.J. Meller, J.P. O'Connell and M. Krihak, *Anal. Chem.*, 73 (2001) 1549.
18 J. Yang, Y. Huang, X.B. Wang, F.F. Becker and P.R.C. Gascoyne, *Anal. Chem.*, 71 (1999) 911.
19 T. Muller, T. Schnelle, T. Gradl, S.G. Shirl and G.J. Fuhr, *J. Liq. Chromatogr. Rel. Technol.*, 23 (2000) 47.
20 G. Blankenstein and U.D. Larsen, *Biosensors Bioelectron.*, 13 (1998) 427.
21 P.C.H. Li and D.J. Harrison, *Anal. Chem.*, 69 (1997) 1564.
22 M.R. Emmert-Buck, R.F. Bonner, P.D. Smith, R.F. Chuaqui, Z. Zhuang, S.R. Goldstein, R.A. Weiss and L.A. Liotta, *Science*, 274 (1996) 998.
23 V.A.M. Vincent, J.J. DeVoss, H.S. Ryan and G.M. Murphy, Jr., *J. Neurosci. Res.*, 69 (2002) 578.
24 H.M. Shapiro, *Practical Flow Cytometry*, Wiley-Liss, New York, 1995.
25 S. Miltenyi, W. Mueller, W. Weichel and W. Radbruch, *Cytometry*, 11 (1990) 231.

26 P. Anker, H. Mulcahy, X. Chen and M. Stroun, *Cancer Metastasis Rev.*, 18 (1999) 65.
27 J. Jen, L. Wu and D. Sidransky, *Ann. N.Y. Acad. Sci.*, 906 (2000) 8.
28 Y. Huang, E.L. Mather, J.L. Bell and M. Madou, *Anal. Bioanal. Chem.*, 372 (2002) 49.
29 J.A. Jordan and M.B. Durson, *J. Clin. Microbiol.*, 38 (2000) 2574.
30 P. Belgrader, W. Benette, D. Hadley, J. Richards, P. Statton, R. Mariella, Jr. and F. Milanovich, *Science*, 284 (1999) 449.
31 J. Cheng, E.L. Sheldon, L. Wu, M.J. Heller and J.P. O'Connell, *Anal. Chem.*, 70 (1998) 2321.
32 J. Sambrook, E.F. Fritsch and T. Maniatis, *Molecular Cloning*, Cold Spring Harbor Laboratory Press, New York, 2nd Edn. 1989.
33 J. Blanchard, *J. Chromatogr.*, 226 (1981) 455.
34 R.D. McDowall, *J. Chromatogr.*, 495 (1989) 3.
35 R. Boom, C.J. Sol, M.M. Salimans, C.L. Jansen, P.M. Wertheim-van Dillen and J. Van der Noordaa, *J. Clin. Microbiol.*, 28 (1990) 1875.
36 J.D. Harding, G. Gebeyehu, R. Bebee, D. Simms and L. Klevan, *Nucleic Acid Res.*, 17 (1989) 6947.
37 G. Konat, G. Gantt, I. Laszkiewicz and E.L. Hogan, *Exp. Cell Res.*, 190 (1990) 294.
38 P. Chomczynski, K. Mackey, R. Drews and W. Wilfinger, *Biotechniques*, 22 (1997) 550.
39 D.V. Morrissey, M. Lombardo, J.K. Eldredge, K.R. Kearney, E.P. Groody and M.L. Collins, *Anal. Biochem.*, 181 (1989) 345.
40 Y. Miura, Y. Ichikawa, T. Ishikawa, M. Ogura, R. de Fries, H. Shimada and M. Mitsuhashi, *Clin. Chem.*, 42 (1996) 1758.
41 J.M. Wages, Jr., G.M. Wages, P. Matthews, D. Weller and J. Summerton, *Biotechniques*, 23 (1997) 1116.
42 P.E. Nielsen, M. Egholm, R.H. Berg and O. Buchardt, *Science*, 254 (1991) 1497.
43 D.P. Chandler, J.R. Stults, K.K. Anderson, S. Cebula, B.L. Schuck and F.J. Brockman, *Anal. Biochem.*, 283 (2000) 241.
44 H. Orum, P.E. Nielsen, M. Jorgensen, C. Larsson, C. Stanley and T. Koch, *Biotechniques*, 19 (1995) 472.
45 T.C.H. Hsuih, Y.N. Park, C. Zaretsky, F. Wu, S. Tyagi, F.R. Kramer, R. Sperling and D. Zhang, *J. Clin. Microbiol.*, 34 (1996) 501.
46 G.T. Walker, M.S. Fraiser, J.L. Schram, M.C. Little, K.G. Nadeau and D.P. Malinowski, *Nucleic Acids Res.*, 20 (1992) 1691.
47 D.L. Kacian and T.J. Fultz, *US Patent*, 5,399,491, 1995
48 T. Notomi, H. Okayama, H. Masubuchi, T. Yonekawa, K. Watanabe, N. Amino and T. Hase, *Nucleic Acids Res.*, 28 (2000) e63.
49 C.H. Mastrangelo, M.A. Burns and D.T. Burke, *Proc. IEEE*, 86 (1998) 1769.
50 D.D. Cunningham, *Anal. Chim. Acta*, 429 (2001) 1.
51 E. Verpoorte, *Electrophoresis*, 23 (2002) 677.
52 G.J. Kellogg, T.E. Arnold, B.L.D.C. Duffy and N.F. Sheppard, Jr., in A. Van der Berg, W. Olthius and P. Bergveld (Eds.), *Micro Total Analysis Systems*, Kluwer, Dordrecht, 2000, p. 239.
53 M. Gilar, A. Belenky and B.H. Wang, *J. Chromatogr. A*, 921 (2001) 3.
54 P.R. Brown, C.S. Robb and S.E. Geldart, *J. Chromatogr. A*, 965 (2002) 163.
55 W.E. Cohn, *Science*, 109 (1949) 377.
56 C.G. Horvath, B.A. Preiss and S.R. Lipsky, *Anal. Chem.*, 39 (1967) 1422.
57 C.G. Horvath and S.R. Lipsky, *Anal. Chem.*, 41 (1969) 1227.
58 P.R. Brown, *J. Chromatogr.*, 52 (1970) 257.
59 P.R. Brown, in P.R. Brown (Ed.), *HPLC in Nucleic Acid Research*, Dekker, New York, 1984, p. 49.

60 C.G. Horvath, *J. Chromatogr. A*, 806 (1998) 3.
61 C.G. Horvath and S.R. Lipsky, *Anal. Chem.*, 41 (1969) 1227.
62 C.A. Bruins, M.N. Munk and F.R. MacDonald, *Clin. Chem.*, 16 (1970) 201.
63 R.A. Hartwick and P.R. Brown, *J. Chromatogr.*, 112 (1975) 651.
64 H. Staghlberg, B. Jonsson and Cs. Horvath, *Anal. Chem.*, 63 (1991) 1867.
65 Y. Kato, Y. Yamasaki, A. Onaka, T. Kitamura, T. Hashimoto, T. Murotsu, S. Fukushige and M. Matsubara, *J. Chromatogr.*, 478 (1989) 264.
66 W. Mueller, *Eur. J. Biochem.*, 155 (1986) 203.
67 M. Merion, W. Warren, C. Stacey and M.E. Dwyer, *BioTechniques*, 6 (1998) 246.
68 H. Yamakawa, K.I. Higashino and O. Ohara, *Anal. Biochem.*, 240 (1996) 242.
69 F. Fack and V. Sarantoglou, *Nucleic Acids Res.*, 19 (1991) 4181.
70 W. Bloch, *Eur. Patent*, Publication No. 0507591A2.
71 M.W. Dong, J.R. Grant and B.R. Larsen, *BioChromatography*, 4 (1989) 19.
72 P.J.M. Van Haastert, *J. Chromatogr.*, 210 (1981) 241.
73 M.P. Henry, in W.S. Hancock (Ed.), *High-performance Liquid Chromatography in Biotechnology*, Wiley, New York, 1990, p. 205.
74 Y. Baba and M.K. Ito, *J. Chromatogr.*, 485 (1989) 647.
75 Y.F. Ma, S.C. Lin, C. Horvath, U. Yang and D.M. Crothers, *J. Chromatogr.*, 508 (1990) 61.
76 S. Nakatani, T. Tsuda, Y. Yamasaki, H. Moriyama, H. Watanabe and Y. Kato, presented at the *15th International Symposium on Protein, Peptide and Polynucleotide Analysis*, Boston, MA, 18–21 November 1995, poster.
77 R.A. Hartwick and P.R. Brown, *J. Chromatogr.*, 126 (1976) 679.
78 H.A. Scoble, in P.R. Brown (Ed.), *HPLC in Nucleic Acid Research*, Dekker, New York, 1984, p. 17.
79 S. Assenza and P.R. Brown, *J. Chromatogr.*, 181 (1980) 1210.
80 R.A. Hartwick, C. Grill and P.R. Brown, *Anal. Chem.*, 51 (1979) 34.
81 P.R. Brown, A.M. Krstulovic and R.A. Hartwick, *J. Clin. Chem. Clin. Biochem.*, 14 (1976) 282.
82 R.A. Hartwick, S.P. Assenza and P.R. Brown, *J. Chromatogr.*, 186 (1979) 647.
83 J.M. Karle, L.W. Anderson, D.D. Dietrick and R.L. Cysyk, *Anal. Biochem.*, 109 (1980) 41.
84 K. Nakano, S.P. Assenza and P.R. Brown, *J. Chromatogr.*, 233 (1982) 51.
85 C.W. Gehrke, K.C. Kuo and R.W. Zumwalt, *J. Chromatogr.*, 188 (1980) 129.
86 C.W. Gehrke and F.C. Kuo, *J. Chromatogr.*, 471 (1989) 3.
87 K.H. Schram, *Mass Spectrom. Rev.*, 17 (1998) 131.
88 C. Lengger, G. Schoech and H. Topp, *Anal. Biochem.*, 287 (2000) 65.
89 N.E. Hoffman and J.C. Liao, *Anal. Chem.*, 49 (1977) 2231.
90 P.J.M. Van Haastert, *J. Chromatogr.*, 210 (1981) 229.
91 P.A. Doris, P.J. Oefner, B.S. Chilton and A. Hayward-Lester, *J. Chromatogr. A*, 806 (1998) 47.
92 G. Zon, in W.S. Hancock (Ed.), *High-performance Liquid Chromatography in Biotechnology*, Wiley, New York, 1990, p. 301.
93 K. Yamazaki, H. Tomizawa, A. Miyanaga, O. Ishikawa, S. Nakatani and H. Moriyama, *Nucleic Acids Symp. Ser.*, 34 (1995) 149.
94 C.G. Huber, P.J. Oefner and G.K. Bonn, *Anal. Chem.*, 67 (1995) 578.
95 W. Xiao and P.J. Oefner, *Hum. Mutat.*, 17 (2001) 439.
96 S.H. Ke and R.M. Wartell, *Nucleic Acid Res.*, 21 (1993) 5137.
97 F. Abou-ela, D. Koh, I. Tinoco and F.H. Martin, *Nucleic Acid Res.*, 13 (1985) 4811.
98 C.G. Huber, P.J. Oefner, E. Preuss and G.K. Bonn, *Nucleic Acid Res.*, 21 (1993) 1061.
99 C.G. Huber, P.J. Oefner and G.K. Bonn, *Anal. Biochem.*, 212 (1993) 351.
100 C.G. Huber, A. Premstaller, H. Oberacher, H. Xiao, G.K. Bonn and P.J. Oefner, *J. Biochem. Biophys. Meth.*, 47 (2001) 5.

101 E. Gross, N. Arnold, J. Goetter, U. Schwarz-Boeger and M. Kiechle, *Hum. Genet.*, 105 (1999) 72.
102 J.I. Spiegelman, M.N. Mindrions and P.J. Oefner, *Biotechniques*, 29 (2000) 1084.
103 A. Hayward-Lester, C.A. Hinojos, B. Nurowska, A. Hewetson, S. Sabatini, P.J. Oefner and P.A. Doris, *J. Hypertens.*, 17 (1999) 1081.
104 P.A. Underhill, G. Passarino, A.A. Lin, P. Shen, R.A. Foley, M. Lahr, P.J. Oefner and L.L. Cavalli-Sforza, *Ann. Hum. Genet.*, 65 (2001) 43.
105 M. Giordano, P.J. Oefner, P.A. Underhill, L.L. Cavalli, R. Tosi and P.M. Richiardi, *Genomics*, 56 (1999) 247.
106 E. Schaeffeler, T. Lang, U.M. Zanger, M. Eichelbaum and M. Schwab, *Clin. Chem.*, 47 (2001) 548.
107 P.J. Oefner, *J. Chromatogr. B*, 739 (2000) 345.
108 J.E. Larson, S.C. Hardies, R.K. Patient and R.D. Wells, *J. Biol. Chem.*, 254 (1979) 5535.
109 R. Bischoff and L.W. McLaughlin, *J. Chromatogr.*, 270 (1983) 117.
110 L.W. McLaughlin, *Chem. Rev.*, 89 (1989) 309.
111 Y. Kato, M. Sasaki, T. Hashimoto, T. Murotsu, S. Fukushige and K. Matsubara, *J. Chromatogr.*, 266 (1983) 341.
112 T. Anderson, M. Carlsson, L. Hagel, P.-A. Pernemalm and J.-C. Janson, *J. Chromatogr.*, 326 (1985) 33.
113 H.G. Barth and B.E. Boyes, *Anal. Chem.*, 62 (1990) 381R.
114 T.A. Goss, M. Bard and H.W. Jarrett, *J. Chromatogr.*, 588 (1991) 157.
115 S.J. Burton, *Am. Biotech. Lab.*, 14 (1996) 64.
116 E. Bernal-Mendez and C.J. Leumann, *J. Biol. Chem.*, 276 (2001) 35320.
117 G. Kahl (Ed.), *Dictionary of Gene Technology*, Wiley-VCH, Weiheim, 2001, 2nd Edn. p. 315.
118 D. Rickwood and B.D. Hames (Eds.), *Gel Electrophoresis of Nucleic Acids*, Irl Press, 1990.
119 J.R. Cann, *Anal. Biochem.*, 15 (1996) 1.
120 M.D. Miller, Jr., R.A. Acey, L.Y.-T. Lee and A.J. Edwards, *Electrophoresis*, 22 (2001) 791.
121 B. Cedervall and T. Radivoyevitch, *Electrophoresis*, 17 (1996) 1080.
122 J. Maule, *Mol. Biotechnol.*, 9 (1998) 107.
123 H. Lodish, A. Berk, S.L. Zipursky, P. Matsudaira, D. Baltimore and J.E. Damell, *Molecular Cell Biology*, W.H. Freeman, New York, 4th Edn. 2000.
124 R.M. Wartell, S.H. Hosseini and J.D. Moran, *Nucleic Acid Res.*, 18 (1990) 2699.
125 R.M. Wartell, S. Hosseini, S. Powell and J. Zhu, *J. Chromatogr. A*, 806 (1998) 169.
126 R. Fodde and M. Losekoot, *Hum. Mutat.*, 3 (1994) 83.
127 G. Muyzer and K. Smalla, *Antonie van Leeuwenhoek*, 73 (1998) 127.
128 H. Schickle, B.J. Lamb and S.M. Hanash, *Electrophoresis*, 20 (1999) 1233.
129 J. Vijg and N.J. van Orsouw, *Electrophoresis*, 20 (1999) 1239.
130 Y. Wu, R. Hofstra, H. Scheffer, A.G. Uitterlinden, E. Mullaart, C.H.C.M. Buys and J. Vijg, *Hum. Mut.*, 8 (1996) 160.
131 N.J. van Orsouw, D. Li, P. van der Vlies, H. Scheffer, C. Eng, C.H.C.M. Buys, F.P. Li and J. Vijg, *Hum. Mol. Genet.*, 5 (1996) 755.
132 M. Nystroem-Lahti, Y. Wu, A.-L. Moisio, R.M.W. Hofstra, J. Osinga, J.-P. Mecklin, H.J. Jarvinen, J. Leisti, C.H.C.M. Buys, A. de la Chapelle and P. Peltomaki, *Hum. Mol. Genet.*, 5 (1996) 763.
133 N.J. van Orsour, X. Zhang, J.Y. Wei, D.R. Johns and J. Vijg, *Genomics*, 52 (1998) 27.
134 A. Guttman and Z. Ronai, *Electrophoresis*, 21 (2001) 3295.
135 A. Guttman, *LC–GC*, 17 (1999) 1020.
136 I.N.M. Day, E. Spanakis, X. Chen and S.D. O'Dell, *Electrophoresis*, 20 (1999) 1250.

137 C. Heller (Ed.), *Analysis of Nucleic Acids by Capillary Electrophoresis*, Vieweg, Braunschweig/Wiesbaden, 1997.
138 K.R. Mitchelson and J. Cheng (Eds.), *Capillary Electrophoresis of Nucleic Acids, Vol. I: Introduction to the Capillary Electrophoresis of Nucleic Acids*, Humana, Totowa, 2001.
139 K.R. Mitchelson and J. Cheng (Eds.), *Capillary Electrophoresis of Nucleic Acids, Vol. II Practical Applications of Capillary Electrophoresis*, Humana, Totowa, 2001.
140 C. Heller, *Electrophoresis*, 22 (2001) 629.
141 M.N. Albarghouthi and A.E. Barron, *Electrophoresis*, 21 (2000) 4096.
142 I. Kheterpal and R.A. Mathies, *Anal. Chem.*, 71 (1999) 31A.
143 Y. Kiba and Y. Baba, in J. Cazes (Ed.), *CE in Encyclopedia of Chromatography*, Dekker, New York, 2001, p. 556.
144 P.G. Righetti, C. Gelfi and M.R. D'Acunto, *Electrophoresis*, 23 (2002) 1361.
145 A.S. Cohen, D.R. Najarian, A. Paulus, A. Guttman, J.A. Smith and B.L. Karger, *Proc. Natl Acad. Sci. USA*, 85 (1988) 9660.
146 D.N. Heiger, A.S. Cohen and B.L. Karger, *J. Chromatogr.*, 516 (1990) 33.
147 H. Drossman, J.A. Lucky, A.J. Kostichka, J. D'Cunha and L.M. Smith, *Anal. Chem.*, 62 (1990) 900.
148 K. Ulfelder, H.E. Schwartz, J.H. Hall and F.J. Sunzeri, *Anal. Biochem.*, 200 (1992) 260.
149 H.E. Schwartz, K. Ulfelder, F.J. Sunzeri, M.P. Busch and R.G. Brownlee, *J. Chromatogr.*, 559 (1991) 267.
150 C. Heller, in K.R. Mitchelson and J. Cheng (Eds.), *Capillary Electrophoresis of Nucleic Acids*, *Vol. I*, Humana, Totowa, 2001, p. 111.
151 H.J. Issaq, in K.R. Mitchelson and J. Cheng (Eds.), *Capillary Electrophoresis of Nucleic Acids*, *Vol. I*, Humana, Totowa, 2001, p. 189.
152 H. Swerdlow and R. Gesteland, *Nucleic Acid Res.*, 18 (1990) 1415.
153 A.S. Cohen, D.R. Najarina and B.L. Karger, *J. Chromatogr.*, 516 (1990) 49.
154 R.A. Mathies and X.C. Huang, *Nature*, 359 (1992) 167.
155 H. Kamabara and S. Takahashi, *Nature*, 361 (1993) 565.
156 L. Mitnik, M. Novotny, C. Felten, S. Buonocore, L. Koutny and D. Schmalzing, *Electrophoresis*, 22 (2001) 4104.
157 J.Z. Zhang, Y. Fang, J.Y. Hou, H.J. Ren, R. Jiang, P. Roos and N.J. Dovichi, *Anal. Chem.*, 67 (1995) 4589.
158 M.J. Rocheleau, R.J. Grey, D.Y. Chen, H.R. Harke and N.J. Dovichi, *Electrophoresis*, 13 (1992) 484.
159 Y. Endo, C. Yoshida and Y. Baba, *J. Biochem. Biophys. Methods*, 41 (1999) 133.
160 G. Xue and E.S. Yeung, *Electrophoresis*, 23 (2002) 1490.
161 C. Heller, in K.R Mitchelson and J. Cheng (Eds.), *Capillary Electrophoresis of Nucleic Acids*, *Vol. I*, Humana, Totowa, 2001, p. 111.
162 P.G. Righetti and C. Gelfi, *Electrophoresis*, 18 (1997) 1709.
163 Y. Baba, *J. Chromatogr. B*, 687 (1996) 271.
164 J. Ren, *J. Chromatogr. B*, 741 (2000) 115.
165 I.V. Kourkine, C.N. Hestekin and A.E. Barron, *Electrophoresis*, 10 (2002) 1375.
166 H. Tian, A. Jaquins-Gerstl, N. Munro, M. Trucco, L.C. Brody and J.P. Landers, *Genomics*, 63 (2000) 25.
167 I.V. Kourine, C.N. Hestekin, B.A. Buchholz and A.E. Barron, *Anal. Chem.*, 74 (2002) 2565.
168 W. Liu, D. Han, J. Yuan and J. Andrieu, *Nature*, 368 (1994) 269.
169 K. Okano and H. Kambara, *Gene*, 176 (1996) 231.
170 M.O. Dorschner, D. Barden and K. Stephen, *J. Mol. Diagn.*, 4 (2002) 108.
171 C.E. Mitchell, S.A. Belinsky and J.F. Lechner, *Anal. Biochem.*, 224 (1995) 148.

172 J.M. Butler and D.J. Reeder, in K.R. Mitchelson and J. Cheng (Eds.), *Capillary Electrophoresis of Nucleic Acids*, *Vol. II*, Humana, Totowa, 2001, p. 49.
173 K. Lazaruk, P.S. Walsh, F. Oaks, D. Gilbert, B.B. Rosenblum, S. Menchen, D. Scheiber, H.M. Wenz, C. Holt and J. Wallin, *Electrophoresis*, 19 (1998) 86.
174 N. Zhang, H. Tan and E.S. Yeung, *Anal. Chem.*, 71 (1999) 1138.
175 T.R. Moretti, A.L. Baumstark, D.A. Defenbaugh, K.M. Keys, A.L. Brown and B. Budowle, *J. Forensic Sci.*, 46 (2001) 661.
176 Y. Baba, M. Tsuhako, T. Sawa, M. Akashi and E. Yashima, *Anal. Chem.*, 64 (1992) 1920.
177 Y. Baba, T. Sawa, A. Kishida and M. Akash, *Electrophoresis*, 19 (1998) 433.
178 Y. Baba, in K.R. Mitchelson and J. Cheng (Eds.), *Capillary Electrophoresis of Nucleic Acids*, *Vol. II*, Humana, Totowa, 2001, p. 347.
179 M.J. Pearce and N.D. Watson, in K.R. Mitchelson and J. Cheng (Eds.), *Capillary Electrophoresis of Nucleic Acids*, *Vol. I*, Humana, Totowa, 2001, p. 347.
180 J.M. Leeds, M.J. Graham, L. Truong and L.L. Cummins, *Anal. Biochem.*, 235 (1996) 36.
181 J. Xian, in K.R. Mitchelson and J. Cheng (Eds.), *Capillary Electrophoresis of Nucleic Acids*, *Vol. II*, Humana, Totowa, 2001, p. 355.
182 M.D. Morris, J.J. Schwinefus and O. de Carmejane, in K.R. Mitchelson and J. Cheng (Eds.), *Capillary Electrophoresis of Nucleic Acids*, *Vol. I*, Humana, Totowa, 2001, p. 307.
183 H.M. Liebich, G. Xu, C. Di Stefano and R. Lehmann, *J. Chromatogr. A*, 793 (1998) 341.
184 R. Zhao, G. Xu, B. Yue, H.M. Liebich and Y. Zhang, *J. Chromatogr. A*, 828 (1998) 489.
185 E. Katsivela and M.G. Hoefle, *J. Chromatogr. A*, 700 (1995) 125.
186 E. Katsivela and M.G. Hoefle, *J. Chromatogr. A*, 717 (1995) 91.
187 J. Ding and P. Vouros, *Anal. Chem.*, 69 (1997) 379.
188 J. Ding and P. Vorous, *J. Chromatogr. A*, 887 (2000) 103.
189 B. Behnke and E. Bayer, *J. Chromatogr. A*, 680 (1994) 93.
190 V. Dolnik, S. Liu and S. Jovanovich, *Electrophoresis*, 21 (2000) 41.
191 A. Manz, N. Graber and H.M. Widmer, *Sens. Actuat. B*, 1 (1990) 244.
192 G.J.M. Bruin, *Electrophoresis*, 21 (2000) 3931.
193 A.J. Gawron, R.S. Martin and S.M. Lunte, *Eur. J. Pharm. Sci.*, 14 (2001) 1.
194 J. Khandurina and A. Guttman, *J. Chromatogr. A*, 943 (2002) 159.
195 D.R. Reyes, D. Iossifidis, P. Auroux and A. Manz, *Anal. Chem.*, 74 (2002) 2623.
196 P. Auroux, D. Iossifidis, D.R. Reyes and A. Manz, *Anal. Chem.*, 74 (2002) 2637.
197 T.D. Boone, Z.H. Fan, H.H. Hooper, A.J. Ricco, H. Tan and S.J. Williams, *Anal. Chem.*, 74 (2002) 78A.
198 D.C. Duffy, J.C. McDonald, O.J.A. Shueller and G.M. Whitesides, *Anal. Chem.*, 70 (1998) 4974.
199 M. Ueda, H. Abe, H. Kuyama, H. Nakanishi, A. Arai and Y. Baba, *Bioimages*, 7 (1999) 157.
200 J.P. Alarie, S.C. Jacobson, C.T. Culbertson and J.M. Ramsey, *Electrophoresis*, 21 (2000) 100.
201 S.C. Jacobson, R. Hergenoeder, L.B. Koutny, R.J. Warmack and J.M. Ramsey, *Anal. Chem.*, 66 (1994) 1107.
202 L.L. Shultz-Lockyear, C.L. Colyer, Z.H. Fan, K.I. Roy and D.J. Harrison, *Electrophoresis*, 20 (1999) 529.
203 Z. Ronai, C. Barta, M. Sasvari-Szekely and A. Guttman, *Electrophoresis*, 22 (2001) 294.
204 Q. Gao, Y. Shi and S. Liu, *Fresenius J. Anal. Chem.*, 371 (2001) 137–145.
205 I.L. Medintz, B.M. Paegel, R.G. Blazej, C.A. Emrich, L. Berti, J.R. Scherer and R.A. Mathies, *Electrophoresis*, 22 (2001) 3845.
206 A.T. Woolley, G.F. Sensabaugh and R.A. Mathies, *Anal. Chem.*, 69 (1997) 2181.
207 R.A. Mathies, C.A. Emrich, H. Tian, I. Medintz, L. Berti and J. Tom, in J.M. Ramsey and A. van der Berg (Eds.), *Micro Total Analysis Systems*, Kluwer, Dordrecht, 2001, p. 13.

208 C.S. Effenhauser, A. Paulus, A. Manz and H.M. Widmer, *Anal. Chem.*, 66 (1994) 2949.
209 H. Chou, C. Spence, A. Scherer and S. Quake, *Proc. Natl Acad. Sci. USA*, 96 (1999) 11.
210 M. Ueda, Y. Endo, H. Abe, H. Huyama, H. Nakanishi, A. Arai and Y. Baba, *Electrophoresis*, 22 (2001) 217.
211 A.T. Wooley and R.A. Mathies, *Proc. Natl Acad. Sci. USA*, 91 (1994) 11348.
212 Y. Shi, P.C. Simpson, J.R. Scherer, D. Wexler, C. Skibola, M.T. Smith and R.A. Mathies, *Anal. Chem.*, 71 (1999) 5354.
213 L. Zhang, F. Dang and Y. Baba, *Electrophoresis*, 23 (2002) 2341.
214 C.L. Colyer, T. Tang, N. Chiem and D. Jed Harrison, *Electrophoresis*, 18 (1997) 1733.
215 L.J. Jin, J. Ferrance and J.P. Landers, *BioTechniques*, 31 (2001) 1332.
216 E. Verpoorte, *Electrophoresis*, 23 (2002) 677.
217 A.P. Sassi, A. Paulus, I.D. Cruzado, T. Bjornson and H.H. Hooper, *J. Chromatogr. A*, 894 (2000) 203.
218 D. Schamlzing, L. Koutny, D. Chisholm, A. Adourian, P. Matsudaira and D. Ehrlich, *Anal. Biochem.*, 270 (1999) 148.
219 Y. Chen, W. Wang, K. Young, T. Chang and S. Chen, *Clin. Chem.*, 45 (1999) 1938.
220 H. Tian, L.C. Brody and J.P. Landers, *Genome Res.*, 10 (2000) 1403.
221 H. Tian, L.C. Brody, S. Fan, Z. Huang and J.P. Landers, *Clin. Chem.*, 47 (2001) 173.
222 I. Medintz, W.W. Wong, G. Sensabaugh and R.A. Mathies, *Electrophoresis*, 21 (2000) 2352.
223 I. Medintz, W.W. Wong, L. Berti, L. Shiow, J. Tom, J. Scherer, G. Sensabaugh and R.A. Mathies, *Genome Res.*, 11 (2001) 413.
224 D. Schmalzing, A. Belenky, M.A. Novotny, L. Koutny, O. Salas-Solano, S. El-Difrawy, A. Adourian, P. Matsudaira and D. Ehrlich, *Nucleic Acid Res.*, 28 (2000) e43.
225 H. Tian, A. Jaquins-Gerstl, N. Munro, M. Trucco, L.C. Brody and J.P. Landers, *Genomics*, 63 (2000) 25.
226 M. Jabasini, L. Zhang, F. Dang, F. Xu, M.R. Almofli, A.A. Ewis, J. Lee, Y. Nakahori and Y. Baba, *Electrophoresis*, 23 (2002) 1537.
227 A.T. Woolley and R.A. Mathies, *Anal. Chem.*, 67 (1995) 3676.
228 L. Mitnik, M. Novotny, C. Felten, S. Buonocore, L. Koutny and D. Schmalzing, *Electrophoresis*, 22 (2001) 4104.
229 D. Schmalzing, L. Kountny, O. Salas-Solano, A. Adourian, P. Matsudaira and D. Ehrlich, *Electrophoresis*, 20 (1999) 3066.
230 E. Carrilho, *Electrophoresis*, 21 (2000) 55.
231 S. Liu, Y. Shi, W.W. Ja and R.A. Mathies, *Anal. Chem.*, 71 (1999) 566.
232 D. Schmalzing, A. Adourian, L. Koutny, L. Ziaugra, P. Matsudaira and D. Ehrlich, *Anal. Chem.*, 70 (1998) 2303.
233 L. Koutny, D. Schmalzing, O. Salas-Solano, S. El-Difrawy, A. Adourian, S. Buoncore, K. Abbey, P. McEwan, P. Matsudaira and D. Ehrlich, *Anal. Chem.*, 72 (2000) 3388.
234 B.A. Buchholz, E.A. Doherty, M.N. Albarghouthi, F.M. Bogdan, J.M. Zahn and A.E. Borron, *Anal. Chem.*, 73 (2001) 157.
235 B.M. Paegel, C.A. Emrich, G.J. Wedemayer, J.R. Scherer and R.A. Mathies, *Proc. Natl Acad. Sci.*, 99 (2002) 574.
236 V. Studer, P. Youinou, A. Pepin, A. Lebib, Y. Chen and Y. Baba, in J.M. Ramsey and A. van der Berg (Eds.), *Micro Total Analysis Systems*, Kluwer, Dordrecht, 2001, p. 201.
237 S.A. Soper, B.L. Legendre and D.C. Williams, *Anal. Chem.*, 67 (1995) 4358.
238 S.C. Hung, R.A. Mathies and A.N. Glazer, *Anal. Biochem.*, 255 (1998) 32.
239 S. Takahashi, K. Murakami, T. Anazawa and H. Kambara, *Anal. Chem.*, 66 (1994) 1021.
240 J.R. Scherer, I. Kheterpal, A. Radhakrishnan, W.W. Ja and R.A. Mathies, *Electrophoresis*, 20 (1999) 1508.

241 C.G. Huber and H. Oberacher, *Mass Spectrom. Rev.*, 20 (2001) 310.
242 C.G. Huber and A. Krajete, *Anal. Chem.*, 71 (1999) 3730.
243 H.J. Gaus, S.R. Owens, M. Winniman, S. Cooper and L.L. Cummins, *Anal. Chem.*, 69 (1997) 313.
244 E.T. Gangl, R.J. Turesky and P. Vouros, *Anal. Chem.*, 73 (2001) 2397.
245 H. Oberacher, W. Parson, R. Muehlmann and C.G. Huber, *Anal. Chem.*, 73 (2001) 5109.
246 H. Oberacher, P.J. Oefner, W. Parson and C.G. Huber, *Angew. Chem. Int. Ed.*, 40 (2001) 3828.
247 A.H. Fang, W.A. Smith, P. Vouros and R.C. Gupta, *Biochem. Biophys. Res. Commun.*, 23 (2001) 281.
248 J.C. Lindon, J.K. Nicholson and I.D. Wilson, *Adv. Chromatogr.*, 63 (1995) 315.
249 S. Bourne, *Am. Lab.*, 30 (1998) 17F.
250 J. Wang, *Talanta*, 56 (2002) 223.
251 R.F. Service, *Science*, 287 (2000) 1954.
252 A.D. Roses, *Nature*, 405 (2000) 857.
253 Y. Baba and D. Figeys, *Genomics and Proteomics for Analytical Chemists*, Wiley, New York, 2001.

This page is intentionally left blank

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 20

Drugs

IAN D. WILSON

CONTENTS

20.1 INTRODUCTION

The heterogeneous chemical nature of drugs makes a comprehensive treatment of their chromatography somewhat problematic. Taken together with the vast number of potential applications, in areas as diverse as preparative separations, drug purity determination, drug testing for abuse and forensic toxicology, biological fluid analysis in drug discovery and development as well as therapeutic monitoring, the task of summarizing the general practice and advances in this area in a single chapter becomes totally impractical. Layered upon this already complex matrix are additional issues, such as chiral separations, which have also been of considerable interest to drug analysts. It is also clear, from even a brief examination of the literature, that the problems faced in trying to determine low levels of impurities in a bulk drug or formulation, where many, or all, of the components of interest are structurally related, are quite different from those faced when trace amounts of drug are to be quantified in complex biological fluid samples. Similarly, the difficulties of the forensic toxicologist, who tries to identify unknown drugs in a biological fluid are different from those faced in therapeutic drug monitoring. No attempt has therefore been made to provide an exhaustive treatment of the subject of the chromatography of drugs and, in contrast to the treatment provided in the previous edition of this work [1], coverage of individual compound classes (*e.g.*, steroids or anti-depressants) has not been undertaken. This is for the very simple reason that with current, highly efficient, methods for interrogating the literature any researcher requiring information on the chromatography of specific compounds can obtain this within a very short time. Instead, topics have been chosen that highlight areas of rapid development, or that are of general interest.

While there is a clear trend toward the dominance of high-performance liquid chromatography (HPLC) in the separation and analysis of drugs, other techniques, such as thin-layer (or planar) (TLC) and gas/liquid chromatography (GLC) continue to have many applications in the separation and analysis of drugs. Other techniques that were once considered to show great promise, such as supercritical-fluid chromatography, have in reality made little impact and are still confined to a few niche applications. This trend towards increasing use of HPLC has been evident for some time and was highlighted in the previous edition of this volume [1]. In fact, much of what was said in the previous edition remains true of current practice. Perhaps the single most important advance that has been seen in the chromatographic analysis of drugs since the last edition has been the rise of the combined techniques, which barely rated a mention in the previous work. This mainly applies to the combination of HPLC with mass spectrometry (HPLC/MS) and tandem MS (MS/MS), but significant advances have also resulted from the combination of HPLC with nuclear magnetic resonance (NMR) spectroscopy. There is no doubt that the successful interfacing of HPLC with MS has revolutionized the way drugs are detected and identified. Further, as a result of the specificity of detection that MS techniques provide, the need for chromatographic resolution and total analysis time has been reduced. Naturally, the higher capital cost of a tandem mass spectrometer compared to, *e.g.*, a UV detector has also resulted in pressure for more rapid analysis time to ensure the most efficient use of the equipment. This coupling of methods (sometimes referred to as "hyphenation") has had a

particularly profound effect in the area of the analysis of drugs and their metabolites in biological fluids ("bioanalysis") where sensitivity and specificity are of paramount importance. Another aspect that has received much attention is sample preparation. This topic is also considered below, with emphasis on newer developments.

20.2 PREPARATION OF BIOLOGICAL SAMPLES

There are many fields of application for the analysis of drugs and their metabolites in biological fluids, including forensic analysis for drug abuse or intoxication (Chap. 23), all the various stages of drug discovery and development (including drug metabolism studies), and therapeutic drug monitoring. Sample preparation is used to provide samples in a form compatible with the chromatographic system, bringing analytes into the range required by the detector. It also serves to remove substances, such as proteins and other interfering material, that would lead to a degradation in performance. Sometimes all of these factors will be important, while in other cases only one will be significant, and the type of sample preparation performed will vary accordingly. In the majority of cases, some form of sample preparation is needed in order to remove matrix interferences and protect both column and mass spectrometer from contamination. Sample preparation techniques provide an important means for the introduction of specificity into the analysis of biological fluids. The balance between the amount of purification and concentration required is defined by the sensitivity and specificity of the detector, and this is clearly different for, *e.g.*, mass spectrometry and UV detection. The balance can also change during the course of drug development. Thus, methods used early on, in the discovery and optimization phases, are generally not required to be as sensitive as those that must be employed in the first human volunteer studies, or in the later stages of clinical development. Several good sources of reference are now available on various aspects of sample preparation [2–8].

20.2.1 Protein precipitation

Protein precipitation, where the sample is mixed with an organic solvent, such as acetonitrile, methanol, or ethanol, or with an acid, such as perchloric acid, is often the method of choice when drug concentrations are high and detection is by a sensitive and specific method, such as MS or MS/MS, so that the dilution of the sample is not a limiting factor. The technique is often well suited to the early stages of drug discovery, where doses, and therefore plasma concentrations, are high. Systems now available for the automation of protein precipitation in 96-well-plate format greatly increase the potential of the technique. The advantage of protein precipitation is its simplicity, but the resulting extracts still contain large amounts of low-molecular-mass contaminants. Care must therefore be taken to introduce the minimum amount of sample into the system to avoid deterioration in the performance of column and detector (hence the requirement for sensitive detection or high initial sample concentration). There can also be problems associated with the suppression of ionization (discussed below). Examples of methods involving automated protein precipitation in the 96-well format include an assays for

salbutamol [9], a 5-hydroxytryptamine 1D agonist in rat plasma [10], and the highly protein-bound metal chelator ICL670 [11].

20.2.2 Liquid/liquid extraction

Although it has fallen out of fashion to some extent because of the widespread misconception that that liquid/liquid extraction (LLE) is difficult to automate, the technique still offers many benefits. Indeed, an example of a recent method employing automated LLE for the EGFR tyrosine kinase inhibitor irressa has been described, extraction being performed with a Xymate robot [12]. As well as such robotic methods, there are still many applications in the literature of "conventional" LLE. More recently, advances in the automation of the technique have resulted in the development of micro-LLE in a 96-well format [13]. Another recent example of this type of automated LLE is provided by studies on the extraction of diphenylhydramine, desipramine, chlorpheniramine, trimipramine and lidocaine from rat plasma with ethyl acetate [14].

20.2.3 Solid-phase extraction

20.2.3.1 Conventional approaches

Solid-phase extraction (SPE) can be performed off-line, either manually or automatically with cartridges, automatically in the 96-well format, or on-line with a variant of the column-switching technique (see below). Like LLE extraction, SPE can by now be considered to be a mature technique, most of it performed with alkyl-bonded silicas (C_2, C_8, C_{18}, etc.), either in cartridges or, increasingly, in 96-well (or greater) format. However, areas where innovations have been made include the introduction of mixed-mode stationary phases, where a combination of ion exchange and hydrophobic interactions are used to obtain greater specificity of extraction, and polymeric materials, such as the Oasis phase [15]. In terms of instrumentation and automation, the development of SPE in the 96-well (and higher) format has greatly helped in automating the technique. Recent examples of methods employing SPE in the 96-well format include methods for betamethasone in rat plasma, extracted onto the Oasis phase [16], and an alpha-1a receptor antagonist in human plasma with extraction *via* a mixed-phase cation SPE material [17]. The Gilson ASPEC instrument also provides a method for automated SPE. An example is provided by the analysis of atovaquone in plasma and whole blood. In this method, proteins were precipitated from plasma and centrifuged. The liquid phase was then mixed with phosphate buffer prior to application to C_8 SPE cartridges. The extracts from these cartridges were then analyzed by HPLC with UV detection [18]. Another recent example of the use of this type of automated sample preparation is the determination of nortriptyline in human serum. The analysis was also accomplished by on-line SPE onto cyanopropyl SPE cartridges, followed by HPLC on a C_{18}-bonded column with UV detection [19]. Comparison with a LLE method showed good agreement. Alternative automated SPE methods, employing conventional cartridges with instruments such as the

Zymark "RapiTrace" system, have also been used for bioanalytical sample preparation [20]. However, the facile automation of SPE is still hampered by the potential of the columns to become blocked by particulate matter in the samples, and this frequently necessitates a centrifugation or filtration step prior to extraction.

20.2.3.2 On-line extraction and column switching

Another means of automated sample preparation with the potential to reduce sample handling and increase throughput is to perform the sample clean-up on-line by means of column switching. In this type of method the sample is injected into the system with minimal sample preparation, and the analyte is extracted onto an initial trapping column, any unretained material being pumped to waste. After a washing step, designed to remove as much of the unwanted co-extracted interfering substances as possible (which are also flushed to waste), a switching valve is activated and the analytes are eluted (usually by back-flushing) on to the analytical column for separation from residual contaminants and quantification. While this analytical separation is being undertaken, the extraction column is being reconditioned and the next sample loaded. The recent literature contains numerous examples of the use of HPLC with automated on-line extraction, including methods for immunosuppressants, such as cyclosporine, tacrolimus, SDZ-RAD, and sirolimus [21]. In this method, the protein in samples, following the addition of an internal standard, was precipitated and the drugs were extracted on-line, using a C_{18}-bonded phase. After a wash step to remove interferences, the analytes were back-flushed onto a C-8 analytical column and detected by MS. An example of the results obtained for cyclosporin is shown in Fig. 20.1.

A method for promethazine and 3 metabolites in urine was developed, using an Oasis HLB extraction column onto which the analytes were extracted. After application of the sample (1–2 mL), interferences were removed by using a water/methanol/ammonium acetate gradient at 4 mL/min before back-flushing the analytes onto a Zorbax SB-CN analytical column for analysis with UV detection. This set-up enabled detection of low-ng/mL concentrations of all four analytes [22]. Similarly, the analysis of ephedrines in urine was accomplished by HPLC with UV/DAD (diode-array detector) after in-line extraction of urine samples on a C_{18} extraction column and back-flushing of the analytes onto a C_{18}-bonded analytical column [23]. A comparison of off-line sample preparation with on-line extraction has been described, where sample preparation was performed using an alkyl-diol silica phase. This study compared the on- and off-line methods for the racemic anti-depressant citalopram and its metabolites in plasma. It showed that both approaches gave comparable results, but that the on-line method was faster [24]. Another anti-depressant, reboxetine, has also been analyzed in plasma and serum using column switching, with extraction onto a C-8 phase and analysis on a CN column [25]. Indomethacin in small volumes (50 μL) of rat plasma has been determined in a fully automated column-switching system, following direct injection of the sample. Concentrations as low as 50 ng/mL could be determined with excellent accuracy and precision by UV detection [26]. As well as in biological fluids, the analysis of drugs in tissue samples has also been carried out using column-switching techniques.

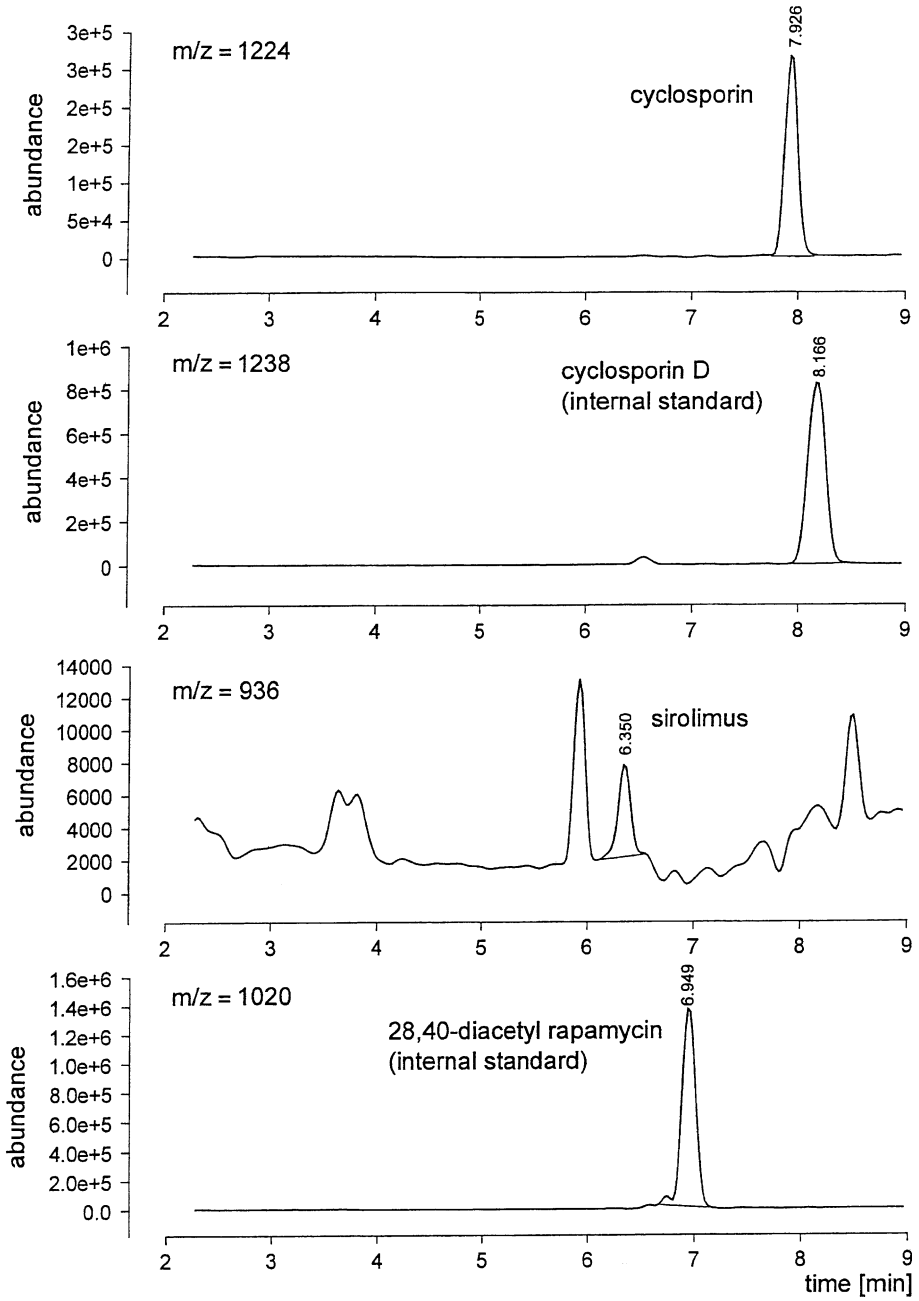


Fig. 20.1. Representative ion chromatograms of a blood sample from a kidney-graft patient 12 h after oral administration of cyclosporin and 8 h after oral administration of sirolimus. 500 μg/l cyclosporin D and 100 μg/l 28,49-*O*-diacetyl rapamycin were added as internal standards for cyclosporine and sirolimus, respectively. The concentration of cyclosporine was 162 μg/mL and that of sirolimus was 0.55 μg/mL. (Reproduced from Ref. 21, with permission.)

Examples include the quantification of tazobactam and piperacillin in serum and in extracts of fatty tissue [27] and a number of drugs in tissue extracts as diverse as brain, fat, liver, lung, muscle, skin, and spleen [28].

One of the problems associated with the direct injection of plasma or serum samples is that the adsorption of proteins on the extraction column can markedly shorten the column lifetime. To overcome this problem, so-called "restricted access" media (RAMs) have been developed. Their surface has little affinity for the proteins in the sample, and the analytes are extracted onto a suitable stationary phase in the pores of the silica, which are inaccessible to the proteins [29]. An example of a method based on the use of RAMs is the analysis of the enantiomers of a number of structurally related anticholinergic drugs in serum [30]. In this method the drugs trihexyphenidyl, procyclidine, and biperiden were determined in the low-ng/mL range, using on-line extraction, followed by a chiral separation on a β-cyclodextrin column with MS detection. A recent study examined the use of two different types of RAM, a protein-covered phase (BioTrap 500 MS) and a more conventional alkyl-diol phase (LiChrosphere RP-18 ADS) for the analysis of flunitrazepam and its metabolites in plasma [31]. The authors concluded that it was possible to perform >1500 injections of 50 μL of plasma on the former and *ca*. 800 injections of the same amount on the latter material without significant loss of performance. The glucuronide of entacapone (a catechol-*O*-methyltransferase inhibitor), present as *Z*- and *E*-isomers, was analyzed in rat plasma by HPLC/ESI/MS/MS on the Lichrosphere RP-18 ADS material and column switching with good accuracy and precision from 2.5 ng to 100 μg/mL, using 5- to 100 μL samples [32].

20.2.3.3 Solid-phase micro-extraction

The technique of solid-phase micro-extraction (SPME) was introduced in the early 1990s [33–35], and applications to drugs have slowly accumulated since then. In SPME, a small amount of stationary phase, covering a short fiber, is used to adsorb the analytes, and sampling takes place either by exposure of the SPME fiber to the headspace above the sample in the case of volatile compounds, or by direct immersion in the case of involatile analytes. The technique works most easily when interfaced with GC and, therefore, it is most suitable for volatile compounds or compounds made volatile by *in situ* derivatization, although attempts at liquid interfacing to HPLC have been reported [35]. In addition, a technique called "in-tube" SPME, where the sample is passed through a thin capillary tube, with a sorbent coating the inside, offers the potential for interfacing with HPLC systems [35]. Compared to conventional SPE, there have been relatively few applications of SPME to drugs in biological matrices [36], but these examples cover a fair range of drug classes: amphetamines [37–39], analgesics [40,41], anesthetics [42,43], anti-depressants [44–46], barbiturates [47–49], benzodiazepines [50–52], and isoflavones [53] in biological matrices, such as urine, plasma, and saliva. Another area where SPME has found application in pharmaceutical analysis is in the determination of organic volatile impurities in drug substances, including a method for 13 commonly found solvents [54].

20.2.3.4 Immuno-affinity extraction

Immuno-affinity extraction is based upon the use of antibodies, bonded to a suitable support, for the specific extraction of drugs from the sample. This approach has great appeal for the trace analysis of drugs in biological matrices, but the major problem is the time it takes to generate a suitable antibody. The development and use of antibody-mediated extraction, in both on- and off-line mode, has been the subject of a number of reviews [55–57]. While there have been a number of reported applications in the environmental field, there have been relatively few for drugs and metabolites. However, applications of immuno-affinity extraction to morphine in urine [58] and clenbuterol in plasma [59] have been described, which illustrate the potential of the methodology.

20.2.3.5 Molecularly imprinted polymers

Molecularly imprinted polymers (MIP) are polymers prepared in the presence of a "template" molecule (the intended analyte, or a close structural analog) [60–62]. After polymerization, the template is removed by extraction to leave "imprinted" sites that retain an affinity for the template and related structures. Such polymers are therefore analogous to antibodies and indeed have also been termed "plastibodies". One of the applications of MIP has been in the area of sample preparation where they have been employed as phases for SPE (both in- and off-line). As a result of the interest in molecular-imprint SPE (MISPE) a number of reviews have been published on the subject [63–66]. Currently, the field is in its infancy, and most of the existing applications can be considered to be "proof-of-concept" studies with few thoroughly validated methods published to date. However, these materials seem to be suited to a wide range of analytes and matrices, including pentamidine [67], theophylline [68–70], bupivacaine [71], tramadol [72], ibuprofen and naproxen [73], propranolol and analogs [74–79], clenbuterol [80], tamoxifen [81], darifenacin [82] and phenytoin [83].

It is generally accepted that the affinity of polymers for the template is greatest under conditions in which the polymer was prepared, which was usually in an organic solvent rather than an aqueous biological fluid. Nevertheless, analytes can be extracted from aqueous solution using a mixture of specific and non-specific interactions, and the binding can then be converted to the specific form by washing with the appropriate organic solvent (the so-called "solvent switch"). Careful selection of the subsequent elution conditions is required in order to maximize the affinity interaction and ensure selectivity. Currently, a number of problems are associated with the use of these MIP for SPE. These include non-specific interactions, which result in the co-extraction of interferents, the non-specific extraction of the analyte itself, and also problems resulting from the difficulty of ensuring the complete removal of the template from the MIP. If this is not achieved, templates can leach from the polymers during the analytical procedure and, if the template has been prepared using the analyte itself, trace analysis becomes impossible. One way around this problem is to use a close structural analog of the analyte to prepare the MIP, relying on the subsequent separation to separate the analyte from any leached template.

20.2.4 Turbulent-flow chromatography

An interesting innovation in the technique of introducing biological fluids, such as plasma, without pretreatment has been the development of turbulent-flow chromatography. The technique, which employs high flow-rates and comparatively large-grained packing materials, allows the direct injection of plasma samples without the need for prior removal of protein. Samples are loaded onto the column with a highly aqueous mobile phase to encourage retention of the analytes while promoting the elution of proteins. As the sample moves through the column, the improved mass-transfer characteristics of this type of system, when optimized for low-molecular-mass compounds, results in the retention of the analytes, while interactions of the stationary phase with proteins are minimized so that they are rapidly eluted without blocking the column. During this phase of the analysis, the eluate from the column is usually sent to waste. Having achieved analyte retention, the mobile-phase composition is adjusted to elute it into the detector (usually a mass spectrometer) or a second column. Analytical applications to a range of drug classes [84–93], including methods for pyridineisoquinolone [84,85], doxazosin, dofetilide and CP-122,288 [86], terbinafine [92], and others have been described. More are to be expected as the technique gains popularity.

20.3 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

20.3.1 Separation techniques

20.3.1.1 Conventional methods

At the time of the last review of the chromatography of drugs in this series [1] HPLC was already a mature and well-established technique, and many of the separations described in the current literature are very similar to those described in that work. Thus, the bulk of routine separations still appear to be carried out in the reversed-phase mode on alkyl-bonded silicas (most often C_{18}) on 4.6-mm ID columns, between 10 and 25 cm in length. The major exceptions to this general observation are in the field of drug analysis in biological fluids, discussed below, where the need for high throughput, combined with mass-spectrometric detection has, in many instances, led to the use of shorter columns, combined with rapid gradients. Therefore, instead of attempting to provide a comprehensive coverage of current practice, a limited number of areas will be discussed, where developments in column technology that may become important in the next few years can be identified.

20.3.1.2 Narrow- and micro-bore separations

An active topic of the chromatography of drugs is the use of miniaturized systems, although the relative number of examples is still small. Examples of the use of such

methods include an application to the drug bosentan and three of its metabolites in matrices such as plasma, bile, and liver tissue. HPLC with gradient elution was performed on a 150 × 2.1-mm column with a mobile phase composed of acetonitrile/methanol/ammonium acetate at 0.25 mL/min. Using MS/MS, detection limits in the low-ng range were achieved [94]. An example of a fully automated method for the analysis of the anti-inflammatory drugs aceclofenac and diclofenac in human plasma, by narrow-bore HPLC, has been described [95]. In this method, a column-switching approach was adopted, the analytes being directly extracted from the plasma samples (100 μL) onto a Capcell Pak Ph-1 column, followed by elution and focusing on a second "intermediate" column (Capcell C18) and then separation and quantification *via* a 100 × 2-mm-ID phenyl-hexyl column. The same research group produced a similar method for sildenafil and a metabolite in human plasma [96]. In another example, the $GABA_B$ receptor agonist, CGP 44532, was analyzed in rat plasma samples, using a combination of pre-column derivatization, on-line SPE, microbore HPLC and MS/MS. Derivatization of this alkylphosphinic acid was undertaken not for the purposes of detection but to reduce the polarity of the compound so as to obtain retention. Derivatization was performed by treating plasma ultrafiltrates with 4-nitrobenzylchloroformate. On-line SPE was then used to extract the derivative and remove the excess of derivatizing agent prior to elution onto the 150 × 1-mm-ID analytical column, which was packed with a 3-μm C_{18}-bonded phase. Using this approach, an accurate and precise assay was obtained from 10 to 500 ng/mL [97]. An example of the results obtained in this study is provided in Fig. 20.2.

Another good example of the use of 1-mm-ID columns for HPLC, combined with SPE in the 96-well-plate format, is provided by studies on a drug candidate, where the

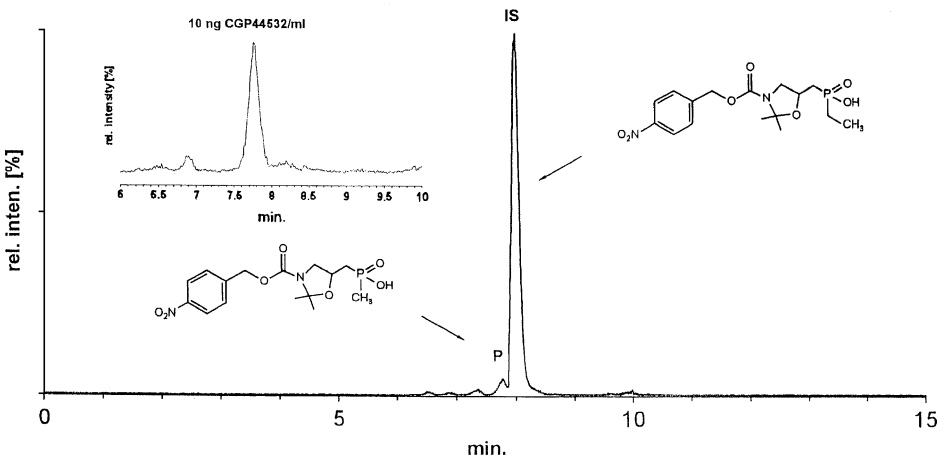


Fig. 20.2. A typical selected-reaction monitoring chromatogram for CGP-44532, 3-amino-2(S)-hydroxypropylmethyphosphinic acid, and its internal standard (IS) derivatized with 4-nitrobenzyl-chloroformate from a plasma/water sample containing 10 ng of the analyte. Following precolumn derivatization, on-line SPE was performed prior to separation on a microbore HPLC column (150 × 1 mm ID). (Reproduced from Ref. 97 with permission.)

performance of 1- and 2-mm-ID columns was compared. Chromatography was performed on 5-μm Symmetry C_{18}-bonded material with MS/MS for detection. It was found that results with the 1-mm-ID column were as good as with the 2-mm-ID column in terms of sensitivity, accuracy, and precision. When used with a guard column, the 1-mm-ID column could be used for 2500 injections [98]. Applications of capillary chromatography are still sparse in drug analysis, but the use of a reversed-phase capillary column (50 × 0.3 mm) with UV detection, coupled off-line to MALDI *via* micro-fraction collection for the screening of single-bead combinatorial libraries, has been described. This methodology enabled analysis of libraries for identification and purity at the 100-fm to 1-pm level [99]. In another recent example, size exclusion on a microscale in 96-well Sephadex plates was used in combination with capillary HPLC/MS to assess ligand binding. The ligands were incubated with the target proteins and the protein-bound material was separated from free ligands by size exclusion. The latter were then recovered from the Sephadex columns, and the amount was determined by capillary HPLC/MS, using reversed-phase chromatography on a C_{18}-bonded phase (150 × 0.32 mm) with a linear gradient [100].

20.3.1.3 Monolithic columns

Unlike conventional columns, which are packed with particles of stationary phase, monolithic columns are formed as a single porous unit. Recently, such monolithic columns have been commercialized. These columns display enhanced permeability and can thus be used at high flow-rates, providing the potential for faster analysis. While the use of monolithic columns for drug separations is still in its early stages, some applications to the chromatography of drugs are beginning to be seen [101,102]. In the first example of this type of application [101], HPLC on a monolithic column was used in combination with MS/MS to identify metabolites of debrisoquine, produced *in vitro*. When the results were compared with more conventional methods, such as short-column rapid-gradient HPLC/MS, the monolith was found to provide superior performance. Other studies from different laboratories [103,104] have also demonstrated that monolithic columns can be used for the rapid analysis of drugs and metabolites in plasma. In a recent example [105], such columns were used for the analysis of some COX-II inhibitors in human plasma with chromatographic analysis times of less than one minute (Fig. 20.3). This represents an up to five-fold reduction in analysis time. Not only MS but also UV and fluorescence detection have been used with these columns to investigate the metabolism of a variety of drugs used as test probes, *e.g.*, phenacetin, coumarin, and paclitaxel, by a range of cytochrome P450s [106]. Use of monolithic columns has provided maximum analysis cycle times of *ca*. 4.5 min for this type of assay. Another recent application involved the use of four monolithic columns, connected in parallel to a tandem mass spectrometer, for the analysis of oxazepam in solid-phase extracts of plasma [107]. This arrangement enabled the parallel analysis of samples with an overall analysis time of only 30 sec per sample.

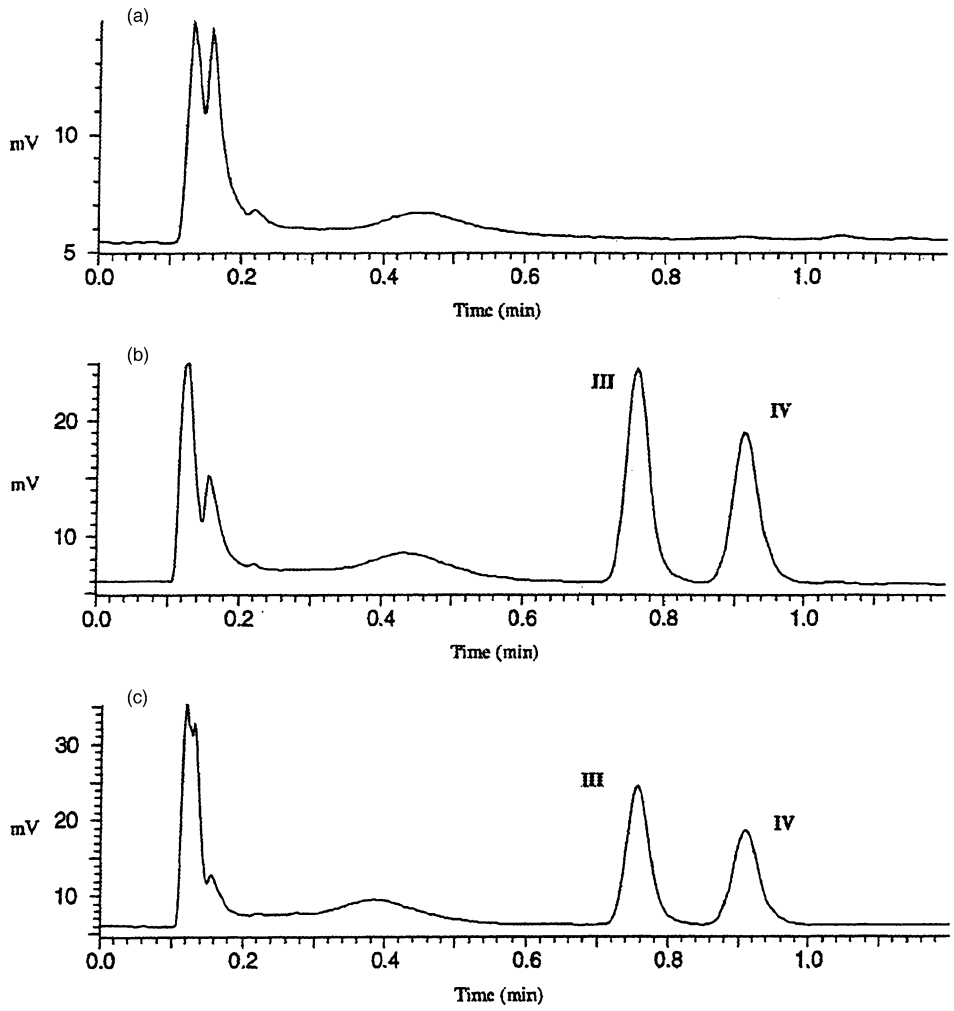


Fig. 20.3. Representative LC/fluorescence chromatograms from the assay of 3-isopropoxy-4-(methanesulfonylphenyl)-5,5′-dimethyl-5*H*-furan-2-one (III) in human plasma, (A) control plasma, (B) plasma spiked with 100 ng/mL of III and 100 ng/mL of internal standard (IV), and (C) a human clinical sample collected 0.5 h after dosing with III. Separation was performed on a Chromolith Speed Rod monolithic column (4.6 × 50 mm ID). (Reproduced from Ref. 105 with permission.)

20.3.1.4 Polymeric column packings and hybrid silicas

While conventional silica-based bonded phases have proved immensely successful when applied to the separation of drugs, they have a number of well-recognized limitations. In particular, the limited pH and temperature ranges over which silica is stable restricts the conditions that can be employed in separations based on these materials.

A range of packings are now available which do not suffer from these problems, or not to such an extent. Such materials include graphitized carbon, zirconia-based phases, organic polymer phases and the so-called inorganic/organic hybrid materials, exemplified by the XTerra phase. The Oasis packing material, one of the newer polymer phases, has recently been used in both micro-bore (50×1 mm) and capillary (50×0.18 mm) HPLC, in combination with MS/MS detection, for the analysis of a novel isoquinoline in plasma [108]. In this example, multiple parallel separations on four columns were used, combined with relatively high flow-rates into a four-way, multiple-sprayer interface on the mass spectrometer in order to generate a high-throughput system. (This approach is described in more detail Sec. 20.3.2.1) An example of drug separations on the inorganic/organic hybrid C_{18}-bonded XTerra phase is the application of this type of column to the analysis of doxepin, imipramine, and nortriptyline [109]. While the impact of these new phases is difficult to judge at present, there is no doubt that continuing innovation in this area is extending the boundaries of HPLC to provide robust and reliable separations for most classes of drug.

20.3.1.5 Elevated temperature

An interesting recent development in HPLC, which has been applied to the separation of a range of drugs and model compounds, is the application of high temperature (often in excess of 100°C) to HPLC separations [110–112]. Water above 100°C has been termed "superheated water", and although its use as an eluent is still in its infancy, some interesting examples have been presented, where the organic component has been greatly reduced or even completely eliminated. While the number of applications to date is not large, such methods do seem to have promise, offering faster or more efficient separations. The use of pure water as a mobile phase also has obvious attractions as an "environmentally friendly" solvent system. However, the use of elevated temperature is not without difficulty, as there can be problems with the stability of both analyte and column (if it is silica-based). Nevertheless, separations of drugs on zirconia-based phases, polymer columns, and graphitized carbon have been reported, as well as work on more stable silica-based materials, such as XTerra. As an example, a mixture of drugs and model compounds, including paracetamol (acetaminophen), antipyrine, caffeine, phenacetin, and dimethylantipyrine, were separated on a range of stationary phases with pure water as the mobile phase at a temperature ranging from 225°C for graphitized carbon to 165°C for BDS Hypersil and XTerra [113].

20.3.1.6 Chiral separations

In most optically active drugs only one of the two enantiomers of the racemate has the desired biological activity, while alternative pharmacological properties and sometimes toxic manifestations may be associated with the other. There has, thus, been a general acceptance in recent years of the benefits of taking only single enantiomers into development. This has produced requirements for preparative and analytical separations of synthetic intermediates and bulk drugs to control purity and for analytical methods to

detect any propensity for *in vivo* metabolic inversion. It is difficult to overestimate the importance of chiral separations in the field of pharmaceuticals and, as a result, the literature covering this subject is huge. Because it is impractical to give full coverage to this complex topic here, the reader is directed to a number of recent reviews. A detailed consideration of chiral drug separations is given in Ref. 114, the use of enantiomeric separations for chiral impurities is considered in Ref. 115, and a useful, brief overview, concentrating on biological applications of chiral separations, has recently been provided by Haginaka [116]. Although there are many examples of chiral drug separations by TLC, SFC, GLC, and capillary electrophoresis, the majority of these applications are performed by HPLC.

A range of strategies have been adopted for the chiral separation of drugs, based on either direct separations on a chiral column, with chiral mobile-phase additives, or by an indirect method *via* the formation of diastereoisomers with a chiral derivatizing agent. In general, because of the perceived disadvantages of chiral derivatizations it would seem that the use of chiral columns or mobile-phase additives is conducive to obtaining reliable enantioseparations. The most popular mobile-phase additives have been cyclodextrins, preponderantly β-cyclodextrin or its sulfated, hydroxypropyl, methyl or carboxymethyl derivatives. Examples of the use of cyclodextrins as mobile phase additives for drug enantiomers include the separation of thalidomide [117] and flobufen with β-cyclodextrin [118], doxazosin with carboxymethyl-β-cyclodextrin [119], and amlodipine with sulfobutylether-β-cyclodextrin [120]. Alternative strategies involve the use of chiral ion-pair reagents, such as benzoxycarbonyl-glycyl-L-alanine, (+) or (−)-10-camphor-sulfonic acid and (2*S*,3*S*)-dicyclohexyl tartrate, which have been applied to propranolol [121], vincamine [122], and (*S*)-atropine [123], respectively. Examples of the separation of drugs on chiral stationary phases (CSPs) have been very numerous [114–116]. These include separations on polymeric phases, based on materials such as derivatized cellulose, phases built around "chiral cavities" such as cyclodextrins, molecular imprints, macrocyclic glycopeptide antibiotics (*e.g.*, vancomycin) and crown-ether-based CSP. There have also been applications of the Pirkle-type CSP, of protein-based phases, and of chiral ion exchangers. These separations are now sufficiently well established that examples of the use of chiral analysis *via* chromatography have begun to appear as pharmacopoeial methods [124].

For low loadings and where a "reversed-phase" type of separation is required, the protein phases, particularly the CSP based on human α 1-acid glycoprotein (AGP), have been demonstrated to have broad utility. AGP has thus been used to resolve the enantiomers of β-blockers [125–127], verapamil and gallopamil [128], benzapril [129] mosapride [130], valsartin [131], and felodipine [132,133], among others. CSPs made from human serum albumin (HSA), bovine serum albumin (BSA), and ovomucoid (OVM) have also produced useful separations. Examples of the use of these types of protein-based columns include the use of HSA for ketoprofen [134] and warfarin [135], of BSA for ketoprofen [136], and of OVM for fluoxetine [137] and promethazine and related compounds [138].

The cyclodextrin phases (including derivatives), in general, exhibit a higher load capacity than the protein-based CSP and are regarded as being more rugged. They can be used with both reversed- and normal-phase eluents, and there are very many applications

of their use in the field of drug separations [114–116]. Typical examples of the use of cyclodextrin-based CSP include the separation of β-blockers on the β-cyclodextrin-based phase Cyclobond 1 [125] and of propranolol, which was determined in plasma and urine, using racemic alprenolol as internal standard, on a Cyclobond 1 2000 column [139]. Similarly drugs, such as methadone and metabolites, were analyzed in biological fluids using a Cyclobond 1 2000 RSP column [140], and simendan and warfarin enantiomers were analyzed in such fluids on Cyclobond 1 2000 columns [141,142]. Like the cylcodextrin CSP, the cellulose-derived phases have also been very widely applied to drug separations. Examples include the analysis of metoprolol in plasma, following SPE, on Chiracel OD-H [143], oxprenolol in plasma on Chiracel OD-R [144], and both verapamil and norverapamil on Chiralpak AD [145].

20.3.2 Detectors

20.3.2.1 Mass spectrometry and tandem mass spectrometry

Probably the greatest change in the chromatographic analysis of drugs that has occurred since the last edition of this work has been the phenomenal rise of the application of mass spectrometry as a means of detection following HPLC (Chap. 10). This has been the direct result of the development of MS interfaces, such as atmospheric-pressure chemical ionization (APCI) and electrospray ionization (ESI) that are compatible with aqueous eluents and the flow-rates associated with reversed-phase HPLC. The resulting specificity and sensitivity of MS-based HPLC systems for the quantitative analysis of polar, and generally involatile, drug molecules has made such systems ideal for a range of sample types. When the ability of these systems to also provide spectroscopic information is taken into account, which aids in the identification of unknowns in areas such as impurity profiling and drug metabolism, it becomes easy to understand the popularity of this approach.

The role of HPLC/MS in impurity identification has been described in a recent volume devoted to the determination of drug impurities [146]. An example of the application of HPLC, together with HPLC/NMR, for the identification of pharmaceutical degradation products is provided by studies on a protease inhibitor in dosage formulations [147]. A total of six degradents were characterized by this approach. A further example where MS shows its value in detecting impurities, is the use of HPLC/UV/MS for the determination of impurity profiles of ethynodiol diacetate [148]. In this study, the combination of on-line UV, electrospray positive-ion API/MS and off-line ^{13}C and ^{1}H NMR enabled the confirmation of the presence of the *E*- and *Z*-isomers of 17-α-ethinyl-ene-4-estra-3-β,17-diol 3-acetate-17(3′-acetoxy-2′-butenoate) and also provided the identification of a previously uncharacterized impurity, 17α-ethynyl-ene-4-estr-3-β,17-diol-3-acetate-17-(3-oxo-butanoate). Photodegradation products have been characterized for the drug clinafloxacin, a fluoroquinolone antibiotic, by using HPLC/MS and subsequent NMR of these products, following isolation by preparative HPLC. This led to the identification of eight new degradation products [149]. HPLC/MS and HPLC/MS/MS have been applied to the identification of degradation products of sumatriptan succinate. Separations were

carried out on an ethylsilane column, by isocratic reversed-phase chromatography [150]. In another example, HPLC/MS^n with gradient reversed-phase chromatography has been applied successfully to the identification of degradation products of a novel carbapenem antibiotic in an aqueous matrix. This study revealed the presence of eight potential degradents [151].

One of the other driving forces that has ensured the increasing adoption of HPLC/MS-based analysis in the pharmaceutical sector has been the ever-increasing need for speed. Developments in synthetic chemistry, centered on robotic synthesis, multiple-parallel synthesis, combinatorial chemistry, etc., have led to a huge increase in the number of chemicals being prepared that require identification. A useful consideration of the application of HPLC/MS techniques for the analysis of these "combinatorial libraries" was presented by Lane [152]. An innovative method for providing the high-throughput required for combinatorial chemistry has been the approach of "multiplexing" (MUX) several chromatographic systems into one mass spectrometer *via* special interfaces. MUX systems have been described that allow the eluent from four or eight columns to be analyzed simultaneously (Fig. 12.3). The application of the use of an eight-channel MUX system to combinatorial chemistry has been described [153], where it was found to be possible to analyze 3000 samples per day, using a rapid gradient separation (with a cycle time of 3.5 min/sample). The increased synthetic productivity has been matched by the rise of high-throughput screening (HTS) of the huge chemical libraries that most companies have amassed. Such screens, with their ability to identify many hundreds of active molecules, have demanded a corresponding increase in the *in vivo* testing. The consequence of this has been the development of a range of strategies to improve productivity in the generation of pharmacokinetic and metabolic data during drug discovery.

Both HPLC/MS and HPLC/MS/MS have been enlisted in the analysis of biological samples in drug discovery. To ensure the most efficient use of instrument time, and to minimize the amount of effort required for method development, most groups have opted for so-called "generic methods". The idea is that a single method of (minimal) sample preparation, such as protein precipitation, a single column, and a single MS-compatible eluent (generally a rapid gradient) [154], capable of dealing with a wide range of analytes (acidic, basic, neutral, and amphoteric), is used to analyze all samples. Clearly, such a method is not achievable for all compounds, but, nevertheless, it is possible to devise schemes that do come very close. Another means of increasing productivity is the simultaneous analysis of several compounds in a single run. Various methods have been advocated for this, with multiple ("cocktail") dosing of up to *ca.* 10 compounds being administered to a single experimental animal, representing one approach [155–157]. This approach has also been used for *in vitro* studies [158–159]. The cocktail approach has the advantage of minimizing animal usage, but it is open to the risk that administered compounds will interfere with each other's metabolism and elimination, thus giving rise to a false estimate of pharmacokinetic properties. In addition, doses that can be given tend to be lower. An alternative approach, which avoids these problems, albeit with the increased use of experimental animals, involves samples from a number of animals, each treated with a different compound, being pooled prior to analysis [160,161]. Clearly, when selecting compounds for analysis in this way, much care needs to be taken to ensure that

isobaric compounds are not present, and some thought needs to be given to the potential of metabolites to interfere. Another method for increasing through-put and maximizing the use of the mass spectrometer is the use of the multiple-parallel column approach described above for combinatorial chemistry. This has found increasing application for the analysis of biological fluids [107,162–168], combinations from 2 to 8 separations being conducted at once.

As indicated above, sample preparation methods for this type of work tend to be limited to protein precipitation (sometimes carried out by 96-well-plate technology [169]. Separations are then usually performed on a relatively short column (2–5 cm), often with high flow-rates and short retention times [170–172]. In addition to the use of such techniques for biological fluid samples, there have also been applications to the assessment of metabolic stability [173], metabolic inhibition [174], and drug transport [175]. While suitable for preliminary work on drugs, this minimalist approach can be subject to problems associated with ion suppression due to contaminants in the eluate. Such ion-suppression effects are now widely recognized [176–179] and means for their detection have been devised. An interesting approach to assessing ion suppression involves the infusion of the analyte, post-column, into the eluent, following the injection of a blank sample. Any ion suppression will be seen as a negative inflection in the ion current for the compound of interest and, if this should also coincide with its retention time in the chromatographic system under study, then interference in its determination in biological samples can be expected. The elimination of such effects becomes increasingly important as compounds move through the discovery process and enter full development and subsequent clinical use, and studies of suppression are critical parts of method development. This inevitably means more specialized sample preparation and more selective chromatography, so that the generic approach used in early discovery is not appropriate at later stages. The number of HPLC/MS/MS methods for drugs, of all classes, in samples, such as plasma and urine, is now huge.

Typical recent examples of validated methods for drugs in plasma include the detection of sildenafil and its metabolite, desmethylsildenafil, in human plasma. This analysis was carried out, with a structural analog as the internal standard, using SPE in the 96-well-plate format, by chromatography on a silica column with an aqueous organic mobile phase. A limit of quantification (LOQ) of 1.0 ng/mL was achieved for both compounds [180]. HPLC/MS/MS was also used in an interesting study on the simultaneous analysis of the COX-2 inhibitor rofecoxib by reversed-phase chromatography with acetonitrile/water on a narrow-bore (100 × 3-mm-ID) C_{18} column at a flow-rate of 0.4 mL/min. Detection was by MS/MS with an APCI interface in negative ionization mode. A methyl analog was used as the internal standard, and the analytes were isolated from plasma by LLE. The analysis had a LOQ of 0.1 ng/mL and was linear up to 100 ng/mL [181]. More recently, the same authors have produced a method for this drug and its [$^{13}C_7$] stable-isotope-labeled form in order to compare the oral bioavailabilities of the drug. Thus, the unlabelled rofecoxib was administered orally at either 12.5 or 25 mg/kg with a simultaneous intravenous administration of the stable-isotopically labeled drug. As before, a reversed-phase chromatographic separation was performed, using acetonitrile/water (1:1) as mobile phase and a structural analog as internal standard. By using mass spectrometry as the means of detection, these investigators were thus able to determine both intravenous and oral

pharmacokinetic parameters simultaneously, greatly reducing the number of volunteers and samples required for such studies [182]. A reversed-phase HPLC/MS/MS method for rosuvastatin, a HMG-CoA reductase inhibitor, achieved a LOQ of 0.1 ng/mL [183]. After addition of a deuterated internal standard, automated SPE with the Oasis HLB polymeric sorbent in 96-well format was used, followed by separation on a C_{18} bonded phase with a typical mobile phase of methanol/0.2% formic acid (70:30) at 1 mL/min. A retention time of 3.5 min was used for the analyte in order to minimize the total analysis time, and for the first 2 min of the analysis, the eluent from the column was vented to waste rather than introduced into the ion source of the mass spectrometer. Such procedures reduce the amount of contamination of the spectrometer and, therefore, reduce the need for system maintenance.

Recently, a method was described for the anti-leukemia drug gleevec and its major de-methylated metabolite in human plasma, which were analyzed by HPLC/MS/MS following protein precipitation with methanol. Sample preparation was performed in a 96-well plate on 200 μL of sample. Using a deuterated internal standard, LOQs of 4 ng/mL (corresponding to *ca*. 40 pg on-column) were obtained with an analysis time of less than 2 min [184]. Another useful example of the use of HPLC/MS is found in the analysis of the semi-synthetic macrolide antibiotic clarithromycin in human plasma. This was performed following LLE by RP-LC on a C_{18} bonded column with acetonitrile/methanol/0.1% acetic acid (1:1:2) and MS/MS detection. This type of analysis is particularly suited to this analyte, as it lacks a suitable chromophore, precluding sensitive UV detection. In contrast, the HPLC/MS/MS method developed by the same authors, with roxithromycin as an internal standard, enabled sensitive quantification in 300 μL of plasma down to *ca.* 3 ng/mL [185].

20.3.2.2 Inductively coupled-plasma mass spectrometry

The relatively recent introduction of HPLC combined with inductively coupled-plasma mass spectrometry (ICP-MS) has opened up a number of possibilities for the speciation of elements rather than their mere detection (*e.g.*, As III, As V, and organo-arsenic species, such as arsenobetaine, etc., rather than simply total arsenic). ICP-MS enables the sensitive, specific, and quantitative detection of particular atoms present in molecules, thereby providing complementary information compared to molecular MS, and enabling quantification based on the presence of that element in the analyte. This makes HPLC/ICP-MS particularly well suited to the quantification of "unknown" impurities or metabolites where standards are not available. While there are relatively few classes of drugs that contain metals, obvious candidates for this technique are the platinum anti-cancer drugs and the gadolinium metal complexes. Examples of the use of HPLC/ICP-MS for these compounds in formulations and biological fluids have recently been reported [186,187]. These include the use of the technique for the determination of a gadolinium metal complex (gadiodiamide) at the level of 32 pg (52 fmol) on-column [187] and the determination of Pt of a platinum anti-cancer drug in formulations and biological fluids with high sensitivity and specificity [186]. An example of the use of HPLC/ICP-MS for a radio-labeled platinum anti-cancer drug is shown in Fig. 20.4.

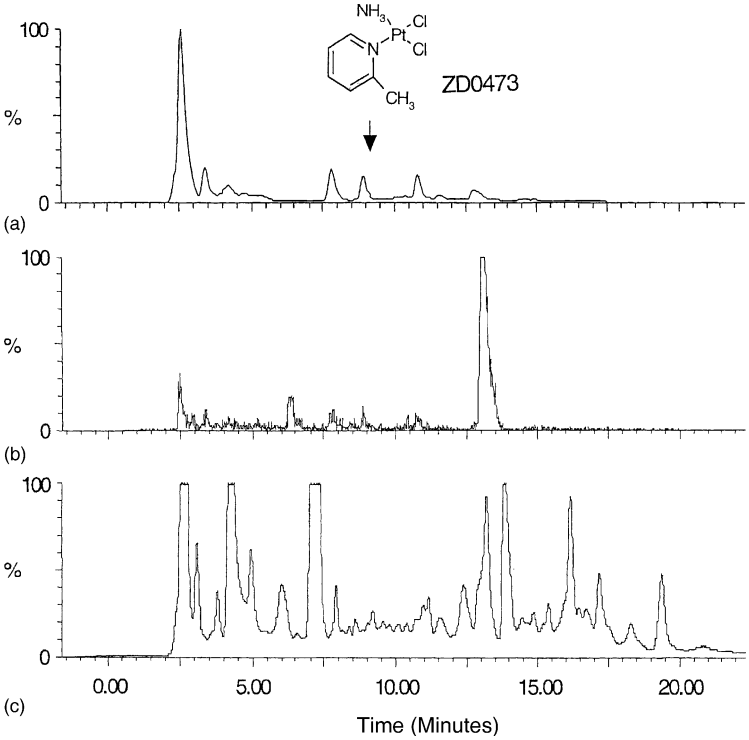


Fig. 20.4. Liquid column chromatograms of the experimental [^{14}C]-labeled platinum anti-cancer drug ZD0473 A) by ICP-MS B) by ^{14}C-radioactivity and C) by UV adsorption. (Reproduced from Ref. 186 with permission.)

In addition to metals, ICP-MS can also be used to detect bromine- [188–191], chlorine- [192,193], iodine- [187] sulfur- [189,192,194] and phosphorous- [187] containing drugs, which are much more common. HPLC/ICP-MS for the detection of sulfur-containing compounds has been described for the detection of cimetidine and a number of structurally related impurities at well below the 0.1% level, with detection limits at the 4–20-ng/g level [194]. Applications for sulfur-specific detection also include the detection of sulfur-containing drug metabolites in compounds that originally did not contain this element (see below). Phosphorous has also been specifically detected in both phospholipids and a range of phosphopeptides, obtained either by synthesis or from a tryptic digest of β-casein [187]. Detection limits in the region of 20 ng on-column were estimated for the phospholipids. In the case of the halogens, specific detection of the metabolites of 4-bromoaniline in rat urine was the first example to be reported [188]. Other examples of the use of Br-specific detection have followed, often coupled with simultaneous MS detection to provide structural information [189–191]. Thus, while HPLC/ICP-MS enables specific detection of compounds containing these elements, the greatest benefits arise when the system is also coupled to conventional mass spectrometers that can provide molecular data as well. Examples of the combination of HPLC/ICP-TOF-MS/MS in the area of drug metabolism have been described, where ICP-MS was used for speciation and quantification, while

TOFMS was used for metabolite identification. The first such example was the investigation of the metabolism of 2-bromo-4-trifluoromethylacetanilide [189]. Rat urine samples obtained for the first 8 h after administration was analyzed by reversed-phase gradient HPLC. The effluent from the column, monitored with a photodiode-array (PDA) detector provided UV spectra of the metabolites. Immediately after UV detection, the flow was split, *ca.* 95% being sent to ICP-MS for bromine-selective detection, while the remaining 5% was directed to an orthogonal acceleration (oa) TOF mass spectrometer. More recent applications of this approach have used Br-selective detection to investigate the metabolites of bromobradykinin [190] in plasma and of 4-bromoaniline metabolites in urine [191]. In the latter study, S-specific detection was also utilized to look for sulfur-containing metabolites. It is also possible to detect chlorine by HPLC/ICP-MS, albeit with less sensitivity than bromine. The same instrumental set-up that had been used for the detection of 4-bromoaniline-related material was subsequently used for the detection of diclofenac metabolites in rat urine, using chlorine-specific detection [192]. In addition to chlorine, sulfur was also monitored to enable S-containing metabolites of the drug to be detected. Identification was performed *via* oa-TOFMS. HPLC/ICP-MS has been applied not only to bromine and chlorine, but also to the detection of iodine-containing components present in a crude mixture consisting of an iodinated X-ray contrast medium and various impurities [187], with limits of detection levels corresponding to *ca.* 0.4 ng of iodine on-column.

20.3.2.3 Nuclear magnetic resonance spectroscopy

While the sensitivity of nuclear magnetic resonance (NMR) spectroscopy is generally considered to be insufficient for the trace analysis and quantification of drugs in biological fluids, HPLC/NMR has been applied with benefit to the characterization of drug impurities [195,196] and to synthetic chemistry in the form of combinatorial peptide libraries [197, 198]. Other applications have included vitamin derivatives [199] and the identification of the products of drug metabolism [200–219]. These areas have been the subject of a number of reviews [219,220], and, recently, a volume devoted to this technique has been published [221]. The first application of HPLC/NMR to the identification of drug impurities employed a 400-MHz NMR spectrometer, fitted with an HPLC flow-cell. A 3% impurity in a synthetic drug precursor [195] was investigated using stopped-flow ^{1}H-NMR spectroscopy. Other studies include one where a combination of RP-HPLC/NMR and HPLC/MS was used to examine and identify impurities in batches of the steroidal drug fluticasone propionate [196]. This work was carried out with a 600-MHz instrument and reversed-phase gradient chromatography. Stopped-flow NMR and "time slicing" (where the chromatographic peak is gradually moved through the flow cell by a series of small incremental steps, stopped-flow spectroscopy being performed after each step) were used to obtain good-quality ^{1}H-NMR spectra (Fig. 20.5). These data, together with the MS results, enabled the identification of a number of dimeric impurities of the drug. Chiral HPLC, coupled to ^{1}H-NMR and circular dichroism (CD) detection, was used as a means of investigating the isomers of atracurium besylate [197], which occurs as a mixture of 10 isomers, in different proportions. For this study, following separation on a chiral column,

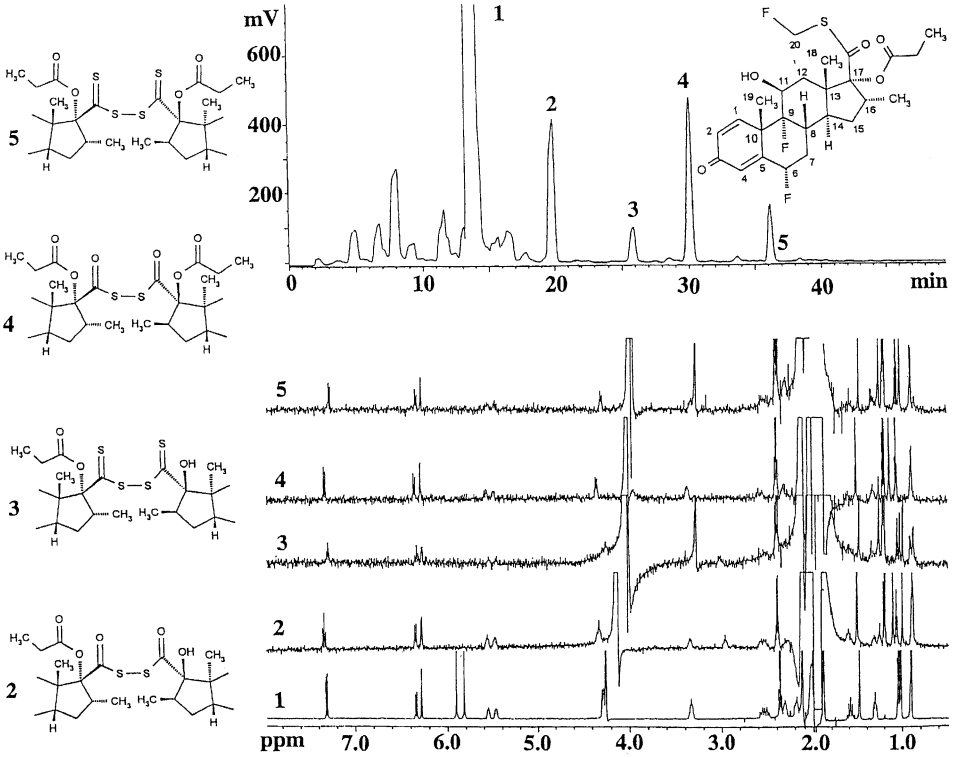


Fig. 20.5. Liquid column chromatograms and ^{1}H-NMR spectra of fluticasone (see inset) and dimeric impurities (partial structures on the left). Top UV absorption, bottom ^{1}H-NMR spectra. The numbering of the UV peaks and NMR spectra corresponds to that used for the structures. (Reproduced from Ref. 196 with permission.)

stopped-flow ^{1}H-NMR spectra were obtained, with a 750-MHz instrument. There have also been applications to a GART inhibitor [198], degradation products of a HIV protease inhibitor [199], an impurity in the drug naftopidil [200], the auto-oxidation and photodegradation products of ethinylestradiol (also by HPLC/MS) [201] and the degradation products of an antifungal agent [202]. A study by Mistry *et al*. [203] has demonstrated that HPLC/NMR could be used to characterize impurities below the 0.1% level, which is the relevant limit for regulatory submissions.

In the case of combinatorial chemistry, there have been two reported studies, one dealing with a mixture of 27 peptides [204] and the other examining two different mixtures, one of four aromatic compounds and the other of three pentapeptides [205]. One of the major areas of application of HPLC/NMR has been to the identification of drug metabolites. The first such study reported the identification of the metabolites of ibuprofen in urine samples [207]. This study was rapidly followed by investigations on a number of other drugs, including flurbiprofen [208] (using both ^{1}H- and ^{19}F-NMR), antipyrine [209], paracetamol [210,211], tolfenamic acid [212], naproxen [213], and the anti-cancer drug

iphosfamide (by ^{31}P- as well as ^{1}H-NMR) [214]. Other metabolic studies by means of HPLC/NMR include BW935U83 [215] (a HIV-1 reverse-transcriptase inhibitor), GW524W91 (an anti-HIV compound) [216], paracetamol [210,217], phenacetin [218], DPC423 [219], LY335979 [220], roxifiban [221], trifluorperazine [222], GW420867 [223], GW273629 [224], and iloperidone [225]. The field strength of the NMR spectrometers used in the examples cited here ranged from 500 to 800 MHz, and reversed-phase chromatography was generally used, with D_2O, rather than H_2O, in the mobile phase. However, usually non-deuterated acetonitrile was employed as the organic modifier, owing to the high cost of the deuterated form of this solvent. Various combinations of on-flow and stopped-flow experiments were used to obtain spectra.

20.3.2.4 Multiple detectors

In addition to studies where HPLC has been coupled to NMR, there have also been a number of studies where multiple spectroscopic methods were combined. The bulk of these studies have involved the simultaneous linking of HPLC with both NMR and MS. An early experiment, involving HPLC/NMR/MS was performed on a mixture of fluconazole and two related triazole structures, where on-flow mass and ^{1}H-NMR spectra were obtained for all three components [229]. The eluent from the HPLC column was split 60:40, the larger portion being directed to the flow-probe of a 500-MHz NMR spectrometer and the rest sent to the particle-beam interface of a Fisons Trio 1000 mass spectrometer (chemical ionization mode). This example was followed shortly afterwards by a HPLC/NMR/MS study on metabolites of acetaminophen (paracetamol), isolated by SPE from urine [230]. More recently, there has been a further study, where the identification of acetaminophen metabolites in urine by means of HPLC/NMR/MS was described [231]. Other examples of the application of this approach to the metabolism of drugs include a HPLC/NMR/MS analysis of ibuprofen metabolites in a urine extract [232]. Both NMR and MS easily detected the major glucuronide metabolites, but the greater sensitivity of MS to certain minor ibuprofen-related components also directed NMR analysis in a way that NMR spectra were obtained on peaks that might otherwise have gone undetected by NMR alone. Other information obtained by ^{1}H-NMR that would not have been found by MS concerned the ratio of diastereoisomers in the glucuronide conjugates of various metabolites of ibuprofen, which was administered as a racemate. Dear *et al.* [233] investigated the fate of the novel non-nucleoside reverse-transcriptase inhibitor GW 420867 by HPLC/NMR/MS. Following the collection of the peaks of interest in a peak-sampling unit, NMR spectra were obtained and the peaks were subsequently subjected to MS. This work was performed with an in-line-configuration NMR/MS layout rather than an in-parallel system. In this way, several hydroxylated and glucuronidated metabolites were identified.

A HPLC/NMR/MS study on a [^{14}C]-labeled β-blocker, with the use of an on-line radioactivity monitor for direct spectroscopic analysis of the peaks of interest, has also been reported [234]. In this work, the resulting ^{1}H-NMR and MS data allowed the parent drug, its ring-hydroxylated metabolite, and the ring-hydroxyglucuronide to be identified. The drug had been given as the racemate, and the NMR spectrum of the latter metabolite

clearly showed that enantioselective metabolism (or excretion) had occurred judging from the different intensities of the anomeric proton resonances of the two diastereoisomeric glucuronides. Further, the de-acetylation and subsequent re-acetylation of the drug and its metabolites was also studied by the expedient of incorporating a [^{13}C]-labeled acetyl group into the compound. Both NMR and MS showed that *ca.* 10% of the [^{13}C]-acetyl group had been removed and replaced by its [^{12}C] equivalent. HPLC/NMR/MS has also been investigated as a potential approach in combinatorial chemistry [235], using a model peptide library of some 10 compounds. Application of 100 μg of each analyte to the column enabled both NMR and MS data to be obtained on-flow.

Recently, there have been a number of studies where superheated water was used as the mobile phase. The simplicity of using water (or D_2O) as a solvent for HPLC/NMR/MS is very attractive, as it is easily compatible with both types of detector. However, it should be noted that this technique is not suitable for all compounds, and an obvious potential drawback is that the elevated temperature may cause the analytes to decompose during chromatography. Despite this potential problem, superheated water as a chromatographic eluent with HPLC/NMR/MS has been tested on several pharmaceuticals, such as caffeine, salicylamide, salicylic acid, phenacetin [236], and sulfonamides [237]. In the latter study, a noteworthy finding was the quantitative *trans*-deuteration of methyl groups adjacent to the ring nitrogens of these drugs. More recently, superheated water has been used with even more complex concatenations of detectors (see below). Additional applications of HPLC/NMR/MS to medicinal plant products have included the characterization of extracts of *Hypericum preforatum* L. [238]. In this example, RP-HPLC, combined with NMR and MS, resulted in the identification of a wide range of compounds in the extract. These included the arabinoside and galacturonides of quercetin, which had not previously been described for this species. In addition, the known rutinoside, glucoside, rhamnoside, and galactoside of quercetin and hypericin, protohypericin, pseudohypericin, and protopseudohypericin were positively identified.

While HPLC/NMR/MS systems provide a large amount of spectroscopic information, they do not represent the limit of concatenation. Indeed, system have been constructed that provide for UV, NMR, MS, and IR data to be obtained, on-line, on a single separation. The first application of a HPLC/IR/NMR/UV/MS system for on-line spectroscopy was to the analysis of a model mixture of non-steroidal anti-inflammatory drugs (NSAIDs) (ibuprofen, flubiprofen, naproxen, and indomethacin) [239]. The MS data were obtained using TOFMS, which allowed accurate mass data to be obtained for each of the NSAIDs. This "proof-of-concept" system was designed only to show that linking the various components together was possible. The overall sensitivity of the combination was not investigated and, indeed, the concentration of components in the test mixture was quite high (2 mg on-column of each of the 4 NSAIDs). In a further investigation of the use of superheated water as an eluent the combination HPLC/IR/UV/NMR/MS was examined with a model mixture of antipyrine, caffeine, and phenacetin [240]. Chromatographic separations were performed with D_2O as the mobile phase on both C8 XTerra and Oasis HPLC columns at 85 and 185°C, respectively. D_2O as the sole component of the mobile phase at elevated temperature proved to be an excellent eluent for multiple spectroscopic determinations. In the case of the XTerra phase, with an oven temperature of 85°C, the limit of spectroscopic identification (LOSI) for caffeine was *ca.* 46 μg on-column, while

the limit of detection (LOD) was perhaps in the region of 10 μg. However, as noted above, there is little doubt that this could have been greatly improved upon with suitable optimization, and state-of-the-art probes. An example of the results obtained for phenacetin, using this system, is given in Fig. 20.6. Although both hot and superheated water proved to be an excellent eluent for this type of work, the spectrometers in this system were quite capable of obtaining data with conventional reversed-phase eluents.

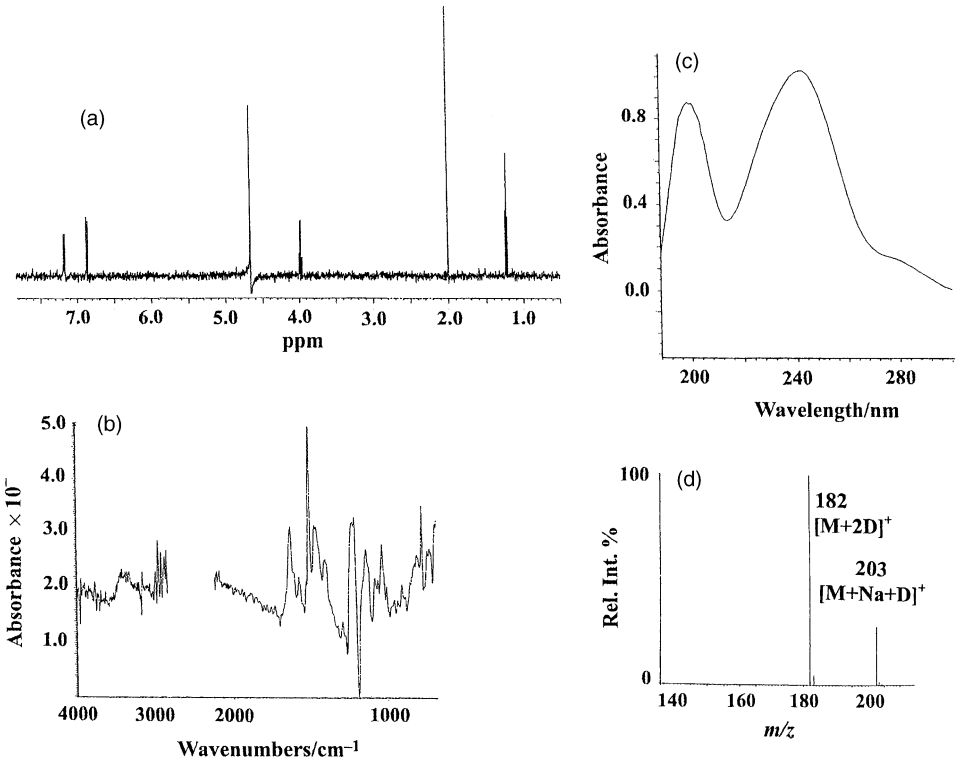


Fig. 20.6. Spectra obtained on-flow (1 mL/min) for phenacetin (72 μg on column) following chromatography on XTerra at an oven temperature of 85°C A) ^{1}H-NMR, B) IR, C) UV and D) MS (Reproduced from Ref. 240 with permission).

20.4 SUPERCRITICAL-FLUID CHROMATOGRAPHY

Once considered to be a technique of great promise, SFC (and the related technique of sub-critical fluid chromatography) has not fulfilled its potential and, by no stretch of the imagination, can SFC be said to be an important technique for the separation of drugs. However, SFC does have some applicability to certain niche applications, such as chiral separations. For application to the separation of drugs, packed-column SFC is generally used [241–243]. A recent treatment of the topic, as it relates to impurities in drugs, was

provided by Gyllenhaal [244]. One of the advantages of SFC, in addition to a somewhat greater efficiency than HPLC, is that it often provides access to a "normal-phase" separation that can complement the more usual reversed-phase separations obtained in HPLC. Separations are usually performed on relatively polar stationary phases, such as silica, cyanopropyl or diol, and the eluotropic strength of the carbon dioxide mobile phase is adjusted with an organic solvent, such as methanol, together with acidic and basic modifiers, such as citric acid or triethylamine. Other stationary phases, such as porous graphitic carbon, have also been used. Another advantage of SFC is the low back-pressure resulting from the use of a supercritical fluid as the mobile phase. This means that it is practical to couple a number of columns in series to obtain a large number of theoretical plates. A recent example of this approach, where the results of SFC for one, four, six, and eight columns were compared, is a study of the impurities of SC-65872 [245]. In this work, cyanopropyl columns (250 × 4.6 mm ID) were used at a flow-rate of 3 mL/min, with a solvent gradient based on carbon dioxide, modified with methanol and isopropylamine (outlet pressure 120 bar) and a temperature gradient from − 32°C to 450°C. An example of the result obtained with the four-column configuration is shown in Fig. 20.7. In SFC, UV detection is the most common means of monitoring the eluent, but fluorescence [246], NMR [247], and MS [248] have also been used.

Examples of the use of packed-column SFC for drugs include methods for metoprolol and impurities [249], clevidipine impurities [250] and dosage forms [251], isosorbide mononitrate from tablets [252], and paclitaxel and impurities [253]. When used in combination with MS to direct fraction collection, semi-preparative packed-column SFC can be used as an alternative to HPLC for the purification of drugs [254]. Another field of application for SFC, where the increased efficiency of the technique shows benefits compared to HPLC, is in the area of enantiomer separations and tests of enantiomeric purity. These separations can be achieved either with a chiral column or by adding chiral modifiers (*e.g.*, tartatic acid or L-*N*-benzoyloxyarginine [244]) to the mobile phase. Recent examples of the use of chiral SFC for drugs include the resolution of metoprolol on Chiracel OD [244] and of clevidipine on Chiracel OD + Chirapak AD [244].

20.5 GAS CHROMATOGRAPHY

Except perhaps in drug analysis for forensic toxicology, where capillary GC and GC/MS are firmly established, and in the analysis of volatile drugs (mainly anesthetics), the use of GC for drug analysis continues to diminish. This is probably because many drugs are rather polar and involatile and thus often require derivatization before gas chromatography is possible. When this requirement is combined with the ready availability of HPLC/MS, with its sensitive detection of unmodified drugs there is not so much need for GC. Indeed, in many pharmaceutical laboratories gas chromatographs have been completely replaced by HPLC equipment. However, numerous examples of the use of GC in drug analysis are still being published. A good discussion of the use and limitations of GLC for drug impurity profiling was presented by Lauko [255].

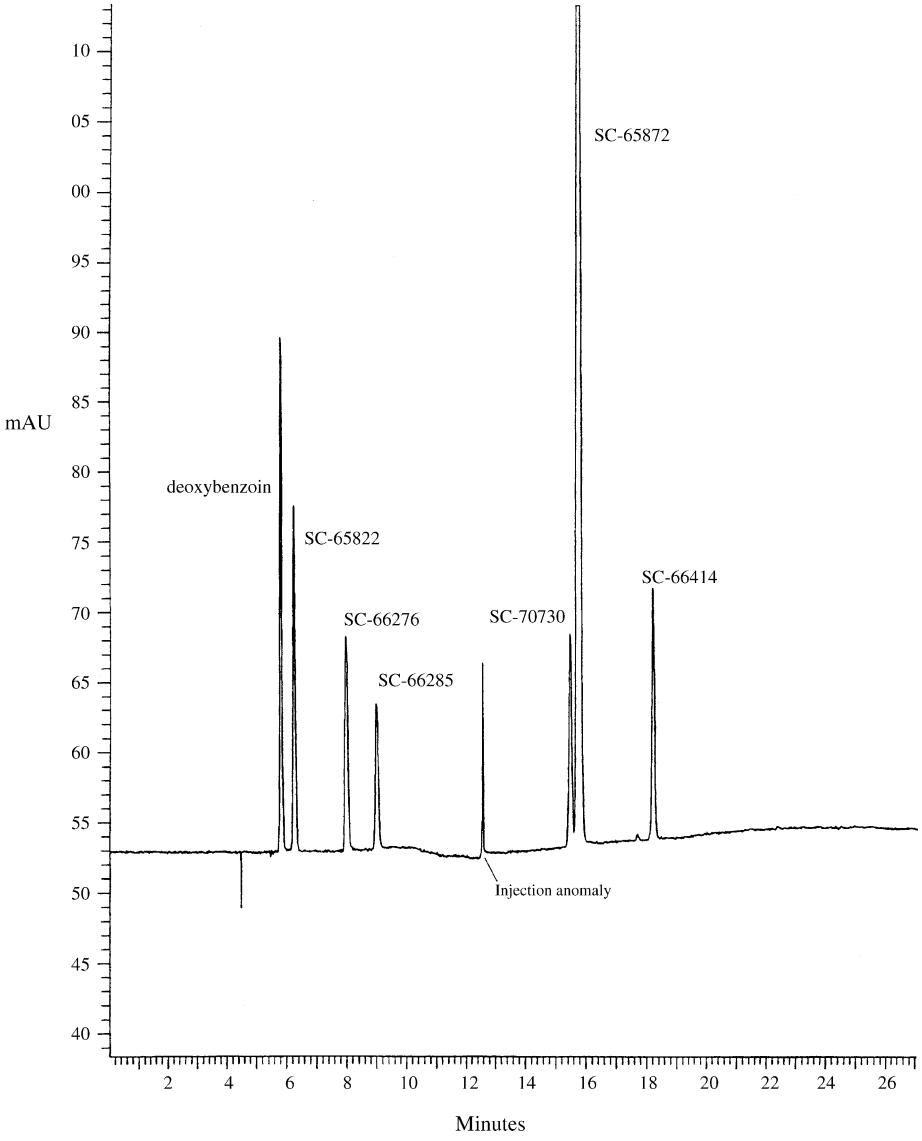


Fig. 20.7. SFC on four CN columns (each 250 × 4.6 mm ID), linked in series, showing for a drug and a range of impurity peaks. (Reproduced from Ref. 245 with permission.)

20.5.1 Following derivatization

Illustrative recent examples of the application of GC to drug analysis include the determination of the anti-depressant sertraline in plasma by capillary GC/MS [256], following extraction and derivatization with heptafluorobutyric acid. Detection limits of 0.1 ng/mL were achieved, using single-ion monitoring (SIM). A semi-automated method

for the analysis of the prostaglandin analog fluprostenal in plasma was developed with sample preparation by SPE, on ODS silica gel in a 96-well format, followed by derivatization to form a trimethylsilyl derivative, and quantification by capillary GC and negative-ion CI-MS/MS. This procedure allowed quantification of from 30 to 5,800 pg/mL of the drug in 0.1 mL of sample [257].

Fluoxetine and one of its metabolites (*p*-trifluoromethylphenol) have been determined by capillary GC with electron-capture detection, after derivatization with pentafluorobenzenesulfonyl chloride in order to study its fate upon incubation with microsomes. This methodology provided a LOD in the low-pg region (34.6 pg and 8.1 pg for the drug and its metabolite, respectively) [258]. The bronchodilator and anti-inflammatory drug fenspiride and two of its *in vivo* metabolites were detected after mixed-mode SPE from plasma and urine, followed by trimethylsilylation and capillary GC/MS [259]. Another example of the use of GC/MS for the analysis of drugs and their metabolites is provided by studies on the Phase I and Phase II metabolites of the anabolic steroid methandrosterone in urine. Following a variety of sample pretreatment techniques, including LLE, SPE, enzyme and chemical hydrolysis, analysis was performed by capillary GC/MS of their *O*-methyloxime trimethylsilyl derivatives [260].

An interesting example of the use of SPME, *in situ* derivatization followed by GC/MS, has been developed for the detection of drugs in urine. Initially, the urine sample was extracted with hexane/ethyl acetate in alkaline medium. The organic phase was then evaporated to dryness in a small headspace-analysis vial with the SPME fiber, "doped" with acetic anhydride/pyridine to effect acetylation of the analytes. After heating it at 200°C for 10 min, the SPME fiber was introduced into the gas chromatograph for thermal desorption of the derivatized drugs. With modification to remove lipids, the same procedure was also used for plasma samples. While used in this instance for a drug screening application, such a procedure could also be used for more conventional drug analysis [261].

20.5.2 Without derivatization

When compounds are volatile, derivatization may be dispensed with. An example of such a method is the analysis of clemastine, an anti-rhinitis compound, in plasma [262]. Following LLE with toluene, the compound was analyzed by capillary GC with a nitrogen/phosphorus detector (NPD). This method provided sensitivity down to 100 pg/mL and quantification up to 12.8 ng/mL. Another example is the analysis of fentanyl, a nitrogen-containing opiate, in plasma. LLE was also used for sample preparation, followed by capillary GC with a NPD. This method provided a LOD and LOQ of 0.1 and 0.5 ng/mL, respectively, based on a 200-μL sample [263]. The halogenated anesthetics halothane and desflurane were assayed by capillary GC/MS detection with a LOQ ranging from 2.9 μg/mL for halothane up to 9.2 μg/mL for desflurane (the pharmacological range being *ca.* 100 μg/mL). An example of the chromatograms obtained for this method is shown in Fig. 20.8 [264]. Sameridine, a local anesthetic with analgesic properties, was analyzed by GC, following LLE from plasma. Capillary GC with splitless injection was used with selective detection by a NPD [265].

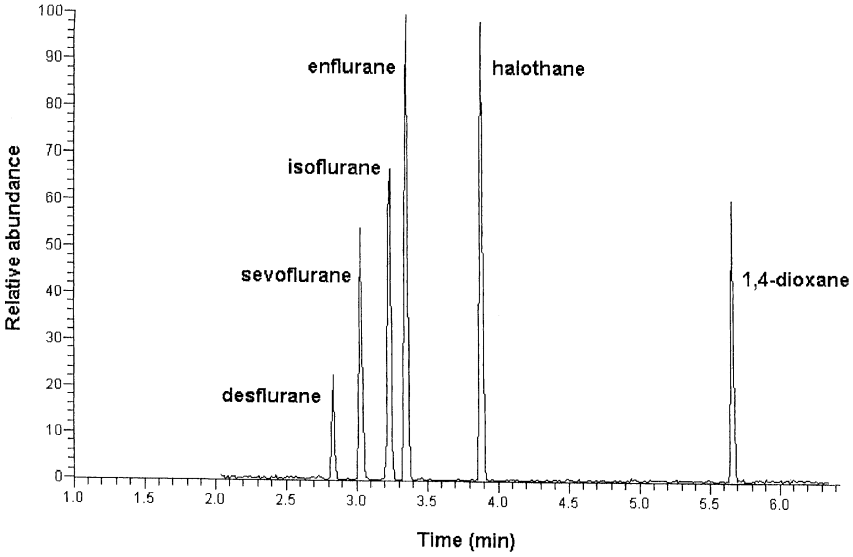


Fig. 20.8. Total ion chromatogram for a range of volatile anesthetics obtained by GC/MS. (Reproduced from Ref. 264 with permission.)

Tramadol, a centrally acting analgesic, and its *O*-desmethyl metabolite were analyzed by capillary GC/NPD on a DB-1 column, after LLE from plasma and brain extracts [266]. This assay gave a good linear range and enabled quantities of between 10 ng and 10 μg/mL plasma (or per gram of tissue) to be determined. A linear temperature gradient from 135 to 179°C at 4°C/min was employed. Nevirapine, a nitrogen-containing HIV-1 nucleoside reverse-transcriptase inhibitor, was also determined in human plasma by capillary GC without the need for derivatization. Using the selective NPD, a LOQ of 10 ng/mL was obtained (LOD 2 ng/mL) [267].

20.6 THIN-LAYER CHROMATOGRAPHY

Since TLC is well suited to the separation of most classes of drugs, there is a vast number of applications in the literature. Much of this information has been collected in books and reviews, which provide an excellent starting point for anyone wishing to perform or develop a separation [268–271]. TLC, therefore, remains an important "work horse" for the chromatographic separation of drugs and their metabolites, and is extensively treated in pharmacopoeias. In addition, TLC is widely used to analyze complex mixtures derived from medicinal plants. A particularly good coverage of this topic is provided by Wagner and Bladt [272] and also by Nyiredy and Glowniak [273].

However, with the growing use of HPLC, TLC is increasingly being confined to niche applications. Although not as easy to automate as either HPLC or GC, TLC has remained a popular method for the separation of drugs for the reasons that were given in the previous

edition of this work and in Chap. 6. It is inexpensive, undemanding in terms of equipment, flexible, and capable of high sample throughput because of the parallel nature of the separation. TLC at its simplest relies only on the human eye, and for rapid and efficient qualitative analysis it is probably the technique of choice. In addition to visual detection based on native color or UV quenching, treatment of the developed TLC plates with detection reagents of varying degrees of specificity can be used to extend the scope of the technique. TLC can also be used quantitatively, and can be combined with a wide range of detection methods of varying degrees of sensitivity and specificity (UV, fluorimetry, radioactivity detection, FTIR, Raman spectroscopy, and MS). The bulk of TLC separations continue to be performed in the normal-phase mode on silica gel, although there are a fair number of reports of separations on modified, usually C_{18}-bonded, layers. Probably the major change in pharmaceutical practice that has been observed over the last decade has been the gradual rise in the use of high-performance (HP) TLC. Recent accounts of the use of planar chromatography in drug synthesis and purification, and in the assessment of drug purity, are given in the reviews of Kalasz and Bathori [274], and Ferenczi-Fodor and Vegh [275]. An illustration of the use of HP-TLC, combined with scanning densitometry, for impurity profiling is provided by studies on the neuroleptic fluoxetine, where a method was developed for assessing the amount of (1-*R,S*)-3-methylamino-1-phenylpropan-1-ol, *N*-methyl-3-phenylpropan-1-amine, and an impurity in tablets or capsules [276]. Similarly, the drug gliclazide, a hypoglycemic drug, and its impurities were determined in formulations by TLC on silica gel, combined with scanning densitometry at 226 nm [277]. A typical recent example of the use of quantitative TLC, employing scanning densitometry for drugs in formulations can be found in the determination of doxazosin, an α_1-antagonist. This application, performed on silica gel HP-TLC plates with UV detection at 277 nm, resulted in a highly accurate and reproducible method [278]. Similarly methods for acyclovir [279,280], dexamethasone, neomycin, ketoconazole and coenzyme Q_{19} [281], tripolidine hydrochloride and methscopolamine [282], and a mixture of benazepril hydrochloride and hydrochlorothiazide [283] have been described.

An area where an increasing number of applications can be expected in drug analysis is the use of the technique of OPLC (overpressured-layer chromatography) (Chap. 6). In OPLC an impermeable layer is placed over the stationary phase under an "overpressure" such that the plate effectively becomes a planar column. This configuration allows solvents to be forced through the layer, effecting rapid and efficient analysis. Recent examples of its use for purity testing are provided by a series of studies on steroids [284–286]. Another frequently reported use of TLC is the investigation of hydrophobicity by means of reversed-phase (RP) layers (generally C_{18}-bonded) and relating R_F to lipophilicity. Typical examples of this type of application are the use of RP-TLC to investigate the hydrophobicity of novel fungicidal compounds [287], NSAIDs drugs [288] and a range of *N*-substituted α-piperazine-γ-hydroxybutyric acids [289].

While chiral separations have featured strongly in HPLC, developments in TLC have been less dramatic. There have been relatively few applications of chiral separations for drugs by TLC, and this is unlikely to change. The subject of enantiomer separation in TLC has been subject to several reviews [290,291], and recent publications report the use of

molecularly imprinted stationary phases for the separation of ephedrine-related compounds [292,293], β-blockers and NSAIDs [294,295]. β-Blockers have also been resolved on plates prepared from cellulose and cellulose triphenylcarbamate [296]. TLC, when combined with radioactivity detectors, continues to be important as a means of examining the metabolic fate of radio-labeled drugs in biological systems. This topic has been reviewed [297,298], and an example of the application of OPLC to such studies is provided by a series of investigations on the metabolic fate of the drug deramciclane [299,300].

20.6.1 Detectors

In general, much of the spectroscopic characterization that has been undertaken on substances separated on TLC plate has continued to be performed by means of the traditional "scrape-and-elute" methods and very little by *in situ* characterization.

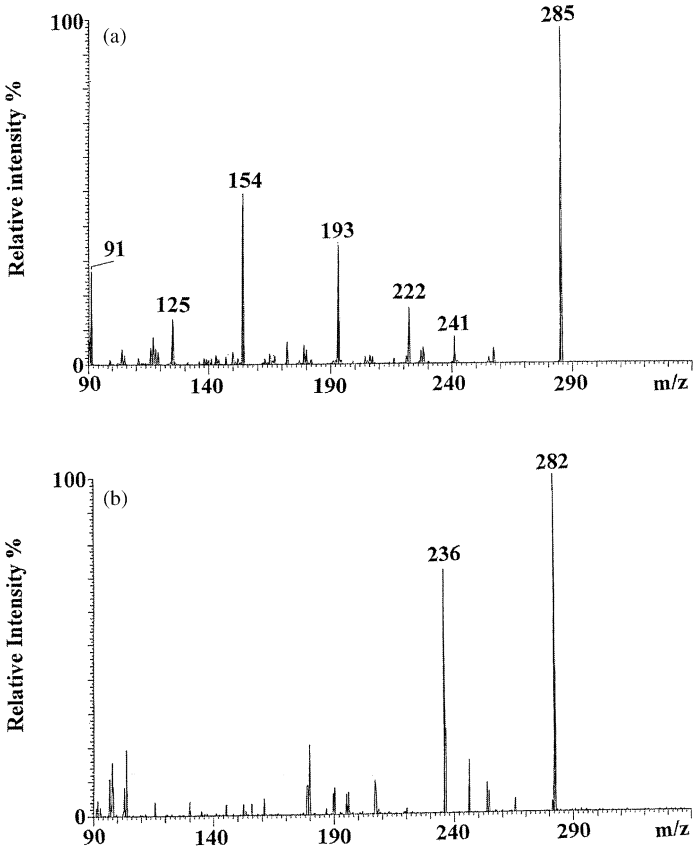


Fig. 20.9. FAB/MS/MS spectra for (a) diazepam (m/z 285) and (b) nitrazepam (m/z 282) obtained from the silica gel of a HP-TLC plate. (Reproduced from Ref. 305 with permission.)

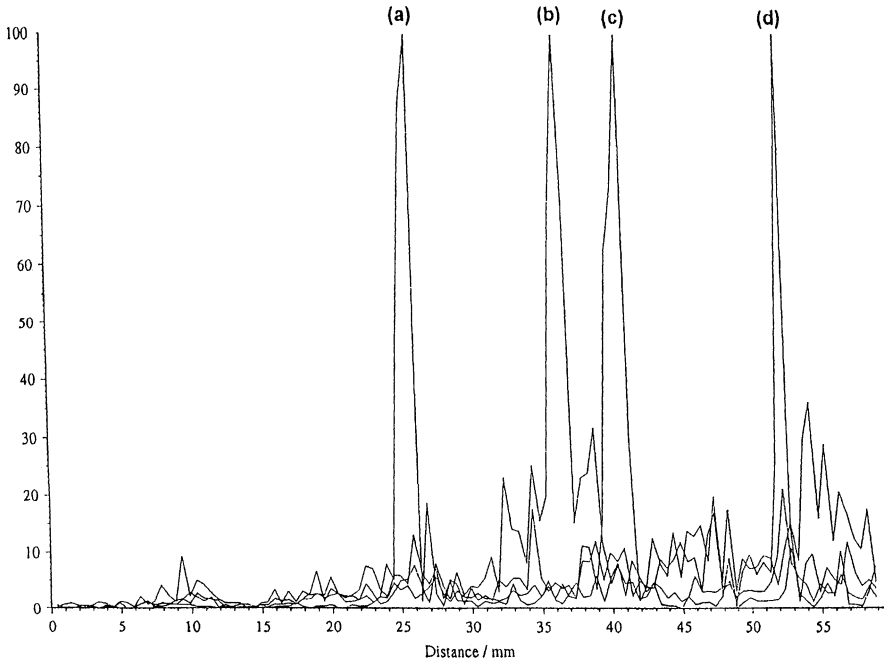


Fig. 20.10. Overlaid ion chromatograms obtained via TLC/MALDI/MS analysis of a mixture of four compounds (a, UK-224,671; b, UK-256,327; c, UK-253,501 and d, UK-260,489 (25 μg/component). (Reproduced from Ref. 306 with permission.)

However, while it is more difficult to couple TLC directly with MS and other forms of spectroscopy, there are a number of examples of where this has been successfully implemented. In particular, the ability to obtain UV (and fluorescence spectra) from TLC plates has been available for many years. More informative forms of spectroscopy, such as IR and MS, have also been combined with TLC. The area of TLC/MS has been recently reviewed [301,302], and illustrative examples include the analysis of NSAIDs [303], morphine [304], and benzodiazepines [305] in biological fluids with fast-atom bombardment (FAB)-MS/MS, and TLC/MALDI/MS for some experimental compounds [306]. For FAB-MS/MS, silica was removed from the plate and mixed with the FAB matrix prior to spectroscopy. Typical results for diazepam and nitrazepam in urine, obtained from the silica gel of a HP-TLC plate [305] are shown in Fig. 20.9. However, the TLC/MALDI procedure [306] does not require the removal of the stationary phase enabling ion-chromatograms to be constructed, as shown in Fig. 20.10.

FTIR has also been applied to the detection and identification of substances *in situ* on TLC plates. TLC/FTIR has been reviewed [307,308]. Applications to drugs include the identification of impurities of benzodiazepines, such as chlordiazepoxide [309], nordiazepam and flurazepam [307], and caffeine, paracetamol, and phenazone [310]. Both qualitative and quantitative applications of TLC/FTIR are described in these studies.

REFERENCES

1 K. Macek and J. Macek, in E. Heftmann (Ed.), *Chromatography*, 5th Edn., Elsevier, Amsterdam, 1992, pp. B394–B445.
2 D. Stevenson and I.D. Wilson (Eds.), *Sample Preparation for Biomedical and Environmental Analysis*, Plenum, New York, 1994.
3 C.F. Poole and I.D. Wilson (Eds.), Solid-Phase Extraction, *J. Chromatogr. A*, 885 (2000).
4 J. Pawliszyn, *Solid-Phase Microextraction, Theory and Practice*, Wiley-VCH, New York, 1997.
5 E.M. Thurman and M.S. Mill, *Solid-Phase Extraction, Principles and Practice*, Wiley, New York, 1998.
6 A. Handley (Ed.), *Extraction methods in Organic Analysis*, Sheffield Academic Press/CRC Press, Sheffield, 1999.
7 J.S. Fritz, *Analytical Solid-Phase Extraction*, Wiley-VCH, New York, 1999.
8 N.J.K. Simpson (Ed.), *Solid-Phase Extraction, Principles, Techniques and Applications*, Dekker, New York, 2000.
9 R.A. Biddlecombe and S. Pleasance, *J. Chromatogr. B*, 734 (1999) 257.
10 K.L. locker, D. Morrison and A.P. Watt, *J. Chromatogr. B*, 750 (2001) 13.
11 M.C. Rouan, C. Buffet, F. Marfil, H. Humbert and G. Maurer, *J. Pharm. Biomed. Anal.*, 25 (2001) 995.
12 H.K. Jones, L.E. Stafford, H.C. Swaisland and R. Payne, *J. Pharm. Biomed. Anal.*, 29 (2002) 221.
13 H. Simpson, A. Berthemy, R. Burton, J. Newton, M. Kealy, D. Wells and D. Wu, *Rapid Commun. Mass Spectrom.*, 12 (1998) 75.
14 N. Zhang, K.L. Hoffman, W. Li and D.T. Rossi, *J. Pharm. Biomed. Anal.*, 22 (2000) 131.
15 T. Lemoine, D. Breilh, D. Ducint, J. Dubrez, J. Jougon, J.F. Velly and M.C. Saux, *J. Chromatogr. B*, 742 (2000) 247.
16 C.S. Tamvakopoulos, J.M. Neugebauer, M. Donnelly and P.R. Griffin, *J. Chromatogr. B*, 776 (2002) 161.
17 R.C. Simpson, A. Skarbek and B.K. Matuszewski, *J. Chromatogr. B*, 755 (2002) 133.
18 N. Lindegardh and Y. Bergqvist, *J. Chromatogr. B*, 744 (2000) 9.
19 O.V. Olesen, P. Plougmann and K. Linnet, *J. Chromatogr. B*, 746 (2000) 233.
20 N.H. Huang, J.R. Kagel and D.T. Rossi, *J. Pharm. Biomed. Anal.*, 19 (1999) 613.
21 U. Christians, W. Jacobsen, N. Serkova, L.Z. Benet, C. Vidal, K.-F. Sewing, M.P. Manns and G.I. Kirchner, *J. Chromatogr. B*, 748 (2000) 41.
22 Q. Song and L. Putcha, *J. Chromatogr. B*, 763 (2001) 9.
23 G. Gmeiner, T. Geisendorfer, J. Kainzbauer, M. Nikolajeveic and H. Tausch, *J. Chromatogr. B*, 768 (2002) 215.
24 D. Ohman, B. Carlsson and B. Norlander, *J. Chromatogr. B*, 753 (2001) 365.
25 S. Hartter, H. Weigmann and C. Hiemke, *J. Chromatogr. B*, 740 (2000) 135.
26 S. Liu, M. Kamijo, T. Takayasu and S. Takayama, *J. Chromatogr. B*, 767 (2002) 53.
27 R. Tritler, M. Erlich, T.J. Galla, R.E. Horch and K. Kummerer, *J. Chromatogr. B*, 775 (2002) 127.
28 K. Heinig and F. Bucheli, *J. Chromatogr. B*, 769 (2002) 9.
29 K.-S. Boos and A. Rudolphi, *LC–GC Int.*, 11 (1998) 84.
30 V. Capka and Y. Xu, *J. Chromatogr. B*, 762 (2001) 181.
31 A. El Mahjoub and C. Staub, *J. Chromatogr. B*, 754 (2001) 271.

32 H. Keski-Hynnila, K. Raanaa, M. Forsberg, P. Mannisto, J. Taskinen and R. Kostiainen, *J. Chromatogr. B*, 759 (2001) 227.
33 C. Arthur and J. Pawliszyn, *Anal. Chem.*, 62 (1990) 2145.
34 J. Pawliszyn (Ed.), *Applications of Solid Phase Microextraction*, Royal Society of Chemistry, London, 1999.
35 H. Lord and J. Pawliszyn, *J. Chromatogr. A*, 885 (2000) 153.
36 N.H. Snow, *J. Chromatogr. A*, 885 (2000) 445.
37 H.G. Ugland, M. Krogh and K. Rasmussen, *J. Chromatogr. B*, 701 (1997) 29.
38 H. Lord and J. Pawliszyn, *Anal. Chem.*, 69 (1997) 3889.
39 C. Battu, P. Marquet, A. Fauconnet, E. Lecassie and G. Lachatre, *J. Chromatogr. Sci.*, 36 (1998) 1.
40 S.-W. Myung, S. Kim, J.-H. Park, M. Kim, J.-C. Lee and T.-J. Kim, *Analyst*, 124 (1999) 1283.
41 H. Seno, T. Kumazawa, A. Ishii, M. Nishikawa, H. Hattori and O. Suzuki, *Jpn. J. Forensic Toxicol.*, 13 (1995) 211.
42 T. Watanabe, A. Namera, M. Yashiki, Y. Iwasaki and T. Kojima, *J. Chromatogr. B*, 709 (1998) 225.
43 G.J. de Jong and E.H.M. Koster, *Chromatographia*, 52 (2000) S-12.
44 S. Ulrich and J. Martens, *J. Chromatogr. B*, 696 (1997) 217.
45 X.P. Lee, T. Kumazawa, K. Sato and O. Suzuki, *J. Chromatogr. Sci.*, 35 (1997) 302.
46 A. Nanera, T. Watanabe, M. Yashiki, Y. Iwasaki and T. Kojima, *J. Anal. Toxicol.*, 22 (1998) 297.
47 S. Li and S. Weber, *Anal. Chem.*, 69 (1997) 1217.
48 B. Hall and J. Brodbelt, in J. Pawliszyn, (Ed.), *Applications of Solid Phase Microextraction*, Royal Society of Chemistry, London, p. 497
49 B. Hall and J. Brodbelt, *J. Chromatogr. A*, 777 (1997) 275.
50 K. Jinno, M. Taniguchi, H. Sawada and M. Hayashida, in J. Pawliszyn (Ed.), *Applications of Solid Phase Microextraction*, Royal Society of Chemistry, London, 1999.
51 M. Krogh, H. Grefslie and K. Rasmussen, *J. Chromatogr. B*, 689 (1997) 357.
52 Y. Luo, L. Pan and J. Pawliszyn, *J. Microcol. Sep.*, 10 (1998) 193.
53 M. Satterfield, D.M. Black and J.S. Brodbelt, *J. Chromatogr. B*, 759 (2001) 33.
54 A.R. Raghani, *J. Pharm. Biomed. Anal.*, 29 (2002) 507.
55 D. Stevenson, *J. Chromatogr. B*, 745 (2000) 39.
56 N. Delaunay, V. Pichon and M.-C. Hennion, *J. Chromatogr. B*, 745 (2000) 15.
57 D. Stevenson, in I.D. Wilson, E.R. Adlard, M. Cooke and C.F. Poole (Eds.), *Encyclopaedia of Separation Science*, Academic Press, London, 2000, pp. 3060.
58 B.A. Rashid, G.W. Aherne, M.F. Katmeh, P. Kwasowskia and D. Stevenson, *J. Chromatogr. A*, 797 (1998) 245.
59 B.A. Rashid, P. Kawasoki and D. Stevenson, *J. Pharm. Biomed. Anal.*, 21 (1999) 635.
60 J. Olsen, P. Martin and I.D. Wilson, *Anal. Commun.*, 35 (1998) 13H.
61 D. Stevenson, *Trends Anal. Chem.*, 18 (1999) 154.
62 L.I. Anderson, *J.Chromaogr. B*, 739 (2000) 163.
63 L.I. Anderson, *J.Chromatogr. B*, 745 (2000) 3.
64 F. Lanza and B. Sellergren, *Chromatographia*, 53 (2001) 99.
65 N. Masque, R.M. Marce and F. Borrull, *Trends Anal. Chem.*, 20 (2001) 477.
66 K. Haupt, *Analyst*, 126 (2001) 747.
67 B. Sellergren, *Anal. Chem.*, 66 (1994) 1578.
68 W.M. Mullet and E.P.C. Lai, *Anal. Chem.*, 70 (1998) 3636.
69 W.M. Mullet and E.P.C. Lai, *Microchem. J.*, 61 (1999) 143.
70 W.M. Mullet and E.P.C. Lai, *J. Pharm. Biomed. Anal.*, 21 (1999) 835.

71 L.I. Andersson, *Analyst*, 125 (2000) 1515.
72 K.-S. Boos and C.T. Fleischer, *Fresenius J. Anal. Chem.*, 371 (2001) 16.
73 J. Haginaka and H. Sanabe, *Anal. Chem.*, 72 (2000) 5206.
74 W.M. Mullet, P. Martin and J. Pawliszyn, *Anal. Chem.*, 73 (2001) 2383.
75 P. Martin, I.D. Wilson, E.D. Morgan, G.R. Jones and K. Jones, *Anal. Commun.*, 34 (1997) 45.
76 J. Olsen, P. Martin, I.D. Wilson and G.R. Jones, *Analyst*, 124 (1999) 467.
77 P. Martin, I.D. Wilson and G.R. Jones, *J. Chromatogr.*, 889 (2000) 143.
78 P. Martin, I.D. Wilson and G.R. Jones, *Chromatographia Suppl.*, 52 (2000) S-19.
79 P. Martin, T.D. Wilson, I.D. Wilson and G.R. Jones, *Analyst*, 126 (2001) 757.
80 C. Bergen, S. Bayoudth, D. Sherrington and K. Ensing, *J. Chromatogr. A*, 889 (2000) 105.
81 B.A. Rashid, R.J. Briggs, J.N. Hay and D. Stevenson, *Anal. Commun.*, 34 (1997) 303.
82 R.F. Venn and R.J. Goody, *Chromatographia*, 50 (1999) 407.
83 A. Bereczki, A. Tolokan, G. Horvai, V. Horvath, F. Lanza, A.J. Hall and B. Sellergren, *J. Chromatogr. A*, 930 (2001) 31.
84 J. Ayrton, *Rapid Commun. Mass Spectrom.*, 11 (1997) 1953.
85 J. Ayrton, G.J. Dear, W.J. Leavens, D.N. Mallet and R.S. Plumb, *Rapid Commun. Mass Spectrom.*, 11 (1999) 1953.
86 C. Chassaing, P. Macrae, P. Wright, A. Harper, J. Luckwell, K. Saunders and R. Venn, *Chromatographia*, 53 (2001) 122.
87 D. Zimmer, V. Packard, W. Czembor and C. Muller, *J. Chromatogr. A*, 584 (1999) 22.
88 J.L. Herman, *Rapid Commun. Mass Spectrom.*, 16 (2002) 1.
89 J. Ayrton, G.J. Dear, W.J. Leavens, D.N. Mallet and R.S. Plumb, *Rapid Commun. Mass Spectrom.*, 13 (1999) 1657.
90 M. Jemal and W. Yuan-Qing, *Rapid Commun. Mass Spectrom.*, 12 (1998) 1389.
91 J.-T. Wu, H. Zeng, M. Qian, B.L. Brogdan and S.E. Unger, *Anal. Chem.*, 72 (2000) 61.
92 N. Brignol, R. Bakhtiar, L. Dou, T. Majumdar and F.L.S. Tse, *Rapid Commun. Mass Spectrom.*, 14 (2000) 141.
93 J.M. Long, C.A. James, B.J. Clark, M.G. Castelli and S. Rolando, *Chromatographia*, 55 (2002) S31.
94 B. Lausecker, B. Hess, G. Fischer, M. Mueller and G. Hopfgartner, *J. Chromatogr. B*, 749 (2000) 67.
95 H.S. Lee, C.K. Jeong, S.J. Choi, S.B. Kim, M.H. Lee, G.I. Ko and D.H. Sohn, *J. Pharm. Biomed. Anal.*, 23 (2000) 775.
96 C.H. Jeong, H.-Y. Lee, M.-S. Jang, W.B. Kim and H.S. Lee, *J. Chromatogr. B*, 752 (2001) 141.
97 W. Blum, R. Aichholz, P. Ramstein, J. Kuhnol, W. Froestl and S. Desrayaud, *J. Chromatogr. B*, 748 (2000) 349.
98 S. Ludtke, H. Switek, W. Roth, S. Wild and J. Schmid, *Chromatographia*, 55 (2002) S-45.
99 O. Keil, T. LeRiche, H. Deppe and D.A. Volmer, *Rapid Commun. Mass Spectrom.*, 16 (2002) 814.
100 P.A. Wabnitz and J.A. Loo, *Rapid Commun. Mass Spectrom.*, 16 (2002) 85.
101 G. Dear, R. Plumb and D. Mallet, *Rapid. Commun. Mass Spectrom.*, 15 (2001) 152.
102 R. Plumb, G. Dear, D. Mallet and J. Ayrton, *Rapid Commun. Mass Spectrom.*, 15 (2001) 986.
103 J.-T. Wu, H. Zeng, Y. Deng and S. Unger, *Rapid Commun. Mass Spectrom.*, 15 (2001) 1113.
104 Y. Hsieh, G. Wang, Y. Wang, S. Chackalamannil, J.-M. Brisson, K. Ng and W. Korfmacher, *Rapid Commun. Mass Spectrom.*, 16 (2002) 944.
105 P.T. Vallano, R.S. Mazenko, E.J. Woolf and B.K. Matuszewski, *J. Chromatogr. B*, 779 (2002) 249.
106 E.S.M. Lutz, M.E. Markling and C.M. Masimirembwa, *J. Chromatogr. B*, 780 (2002) 205.

107 Y. Deng, J.-T. Wu, T. Lloyd, C.L. Chi, T.V. Olah and S.E. Unger, *Rapid Commun. Mass Spectrom.*, 16 (2002) 1116.
108 M.K. Bayliss, D. Little, D.N. Mallet and R.S. Plumb, *Rapid Commun. Mass Spectrom.*, 14 (2000) 2039.
109 U.D. Neue, C.H. Phoebe, K. Tran, Y.-F. Cheng and Z. Lu, *J. Chromatogr. A*, 925 (2001) 49.
110 R.M. Smith and R.J. Burgess, *Anal. Comm.*, 33 (1996) 327.
111 D.J. Miller and S.B. Hawthorne, *Anal. Chem.*, 69 (1997) 623.
112 R.M. Smith and R.J. Burgess, *J. Chromatogr. A*, 785 (1997) 49.
113 I.D. Wilson, *Chromatographia*, 52 (2000) S-28.
114 M. Lammerhofer and W. Lindner, in K. Valko (Ed.), *Separation Methods in Drug Synthesis and Purification*, Elsevier, Amsterdam, 2000, p. 337.
115 S. Gorog, in S. Gorog (Ed.), *Identification and Determination of Impurities in Drugs*, Elsevier, Amsterdam, 2000, p. 525.
116 J. Haginaka, *J. Pharm. Biomed. Anal.*, 27 (2002) 357–372.
117 J.C. Reepmeyer, *Chirality*, 8 (1996) 11.
118 E. Tesarpova, M. Gilar, A. Jegorov, M. Uhrova and Z. Deyl, *Biomed. Chromatogr.*, 11 (1997) 321.
119 P.K. Owens, A.F. Fell, M.W. Coleman and J.C. Berridge, *Chirality*, 9 (1997) 184.
120 P.K. Owens, A.F. Fell, M.W. Coleman and J.C. Berridge, *Chirality*, 8 (1996) 466.
121 C. Petterson, A. Karlsson and C. Gioeli, *J. Chromatogr.*, 407 (1987) 217.
122 G. Szepesi, M. Gazdag and R. Ivancsics, *J. Chromatogr.*, 244 (1982) 33.
123 E. Heldin, H.H. Huynh and C. Pettersson, *J. Chromatogr.*, 450 (1992) 339.
124 British Pharmacopoeia 1998, Her Majesties Stationary Office, London, 1998, p. 1153.
125 J. Ekelund, A. van Arkens, K. Bronnum-Hansen, K. Fich, L. Olsen and P.V. Petersen, *J. Chromatogr. A*, 708 (1995) 253.
126 A. Ceccato, P. Hubert and J. Cromen, *J. Chromatogr. A*, 760 (1997) 193.
127 H. Makamba, V. Andriasano, R. Gotti, V. Cavrini and G. Felix, *J. Chromatogr. A*, 818 (1998) 43.
128 C. Rustichelli, V. Ferioli and G. Gamberini, *Chromatographia*, 44 (1997) 477.
129 R. Cirilli and F. Latorre, *J. Chromatogr. A*, 818 (1998) 53.
130 A. Karlsson and A. Aspegren, *Chromatographia*, 47 (1998) 189.
131 E. Francotte, A. Davatz and P. Richert, *J. Chromatogr. B*, 686 (1996) 77.
132 H. Haupt, C. Pettersson and D. Westerlund, *Chirality*, 7 (1995) 23.
133 A. Karlsson, D. Petterson and K. Hernqvist, *Chirality*, 7 (1995) 147.
134 Z.D. Zhivkova and V.N. Russeva, *J. Chromatogr. B*, 714 (1998) 277.
135 B. Loun and D.S. Hage, *Anal. Chem.*, 68 (1996) 1218.
136 H. Hofstetter, O. Hofstetter and V. Shurig, *J. Chromatogr. A*, 764 (1997) 35.
137 B.A. Olsen, D.D. Wirth and J.S. Larew, *J. Pharm. Biomed. Anal.*, 17 (1998) 623.
138 G.W. Ponder, S.L. Butram, A.G. Adams, C.S. Ramanathum and J.T. Stewart, *J. Chromatogr. A*, 692 (1995) 173.
139 H.L. Zhang, J.T. Stewart and M. Ujhelyi, *J. Chromatogr. B*, 688 (1995) 309.
140 K. Petritis, A. Valleix, C. Elfakir and M. Dreux, *J. Chromatogr. A*, 913 (2001) 331.
141 F. Mangani, T. Korkolainen and M. Karlsson, *Chirality*, 8 (1996) 511.
142 K.M. Rentsch, U. Gutteck-Amsler, R. Buehrer, K.E. Fattinger and D.J. Vonderschnitt, *J. Chromatogr. B*, 742 (2000) 131.
143 K.H. Kim, H.J. Ki, J.H. Kim and S.D. Shin, *J. Chromatogr. B*, 751 (2001) 69.
144 B. Mistry, L.J. Leslie and N.D. Eddington, *J. Chromatogr. B*, 758 (2001) 153.
145 E. Bransteterova and I.W. Wainer, *J. Chromatogr. B*, 732 (1999) 395.

146 L. Tollsten, in S. Gorog (Ed.), *Identification and Determination of Impurities in Drugs*, Elsevier, Amsterdam, 2000, p. 266.
147 S.X. Peng, B. Borah, R.L.M. Dobson, Y.D. Liu and S. Pikul, *J. Pharm. Biomed. Anal.*, 20 (1999) 75.
148 M. Babjak, G. Balogh, M. Gazdag and S. Gorog, *J. Pharm. Biomed. Anal.*, 29 (2002) 1153.
149 M.J. Lovdahl and S.R. Priebe, *J. Pharm. Biomed. Anal.*, 23 (2000) 521.
150 X. Xu, M.G. Barlett and J.T. Stewart, *J. Pharm. Biomed. Anal.*, 26 (2001) 367.
151 Z. Zhao, X.-Z. Qin and R.A. Reed, *J. Pharm. Biomed. Anal.*, 29 (2002) 173.
152 I. Chu, F. Liu, A. Soares, P. Kumari and A.A. Nomeir, *Rapid Commun. Mass Spectrom.*, 16 (2001) 1501.
153 S. Lane, in K. Valko (Ed.), *Separation Methods in Drug Synthesis and Purification*, Elsevier, Amsterdam, 2000, p. 127.
154 L. Fang, J. Cournoyer, M. Demee, J. Zhao, D. Tokushige and B. Yan, *Rapid Commun. Mass Spectrom.*, 16 (2002) 1440.
155 D.A. McLoughlin, T.V. Olah and D.J. Gilbert, *J. Pharm. Biomed. Anal.*, 15 (1997) 1893.
156 T.V. Olah, D.A. McLoughlin and D.J. Gilbert, *Rapid Commun. Mass Spectrom.*, 1 (1997) 17.
157 J. Berman, K. Halm, K. Adkinson and J. Schaffer, *J. Med. Chem.*, 40 (1997) 827.
158 H.-Z. Bu, M. Poglod, R.J. Micetich and J.K. Khan, *Rapid Commun. Mass Spectrom.*, 15 (2001) 335.
159 C. Tannergren, P. Languth and K.-J. Hoffmann, *Pharmazie*, 56 (2001) 337.
160 B. Kuo, T.V. Noord, M.R. Feng and D.S. Wright, *J. Pharm. Biomed. Anal.*, 16 (1998) 137.
161 Y. Hsieh, M.S. Bryant, J.-M. Brisson, K. Ng and W.A. Korfmacher, *J. Chromatogr. B*, 767 (2002) 353.
162 M.K. Bayliss, D. Little, D. Mallet and R.S. Plumb, *Rapid Commun. Mass Spectrom.*, 14 (2000) 2039.
163 L. Yang, T.D. Mann, D. Little, N. Wu, R.P. Clement and P.J. Rudewicz, *Anal. Chem.*, 73 (2001) 1740.
164 Y.-Q. Xia, C.E.C.A. Hop, D.Q. Liu, S.H. Vincent and H.L. Chiu, *Rapid Commun. Mass Spectrom.*, 15 (2001) 2135.
165 J.-T. Wu, *Rapid Commun. Mass Spectrom.*, 15 (2001) 73.
166 Y. Deng, H. Zeng, S.E. Unger and J.-T. Wu, *Rapid Commun. Mass Spectrom.*, 15 (2001) 1634.
167 M. Jemal, M. Huang, Y. Mao, D. Whigan and M.L. Powell, *Rapid Commun. Mass Spectrom.*, 15 (2001) 994.
168 R.C. King, C. Miller-Stein, D.J. Magiera and J. Brann, *Rapid Commun. Mass Spectrom.*, 16 (2002) 43.
169 R.A. Biddlecome and S. Pleasance, *J. Chromatogr. B*, 734 (1999) 257.
170 A. Xu, K. Linderholm, L. Peng and J. Hulse, *J. Pharm. Biomed. Anal.*, 14 (1996) 447.
171 Y.-X. Li, K. Neufeld, J. Chastain, A. Curtis and P. Vegelati, *J. Pharm. Biomed. Anal.*, 16 (1997) 961.
172 M.D. Moyer, T. Johansen and R.J. Stubbs, *J. Pharm. Biomed. Anal.*, 17 (1998) 45.
173 L. Romanyshy, P.R. Tiller and C.E.C.A. Hop, *Rapid. Commun. Mass Spectrom.*, 14 (2000) 1662.
174 J. Ayrton, R. Plumb, W.J. Leavens, D. Mallet, M. Dickems and G.J. Dear, *Rapid Commun. Mass Spectrom.*, 12 (1998) 217.
175 F. Leroy, B. Presle, F. Verillon and E. Verette, *J. Chromatogr. Sci.*, 39 (2001) 487.
176 D.L. Burhman, P.I. Price and P.J. Rudewicz, *J. Am. Soc. Mass Spectrom.*, 7 (1996) 1099.
177 B.K. Matuszewski, M.L. Constanzer and C.M. Chavez-Eng, *Anal. Chem.*, 70 (1998) 882.

178 R. Bonfigolio, R.C. King, T.V. Olah and K. Merkle, *Rapid. Commun. Mass Spectrom.*, 13 (1999) 1175.
179 B. Law and D. Temesi, *J. Chromatogr. B*, 748 (2000) 21.
180 A. Eerkes, T. Addison and W. Naidong, *J. Chromatogr. B*, 768 (2002) 277.
181 C.M. Chavez-Eng, M.L. Constanzer and B.K. Matuszewski, *J. Chromatogr. B*, 748 (2000) 31.
182 C.M. Chavez-Eng, M.L. Constanzer and B.K. Matuszewski, *J. Chromatogr. B*, 767 (2002) 117.
183 C.K. Hall, A.D. Penman, C.K. Smith and P.D. Martin, *J. Chromatogr. B*, 772 (2002) 219.
184 B. Baktiar, J. Lohne, L. Ramos, L. Khemi, M. Hayes and F. Tse, *J. Chromatogr. B*, 768 (2002) 325.
185 G.F. van Rooyen, M.J. Smit, A.D. de Jager, H.K.L. Hundt, K.J. Swart and A.F. Hundt, *J. Chromatogr. B*, 768 (2002) 223.
186 C.J. Smith, I.D. Wilson, F. Abou-Shakra, R. Payne, H. Grisedale, A. Long, D. Roberts and M. Malone, *Chromatographia*, 55 (2002) S151.
187 B.-O. Axelsson, M. Jornten-Karlsson, P. Michelsen and F. Abou-Shakra, *Rapid Commun. Mass Spectrom.*, 15 (2001) 375.
188 J.K. Nicholson, J.C. Lindon, G. Scarfe, I.D. Wilson, F. Abou-Shakra, J. Castro Perez, A. Eaton and S. Preece, *Analyst*, 125 (2000) 235.
189 J.K. Nicholosn, J.C. Lindon, G.B. Scarfe, I.D. Wilson, F. Abou-Ahakra, A.B. Sage and J. Castro-Perez, *Anal. Chem.*, 73 (2001) 1491.
190 P. Marshall, O. Heudi, S. Mckeown, A. Amour and F. Abou-Shakra, *Rapid Commun. Mass Spectrom.*, 16 (2002) 220.
191 F.R. Abou-Shakra, A.B. Sage, J. Castro-Pereez, J.K. Nicholson, J.C. Lindon, G.B. Scarfe and I.D. Wilson, *Chromatographia*, 55 (2002) S9.
192 O. Corcoran, J.K. Nicholson, J.K. Lenz, F. Abou-Shakra, J. Castro-Perez, A.B. Sage and I.D. Wilson, *Rapid Commun. Mass Spectrom.*, 14 (2000) 2377.
193 C.J. Duckett, N.J.C. Bailey, H. Walker, F. Abou-Shaker, I.D. Wilson, J.C. Lindon and J.K. Nicholson, *Rapid Commun. Mass Spectrom.*, 16 (2002) 245.
194 E.H. Evans, J.-C. Wolff and C. Eckers, *Anal. Chem.*, 73 (2001) 4722.
195 K. Roberts and R.J. Smith, *J. Chromatogr. A*, 677 (1994) 385.
196 N. Mistry, I.M. Ismail, M.S. Smith, J.K. Nicholson and J.C. Lindon, *J. Pharm. Biomed. Anal.*, 16 (1997) 697.
197 N. Mistry, A.D. Roberts, G.E. Tranter, P. Francis, I. Barylski, I.M. Ismail, J.K. Nicholson and J.C. Lindon, *Anal. Chem.*, 71 (1999) 2838.
198 B.C.M. Potts, K.F. Albizati, M.O. Johnson and J.P. James, *Magn. Reson. Chem.*, 37 (1999) 393.
199 S.X. Peng, B. Borah, R.L.M. Dopbson, Y.D. Liu and S. Pikul, *J. Pharm. Biomed. Anal.*, 20 (1999) 75.
200 C. Yang, Y.K. Si, W.Y. He., *Chin. J. Anal. Chem.*, 29 (2001) 796.
201 B.E. Segmuller, B.L. Armstrong, R. Dunphy and A.R. Oyler, *J. Pharm. Biomed. Anal.*, 23 (2000) 927.
202 W. Feng, H. Liu, G. Chen, R. Malchow, F. Bennet, E. Lin, B. Pramanik and T.C. Chan, *J. Pharm. Biomed. Anal.*, 25 (2001) 545.
203 N. Mistry, I.M. Ismail, M.S. Smith, J.K. Nicholson and J.C. Lindon, *J. Pharm. Biomed. Anal.*, 16 (1997) 697.
204 J.C. Lindon, R.D. Farrant, P.N. Sanderson, P.M. Doyle, S.L. Gough, M. Spraul and M. Hofmann, *Magn. Reson. Chem.*, 33 (1995) 857.
205 J. Chin, J.B. Fell, M. Jarosinski, M.J. Shapiro and J.R. Warring, *J. Org. Chem.*, 63 (1998) 386.
206 K. Albert, G. Schlotterbeck, U. Braumann, H. Handel, M. Spraul and G. Krack, *Angew. Chem. Int. Ed. Engl.*, 34 (1995) 1014.

207 M. Hofmann, P. Dvorstak, J.K. Nicholson and I.D. Wilson, *Anal. Chem.*, 65 (1993) 327.
208 M. Spraul, M. Hofmann, I.D. Wilson, E. Lenz, J.K. Nicholson and J.C. Lindon, *J. Pharm. Biomed. Anal.*, 11 (1993) 1009.
209 I.D. Wilson, J.K. Nicholson, M. Hofmann, M. Spraul and J.C. Lindon, *J. Chromatogr.*, 617 (1993) 324.
210 M. Spraul, M. Hofmann, J.C. Lindon, J.K. Nicholson and I.D. Wilson, *NMR Biomed.*, 7 (1994) 295.
211 A.W. Nicholls, R.D. Farrant, J.P. Shockcor, S.E. Unger, I.D. Wilson, J.C. Lindon and J.K. Nicholson, *J. Pharm. Biomed. Anal.*, 15 (1997) 901.
212 U.G. Sidelmann, U. Braumann, M. Hofmann, M. Spraul, J.C. Lindon, J.K. Nicholson and S.H. Hansen, *Anal. Chem.*, 69 (1997) 607.
213 U.G. Sidelmann, I. Bjornsdottir, J.P. Shockor, S.H. Hansen, J.C. Lindon and J.K. Nicholson, *J. Pharm. Biomed. Anal.*, 24 (2001) 569.
214 P.J.D. Foxall, E.M. Lenz, J.C. Lindon, G.H. Nield, I.D. Wilson and J.K. Nicholson, *Ther. Drug Monitor.*, 18 (1996) 498.
215 J.P. Shockcor, S.E. Unger, P. Savina, J.K. Nicholson and J.C. Lindon, *J. Chromatogr. B*, 748 (2000) 269.
216 J. Shockor, R.W. Wurm, L.W. Frick, P.N. Sanderson, R.D. Farrant, B.C. Sweatman and J.C. Lindon, *Xenobiotica*, 26 (1996) 189.
217 A.W. Nicholls, S.T. Caddick, I.D. Wilson, R.D. Farrant, J.C. Lindon and J.K. Nicholson, *Biochem. Pharmacol.*, 49 (1995) 1155.
218 A.W. Nicholls, J.C. Lindon, R.D. Farrant, J.P. Shockor, I.D. Wilson and J.K. Nicholson, *J. Pharm. Biomed. Anal.*, 20 (1999) 865.
219 A.E. Mutlib, J.P. Shockcor, S.Y. Chen, R.J. Espina, D.J. Pinto, M.J. Orwat, S.R. Prakash and L.S. Gan, *Chem. Res. Tox.*, 15 (2002) 48.
220 W.J. Ehlardt, J.M. Woodland, T.M. Baughman, M. Vandenbranden, S.A. Wrighton, J.S. Kroin, B.H. Norman and S.R. Maple, *Drug Met. Dispos.*, 26 (1998) 42.
221 A.E. Mutlib, S. Diamond, J.P. Shockcor, R. Way, G. Nemeth, L. Gan and D.D. Christ, *Xenobiotica*, 30 (2000) 1091.
222 M. Dachtler, H. Handel, T. Glaser, D. Lindquist, R.M. Hawk, C.N. Karson, R.A. Komoroski and K. Albert, *Magn. Reson. Chem.*, 38 (2000) 951.
223 G.J. Dear, J. Ayrton, R. Plumb, B.C. Sweatman, I.M. Ismail, I.J. Fraser and P.J. Mutch, *Rapid Commun. Mass Spectrom.*, 12 (1998) 2023.
224 G.J. Dear, R.S. Plumb, B.C. Sweatman, P.S. Parry, A.D. Roberts, J.C. Lindon, J.K. Lindon, J.K. Nicholson and I.M. Ismail, *J. Chromatogr. B*, 748 (2001) 295.
225 A.E. Mutlib, J.T. Strupeczewski and S.M. Chesson, *Drug Metab. Disp.*, 23 (1995) 951.
226 J.C. Lindon, J.K. Nicholson and I.D. Wilson, in K. Albert (Ed.), *On-line LC-NMR and Related Techniques*, Wiley, Chichester, 2002, p. 45.
227 I.D. Wilson, L. Griffiths, J.C. Lindon and J.K. Nicholson, in S. Gorog (Ed.), *Identification and Determination of Impurities in Drugs*, Elsevier, Amsterdam, 2000, p. 299.
228 K. Albert (Ed.), *On-line LC-NMR and Related Techniques*, Wiley, Chichester, 2002.
229 F.S. Pullen, A.G. Swanson, M.J. Newman and D.S. Richards, *Rapid Commun. Mass Spectrom.*, 9 (1995) 1003.
230 J.P. Shockor, S.E. Unger, I.D. Wilson, P.J. Foxall, J.K. Nicholson and J.C. Lindon, *Anal. Chem.*, 68 (1996) 4431.
231 K.I. Burton, J.R. Everett, M.J. Newman, F.S. Pullen, D.S. Richards and A.G. Swanson, *J. Pharm. Biomed. Anal.*, 15 (1997) 1903.
232 E. Clayton, S. Taylor, B. Wright and I.D. Wilson, *Chromatographia*, 47 (1998) 264.

233 G.J. Dear, J. Ayrton, R. Plumb, B.C. Sweatman, I.M. Ismail, I. Fraser and P.J. Mutch, *Rapid Commun. Mass Spectrom.*, 12 (1998) 2023.
234 G.B. Scarfe, B. Wright, E. Clayton, S. Taylor, I.D. Wilson, J.C. Lindon and J.K. Nicholson, *Xenobiotica*, 28 (1998) 373.
235 R.M. Holt, M.J. Newman, F.S. Pullen, D.S. Richards and A.G. Swanson, *J. Mass Spectrom.*, 32 (1997) 64.
236 R. Smith, O. Chienthavorn, I.D. Wilson, B. Wright and S.D. Taylor, *Anal. Chem.*, 71 (1991) 4493.
237 R.M. Smith, O. Chienthavorn, S. Saha, I.D. Wilson, B. Wright and S. Taylor, *J. Chromatogr. A*, 886 (2000) 289–295.
238 S.H. Hansen, A.G. Jensen, C. Cornett, I. Bjornsdotitir, D. Taylor, B. Wright and I.D. Wilson, *Anal. Chem.*, 71 (1999) 5235.
239 D. Louden, A. Handley, S. Taylor, E. Lenz, S. Miller and A. Sage, *Anal. Chem.*, 72 (2000) 3922.
240 O. Louden, A. Handley, S. Taylor, I. Sinclair, E. Lenz and I.D. Wilson, *Analyst*, 126 (2001) 1625.
241 I.D. Wilson, P. Davies and R.J. Ruane, in J.R. Dean (Ed.), *Applications of Supercritical Fluids in Industrial Analysis*, Blackie, Glasgow, 1993, p. 74.
242 A. Salvador, A. Jamie, G. Becerra and M. De La Gaurdia, *Frezenius. J. Anal. Chem.*, 356 (1996) 109.
243 T.A. Berger, *J. Chromatogr. A*, 785 (1997) 3.
244 O. Gyllenall, in S. Gorog (Ed.), *Identification and Determination of Impurities in Drugs*, Elsevier, Amsterdam, 2000, p. 382.
245 D.A. Roston, S. Ahmed, D. Williams and T. Catalano, *J. Pharm. Biomed. Anal.*, 26 (2001) 339.
246 R.M. Smith, O. Chienvathavorn, N. Danks and I.D. Wilson, *J. Chromatogr.*, 798 (1998) 203.
247 K. Albert, *J. Chromatogr.*, 785 (1997) 65.
248 E. Moyano, M. McCullagh, M.T. Galceran and D.E. Games, *J. Chromatogr.*, 777 (1997) 167.
249 O. Gyllenall, A. Karlson and J. Vessman, *J. Chromatogr.*, 862 (1999) 95.
250 L. Karlsson, O. Gyllenhall, A. Karlson and J. Gottfries, *J. Chromatogr.*, 749 (1996) 193.
251 O. Gyllenhall and J. Hulthe, *J. Pharm. Biomed. Anal.*, 29 (2002) 381.
252 J.L. Bernal, M.J. Nozal, J.M. Rivera, M.L. Serna and L. Toribio, *Chromatographia*, 42 (1996) 89.
253 N.K. Jagota, J.B. Nair, R. Frazer, M. Klee and M.Z. Wang, *J. Chromatogr.*, 721 (1996) 315.
254 T. Wang, M. Barber, I. Hardt and D.B. Kassel, *Rapid Commun. Mass Spectrom.*, 15 (2001) 2067.
255 A. Lauko, in S. Gorog (Ed.), *Identification and Determination of Impurities in Drugs*, Elsevier, Amsterdam, 2000, p. 183.
256 K.M. Kim, B.H. Jung, M.H. Choi, J.S. Woo, K-J. Paeng and B.C. Chung, *J. Chromatogr. B*, 769 (2002) 333.
257 R.D. Gauw, P.J. Stoffolano, D.L. Kuhlenbeck, V.S. Patel, S.M. Garver, T.R. Baker and K.R. Wehmeyer, *J. Chromatogr. B*, 744 (2000) 283.
258 Z.-Q. Liu, Z.-R. Tan, D. Wang, S.-L. Huang, L.-S. Wang and H.-H. Zhou, *J. Chromatogr. B*, 769 (2002) 305.
259 M.C. Dumasia, E. Houghton, W. Hyde, D. Greulich, T. Nelso and J. Peterson, *J. Chromatogr. B*, 767 (2002) 131.
260 A.R. McKinney, D. Ridley and C.J. Suann, *J. Chromatogr. B*, 765 (2001) 71.
261 U. Staerk and W.R. Kulpmann, *J. Chromatogr. B*, 745 (2000) 399.

262 N.N. Davydova, S.U. Yasuda, R.L. Woosley and I.W. Wainer, *J. Chromatogr. B*, 744 (2000) 177.
263 H.S. Choi, H.-C. Shin, G. Khang, J.M. Rhee and H.B. Lee, *J. Chromatogr. B*, 765 (2001) 63.
264 N.C. Yang, K.L. Hwang, C.H. Shen, H.F. Wang and W.M. Ho, *J. Chromatogr. B*, 759 (2001) 307.
265 C. Norsten-Hoog, P. Neidenstrom and T. Arvidsson, *J. Chromatogr. B*, 760 (2001) 123.
266 Q. Tao, D.J. Stone, Jr., M.R. Borenstein, V. Jean-Bart, E.E. Codd, T.P. Coogan, D. Desai-Krieger, S. Liao and R.B. Raffa, *J. Chromatogr. B*, 763 (2001) 165.
267 P. Langemann, D. Schirmer, T. Vath, S. Desch, M. Zilly and H. Klinker, *J. Chromatogr. B*, 767 (2002) 69.
268 G. Szepesi and S. Nyiredy, in J. Sherma and B. Fried (Eds.), *Handbook of Thin-Layer Chromatography*, 2nd Edn., Dekker, New York, 1996, p. 819.
269 B. Fried and J. Sherma, *Thin-Layer Chromatography, Techniques and Applications*, 3rd Edn., Dekker, New York, 1994.
270 F. Kreuzig, Antibiotics, in J. Sherma and B. Fried (Eds.), *Handbook of Thin-Layer Chromatography*, 2nd Edn., Dekker, New York, 1996, p. 445.
271 K. Ferennci-Fodor, Z. Vegh and B. Renger, in Sz. Nyiredy (Ed.), *Planar Chromatography, A Retrospective View for the Third Millenium*, Springer, Budapest, 2001, p. 585.
272 H. Wagner and S. Bladt, *Plant Drug Analysis, A Thin layer Chromatographic Atlas*, Springer, Berlin Heidelberg, 1996.
273 Sz. Nyiredy and G. Glowniak (Eds.), *Planar Chromatography, A Retrospective View for the Third Millenium*, Springer, Budapest, 2001, p. 550.
274 H. Kalasz and M. Bathori, in K. Valko (Ed.), *Separation Methods in Drug Synthesis and Purification*, Elsevier, Amsterdam, 2000, p. 439.
275 K. Ferenczi-Fodor and Z. Vegh, in S. Gorog (Ed.), *Identification and Determination of Impurities in Drugs*, Elsevier, Amsterdam, 2000, p. 146.
276 J. Krzek and A. Maslanka, *J. Planar Chromatogr.*, 15 (2002) 50.
277 J. Krzek, M. Dabrowska and U. Hubicka, *J. Planar Chromatogr.*, 14 (2001) 183.
278 R.T. Sane, M. Francis, P.M. Hill, S. Pawar and A.V. Pathak, *J. Planar Chromatogr.*, 15 (2002) 34.
279 S.S. Dubhashi and P.R. Vavia, *J. Pharm. Biomed. Anal.*, 23 (2000) 1017.
280 T.K. Sia, L. Wulanderi and G. Indrayanto, *J. Planar. Chromatogr.*, 15 (2002) 42.
281 D. Agbaba, E. Slavica, G. Markovic, V. Nedeljkovic, S. Veselinovic and M. Vucetic, *J. Planar Chromatogr.*, 13 (2000) 333.
282 H.M. Baseki and J. Sherma, *J. Planar Chromatogr.*, 13 (2000) 16.
283 A. El-Gindy, A. Ashour, L. Abdel-Fattah and M.M. Shabana, *J. Pharm. Biomed. Anal.*, 25 (2001) 171.
284 A. Kassai, A. Szerci, A. Koppany, Z. Vegh and K. Ferenci-Fodor, *J. Planar Chromatogr.*, 13 (2000) 30.
285 A. Wiszkidenszky, S. Maho, Z. Vegh and K. Ferenczi-Fodor, *J. Planar Chromatogr.*, (1998) 11.
286 B. Bagocsi, D. Fabian, A. Lauko, M. Mezei, S. Maho, Z. Vegh and K. Ferenczi-Fodor, *J. Planar Chromatogr.*, 15 (2002) 252.
287 J.K. Rozylo, A. Niewiadomy, A. Zabinska and J. Matysiak, *J. Planar Chromatogr.*, 11 (1998) 450.
288 E. Forgacs, T. Cserhati, R. Kaliszan, P. Haber and A. Nasal, *J. Planar Chromatogr.*, 11 (1998) 383.
289 B. Malawska, *J. Planar Chromatogr.*, 11 (1998) 137.

290 K. Gunther and K. Moller, Enantiomer Separations in J. Sherma and B. Fried (Eds.), *Handbook of Thin-Layer Chromatography*, 2nd Edn., Dekker, New York, 1996, p. 621.
291 L. Lepri, M. Del Bubba and A. Cincinelli, in Sz. Nyiredy (Ed.), *Planar Chromatography, A Retrospective for the Third millennium*, Springer, Budapest, 2001, p. 517.
292 R. Suedee, C. Songkram, A. Petmoreekul, S. Sangkunakup, S. Sankasa and N. Kongyarit, *J. Planar Chromatogr.*, 11 (1998) 272.
293 R. Suedee, C. Songkram, A. Petmoreekul, S. Sangkunakup, S. Sankasa and N. Kongyarit, *J. Pharm. Biomed. Anal.*, 19 (1999) 519.
294 R. Suedee, T. Srichana, J. Saelim and T. Thavornpibulbut, *Analyst*, 124 (1999) 1003.
295 R. Suede, T. Srichana, T. Saelim and T. Thavonpibulbut, *J. Planar. Chromatogr.*, 14 (2001) 194.
296 L. Lepri, A. Cincinelli and M. Del Bubba, *J. Planar Chromatogr.*, 12 (1999) 302.
297 T. Clark and O. Klein, in J. Sherma and B. Fried (Eds.), *Handbook of Thin-Layer Chromatography*, 2nd Edn. Dekker, New York, 1996, p. 341.
298 I. Klebovich, in Sz. Nyiredy (Ed.), *Planar Chromatography, A Retrospective View for the Third Millenium*, Springer, Budapest, 2001, p. 293.
299 J. Szunyog, E. Mincsovics, I. Hazai and I. Klebovich, *J. Planar Chromatogr.*, 11 (1998) 25.
300 I. Klebovich, E. Mincsovics, J. Szunyog, K. Ludanyi, T. Karancsi, K. Ujszaszy, B. Kiss and K. Vekey, *J. Planar Chromatogr.*, 11 (1998) 394.
301 I.D. Wilson, *J. Chromatogr. A*, 856 (1999) 429.
302 K.L. Busch, in Sz. Nyiredy (Ed.), *Planar Chromatography, A Retrospective View for the Third Millenium*, Springer, Budapest, 2001, p. 261.
303 W. Morden and I.D. Wilson, *Rapid Commun. Mass Spectrom.*, 18 (1996) 1951.
304 F. Tames, I.D. Watson, W. Morden and I.D. Wilson, *J. Chromatogr. A*, 729 (1999) 341.
305 F. Tames, I.D. Wilson, W.E. Morden and I.D. Wilson, *J. Planar Chromatogr.*, 13 (2000) 432.
306 A. Crecelius, M.R. Clench, D.S. Richards, J. Mather and V. Parr, *J. Planar Chromatogr.*, 13 (2000) 77.
307 S.A. Stahlman, *Planar Chromatogr.*, 12 (1999) 5.
308 I.O.C. Rager and K.-A. Kovar, in Sz. Nyiredy (Ed.), *Planar Chromatography, A Retrospective for the Third Millenium*, Springer, Budapest, 2001, p. 247.
309 S. Stahlman and K.-A. Kovar, *J. Chromatogr. A*, 813 (1998) 145.
310 O.R. Frey, K.-A. Kovar and V. Hoffman, *J. Planar Chromatogr.*, 6 (1993) 93.

This page is intentionally left blank

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 21

Environmental analysis

DAMIA BARCELÓ, MIRA PETROVIĆ, ETHEL ELJARRAT,
MARÍA JOSÉ LÓPEZ DE ALDA and ADAMANTIA KAMPIOTI

CONTENTS

21.1 INTRODUCTION

Chemical contamination of the environment is an insidious side effect of human population growth and technological development. The industrial development has brought prosperity to millions of people, but has also left a legacy of a polluted environment that continues to impact our natural resources, and ultimately, human well-being. A wide range of man-made chemicals designed for use in industry, agriculture, and consumer goods as well as chemicals unintentionally formed or produced as by-products of industrial processes or combustion are of potential concern. Besides recognized pollutants, numerous new chemicals are synthesized each year and released into the environment with unforeseen consequences. Generally, environmental contaminants fall broadly into three groups:

(a) The first group includes "conventional" priority contaminants, which have long been recognized as posing risks to human health, due to their acute toxicity, carcinogenic or mutagenic effects, and their persistence in the environment. Examples of these compounds include polycyclic aromatic hydrocarbons (PAH), polychlorinated biphenyls (PCB), lead, benzene, and dichlorodiphenyltrichloroethane (DDT) and its metabolites. Legislation as well as monitoring of these compounds follows long-established standards, and certified methods, developed by the Environmental Protection Agency (EPA) and the International Organization for Standardization (ISO), are available. However, these compounds continue to be studied, and research is oriented toward improvements of analytical methodology with the objective to lower detection limits, increase sample throughput, minimize sample manipulation, and increase overall method efficiency in terms of selectivity and sensitivity.

(b) The second group includes compounds that are relatively new on regulatory lists, and evidence regarding their unforeseen adverse health effects and occurrence in the environment are still emerging. Among them, the endocrine disrupting compounds (EDC) have recently become a major area of concern [1,2].

(c) The third group includes unregulated contaminants, which may be candidates for future regulation, depending on research on their potential health effects and monitoring data regarding their occurrence. This group is mainly composed of products used in

everyday life, such as surfactants and surfactant residues, pharmaceuticals and personal care products, gasoline additives, fire retardants, plasticizers, etc. Many believe that of all the emerging contaminants, antibiotics are the biggest concern, however other compounds, especially polar metabolites and complex mixtures (chemical cocktails) present great challenges to toxicologist [3,4].

This chapter attempts to survey the application of modern chromatographic techniques in environmental analysis. Since it is impossible to cover all analytes of interest, we have focused our attention on recent trends in environmental analysis and only selected classes of contaminants. Recent advances in the analysis of persistent organic pollutants (POP) are also surveyed, and general trends in sample preparation and instrumental analysis are discussed. However, this survey does not include applications of well-established techniques such as liquid chromatography (LC) and gas chromatography (GC) coupled with conventional detectors, which are covered in Part A of this book. Similarly, sample preparation is discussed with emphasis on recent advances in this field, rather than routine methods.

21.2 SAMPLING AND SAMPLE PREPARATION

Sampling and sample preparation typically account for more than 75% of analysis time. The analysis of trace components in complex environmental matrices requires multi-step sample preparation. The general problem in the analysis of complex environmental samples is that the extract obtained by exhaustive extraction techniques typically contains a large number of matrix components, which may not separate from the analytes and interfere with the quantitative analysis. The presence of interfering substances demands either a very selective detection or tedious extract clean-up, or even both. Generally, multi-step sample pre-treatment aimed at the reduction of the matrix content and the enrichment of the target compounds still remains the most direct means of obtaining maximum sensitivity. Efforts are aimed at the development of cost-effective sample handling techniques, characterized by efficiency and simplicity of operations and devices. Several prevailing trends can be distinguished:

(a) application of highly specific, tailored sorbents [*i.e.*, molecularly imprinted polymers (MIP), immunosorbents, restricted-access materials (RAM)] for SPE;
(b) integration of several sample preparation steps into one [*i.e.*, application of passive samplers for simultaneous sampling, extraction and enrichment of pollutants from liquid and gaseous samples and matrix solid-phase dispersion (MSPD) for biological matrices]; and
(c) automation through coupling of sample preparation units and detection systems [*i.e.*, on-line SPE/LC, on-line solid-phase micro-extraction (SPME)/GC, and SPME/LC].

21.2.1 Water samples

Surface, ground, and drinking waters are among the less-complicated matrices, in contrast to wastewater samples and solid environmental samples (sludge, sediment, soil,

biological tissue). Therefore, the sampling strategy often includes just simple bottling. For surface waters (river, lake, sea), discrete (grab) sampling has frequently been employed, whereas for wastewaters composite samples are often collected over sampling periods of from 6 h to several days. One of the major drawbacks of conventional "grab" sampling is that it may fail to detect episodic contamination. Long-term monitoring is preferable, but time-consuming and costly. To overcome this problem, modern sampling strategies for long-term monitoring combine sampling, analyte isolation, and pre-concentration into a single step. An example of this strategy is the application of passive samplers.

After collection of water samples, their further pre-treatment, including preservation, depends on the type of analysis, analytes, and their stability in the sample matrix prior to the extraction of target analytes. Several comprehensive stability studies have been performed, comparing standard and alternative preservation methods for different organic pollutants in water matrices: triazine herbicides [5], organophosphorus pesticides [6], phenolic compounds [7], benzene- and naphthalenesulfonates [8,9], ionic and nonionic surfactants [10–12] and estrogens [13]. These studies showed that conventional methods of preservation (addition of formaldehyde, $HgCl_2$, sulfuric acid, or sodium azide) are not always appropriate, and significant losses of some compounds can occur. The use of SPE materials (disks, cartridges, and disposable precolumns) for the stabilization and storage of analytes, pre-concentrated from water samples was found to be more suitable.

Extraction of target analytes from water is usually performed by SPE, SPME, or L/L extraction. Although still prescribed in some official methods of analysis, L/L extraction entails numerous limitations: It requires large volumes of organic solvents, yielding dilute extracts, and it is not ideal for polar and ionic analytes and difficult to automate. In contrast, SPE requires only small volumes of solvents and yields concentrated extracts. It can be highly selective with an appropriate choice of sorbents, and it is suitable for automation [14] and on-line coupling with chromatographic systems [15]. Portable field samplers for SPME are applicable to most analytes, even volatile compounds, and can be coupled to GC or LC equipment. Equilibrium (non-exhaustive) extraction without solvent can be performed on a wide variety of fibers.

21.2.1.1 Passive sampling

Passive sampling is defined as a sampling technique based on the free flow of analyte molecules from the sampled medium to a collecting medium, as a result of a difference in chemical potentials of the analyte between the two media [16]. It requires the deployment of a calibrated device which uses a diffusion gradient to collect pollutants over a period of days to weeks. These systems are used to measure time-averaged or integrated concentrations of water-dissolved pollutants to which the sample was exposed. The passive sampling devices are not affected by short-term fluctuations in analyte concentrations and thus give more adequate information for long-term monitoring of water quality. Passive sampling of water samples has a much shorter history than that of gaseous samples. Devices used today can be divided into two main groups: membrane- and diffusion-based samplers [17]. Membrane-based samplers are more popular. Several designs have been proposed: semi-permeable membrane devices (SPMD), passive *in situ*

concentration/extraction samplers (PICES), supported-liquid membrane devices (SLM), solvent-filled devices, and sorbent-filled devices. Of all passive samplers, SPMD [18–21] proved to be the most effective in mimicking the biological concentration of analytes in fat tissues (organism surrogate), thus allowing the detection of lipophilic substances in low concentrations and reflecting accurately their concentration factors in aquatic organisms. SPMD consist of a sealed, tubular, low-density polyethylene, flat membrane (25- to 250-μm thin), filled with a high-molecular-weight lipid – typically, high-purity synthetic triolein. After a typical sampling time of 30 days, the SPMD is removed from the aquatic environment and washed with KOH. The analytes are recovered by dialysis against nonpolar solvents. Clean-up by size-exclusion chromatography (SEC) or adsorption chromatography is necessary before analyte classes can be concentrated by SPE and then chromatographed. The main disadvantage of the technique is that the procedure for recovering the analytes from the triolein collecting medium and their subsequent purification is quite tedious. Membrane-based passive samplers are a useful tool for determining average or integrated contaminant concentrations during an exposure interval, and, in spite of their drawbacks, their application in water analysis is constantly increasing. Passive sampling, driven by lipid/water partitioning, has found numerous applications in the analysis of a wide spectrum of organic pollutants in surface waters, ground water, agricultural run-offs, and industrial effluents. Contaminants monitored include: PAH [20–23], PCB, dioxins, and furans [24–26], organochlorine pesticides [24,27,28], organotin compounds [29], chlorophenols [30], and polar biocides (diuron, irgarol) [31].

21.2.1.2 Solid-phase extraction

The concept of tailoring extraction and clean-up materials has matured in recent years. The introduction of several new materials, such as immunosorbents, MIP, and RAM, has undoubtedly reduced the cost of analyzing organic contaminants and enhanced the selectivity of sample preparation. The *immunosorbents*, such as polyclonal antibodies, are immobilized on silica-based supports, activated Sephadex gels, synthetic polymers, sol/gel materials, cyclodextrins, or RAM and packed into cartridges or pre-columns [32,33]. Selected applications of immunosorbents in the analysis of aqueous environmental samples are compiled in Table 21.1. Immuno-affinity extraction, coupled with LC/ESI-MS has been used for the analysis of β-estradiol and estrone in wastewater [34]. The highly selectivity immunosorbents removed much of the interfering background material that otherwise would have seriously suppressed ionization of the estrogens during the electrospray process, and achieved low detection limits of 0.8 and 0.07 ng/L for β-estradiol and estrone, respectively.

Molecularly imprinted polymers (MIP) can be considered to be synthetic antibodies, developed for class- or compound-specific extraction. Porous MIP with antibody-like affinities for an imprinted target analyte are prepared by non-covalent and covalent imprinting. Non-covalently imprinted MIP are prepared by dissolving the target analyte in a mixture of one or several monomers and a cross-linker, a porogen (solvent), and a radical starter. After polymerization, the polymer is ground up and extensively extracted to

TABLE 21.1

APPLICATION OF IMMUNO-AFFINITY EXTRACTION IN ENVIRONMENTAL ANALYSIS OF WATER

Analyte	Sample	Ref.
Atrazine and glyphosite	Surface waters	[326]
Phenylurea & triazine herbicides	Ground water, sea water	[53,54]
Triazine biocides (Irgarol)	Sea water	[327]
Triazines	River and wastewater	[328]
Phenylureas	Ground water, river water	[329]
PAH	Wastewater, groundwater	[330–332]
PAH, benzidine, nitroaniline, aromatic amines	Industrial effluents	[333,334]
Benzene derivatives (BTEX)	Gasoline-contaminated waters	[335]
Steroid sex hormones	Wastewater	[34]

remove the soluble analyte, leaving molecular imprints of the analyte in the polymer. Instead of ground polymers, MIP may be in the form of membranes generated by polymerization on a glass filter, by phase-inversion precipitation, or by evaporation of the solvent, and by *in situ* polymerization of MIP in capillaries or columns, resulting in coatings, or macro-porous fillings [35]. Selective binding (molecular recognition) of a given target compound occurs in an aprotic, low-polarity organic solvent (*i.e.*, the solvent used for polymerization), and this results in the formation of specific hydrogen bonds between the analyte and imprinted polymer. MIP offer important advantages: Besides exhibiting selectivity for specific analytes, they are reusable and more stable in various environments and more quickly prepared than antibodies. However, in some cases MIP tend to exhibit class-specific rather than analyte-specific behavior, in combination with some non-specific binding of non-target analytes, and their affinities and capacities are in most cases lower than those of immunoaffinity cartridges or columns [32].

The current research in the molecular imprinting field is concentrated on MIP-SPE which seems to be one of the most promising applications for MIP today and, at the same time, the application that is closest to commercialization [36]. MIP-SPE has been used mainly in biochemical analysis for the extraction of analytes from blood, urine, bile, etc. [37], whereas applications in the environmental field are still rare. Molecularly imprinted polymers were also used as SPE materials for the trace determination of pesticides [38–42], mostly triazines, in water samples [40–42]. Baggiani *et al.* [43] has also used a MIP as a solid-phase extraction column material for the clean-up of the template molecule (2,4,5-trichlorophenoxyacetic acid) and some related herbicides (2,4-dichlorophenoxyacetic acid, fenoprop, dichlorprop) from river water samples at a concentration level of ng/mL. The technique is still under development, and the use of MIP sorbents in environmental applications is expected to increase.

Restricted access materials [RAM] are bifunctional sorbent, tailored for the fractionation of samples into macro-molecular matrix components and

low-molecular-weight analytes. The term "restricted access" expresses the limited accessibility of macro-molecular matrix components to adsorption sites of well-defined porous sorbents [44]. Size-exclusion separation of unwanted matrix components is a result of topographical restriction, achieved either by a physical diffusion barrier (small pore diameter), or by a chemical diffusion barrier (chemically bonded phase). Low-molecular-weight analytes, able to access active adsorption centers (alkyl chains, or ion-exchange groups) at the inner pore surface, are retained by a reversed-phase, affinity, or ion-pair mechanism, respectively [45,46]. RAM have been applied to direct extraction and enrichment of hydrophobic low-molecular-weight analytes from biological fluids and food samples [47,48], rich in proteins. However, RAM have rarely been applied to water analysis. Tailor-made RAM have been successfully applied to the separation of humic substances, interfering in the analysis of triazines [42], acidic herbicides [49], and polar fungicides [50] in river and groundwater. They have also found use in the on-line clean-up of complex samples and high-throughput flow immunoassay of triazines [51].

21.2.1.3 On-line solid-phase extraction

Over the past several years, there has been an increase in the use of automated instruments that integrate extraction, purification, and detection. One option is on-line coupling of SPE and LC, utilizing special sample preparation units, *e.g.*, PROSPEKT (Spark Holland) or OSP-2 (Merck), and disposable extraction cartridges. On-line SPE/LC coupling techniques have been successfully applied to the analysis of pesticides, PAH, and antifouling agents in water, significantly reducing analysis time, increasing sensitivity, and decreasing the amounts of sample and solvent required [52–58]. Similarly, on-line coupling of SPE to GC is a promising approach with good prospects [59,60]. An on-line SPE/LC method has recently been described for the analysis of the most relevant estrogens and progestogens in water samples. The procedure, based on the on-line SPE of the water sample and subsequent analysis by LC/diode-array detection (DAD) [61], or by LC/ESI-MS [62] allowed monitoring of the target compounds at the ng/L level in up to 16 samples, the maximum number that the PROSPEKT system can process, in a fully automated, unattended operation. Koeber *et al.* [42] described a highly innovative approach for selective extraction of triazine pesticides from water samples, using RAM in combination with MIP. This highly selective on-line SPE procedure, the so-called Six-SPE (size-selective sample separation and solvent switch), involves three different chromatographic processes. First, a RAM pre-column eliminates macro-molecular matrix components by SEC. The low-molecular-weight sample components and analytes are enriched by adsorption chromatography (RP-active sites in an inner-pore surface of RAM). Finally, after a solvent switch, the target analytes are selectively adsorbed on the tailor-made MIP sorbents in a process of affinity chromatography.

21.2.1.4 Solid-phase micro-extraction

The use of SPME in water analysis has increased dramatically over the past several years, as it allows efficient extraction, reduced solvent consumption and analysis time, and

it is easily automated. It is applicable to the determination of a wide range of volatile, semi-volatile and non-volatile organic contaminants and is particularly suited for field analyses and for rapid-response applications. Depending on the analyte and matrix, SPME of water samples can be performed in three different modes: direct-immersion extraction (for less volatile compounds and relatively clean samples), headspace extraction (for more volatile compounds and dirtier samples), and membrane-protected SPME (for the extraction of analytes in heavily polluted samples).

The major part of SPME applications has been developed for GC, due to the ease of coupling these two techniques. In HPLC, coupling is more complex and requires specially designed interfaces to desorb the analytes from the fibers. Coupling of SPME to HPLC through a specially designed interface was first introduced in 1995 [63]. The heart of the interface was a custom-made desorption chamber, used for solvent desorption of the extracted analytes instead of the thermal desorption in the GC injector. However, this method suffered from lower productivity and reproducibility as a result of the manual operation. Additionally, the expansion of SPME/HPLC was partially limited by the availability of fiber coatings for LC analysis, since not all the SPME fibers can be used for LC applications [70], due to solubility and swelling of the fiber coatings in organic solvents. The first automated in-tube SPME/HPLC system was successfully applied to the analysis of polar, thermally labile analytes by the Pawliszyn group [64]. This promising method, which has received increased attention over the past several years, has been termed "in-tube" because the extraction phase is not coated on the surface of a fused-silica rod as in syringe-like SPME devices, but is coated inside a section of a fused-silica tubing (*i.e.*, open-tubular capillary GC column, or micro-LC capillary column). The instrumental set up for the in-tube SPME–HPLC system and a detailed scheme of the in-tube SPME capillary are shown in Fig. 21.1. The method offers several advantages over manual SPME/HPLC [65]:

(a) it is fully automated, requiring no sample manipulation between extraction and HPLC analysis;
(b) it affords a high selectivity, as the range of coatings available for GC capillaries is wider than is currently available for SPME fibers; and
(c) it does not present any carryover problems.

Table 21.2 is a compilation of literature data on selected applications of SPME in environmental analysis. Approximately 40% of the more than 1200 literature references found for SPME were in the field of environmental analysis. For detailed information on the theory and application of SPME the reader is referred to several books and reviews [66–70].

21.2.2 Solid samples

The sorption of organic pollutants by sediment, soil, or suspended solids is governed by their relative hydrophobicity. Persistent organic pollutants (POP), in particular, are sorbed under anaerobic conditions, prevailing in the sub-surface layers of river or marine sediments, and undergo photodecomposition and biodegradation. Because the solid matrices are heterogeneous, special care must be taken to ensure that the samples analyzed

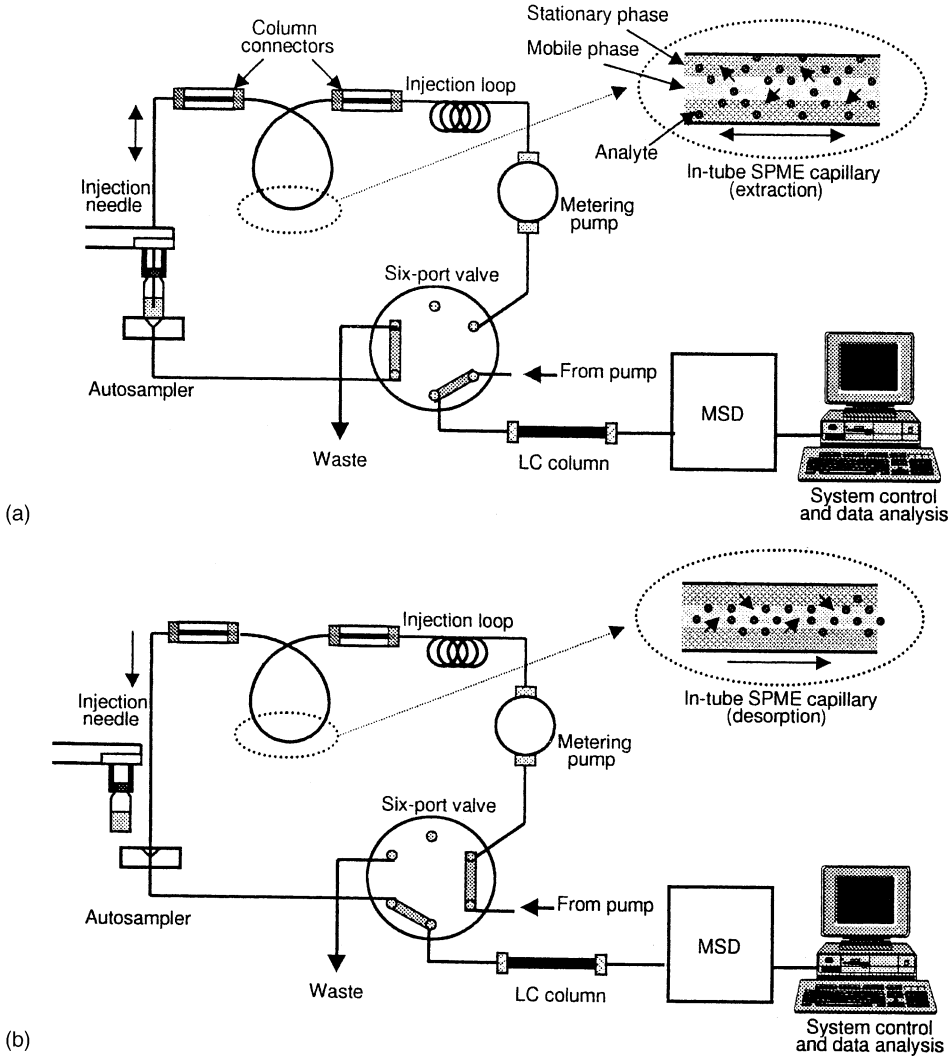


Fig. 21.1. The in-tube SPME/LC/MS system. (a) Extraction (load position); (b) Desorption (injection position). (Reproduced with permission from Ref. 391 ©1999 American Chemical Society.)

are representative of the system studied. In case of sediments or soil samples, depending on the objective of the study (determination of vertical distribution profiles or concentrations in a surface layer), either core samples or grab samples are taken. Usually, water is removed, and the solid matrix and the samples are stored in the dry state. Removal of water from the sediment before extraction was found to be crucial in obtaining good recoveries [71]. Freeze-drying is an accepted and commonly used procedure for drying

TABLE 21.2

APPLICATIONS OF SPME IN ENVIRONMENTAL ANALYSIS OF WATER

Compound	Sample	Mode	Method	Ref.
Butyltin compounds	Lake, river and coastal waters	Headspace	GC/FID	[336,337]
Volatile fatty acids	Urban wastewater	Headspace	GC/FID-MS	[338]
Chlorinated benzenes	Reservoir and tap water	Headspace	GC/MS	[339]
Trihalomethanes	Potable and recreational waters	Headspace	GC/MS	[340]
Organophosphorus insecticides	Natural waters	Headspace	GC/FID-MS	[341]
Phenoxy acids	Water	Direct	GC/MS	[342]
Chlorinated phenoxy acids	Natural waters	In-tube	LC/MS	[343]
Organochlorine pesticides	Groundwater	Direct	GC/EC	[344]
Priority pesticides	River water	Direct	GC/MS	[345]
Carbamate	Surface water	In-tube	LC/UV	[346]
Pyrethroid pesticides	River water			[347]
Fungicides	Natural waters	Direct	GC/EC-MS	[348]
Phthalates	Water	Direct	GC/MS	[349]
Phthalates	Water	In-tube-LC		[350]
Phthalates	River and harbor waters	Direct	GC/MS	[351]
Aldehydes	Drinking water	Headspace	GC/ECD	[352]
PAH	Water samples	Direct/headspace	GC/FID	[353]
PCB	Ocean, wetland and leachate water	Direct	GC/ECD	[354]
Haloethers	Lake and tap water	Direct	GC/FID	[355]
Phenolic compounds	River and waste water	Direct	HPLC/UV/ED	[356]
Chlorophenols	Drinking water	Direct	LC/ED	[357]
Pharmaceuticals and nonylphenol	Ground and river water	Direct		[358]
MTBE	Natural waters	Headspace	GC/MS	[359,360]
Anti-fouling agents	Natural waters	Headspace	GC/EC/MS	[361]
Sex hormones	River and wastewater		LC/ED/UV	[362]
Alkylphenolic compounds	River and drinking water	Direct/headspace	GC/MS	[363,364]
Synthetic musks	Wastewater	Direct/headspace	GC/MS	[365]

sediments and sludges, but it is not known how this affects the levels of target compounds measured, especially those of some relatively volatile compounds [72].

21.2.2.1 Extraction of analytes

Usually, the nature and amounts of organic contaminants in solid environmental samples, such as sediments, soils, and sludges are determined by exhaustive extraction with appropriate solvents. Generally, liquid-phase extraction techniques, such as Soxhlet extraction, sonication, microwave-assisted extraction (MAE), pressurized-liquid extraction (PLE), and supercritical-fluid extraction (SFE) are used. Methods based on solid-phase extraction (*e.g.*, headspace SPME) have also been developed for volatile and semi-volatile compounds. Soxhlet extraction is a reference method, because the equipment is inexpensive and easy to handle. It has the advantage that it has a large matrix capacity and requires no filtration. The drawback is that it requires large volumes of highly purified solvents and long extraction periods, yields impure extracts, and cannot be automated. Demands for new "low-solvent, low-time, low-cost" extraction techniques, amenable to automation, have led to accelerated development of a number of techniques that meet these criteria. Thus, Soxhlet extraction and steam-distillation, used almost exclusively in the 1980s and 1990s, have been partially replaced by more versatile and automatable sonicated extraction systems, SFE, MAE, and PLE.

A wide range of organic compounds can be rapidly extracted by SFE under mild conditions with a considerable saving in the cost and risk of solvent use. However, SFE is not suitable for highly polar organic compounds or matrices with a high water content and may suffer from losses of solutes being trapped in the sorbent. Also, it requires some more costly equipment and a certain amount of optimization. In contrast, older extraction procedures can be easily adapted to PLE. It is easy to use, fast, and requires no filtration and relatively little solvent. Commercial equipment for PLE may be expensive, and some thermolabile compound may suffer degradation. Investment in MAE equipment is more modest, it is also fast and consumes little solvent. It has the advantage that up to 12 extractions can be performed simultaneously, but extracts need to be filtered, and microwave heating is uneven and restricted to matrices that absorb this radiation. An overview of the most representative extraction methods used for selected organic pollutants from solid environmental samples (sediment, soil and sludge) is shown in Table 21.3.

The most advanced extraction techniques use elevated temperature and pressure, resulting in improved mass transfer of the analytes and, consequently, increased extraction efficiency. This approach is employed in several techniques such as SFE, MAE, and PLE. SFE with solid-phase trapping has been tested and used for different groups of organic pollutants. Although excellent results and unique improved selectivity were obtained for selected applications, the method did not find acceptance. This is because the extraction conditions depend on the sample, requiring complicated optimization procedures [73] and also because of the success of PLE. PLE, also termed accelerated solvent extraction (ASE), or pressurized-fluid extraction (PFE), has become widely accepted. A modification of this technique, extraction with pressurized (supercritical) hot water, as well as

TABLE 21.3

EXTRACTION, CLEAN-UP, AND DETECTION METHODS FOR THE QUANTITATIVE DETERMINATION OF SELECTED GROUPS OF ORGANIC POLLUTANTS IN SOLID ENVIRONMENTAL SAMPLES

Analytes	Matrix	Extraction method	Solvent	Clean-up	Detection	Ref.
Non-ionic surfactants						
NPEO, AEO, CDEA	Sewage sludge	Sonication	DCM/MeOH (3:7)	SPE-C_{18}	LC-ESI/APCI-MS	[311]
NPEO, NP	Marine sediment	Sonication	Hexane/ acetone (6:4)	SPE-CN-silica	NP-LC/ESI-MS	[241]
APEO, APEC, AP, halogenated derivatives	River sediment, sludge	Sonication	DCM/MeOH (3:7)	SPE-C_{18}	RP-LC/ESI-MS	[228]
OP, NP NPEO	Sediment	SFE + *in situ* acetylation	CO_2	Silica gel	GC/EI-MS	[230,366]
APEO, AP, BrNP, ClNP	Marine sediment	High-temperature continuous-flow sonication	MeOH	1. SPE-NH_2-silica 2. RP-HPLC	LC/ESI-MS	[245,247]
NP, NPEO	Sediment	PLE	MeOH	Alumina	HPLC/FL	[367]
NP	Sediment	Soxhlet or PLE	DCM	SEC	GC/FID	[368]
APEO, APEC, AP, halogenated derivatives	River sediment, sludge	PLE	MeOH/ acetone (1:1)	SPE or on-line RAM	RP-LC/ESI-MS	[92,369]

NP	STP sludge	Micro steam distillation	Cyclohexane	–	HPLC/UV	[370]
NPEC	STP sludge	Subcritical water extraction	H_2O/EtOH	SAX disks	GC-PCI/MS (NH_3)	[74]
Ionic surfactants						
LAS, SPC	Marine sediment	Soxhlet	MeOH	SPE-C_{18}	RP-LC/FL	[371]
LAS, DATS	River sediment	Sonication	MeOH	SPE-C_{18}	GC/NCI-MS	[372]
Pharmaceuticals and sex hormones						
Antibiotics	Soil	Sonication	MeOH	–	RP-LC/ESI-MS	[373]
Antibiotics	Soil, fertilized with manure	Vortex	Ethyl acetate	–	RP-LC/ESI-MS$^{(3)}$	[374]
Antibiotics	Artificial marine sediment	Homogenization, centrifugation	McIlvaine buffer	SPE-Bond Elut Certify LRC	RP-LC/FAB-MS RP-LC/PB-MS	[375]
Natural and synthetic estrogens	River sediment, sludge	PLE	MeOH/acetone (1:1)	SPE or on-line RAM	RP-LC/ESI-MS	[92]
Natural and synthetic estrogens	Sludge	Sonication	MeOH + acetone	Silica gel, SPE-C_{18}, semi-prep HPLC	GC/ion trap-MS/MS	[376]
Natural and synthetic estrogens	River sediment	Sonication	MeOH + acetone	GPC, silica gel	GC/ion trap-MS/MS	[376]
Personal care products						
Bactericide, triclosan	Sewage sludge	SFE	CO_2	Not required	GC/HRMS	[304]

(*Continued on next page*)

TABLE 21.3 (continued)

Analytes	Matrix	Extraction method	Solvent	Clean-up	Detection	Ref.
Polycyclic musks	River sediment	SDE	Cyclohexane	Not required	GC/IT-MS/MS	[377]
Nitro and polycyclic musks	Sewage sludge	Liquid extraction	Hexane	1. GPC 2. SPE (silica)	GC/IT-MS/MS	[294]
Polychlorinated and polybrominated compounds						
PCDD, PCDF	Spiked solid matrix	SFE	CO_2	Not required	GC/HRMS	[378]
PCB	Soil	MAE	*n*-Heptane	SPE-Florisil	GC/MS	[379]
PCDD, PCDF	Sludge	MAE	Toluene/ water (99:1)	Acid digestion + modified silica/ Florisil and alumina	GC/HRMS	[380]
PCB	Sediment, sludge	PLE	Acetone/ hexane (1:1)	Modified silica	Dual-column GC/ECD	[381]
PCDD/PCDF	Soil	PLE	Toluene/MeOH (3:1)	Modified silica and alumina	GC/HRMS	[382]
PBDE	Marine sediment	Soxhlet	Toluene	Modified silica/ Florisil and active carbon	GC/HRMS	[383]
PBDE	Sediment	Soxhlet	Hexane/DCM (1:1)	SPE/alumina	GC/NCI-MS	[384]
PBDE	Sediment	SFE	CO_2	Not required	GC/MS	[385]

Pesticides						
Triazines, phenylureas, phenoxy acids, benzonitriles	Soil	Subcritical extraction, including SPE trap	Hot water		LC/ESI-NI-MS or LC/ESI-PI-MS	[386]
Triazines, Chloroacetanilide herbicides	Soil	MAE	Acetonitrile		GC/NPD GC/MS	[84]
Carbamates, diflubenzuron, chlofetezine, carbendazim, thiabendazole	Fruits	Soxhlet, concentration solvent switch to acetonitrile	Ethyl acetate	Not required	LC/APCI-NI-MS or LC/APCI-PI-MS	[387]
Chlorinated acid herbicides	Soil	Static Subcritical/ SAX Disk	Hexane	Esterification with MeI, EtI or BF_3/MeOH, silylation	GC/ECD or GC/MS	[388]

extraction with hot water under subcritical conditions, have been used for the analysis of polar alkylphenolic compounds (*e.g.*, carboxylates) [74], PAH [75–79], polychlorinated and polybrominated organic compounds [80,81], and brominated flame retardants [82]. MAE has been used for the determination of triazine and chloroacetanilide herbicides in soils [83,84]. Only a few publications report the application of MAE to the determination of pesticides in soil [83–87] and plant matrixes [88–90]. Stir-bar sorptive extraction (SBSE) and matrix solid-phase dispersion (MSPD), followed by LC, were developed for the extraction of some pesticides in oranges [91].

21.2.2.2 Extract clean-up

Because of the complexity of samples and low selectivity of exhaustive extraction techniques, a substantial amount of interfering substances is still found in crude extracts, and subsequent clean-up and fractionation are indispensable. Often, a multi-step clean-up procedure is necessary to permit separation and detection of trace levels of target compounds. The conventional approach for extract clean-up is based on solid/liquid adsorption chromatography, either in long, open columns or in disposable cartridges, packed with various sorbents. Purification and fractionation can be also performed by off-line SPE in cartridges packed with C_{18}-, NH_2-, or CN-modified silica, by RP or normal-phase (NP) LC, or size-exclusion chromatography (SEC). An advanced sample preparation strategy was developed for the determination of alkylphenolic compounds and steroid sex hormones in solid environmental samples [92]. This novel methodology, based on column-switching LC/MS with LiChrospher ADS RAM pre-columns (Merck) is described for the integrated sample clean-up and analysis of estrogenic compounds in sediment samples. The column-switching LC, combined with an effective PLE, saved hours of sample pre-treatment in comparison with conventional sample preparation technique and gave better results in terms of sensitivity and selectivity. Restricted-access pre-columns efficiently separate high-molecular-weight matrix components (humic substances), polar impurities, and inorganic salts, thus significantly reducing ion-suppression effects in ESI-MS detection and improving the separation of analytes from impurities [92].

21.2.3 Biota

Accumulation of lipophilic organic contaminants has been studied in various biological tissues from fish, mussels, and snails. Organic pollutants in fish can come from water that may be far from the sampling point [93]. When small fish were analyzed, several individual fish were homogenized to form a pool of tissues, from which sub-samples were taken for extraction. From large fish, centered cross-sections were used for further analysis. In some studies, fish liver, bile, or the digestive/excretory system, where lipophilic alkylphenolic compounds are likely to accumulate, were chosen for analysis.

21.2.3.1 Extraction of analytes and extract purification

Isolation of organic compounds from biological tissues is a complicated and laborious task because of the nature of the matrix. Disruption of a cellular structure of biological samples results in an abundance of lipids and proteins. Classical methods, such as Soxhlet extraction, often yield high concentrations of lipids and therefore require further manipulation (*e.g.*, SEC or multiple L/L partitioning), which may lead to low recovery or poor reproducibility. The use of antibody-based affinity columns has been explored as a means of shortening the analysis time for PCB, PCDD, and PCDF [94–96]. Compared to classical clean-up and isolation methods, affinity chromatography is >20 times faster, requires only one-hundredth of the organic solvents, and has a considerably higher selectivity. For the purification of extracts from biological samples, which usually contain a high concentration of high-molecular-weight impurities, treatment with concentrated sulfuric acid is frequently used for the removal of lipids. However, this may destroy some of the target compounds. Adsorption chromatography and SEC, or a combination of both, constitutes a milder treatment. Snyder *et al.* [97] used two SEC columns, connected in tandem, for the clean-up of extracts in the analysis of degradation products of surfactants (alkylphenols), but lipids present in the fish tissue extracts were just partially removed, resulting in an increased baseline and elevated operating pressures in the HPLC system. Therefore, after five injections, the analytical column was purged each time with successively less polar solvents. After extensive trial-and-error method development, the same authors [98] devised a clean-up method based on preparative NP-LC for the removal of extracted lipids and achieved detection limits adequate for analyses at toxicologically relevant concentrations.

Another approach to conducting simultaneous disruption and extraction of solid and semi-solid samples involves matrix solid-phase dispersion (MSPD), a technique that combines in one step extraction, concentration, and clean-up by blending a small amount of sample with the selected sorbent (*e.g.*, C_{18} material) [99–101]. Its main characteristic is the ability to handle solid, semi-solid and viscous samples directly and to disrupt and simultaneously disperse a sample in a bonded-phase sorbent that is subsequently used as column packing, placed above the sorbent layer. Depending on the solubility characteristics of the target compounds, the appropriate solvent or solvent sequence is used to elute the column. The processes of blending and preparing a column for MSPD extraction have proven to be the same for a wide range of matrices and analytes. Therefore, MSPD is widely applicable to the analysis of different organic pollutants in animal tissues, vegetables, and other matrices. It has been applied successfully to analytes in fish and mussel samples. Pollutants studied have included penicillins, sufonamides, and tetracycline antibiotics [102–104], ionic [105,106], and nonionic surfactants [107,108], organochlorine pesticides, PCB, and PAH [109–111]. The major advantage of MSPD over the classical solid/liquid extraction methods is that the isolation and purification are combined and accomplished in one step. Thus, solvent consumption and analysis time are greatly reduced. However, the limitations are the manual packing of MSPD columns and the manual control of elution rate, which can significantly affect reproducibility and recovery.

21.3 QUANTITATIVE ANALYSIS

21.3.1 General trends

Currently, the main advances in improving sensitivity and specificity of environmental analyses are due to the application of LC/MS and LC/MS/MS (Chap. 10). One of the obstacles to routine analytical applications of LC/MS had been the unavailability of rugged and reliable LC/MS interfaces. The development of atmospheric-pressure ionization (API) overcame such limitations as poor structural information or sensitivity experienced with thermospray (TS), or particle-beam (PB) techniques, respectively. API is used as a generic term for soft ionization, obtained by different interface/ionization types, such as atmospheric-pressure chemical (APCI) and electrospray (ESI) ionization, which operate under atmospheric pressure conditions. Nowadays, LC/MS has become a routine analytical tool, allowing the detection of polar and non-volatile compounds not amenable to GC analysis.

21.3.1.1 Chromatographic separation

The overall trend in chromatographic analysis of environmental samples is toward fast chromatographic methods through the use of short, narrow-bore columns, high mobile-phase flow-rates and ultra-high pressures. Shortening the analysis times is important for attaining the high sample throughput often required in monitoring studies. Typically, 2-cm long, high-pressure-packed LC columns, coupled with MS, or tandem MS produce rapid trace-level determination and identification of environmental pollutants in water samples [112–116]. Because the separating power of such short columns is limited, highly selective detection (*e.g.*, by MS/MS) is required, especially for on-line SPE/short-column LC.

Turbulent-flow chromatography (TFC), in the form of SPE cartridges for fast on-line enrichment, achieved with high flow-rates and large particle size, can eliminate the need for time-consuming sample preparation. Because it allows direct high-volume-injection of aqueous samples, this technique has become established in a large number of analytical applications, particularly drug discovery/pharmacokinetics (Chap. 20), metabolite profiling, combinatorial library purification, and pre-clinical and clinical applications, but environmental applications are still rare. TFC columns have turned out to be eminently suitable for enrichment of trace pesticides from water volumes up to 50 mL. Complete recovery of the pesticides from the TFC columns requires only small volumes of organic eluent (200–400 μL methanol at a rate of 200 μL/min), underlining the capabilities of TFC columns for on-line SPE applications.

Recently, *monolithic* silica phases have become commercially available (Chap. 12). LC columns, packed with monoliths, a single piece of porous material, have become the subject of extensive studies because of their hydrodynamic advantages. Monolithic columns are more sparsely filled with solid and have a larger cross-sectional area of through pores (and, consequently, a lower flow-resistance) than particle-filled columns. This allows operation at higher flow-rates and flow-gradients [117,118].

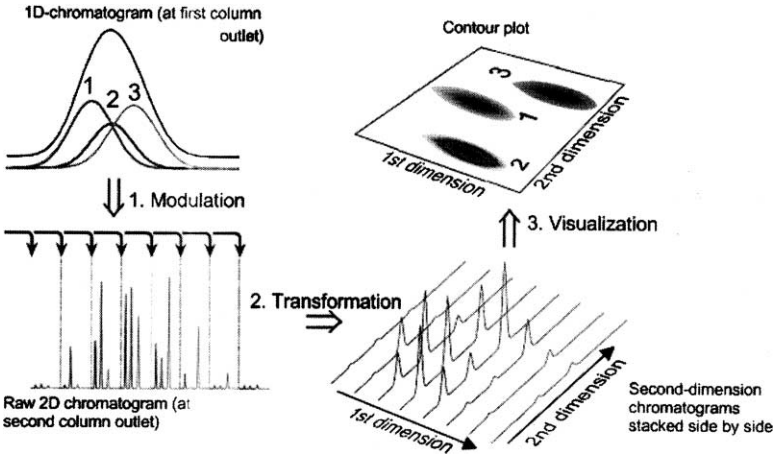


Fig. 21.2. Generation and visualization of a GC × GC chromatogram. (Reproduced with permission from Ref. 392 ©2002 Elsevier.)

Recent improvements in GC analysis involve application of high-speed narrow-bore capillary columns [119–122]. High-speed GC (HSGC), also known as fast, rapid, and ultra-fast GC, can increase efficiency (and, consequently, resolution) and drastically reduce analysis times [123]. The second application of HSGC relates to one of the most promising recent developments in chromatography. Comprehensive two-dimensional GC (GC × GC) (Chap. 8) has a spectacular capability to separate and identify organic compounds in complex environmental samples [124]. In GC × GC the effluent from the first column is separated into a large number of small fraction, and each of these is subsequently separated on the second column (Fig. 21.2). This second separation is much faster than the first one, so that the fractions can be narrow and the separation obtained on the first column can be maintained. The most obvious advantage of GC × GC is the large peak capacity. Because retentions in the two dimensions are almost independent, the peak capacity that can be achieved is close to the product of the peak capacities of the two individual columns [125]. Another advantage of GC × GC systems is the increase in signal-to-noise ratios, which leads to an improvement in detection limits. Finally, all peaks in the chromatogram are described by two time co-ordinates, which make their identification more reliable. The method has been applied successfully to the identification and quantification of methyltetrabutyl ether (MTBE) and other oxygenated and aromatic compounds in gasoline-contaminated groundwaters [126], to PAH [127], and to methyl and ethyl alkenones in marine sediment [128]. The key instrument component that allows the injection of small and narrow fractions from the first column onto the second one is the modulator interface between the two columns. It serves three main goals:

(a) peaks eluted from the first column must be collected and focused,
(b) elution from the first column must be stopped while chromatography is going on in the second column, and, finally,
(c) the collected fraction must be re-injected into the second column.

Recently, cryogenic modulators have been introduced [129,130] as a substitute for the slotted heater, which was used earlier [131]. For GC × GC of dioxins and PCB, the use of cryogenic modulators clearly results in more narrow peaks than with the use of the sweeper system [132].

21.3.1.2 Detection systems

The use of advanced separation systems and narrow-bore columns that produce extremely narrow chromatographic peaks must be accompanied by compatible instrumentation for trace-level analysis. Technologies that have significantly advanced in recent years include tandem (MS/MS) systems, time-of-flight MS (TOF-MS), quadrupole-time-of-flight (Q-TOF-MS) and membrane-introduction MS (MIMS) (Chap. 10). The added power of MS/MS, applying a variety of scan functions and modes, *i.e.*, product-ion scan, precursor-ion scan, neutral loss, multiple reaction monitoring (MRM) has enhanced analytical performance (reliability and sensitivity) and allowed a gradual shift from the detection of parent compounds to the analysis of metabolites and transformation products. Although the sensitivity, selectivity, and efficiency of the MRM approach are excellent, qualitative information, needed to support the structural elucidation of compounds other than the target analytes, is lost. In the full-scan mode, this information can be obtained, but the lack of compound databases and mass-spectral libraries often present an obstacle to the efficient structural elucidation of unknown compounds.

Time-of-flight and magnetic-sector mass spectrometers are the most commonly used instruments for applications where enhanced resolving power is required. Two complementary approaches are available in TOF-MS: One employs instruments that provide high resolution but have a moderate scan speed, the other one employs instruments that feature a high storage speed of, typically, 100–500 spectra/sec but usually provide only unit mass resolution. The first instruments are used in studies that apply GC × GC, whereas the second group is of interest in high-resolution applications. Owing to its non-scanning character, TOF-MS is a valuable tool for fast GC, because this type of instrument can be used to monitor the entire mass range in a very short time with high sensitivity. Scanning-type mass spectrometers are inherently unsuitable for fast separations, since they are, at best, limited to only 5 or 10 data samples/sec. Therefore, when the GC peak widths are below 0.5 sec, a different type of mass detector must be used. TOF-MS is ideally suited to this type of analysis, because high sampling rates can be achieved. The coupling of API technology with TOF-MS combines the high accuracy and excellent sensitivity due to the high-frequency sampling of all ions simultaneously across the full mass range. Orthogonal-acceleration time-of-flight MS (oaTOF MS), coupled to LC, has proved to be a powerful tool for the identification of trace constituents of complex mixtures and/or for confirming their presence. Such instruments provide mass determinations with an accuracy of 5–10 ppm, which is an impressive improvement over the conventional nominal-mass information of a quadrupole instrument. In the field of environmental chemistry, Hogenboom *et al.* [133,134] have demonstrated the capability of oaTOF-MS in multi-residue screening of water specimens and accurate

mass determinations for the confirmation and identification of organic trace contaminants in surface water. Another variation of tandem hybrid instruments that has gained popularity in the last few years is a combination of quadrupole and oaTOF-MS (Q-TOF) instruments. Such instruments enable accurate mass measurement with accuracies of <5 ppm, which dispel interpretation ambiguities and easily differentiate between charge states, even in weak-collisionally-activated decomposition tandem mass spectra [135]. Although environmental applications are still scarce, Q-TOF mass spectrometers are often used in biochemistry for the identification of small molecules (<1000 Da), due to their advantageous ion separation and detection principle. The possibilities of Q-TOF-MS in the LC/MS/MS screening and identification of organic pollutants in surface and sea water [136,137] are being explored, and further development and more environmental applications are expected.

21.3.2 Persistent organic pollutants

Analysis of dioxins and related compounds is a difficult task due to a series of problems such as:

(a) their low concentrations (ppb to ppq),
(b) simultaneous occurrence of a large number of interfering compounds, frequently found in much higher concentrations, and
(c) the need for congener-specific analysis in order to differentiate the most toxic congeners from others with very close chemical and physical properties. Overcoming all these analytical problems has been possible only by applying rigorous clean-up schemes and by using high-resolution (HR)GC, coupled to MS. The method of choice for the determination of many halogenated contaminants is GC, because the volatility of these compounds allows determination by HRGC. The development of capillary columns for HRGC enables congener-specific determination. The combination of high resolution, achieved by capillary GC, and the high sensitivity and selectivity of MS results in a powerful and universal technique for environmental analysis. Finally, an isotopic dilution technique, based on the use of standards, labeled with ^{13}C, provides the reliable quantification needed for the accurate determination of such contaminants.

21.3.2.1 Chlorinated compounds

The method of choice for PCB analysis is GC with sensitive and selective detection by an ECD or by MS (full-scan and SIM modes). Such methods are designed to measure a select list of priority congeners. European regulations often mandate reporting levels of seven "indicator" PCB (PCB 28, 52, 101, 118, 138, 153, and 180) to avoid the complexity involved in analyzing even more congeners. In the last few years, special attention has focused on a few select PCB congeners that are stereochemically similar to the 2378-TCDD and have similar toxicological properties. These congeners, called dioxin-like PCBs, are classified according to the number of chlorine atoms in the

ortho-position: non-*ortho*-Cl and mono-*ortho*-Cl. The World Health Organization has identified 12 of the 209 possible PCBs as having a toxicity similar to that of PCDD/F. The list of these 12 PCBs includes 4 non-*ortho* (PCB 77, 81, 126, and 169) and 8 mono-*ortho* congeners (PCB 105, 114, 118, 123, 156, 157, 167, and 189) [138]. Dioxin-like PCB occur at concentrations lower than the level of "indicator" PCB mentioned above, and are therefore very difficult to analyze. Complex analytical procedures are necessary for the determination of these dioxin-like PCB. Methodologies similar to those used for dioxin analyses are now applied to the determination of the dioxin-like PCBs.

The dioxin and PCB analyses involve detection of multiple congeners at the ppt, or ppq level for which isotope dilution techniques and HRGC/HRMS are currently recommended methods (US EPA Method 1613, US EPA Method 8290, US EPA Method 1668) [139–141]. HRMS was conventionally used, operating in the EI mode (electron energy 38 eV) at a resolving power of 10,000. Under these conditions, different ions (isotopically-labeled ions included) were monitored in SIM mode. Identification of PCDD, PCDF and PCB was based on the following restrictive criteria:

(a) retention times of chromatographic peaks must be within the appropriate chromatographic windows;
(b) simultaneous responses for the two masses monitored must be obtained;
(c) the signal-to-noise ratios must be greater than 3; and
(d) the relative isotopic peak ratios must be within $\pm 15\%$ of the theoretical values. Once these criteria were met, assignment of toxic congeners was performed by comparing retention times with the corresponding labeled internal standards. Quantification was carried out by an isotopic dilution technique, based on the addition of labeled standards. Relative response factors (RRF) were calculated, using different calibration standard solutions containing both native and labeled toxic compounds.

Regarding the chromatographic separation, the isomer-specific elution pattern of PCDD and PCDF is well-established for some widely used columns [142,143]. Analogous studies have been reported for the chromatographic separation of PCB congeners on different columns [144,145]. However, there is evidence of the limitations of single-column capillary HRGC for this type of determination. The chromatographic separation of the toxic isomers from all the non-toxic ones requires the use of at least two columns with different composition and polarity. Chromatography on two different columns is recommended for an unambiguous determination. Recent studies have examined the potential of GC × GC for the qualitative analysis and characterization of complex mixtures of halogenated contaminants, such as PCB [146,147]. Different MS techniques were applied to the analysis of dioxins and related compounds, such as low-resolution (LR)MS, high-resolution (HR)MS, and MS/MS. New approaches including ion-trap (IT) MS/MS and TOF-MS, were developed. The performances of these MS techniques are compared in Table 21.4 [148]. The advantage of IT-MS systems is the much lower price, but their sensitivity is considerably lower than that of HR-MS instruments. The limit of detection for IT-MS systems for TCDD (signal/noise 3/1) is in the range of *ca.* 100 to 300 fg, whereas modern HR-MS instruments have a LOD of about 3 fg [149]. Another disadvantage of the IT-MS is the low reproducibility of quantification. An excess of interfering ions, co-existing with dioxins in the trap, causes space-charge effects and leads

TABLE 21.4

COMPARISON OF MS METHODS FOR DIOXIN AND PCB ANALYSIS

	EI-LRMS-SIM	EI-HRMS-SIM	MS-MS	MS-MS	ToF-LRMS	ToF-HRMS
Analyzer	Quadrupole	Magnetic sector (EBE)	Triple quadrupole	Ion trap	Oa-ToF	oa-ToF
Acquisition rate	10 scans/sec	3 scans/sec	10 scans/sec	10 scans/s	$1-5 \times 10^2$ scans/sec	10 scans/sec
Dynamic range	4 orders	>5 orders	4 orders	3 orders	>5 orders	2.5 orders
Resolution	Unit	$6-8 \times 10^4$	Unit	Unit	Unit	5000–7000
Detection limit	low pg range	3 fg (TCDD)	200–600 fg	100–300 fg (TCDD)	5 pg (PCB-149)	1–4 pg (PCB)
Cleanup required	More exhaustive	Conventional	Less exhaustive	Less exhaustive	Conventional	Conventional
Application (literature)	Dioxins + PCB	Dioxins + PCB	Dioxins + PCB	Dioxins + PCB	PCB	PCB

(Reprinted with permission from Ref. 148 ©2002 John Wiley & Sons.)

to irreproducible results. Ionization conditions are meaningful parameters for the reproducibility. The voltage, the current, and the temperature of the chamber are the parameters for optimizing the ionization conditions. In spite of these drawbacks, MS/MS can now be applied to the ultra-trace detection and quantification of dioxins by means of isotope dilution. IT-MS was developed for the analysis of PCDD and PCDF [150–152] and of PCB [153], as an alternative to HR-MS. Recent studies have demonstrated the applicability of TOF-MS to the analysis of PCB in different types of samples [154–158]. The GC/TOF-MS results were consistent with the GC/HRMS results within the 95% confidence interval limits [155]. Instrumental detection limits for tri- to hepta-chlorinated biphenyls were estimated at 1 pg, whereas the LOD was established as 4 pg, based on deca-chlorinated biphenyls [157]. These LODs were at least one order of magnitude lower than those reported for conventional quadrupole systems, operating in the full-scan mode. These results are very encouraging, and show that GC/TOF-MS can make analysis times one order of magnitude shorter than those with GC/HRMS methods without losses in qualitative or quantitative performance.

21.3.2.2 Brominated compounds

Due to the similarities of polybrominated diphenyl ethers (PBDE) with PCB, both can be analyzed by the same methods. In the past, most of the analyses have concentrated on only a few specific PBDE congeners. Quantitative work has been performed with technical PBDE products, *e.g.*, Bromkal, due to the lack of pure reference standards for most BDE congeners. The major three components in Bromkal have been identified as BDE 47, BDE 99 and BDE 100 [159], and only these congeners could be quantified. Since more than 30 BDE congeners are now available, it has become possible to analyze each individual BDE. Moreover, the availability of some ^{13}C-labeled standards allow the development of a methodology based on the quantification by isotope dilution. The analysis of PBDE constitutes one of the greatest challenges in organic analytical chemistry. This group of compounds has a very broad range of physical and chemical properties, making the analysis of all congeners in a single analytical run quite difficult [160]. Many congeners are thermally labile and decompose before they reach their boiling point, complicating their detection. The molecular-weight distribution of PBDE ranges from 240 to 970 amu, and this makes it relatively difficult to analyze all of them on a single capillary column. This is why many analysts perform the analysis of PBDE in two runs, one for mono- to hepta-BDE and the other for octa- to deca-BDE. The isomer-specific elution pattern of mono- to hepta-PBDE was studied for some widely used columns, ranging from low to high polarity [159]. At least two columns of different polarity should be used to verify peak homogeneity. For the octa-, nona-, and deca-BDE, a short capillary column is used to facilitate the rapid elution of the compounds. The use of simultaneous dual-column chromatography for the analysis of PBDE in order to save analysis time was tested by Robinson *et al.* [161].

Several methods for the qualitative and quantitative analysis of PBDE by GC/negative chemical ionization (NCI)-MS and by GC/electron ionization (EI)-MS have been developed. NCI is the more frequently used ionization mode for the determination of

brominated compounds. This technique offers a higher sensitivity than EI, but it is less selective, since only bromine can be monitored. Furthermore, it does not allow quantification by isotope dilution (^{13}C-labeled PBDE), whereas EI does, and this makes the analysis more reliable at trace levels. An optimization study of the congener-specific analysis of 40 different PBDE by the two approaches, NCI and EI, was carried out [162]. Operating parameters, such as electron energy and source temperature were optimized in order to obtain the maximum sensitivity in the EI-MS study. For NCI-MS analyses, the effect of changing gas (methane or ammonia), source temperature, and system pressure were studied, and the quality parameters of the two approaches tested were compared. The NCI-MS method gave detection limits between 30 fg and 1.72 pg, whereas the EI-MS method gave detection limits between 0.53 and 32.09 pg. Comparison of the detection limits for PBDE clearly indicates that NCI offers better sensitivity than does EI. The main advantage of EI-MS is that it provides better structural information. No structural information on the degree of bromination was obtained by NCI. The mass spectra of all PBDE were dominated by the molecular ion $[Br]^-$ and did not show a molecular ion. However, EI provided better structural information, showing the molecular ions and the sequential losses of bromine atoms. In order to increase selectivity, Pirard *et al.* [163] have chosen MS/MS for detection. With this highly selective method other organohalogen compounds can be determined, and purification can be less rigorous. When collision-induced dissociation (CID) is applied, PBDE behave like PCB and break down with loss of Br_2, except for some congeners, where loss of COBr occurs. These exceptions are the *ortho*-substituted congeners, and for these compounds, the daughter-ions monitored were $M-Br_2$ and $M-COBr$. The detection limits observed in this procedure ranged between 1.51 and 128 pg/g. International inter-laboratory studies on the analysis of PBDE monitor the progress in this important area of environmental analysis [164,165].

21.3.3 Pesticides

The analysis of pesticides is carried out primarily by HPLC or GC, coupled to various selective detectors [166–170]. Among other methods of detection, electrochemical sensors with enzyme-modified electrodes show great promise. Butyryl- and acetylcholinesterases are suitable for the detection of organophosphorus and carbamate insecticides [171]. In the past, biosensors were not accepted in the field of pesticide analysis, due to their low stability and inadequate detection limits. However, Nunes *et al.* [172,173] have reported the development of a highly sensitive amperometric biosensor with cholinesterase for the determination of carbamate pesticides. This method, applied to untreated water and crops, showed high sensitivity and good selectivity. Detection limits varied, depending on the type of the cholinesterase, from 1×10^{-4} to 3.5 mg/kg. Other methods of analysis showing low detection limits and high selectivity are the immunochemical methods, *e.g.*, enzyme-linked immunosorbent assays (ELISA), which are also characterized by high sample throughput, cost effectiveness, simplicity, excellent precision, and accuracy [174].

GC with electron capture (ECD), photometric (FPD), and nitrogen/phosphorous (NPD) detectors has been the workhorse of pesticides analysis since the early 1970s [175]. Mass-spectrometric detection has been used with either electron ionization (EI) or chemical

ionization (CI), in both positive chemical ionization (PCI) and negative chemical ionization (NCI) modes. Comprehensive GC or GC × GC (Sec. 21.3.1.2) (Chap. 8), is a newcomer to the field. There are still few publications on the application of this technique in the determination of pesticides [169,176], and more research is needed for coupling it with MS detectors. GC/MS has been used extensively for the separation and detection of many thermo-stabile polar and nonpolar pesticides with or without derivatization. It is used in both SIM and full-scan mode, allowing identification and quantification of many priority pesticides of different classes [177–180]. The detection limits are *ca.* 2 ng/L. GC/NCI-MS is one of the most valuable reference techniques, providing an optimal compromise between selectivity and sensitivity. The presence of electrophilic residues in the structure of organophosphorous pesticides permits NCI by electron capture, leading to characteristic NCI-MS spectra [181]. Identification of the organophosphorous pesticides is based on only a few diagnostic ions, these being, in some instances, the molecular ion or the deprotonated molecular ion, the M–HCl ion, as well as specific characteristic ions. However, a less selective identification (*e.g.*, limited to a pesticide subclass) is also possible [181].

Three main types of mass spectrometers are used in combination with GC: quadrupole, ion-trap and sector instruments, and time-of-flight instruments. TOF-MS instruments measure the time an ion needs to travel through a field-free region. The major advantage of TOF-MS is the high mass resolution of *ca.* 0.002 Da (10 ppm). The ions are accelerated as discrete packages into the field-free flight tube by a pulsed electrical field. Flight times are proportional to the square root of the *m/z* of an ion and are in the order of microseconds. Consequently, TOF-MS can be operated at very high repetition rates, typically 5–30 kHz, *i.e.*, 5000–30,000 raw mass spectra are generated per second. TOF-MS achieves short analysis times [121,182], as it is the only MS technique yielding several data points across a narrow peak in full-scan operation [183]. It can also give a wide spectral mass range and/or exceptional mass resolution (typically at the expense of speed). Dalluge *et al.* [184], who also used GC/TOF-MS for the analysis of the pesticides, reported detection limits between 1 and 4 pg. Off-line SPE, followed by GC/MS with selected ions, was used by Quintana *et al.* [185] for the determination of pesticides in water. For all 22 pesticides examined the detection limits of the method were less than 0.025 μg/L, except for pendimethalin and metribuzine (0.062 and 0.035 μg/L, respectively) [185]. Azevedo *et al.* [186] optimized a method for the determination of 42 pesticides with off-line SPE, followed by GC/MS. The limits of detection varied from 0.002 to 0.08 μg/L. On-line SPE/GC requires a specific injection technique, such as partially concurrent solvent evaporation (PCSE), fully concurrent solvent evaporation (FCSE) and programmed-temperature vaporization (PTV). The on-line coupling of SPE with LC is now receiving more attention, because with packed liners (full-scan SIM) it is suitable for heat-labile and volatile compounds [187,188]. Pocurull *et al.* [178] used on-line SPE, followed by GC/MS, for the determination of pesticides with different volatilities and properties. MS was used with selected-ion monitoring, and the detection limits ranged from 2 to 20 ng/L.

Most of the modern pesticides and their degradation products are characterized by medium to high polarity and thermal lability. Therefore, LC is the most appropriate separation method [41]. Especially polar pesticides are efficiently separated by RP-LC without derivatization. This has been applied to, *e.g.*, drinking water (detection limit less

than 0.025 μg/L) with, *e.g.*, an ultraviolet/diode array [189–193] or fluorescence detector. The latter improves the selectivity and sensitivity but its application areas are limited, although pre- or post-column derivatization will increase its scope. Pesticide analysis with LC/MS is a fast and efficient way to analyze most of them, especially the polar ones by using reversed-phase systems. The interfaces for pesticide analysis include: thermospray (TS), particle-beam (PB) and atmospheric-pressure ionization (API) interfaces, with the ESI and APCI interfaces as the representatives of the API type. The main difference between the ESI and ACPI is the recommended flow-rate of the column effluent entering the interface of the MS. For ESI it varies form 10 to 100 μL/min, while for the ACPI it is *ca.* 1 mL/min. Azevedo *et al.* [186] analyzed 42 priority pesticides and 33 priority organic pollutants by off-line SPE, followed by LC/APCI-MS, in negative and positive modes, with detection limits of 0.05–0.2 μg/L (polychlorinated phenols) and 0.003–0.07 μg/L (pesticides). Hostetler and Thurman [189] analyzed chloroacetanilide herbicide metabolites in water with a LOD of 0.05 μg/L, and Santos *et al.* [177,178] determined rice herbicides, their transformation products, and clofibric acid by on-line SPE/LC/MS (APCI in negative ionization mode) with a LOD ranging from 0.5 to 0.02 ng/mL. Recent work of Thurman *et al.* [194] showed that the sensitivity of APCI or ESI is different for different classes of pesticides. For example, for neutral and basic compounds (phenylureas, triazines) APCI (especially in the positive-ion mode) is more sensitive, while for cationic and anionic herbicides (*e.g.*, bipyridylium ions, sulfonic acids) ESI (especially in the negative-ion mode) is more sensitive. Parameters like proton affinity and polarity are useful for choosing between ESI and APCI.

LC/MS/MS has been optimized for pesticide analysis [195]. Different modes of MS are summarized in Table 21.5. A rapid determination of pesticides and metabolites in

TABLE 21.5

COMPARISON OF VARIOUS LC/MS METHODS FOR PESTICIDES ANALYSIS

	API-MS^n	MS/MS	TOF-MS	TOF-MS
Analyzer	Ion trap	Triple	oaTOF	TOF
Acquisition rate	50–450	Quadrupole	100–800	2 spectra/sec
Acquisition mode	Full-scan		Continuous	Full-scan
Interface	ESI-APCI	ESI-APCI	ESI	ESI
Detection limit	pg range	<25 ng/L	0.1–1 μg/L	
Reference	[389]	[196]	[133]	[390]

environmental water was achieved by on-line SPE/LC/MS/MS in ESI mode with detection limits of less than 10 ng/L for most of 35 pesticides [196]. However, systematic variation of the flow-rate of the effluent [by flow-injection analysis (FIA)] both with an ESI interface and a heated-nebulizer APCI source, showed significant differences in individual pesticide signal responses. The most hydrophobic pesticides (*e.g.*, chlorpyrifos and trifluralin)

showed drastic losses of sensitivity with increasing flow-rates in both ESI and APCI, while more hydrophilic compounds (*e.g.*, atrazine, simazine, and isoproturon) were concentration-sensitive in ESI and mass-flow-sensitive in APCI [197]. Using LC/ESI-MS/MS after off-line SPE, Yokley *et al.* [198] determined important herbicides, among them chloroacetanilides (metolachlor, alcetochlor and alachlor) and chloroacetamide (dimethenamide) with a LOQ of *ca.* 0.10 ppb. For the analysis of paraquat and diquat in environmental water and plant matrices, a method consisting of off-line SPE, followed by LC/ESI-isotope dilution MS had detection limits of 0.2 and 0.1 mg/L for paraquat and diquat in water matrices, and 0.02 and 0.01 mg/g in plant matrices, respectively [199]. Reports are beginning to appear on the possibilities of screening and identifying water pollutants by the modern data acquisition methods of hybrid quadrupole/orthogonal acceleration/time-of-flight mass spectrometry (Q-TOF), such as data-dependent MS-to-MS/MS switching [133,200]. Using model compounds, a procedure for the LC/MS/MS screening of water extracts was developed, enabling the detection and identification of compounds at levels lower than 0.25 μg/L in surface water [200]. In another study [133], on-line SPE and LC electrospray in combination with oaTOF-MS was shown to be extremely useful for the determination of pesticides of various compound classes in surface water in the concentration range of 0.1–10 μg/L. HPLC/TOF-MS is a promising method for the identification of unknown pesticides or their degradation products, which is almost impossible by simple quadrupole HPLC/MS. The TOF-MS gives the accurate mass of the molecular ion, which may be used to determine the empirical formula of unknown compounds. This is helpful in the determination of pesticides when no standards exist, especially for the ones where the mass of the molecular ion is typically less than 350 amu. HPLC/TOF-MS with the use of both positive- and negative-ion electrospray have been used in the analysis of secondary and tertiary ethanesulfonic acid degradation products of acetochlor and alachlor [201].

21.3.4 Surfactants

A number of books and reviews on the application of LC and GC to the analysis of surfactants in wastewater, surface water, sediments, sludges and biological samples are available [202–206]. Among the various surfactants classes, this chapter will focus on two surfactant types that deserve particular attention because of their extensive use, ubiquity in the environment, and ecotoxicological relevance, *i.e.*, the linear alkylbenzene sulfonates (LAS) and alkylphenol ethoxylates (APEO).

21.3.4.1 Anionic surfactants

Linear alkylbenzene sulfonates (LAS) are the most commonly used anionic surfactants. Commercially available LAS are mixtures of secondary isomers, with alkyl chain-lengths of 10–13 carbon atoms. Routine determination of LAS and their degradation products in environmental samples involves the use of SPE and determination by GC/MS, or LC with either UV, fluorescence (FL), or MS detection [207–212]. Capillary GC of derivatized (or de-sulfonated) LAS allows separation according to alkyl chain-length, the greatest

chain-lengths giving the longest retention times and internal isomers of each homolog being eluted first [213,214]. The major drawback of the analysis of anionic surfactants by GC/MS is that, being nonvolatile, they can only be analyzed after treatment to form volatile derivatives [215]. Owing to the anionic character of LAS, LC, coupled with ESI-MS in negative-ion (NI) mode, is particularly attractive for the quantitative determination of this surfactant type [203]. LAS analysis by ESI-MS under NI conditions led to mass spectra with the four $[M-H]^-$ ions of *m/z* 297, 311, 325, and 339, corresponding to the LAS homologs C_{10} to C_{13}, respectively. With increasing cone voltage, additional fragment ions at *m/z* 183 and 80 were obtained, which were assigned to styrene-4-sulfonate and $[SO_3]^-$, respectively. No side-chain-specific fragment ions were produced under API conditions; thus a mass spectrometric distinction of positional isomers of LAS could not be achieved. Aerobic biodegradation of LAS, as it occurs in wastewater treatment plants and surface waters, leads to the formation of sulfophenyl carboxylates (SPC) [216,217]. Due to their very high polarity, ion-pairing LC was proposed, with equimolar concentrations of acetic acid and triethylamine or tetraethylammonium acetate [218, 219]. To avoid LC conditions involving the use of ion-pairing agents – because of their adverse effects on the process of ion formation [220] – an alternative route was proposed by di Corcia *et al.* [221]. Their method was based on the conversion of the carboxyl groups to methyl esters, resulting in successful separation and a 10-fold enhancement of sensitivity of ESI-MS detection.

21.3.4.2 Non-ionic surfactants

Among different non-ionic surfactant classes, APEO merit special attention with respect to environmental issues, because *ca.* 60% of APEO that enter mechanical and biological sewage and sewage sludge treatment plants are subsequently released into the environment, 85% being in the form of the potentially estrogenic metabolic products, alkylphenols (AP), alkylphenol carboxylates and dicarboxylates (APEC and CAPEC) [222–225]. Moreover, ring-halogenated derivatives of APEO and their degradation products are produced during chlorination in wastewater, or drinking-water treatment plants [226–228]. A large number of analytical methods, mainly based on GC/MS and LC, coupled with UV, FL, or MS, have been developed for the analysis of APEO and their degradation products in wastewater and environmental samples.

21.3.4.2.1 Gas chromatography/mass spectrometry

The analysis of underivatized alkylphenolic compounds by GC/MS is restricted to the most volatile degradation products, such as AP and APEO with less then 4-ethoxy groups. To overcome the volatility problem, various off-line and on-line derivatization procedures have been developed. Off-line derivatization to the corresponding trimethylsilyl ethers, methyl ethers, acetyl esters, pentafluorobenzoyl, or heptafluorobutyl esters was applied in numerous studies, while an alternative derivatization method, applied to analyze NPEO in solid and liquid environmental matrices, consists of *in situ* derivatization and extraction [229–231]. On-line direct GC injection-port derivatization with ion-pair reagents (tetraalkylammonium salts), has also been reported [232]. Two complementary

MS techniques, one, with EI and another, less commonly used, with PCI, for the analysis of APEO, their acidic (APEC) and neutral (AP) metabolites and halogenated derivatives have been evaluated and reviewed [205]. The most significant ions in EI-MS of methylated NPEC were fragments produced by rupture of the benzylic bond in the branched nonyl side-chain [232–234]. GC/CI-MS spectra of the NPEC with isobutane as reagent gas showed characteristic hydride-ion-abstracted fragment ions, shifted by 1 Da from those in the corresponding EI mass spectra [235]. Using ammonia as reagent gas, intense ammonia-molecular ion adducts of the methyl esters, with little, or no secondary fragmentation were obtained from NPEC [236]. There are a few reports on NCI, used to analyze pentafluorobenzyl derivatives of nonylphenol (NP) and NPEO with methane as reagent gas [237,238]. An ion-trap GC/MS system, with low-pressure CI and MS/MS capabilities, was developed for quick switching between EI and CI scans as well as MS/MS modes. The method was successfully applied for the analysis of both NPEO and their degradation products in river water and sewage effluents with large-volume injection [232,239].

21.3.4.2.2 Liquid chromatography/mass spectrometry

By now, LC/MS and LC/MS/MS have become routine methods for the analysis of alkylphenolic nonionic surfactants and their potentially estrogenic degradation products [205,240]. In NP systems, the APEO are separated according to increasing number of ethylene oxide units, while corresponding oligomers with the same number of ethoxy units but different alkyl substituents [*e.g.*, NPEO and octylphenol ethoxylates (OPEO)] are unresolved. NP-LC/ESI-MS was also used for the quantitative determination of individual NPEO in environmental samples [241–243]. Complete chromatographic separation of NPEO oligomers was achieved by gradient elution with non-polar solvents (*e.g.*, toluene as Solvent A and 0.5 m*M* NaOAc in toluene/methanol/water (10:88:2) as Solvent B). However, such mobile phases are not compatible with ESI, and post-column addition of a polar solvent and a modifier is required to facilitate ionization of the target analytes to enhance signal and system stability. Reversed-phase LC allows separation according to the character of the hydrophobic moiety. It is particularly well suited to the separation of surfactants containing various hydrophobic moieties (separation of alkyl homologs). In this case, the length of the ethylene oxide chain does not influence the separation, and the various oligomers containing the same hydrophobic moiety are eluted in one peak. Using RP-LC/ESI-MS, NPE_nO ($n_{EO} = 1–20$) were detected in samples of raw and treated wastewater, river and drinking (tap) water [224,225,228,244–246]. Recently, Ferguson *et al.* [247] reported the application of a mixed-mode HPLC separation, coupled with ESI-MS, to the comprehensive analysis of NPEO and NP in sediment and sewage samples. The mixed-mode separation, which operates by both, size-exclusion and reversed-phase mechanisms, allows the resolution of NPEO ethoxymers prior to introduction into the mass spectrometer when a solvent system is used that is compatible with electrospray. In this method, elution of NPEO is reversed relative to NP-LC, with smaller, less ethoxylated compounds, including NP, being eluted last.

The typical MS spectrum of polyethoxylates yields a characteristic pattern of equally spaced signals with mass differences of 44 Da (one ethylene oxide unit), which is a diagnostic fingerprint for this group of compounds. The nonionic surfactants easily form

adducts with alkaline and other impurities. Using an ESI interface and aprotic solvent, APEO predominantly give evenly spaced sodium adducts $[M + Na]^+$, due to the ubiquity of sodium in the solvents and surfaces [205,241]. Limits of detection reported for APEO were oligomer-dependent. At a given cone voltage, initially, there is an exponential increase in sensitivity with increasing number of EO units in the chain until about 6 EO units. A further increase in the number of EO units results in a slightly decreased response, which was explained by the formation of doubly charged adducts as well as a decrease in transmission of higher-mass ions by the quadrupole mass analyzer [247]. Like their non-halogenated analogs, halogenated APEO, formed during wastewater and drinking water chlorination, show a great affinity for alkali metal ions, and they give exclusively evenly spaced (Δ44 Da) sodium adduct peaks $[M + Na]^+$ with no further structurally significant fragmentation [228]. Fully de-ethoxylated degradation products, octylphenol (OP) and NP, were detected under NI conditions, with both APCI and ESI interfaces. The sensitivity of detection, when an ESI source was used, was *ca.* 40–50 times higher than that obtained with an APCI source [205,248]. Using ESI, AP give exclusively $[M-H]^-$ ions, whereas using APCI at higher voltages (so-called in-source CID), the spectra showed fragmentation that closely resembled that obtained by MS/MS. Alkylphenols gave, in addition to the $[M-H]^-$ ion at *m/z* 205 (for OP) and *m/z* 219 (NP), fragment *m/z* 133, resulting from the loss of a C_5H_{12} (OP) and C_6H_{14} (NP) group [249]. Alkylphenoxy carboxylates (AP_nEC) were detected, in both, the NI mode [223,228,245] and PI mode [224]. In the NI mode, using ESI, APEC gave two types of ions, one corresponding to the de-protonated molecule, $[M-H]^-$, and the other corresponding to de-protonated alkylphenols. The relative abundance of these two ions depended on the extraction voltage [203]. The identity of the dicarboxylated breakdown products was also confirmed by LC/ESI-MS [223,224]. The determination of halogenated NP [228,245] and NPEC [228] by LC/MS under NI conditions has been reported. $ClNPE_1C$ was detected by monitoring the fragment ion at *m/z* 253/255, which also suffered isobaric interferences in some real samples [228].

21.3.4.2.3 Liquid chromatography/tandem mass spectrometry

Although considered to be one of the most powerful techniques for structure interpretation and quantification, LC/MS/MS has been used only rarely in the analysis of APEO and their acidic and neutral metabolites in environmental samples. Several papers describing the application of tandem MS to the unambiguous identification and structural elucidation of alkylphenolic compounds have been published [223,235,250–252]. Compounds detected under negative-ionization conditions (AP, APEC, and their halogenated derivatives) were analyzed by ESI-MS/MS, while for APEO, detected under positive-ionization conditions, no fragmentation was obtained using an ESI source. These compounds were analyzed by APCI-MS/MS. With ESI-MS/MS, CID spectra of NP show fragments at *m/z* 147, 133, 119, and 93, resulting from the progressive fragmentation of the alkyl chain, but *m/z* 117, observed by several authors [240,249], cannot be interpreted. CID spectra of APEC [223,235,240] show an intense signal at *m/z* 219 (for NPEC) that is produced after the loss of the carboxylated (ethoxy) chain, and

sequential fragmentation of the alkyl chain, resulting in ions *m/z* 133 and 147, as mentioned for NP.

Identification of dicarboxylated compounds by LC/MS, under conditions giving solely molecular ions, is difficult, since CA_nPE_mC have the same molecular mass as APEC with one ethoxy unit less and a shorter alkyl chain ($A_{n-1}PE_{m-1}C$). Moreover, since some peaks partially overlap, the unequivocal assignment of individual fragments can only be accomplished by using LC/MS/MS. Jonkers *et al.* [223] studied the aerobic biodegradation of NPEO in a laboratory-scale bioreactor. The identity of the CAPEC metabolites was confirmed by the fragmentation pattern obtained with LC/ES-MS/MS. Of 17 degradation products, 9 were confirmed to be CAPEC metabolites. For chlorinated NP and NPEC [252] the predominant fragmentation occurred on the alkyl moiety, leading to a sequential loss of *m/z* 14 (CH_2 group), the most abundant fragments occurring at *m/z* 167 for ^{35}Cl and *m/z* 169 for ^{37}Cl with a relative ratio of intensities of 3.03.

21.3.5 Pharmaceuticals and personal care products

21.3.5.1 Hormones and contraceptives

Various endocrine-disrupting compounds (EDC), discharged into the aquatic environment have been shown to induce estrogenic responses in fish at concentrations in water (0.1–1 ng/L) [253–255] lower than those commonly detected in the environment (low-ng/L range) and have led to alarming effects on reproduction and developmental processes, such as feminization, decreased fertility, or hemaphoditism [256–258]. While most of the environmental programs carried out to assess the presence and impact of these compounds in the aquatic environment have focused on the investigation of water, soils and sediments have rarely been analyzed. The main analytical steps for the determination of steroids in solid environmental samples are summarized in Table 21.3.

Several reviews have covered the analysis of steroid sex hormones and related synthetic compounds, along with other classes of chemicals, in aquatic environmental samples [240, 259–261]. While GC/MS and immunoassays have been the most commonly used analytical approaches in the past, LC/MS is increasingly favored for the determination of estrogens [262]. The advantage of using LC is that the enzymatic hydrolysis step, required for the immunoassay analysis of both conjugated and unconjugated estrogens, and the derivatization step that normally precedes GC/MS analysis, can be obviated. Because of the very low environmental levels of these compounds and the complexity of some matrices, very efficient extraction/purification methods, in addition to selective and sensitive analytical techniques, are required. The most important purification methods involve the application of

(a) immunosorbents for wastewater extracts in the analysis of estrogens by LC/ESI(NI)-MS [34],
(b) column switching LC/MS with restricted-access materials (ADS C4) for the integrated purification and analysis of estrogens in river sediments [92], and
(c) a fully automated methodology for the on-line SPE and analysis of estrogens and progestogens in water by LC/DAD/ESI(NI)-MS [61,62]. The classical method

for extracting estrogens and progestogens from environmental water samples or for the purification of solid environmental sample extracts, is SPE with octadecylsilane (ODS), polymeric, or graphitized carbon black. The advantages and disadvantages of these types of sorbents and their use in the off-line or on-line analysis of estrogens and progestogens in environmental matrices have been discussed [62].

21.3.5.1.1 Liquid chromatography

The LC separation of both conjugated and unconjugated estrogens and progestogens has invariably been performed on octadecyl silica stationary phases. Both classical, long (250-mm) columns with 4.0- to 4.6-mm ID and 5-μm particle size, and short (55- to 150-mm) columns with smaller (2.0- to 2.1-mm) ID and smaller (3-μm) particle size have been used for separation. As mobile phases, mixtures of water/methanol and, more frequently, water/acetonitrile, with gradient elution from 10–50% to 100% organic solvent have normally been used. For detection, MS has been the technique of choice, although a few authors have reported the use of fluorescence and diode-array detection. Modification of the mobile phase with 0.1% acetic acid [263], 0.2% formic acid [264], 10 m*M* ammonium acetate [265], or by post-column addition of ammonia [13,266] has been performed on various occasions with the aim of improving the sensitivity of the MS detector. The effect of mobile-phase additives on the MS ionization efficiency of estrogens has been evaluated by various authors, but the work conducted by Benjits *et al.* [267] is of particular interest in this respect. They studied in detail the influence of

(a) acetonitrile and methanol as organic modifiers,
(b) volatile bases, such as ammonium hydroxide, isopropylamine, and triethylamine (TEA), and
(c) buffers, such as formic acid/ammonium formate or acetic acid/ammonium acetate, as mobile phase additives, on the ionization efficiency of an ion-spray interface for detection of estradiol. They concluded that a mixture of water and acetonitrile, without the addition of bases or buffer systems is the best choice for optimal ionization of estrogens. TEA and buffers even had a negative impact on ionization, and the use of 100% organic mobile phases was discouraged, as the electrolytic dissociation of analytes and solvation of the resulting ions are reduced under nonaqueous conditions in a gradient system. However, post-column addition of methanolic ammonia drastically increased the response of the ESI-MS system in the analysis of estrogens [13,266].

For the LC/MS determination of estrogens, ESI, operating in the negative-ion mode has been the interface most widely used because of its observed better sensitivity compared to the same interface operating in the positive-ion mode and to APCI operating in the NI mode [13,268]. However, some recent studies indicate that the APCI interface, operating in the PI mode, can furnish sensitivities comparable, in many cases, to that of the negative-ion ESI [263,264,269]. In the LC/MS of estrogens, selected-ion monitoring of the $[M-H]^-$ ion in ESI(NI) and the $[M + H-H_2O]^+$ ion ($[M + H]^+$ for estrone) in APCI(PI) is usually carried out for maximum sensitivity. Because of their low estrogenic potency, estrogen conjugates and progestogens have received less attention

than estrogens. Estrogen conjugates (glucuronides and sulfates) have been analyzed in sewage and river waters by LC/(NI)ESI-MS/MS. Progestogens were determined by LC/ESI(PI)-MS in the SIM mode by recording the $[M + Na]^+$ ion of the analytes [270]. Progestogens can be determined in the positive-ion mode of operation with both ESI and APCI. However, the sensitivity achieved with the APCI interface, is about 10 times lower than that of the ESI interface [268]. In APCI, the $[M + H]^+$ ion of the analyte is recorded.

21.3.5.1.2 Gas chromatography

Gas chromatography, coupled to mass spectrometry, has been the technique most commonly employed in the determination of estrogens in environmental samples. GC has been performed with a variety of capillary columns (DB5-MS, XTI-5, HP Ultra II, etc.) and temperature programs from approximately 45 to 300°C. Both conventional MS and (ion-trap) MS/MS detection, have been accomplished in the EI mode at 70 eV, usually in the SIM mode, and, in most cases, after sample derivatization. The use of NCI has rarely been reported [271]. For derivatization, several reagents, such as *bis*-(trimethylsilyl)trifluoroacetamide [272], *N*-methyl-*N*-(*tert*)-butyldimethylsilyl-trifluoroacetamide (MTBSTFA) [273,274], and heptafluorobutyric anhydride [275] have been used. Derivatization of estrogens normally takes place at the –OH groups of the steroid ring, and the ion masses selected for quantitation depend on the derivatization reaction performed in each case. The sensitivity and general performance of GC/MS/MS, LC/ESI-MS, and LC/ESI-MS/MS for the determination of steroid hormones in complex environmental matrices have been compared [265]. The sensitivity of these techniques increases in the order LC/MS (LOD 200 pg/μL) < GC/MS/MS (LOQ 20 pg/μL) < LC/MS/MS (LOQ 5 pg/μL). LC/APCI-MS/MS, as reported by Lagana *et al.* [269], is *ca.* 2 h faster, less affected by error, and significantly more sensitive than conventional GC/MS methods. An advantage of GC/MS over LC/MS is the availability of extensive libraries of mass spectra, useful for the identification of unknown peaks in estrogenically active fractions. The possibilities of HR-MS for characterizing or identifying environmental contaminants have been discussed [276]. Ten pg of ethynyl estradiol can be detected with a resolution of 20,000. This amount corresponds to a detection limit of 1 ppt if the ethynyl estradiol in 1 liter of water were concentrated to a 0.1-mL extract, followed by injection of 1 μL.

21.3.5.2 Antibiotics and other drugs

Because awareness of the potentially dangerous consequences of the presence of pharmaceuticals in the environment is still recent, their environmental role has not yet been extensively studied. Most of the published methods for the determination of antibiotic drugs are designed for complex biological matrices, like meat, milk, blood, and urine (Chap. 23), and achieve only relatively high detection limits in the range of several hundred ng/kg, or ng/L. Recently, some general reports and reviews have addressed the occurrence, fate, and risk assessment of pharmaceuticals in the environment [3,277–284]. Soils and sediments have been scarcely investigated, in contrast to aqueous media.

A selection of methods developed for the determination of pharmaceuticals in solid environmental samples is presented in Table 21.3.

The principal analytical methods employed in the analysis of pharmaceuticals in aqueous environmental samples has recently been reviewed by Ternes [285]. These methods include both GC and LC, coupled with mass spectrometry. The LC/MS methods have shown lower relative standard deviations than GC/MS methods with derivatization. Ternes considers LC/ESI-MS to be the technique of choice for assaying polar, unstable, and high-molecular-mass compounds. The often incomplete derivatization of highly polar drugs makes their determination by GC inappropriate. Except for a few papers, mentioned below, most of the analytical procedures developed for the environmental determination of pharmaceuticals have been designed for the analysis of a single class of compounds. National surveys of the occurrence of organic wastewater contaminants, including pharmaceuticals, have been published [286,287]. The occurrence of antibiotics in water samples has been investigated by Hirsch *et al.* [288,289]. In all of these studies, analysis was carried out by HPLC/ESI-MS/MS.

Ahrer *et al.* [290], comparing the performance of the ESI and the APCI interface in terms of sensitivity, found that ESI offered better detection limits (average of 0.05 μg/L). For improved sensitivity, MS and MS/MS determinations have been carried out in SIM and MRM mode, respectively. In general, the protonated molecular ion, $[M + H]^+$ of the respective analyte, is selected for quantitation in the SIM mode, or as precursor ion in the MRM mode. Another method [291], based on SPE, derivatization by silylation, and detection by GC/MS, has been developed for the determination of 22 different neutral and weakly basic drugs in wastewater as well as in river and drinking water. This method permits detection of as little as 5 ng/L with recovery rates mostly in excess of 70%. An alternative method, involving SPE and LC/ESI-MS/MS showed detection limits of 10 ng/L in even highly contaminated waters, like sewage-treatment plant effluents.

21.3.5.3 Others

21.3.5.3.1 Fragrances

Musks (nitromusks, polycyclic and macro-cyclic musks) form a class of fragrance ingredients that are added to PPCP, such as detergents, soaps, perfumes, and creams to contribute to their appeal and to maintain the integrity of their scent. Several analytical procedures were developed for their identification and quantification in environmental samples. Sample preparation methods are very similar to those used in pesticide and PCB analysis because of the comparable lipophilicity and polarity of these compounds. GC with NCI-MS or specific detectors (ECD or NPD), were also developed [292,293]. The application of ion-trap MS/MS considerably reduces the background noise and increases selectivity, thus permitting identification and quantification of the nitro and polycyclic musks in very complex matrices, such as sewage sludge samples [294]. Osemwengie and Steinberg [295] reported limits of detection for 7 polycyclic musks, 5 nitro musks, and 3 nitro musk metabolites in the range of 0.02 to 0.30 ng/L, when applying an on-site SPE

(60-L water samples) and laboratory GC/MS analysis of municipal sewage effluents. With SPME/GC/MS, detection limits of 20 ng/L were reported for a nitro musk fragrance (musk ketone) in river water [296] and in the low-ng/L range for polycyclic and macro-cyclic musks [297].

21.3.5.3.2 Sun-screen agents

Sunscreen agents (UV filters), such as 2-hydroxy-4-methoxy-benzophenone, octyldimethyl-*p*-aminobenzoic acid, benzophenone-4, benzo-phenone-3, butyl methoxydibenzoylmethane, octyl methoxycinnamate, homosalate, and octyl salicylate were analyzed in commercial formulations, human plasma and urine, employing conventional SPE enrichment or SFE prior to GC/MS or LC/UV [298–300]. However, the majority of these methods have detection limits in the mg/L range, thus limiting their applicability to the analysis of sun-screen agents in environmental samples. Recently, a method that employs SPME and GC with FID and MS detection was developed for the trace determination (μg/L level) of two sunscreen constituents in natural waters as well as swimming pools and shower wastes [301].

21.3.5.3.3 Antiseptics

Several methods have been proposed for the determination of triclosan (Irgasan DP 300, a chlorinated diphenyl ether) in surface water and wastewaters, as well as in sediments and sludge. Triclosan is used as an antiseptic agent in a vast array of personal care (*e.g.*, toothpaste, acne cream, deodorant, shampoo, toilet soap) and consumer products (children's toys, footwear, kitchen cutting boards), and this results in its direct discharge into sewage systems and receiving surface waters. A method based on diazomethane derivatization and capillary GC/ECD determination was applied to the quantification of triclosan in the wastewater of a slaughterhouse [302]. Triclosan and its 3-chlorinated derivatives were determined in environmental samples (water, sediment, fish tissue) by capillary GC/MS *via* methylation with diazomethane, employing L/L extraction for clean-up [303]. The detection limits in water, sediment, and fish samples were 0.030–0.059 ng/mL, 1.7–4.6 ng/g, and 0.89–2.5 ng/g, respectively. Recently developed methodologies apply continuous L/L extraction with dichloromethane (DCM) and capillary GC/MS [286], SPE of acidified wastewater samples and SFE for lyophilized sludge, followed by derivatization and GC/HR-MS [304]. Occurrence and environmental behavior of triclosan and its methyl derivative in surface waters was studied, using SPMD with triolein [305]. Extracts so obtained were analyzed by GC/MS-EI and showed a *m/z* 288 for triclosan ($M^{+\cdot}$) and *m/z* 302 for methyl triclosan ($M^{+\cdot}$).

21.3.5.3.4 Diagnostic contrast media

Iodinated contrast media, such as iopromide, iomeprol, and diatrizoate, belong to the most frequently used compounds in medicine. Several analytical methods have been developed, mainly designed for the analysis of biological samples and fluids. Generally, they were based on LC, although, because of the strongly polar properties of contrast agents, some authors [306] have proposed ion-pair chromatography on RP-C_{18} and -C_8

columns with detection by UV absorption at 254 nm. More recently, a method based on SPE, with Isolute ENV + material, was developed for the enrichment of five iodinated contrast media in surface and wastewater [307]. Compounds separated on a RP-C_{18} column were detected by ESI-MS/MS, which allowed quantitation in the lower-ng/L range.

21.3.6 Plasticizers

21.3.6.1 Phthalates

Phthalic acid esters (PAE) are a class of chemical compounds widely used in various industrial applications, mainly as plasticizers for polyvinyl chloride (PVC) resins, adhesives, and cellulose film coatings and, to a minor extent, in cosmetics, medical products, and insecticides. They comprise a large group of compounds, several of them considered to be priority pollutants: dimethyl (DMP), diethyl (DEP), dibutyl (DBP), butylbenzyl (BBP), di(2-ethylhexyl) (DEHP) and di-*n*-octyl phthalate(DnOP) [308]. Various methods, based on GC/EI-MS, GC/CI-MS with methane as the reagent gas, either in the positive or negative mode, as well as tandem MS, under PCI conditions with isobutane as reagent gas, LC/APCI-MS, and LC/ESI-MS, have been evaluated for the detection of PAE in water, soil, and sewage sludge samples [309–312]. GC/EI-MS was found to be the most sensitive detection technique, and it is recommended for quantification, although it gives little information on molecular weight and the nature of the alcohol moiety in the molecule. For all phthalates the most abundant ion in the mass spectra is *m/z* 149, corresponding to the protonated phthalic anhydride ion $[C_8H_5O_3]^{+\bullet}$, except for dimethylphthalate, which gives a base ion at *m/z* 163 $[M-31]^{+\bullet}$. However, when analyzing phthalate mixtures, better group profiling is needed with more characteristic ions. In order to prevent misidentification, it is recommended to use, in addition to these two ions, the low-abundance ions in the higher mass range for the assignment of structures [313]. Phthalate esters (DMP, DEP, DBP, and DEHP) were detected in industrial effluents [314] and sewage sludge [311] by LC/APCI-MS under PI conditions. The LC separation of phthalic esters was performed on C_{18} stationary phases. At 20 V, the base ion for DEP, DBP, and DEHP was *m/z* 149 (protonated phthalic anhydride) and *m/z*163 $[M + H-2CH_3]^+$ for DMP. LC/ESI-MS, based on the formation of sodium adducts, can provide molecular-ion information and was found to be a reliable tool for quantitative analysis of phthalate esters in various matrices [312]. Hyötyläinen *et al.* [315] proposed on-line coupling of RP-HPLC to GC/MS by a vaporizer/pre-column solvent split/gas discharge interface for the analysis of phthalates in drinking and surface water. Applying large-volume injection (10 mL), without any sample pre-treatment, limits of detection of 5 to 10 ng/L were achieved.

21.3.6.2 Bisphenol A

Bisphenol A (BPA) is used extensively in the production of polycarbonate, epoxy resins, flame-retardants, and many other products [316]. It is a polar compound, and this

affects the detection limit in GC/MS. Detection limits of 0.1 to 1 μg/L were reported for underivatized BPA [317,318]. To improve the sensitivity, various GC/MS methods, based on pre-concentration by micro L/L extraction [319], and derivatization to silyl BPA [319–321] or pentafluorobenzoylate ester [238] were proposed. NCI-MS with methane as reagent gas afforded very sensitive determinations of BPA-pentafluorobenzoylate in femtogram amounts [238]. This "soft ionization" technique yielded a dominant molecular ion $[M]^-$ and the ^{13}C isotope $[M + 1]^-$ as the second-most intensive ion. A method based on NP-LC and fluorescence detection yielded a LOD of 2 ng/L for water samples and 5 ng/g for sediment samples [322]. LC/MS is an alternative method for the analysis of BPA in environmental matrices. When an ESI interface is used, the main fragment, in addition to the base ion *m/z* 227 $[M-H]^-$, is *m/z* 212, resulting from a cleavage of one of the CH_3 groups [248]. The limits of detection of LC/MS for water samples were in the range of 25 to 100 ng/L [248,249].

21.4 CONCLUSIONS

Because of the chemical diversity of organic contaminants in the environment the range of instrumental techniques applicable to their analysis is also very wide. Among modern analytical techniques, GC and LC, combined with MS and tandem MS, play a pivotal role in providing sufficient selectivity and inherent sensitivity for the analysis of complex environmental matrices. However, further improvements in chemical analysis to lower the limits of detection for some compounds are needed. For example, driven by the estrogenic potency of some compounds (*e.g.*, steroid sex hormones) and their low environmental concentrations, the detection limits required for monitoring of EDC are being pushed from the ng/L-range even lower. With the recent advances in mass spectrometry, *e.g.*, the introduction of TOF-MS and Q-TOF technologies, a new, powerful identification tool has become available. The added power of MS/MS, applied in a variety of scan functions and modes, has enhanced analytical performance (reliability and sensitivity) and allowed a gradual shift from the detection of parent compounds to the analysis of metabolites and transformation products. Furthermore, the introduction of new chromatographic techniques, such as fast LC, fast GC, and GC × GC, has improved the analysis of complex mixtures. Further development of more selective sorbents, such as MIP and immunosorbents, and application of on-line systems for integrated sample preparation and analysis will greatly improve the selectivity of trace level analysis, increase sample throughput, and thus reduce operating costs and contamination risks. However, the question is whether chemical analysis and target-compound monitoring is sufficient to assess contaminants present in the environment. GC/MS screening of fractionated samples has identified several hundreds of individual components in complex samples, such as tannery wastewaters [323,324], and dedicated analytical methods are generally able to detect only a fraction of the dissolved organic carbon (DOC) content [325]. The potential of tandem MS, and especially Q-TOF, has enabled accurate mass measurement and structural elucidation that might be used to identify unknown compounds. However, general screening for unknown compounds is time-consuming and expensive, and is often beset by problems,

such as lack of authentic standards, and lack of mass spectral libraries. Effect-related analysis, focused on relevant compounds, seems to be a more appropriate way to tackle complex environmental contamination problems. Toxicity Identification Evaluation (TIE) procedures, combining chemical analysis and specific bioassays, have become established and powerful methods of determining the causes of the effects observed. The technique employs a small-volume bioassay, followed by LC fractionation of a complex sample in order to isolate active components with a demonstrated effect and characterize them by LC/MS/(MS) or GC/MS.

ACKNOWLEDGMENTS

This work has been supported by the EU Program Copernicus (EXPRESS-IMMUNOTECH) contract Number ICA2-CT-2001-10007, by the Spanish Ministerio de Ciencia y Tecnologia (PPQ 2002-10945-E and PPQ2001-1805-CO3-01). Mira Petrovic and María J. López de Alda acknowledge the Ramon y Cajal contract from the Spanish Ministry of Science and Technology.

REFERENCES

1 Communication from the Commission to the Council and the European Parliament. Community Strategy for Endocrine Disrupters: A Range of Substances Suspected of Interfering with the Hormone Systems of Humans and Wildlife, Brussels, 17-12-99 COMM (1999) 706 final, 31 pages.
2 Towards the Establishment of a Priority List of Substances for Further Evaluation of Their Role in Endocrine Disruption, Final Report, BKH Consulting Engineers, Delft, November 2000.
3 C.G. Daughton and T.A. Ternes, *Environ. Health Perspect.*, 107(Suppl. 6) (1999) 907.
4 B.E. Erickson, *Environ. Sci. Technol.*, 36 (2002) 140A.
5 E. Martinez and D. Barceló, *Chromatographia*, 42 (1996) 72.
6 S. Lacorte, N. Ehresmann and D. Barceló, *Environ. Sci. Technol.*, 29 (1995) 2834.
7 M. Castillo, D. Puig and D. Barceló, *J. Chromatogr. A*, 778 (1997) 301.
8 M.C. Alonso and D. Barceló, *Analyst*, 127 (2002) 472.
9 M.C. Alonso and D. Barceló, *J. Chromatogr. A*, 889 (2000) 231.
10 E. Kubeck and C.G. Naylor, *J. Am. Oil Chem. Soc.*, 67 (1990) 400.
11 C.G. Naylor, J.P. Mieure, W.J. Adams, J.A. Weeks, F.J. Castaldi, L.D. Ogle and R.R. Romano, *J. Am. Oil Chem. Soc.*, 69 (1992) 695.
12 M. Petrović and D. Barceló, *Fresenius J. Anal. Chem.*, 368 (2000) 676.
13 C. Baronti, R. Curini, G. D'Ascenzo, A. Di Corcia, A. Centili and R. Samperi, *Environ. Sci. Technol.*, 34 (2000) 5059.
14 I. Liška, *J. Chromatogr. A*, 885 (2000) 3.
15 D.T. Rossi and N. Zhang, *J. Chromatogr. A*, 885 (2000) 97.
16 T. Gorecki and J. Namiesnik, *Trends Anal. Chem.*, 21 (2002) 276.
17 A. Kot, B. Zbiegala and J. Namiesnik, *Trends Anal. Chem.*, 19 (2000) 446.
18 J.N. Huckins, M.W. Tubergen and G.K. Manuweera, *Chemosphere*, 20 (1990) 533.
19 E.R. Bennett, T.L. Metcalfe and C.D. Metcalfe, *Chemosphere*, 33 (1996) 363.

20 J.D. Petty, B.C. Poulton, C.S. Charbonneau, J.N. Huckins, S.B. Jones, J.T. Caneron and H.F. Prest, *Environ. Sci. Technol.*, 32 (1998) 837.
21 H.F. Prest, L.A. Jacobson and M. Wilson, *Chemosphere*, 12 (1997) 3047.
22 J.A. Lebo, J.L. Zajicek, C.E. Orazio, J.D. Petty, J.N. Huckins and E.H. Douglas, *Polycylic Aromatic Compd.*, 8 (1996) 53.
23 J.N. Huckins, J.D. Petty, C.E. Orazio, J.A. Lebo, R.C. Clark, V.L. Gibson, W.R. Gala and K.R. Echols, *Environ. Sci. Technol.*, 33 (1999) 3918.
24 A. Bergovist, B. Strandberg, R. Ekelund, C. Rappe and A. Granmo, *Environ. Sci. Technol.*, 32 (1998) 3887.
25 A. Rantalainen, M.G. Ikonomou and I.H. Rogers, *Chemosphere*, 37 (1998) 1119.
26 J.A. Lebo, R.W. Gale, J.D. Petty, D.E. Tillit, J.N. Huckins, J.C. Meadows, C.E. Orazio, K.R. Echols, D.J. Schroeder and L.E. Inmon, *Environ. Sci. Technol.*, 29 (1995) 2886.
27 P.A. Brgqvist, B. Strandberg, R. Ekelund, C. Rappe and A. Granmo, *Environ. Sci. Technol.*, 32 (1998) 3887.
28 G.S. Ellis, J.N. Huckins, C.E. Rostad, C.J. Schmitt, J.D. Petty and P. MacCarthy, *Environ. Toxicol. Chem.*, 14 (1995) 1875.
29 N. Folsvik, E. Brevik and J.A. Berge, *J. Environ. Monit.*, 2 (2000) 281.
30 A. Granmo, R. Ekelund, M. Berggren, E. Brorstrom-Lunden and P.A. Bergvist, *Environ. Sci. Technol.*, 34 (2000) 3323.
31 J.K. Kingston, R. Greenwood, G.A. Mills, G.M. Morrison and L.B. Persson, *J. Environ. Monit.*, 2 (2000) 487.
32 M.G. Weller, *Fresenius J. Anal. Chem.*, 366 (2000) 635.
33 N. Delaunay, V. Pichon and M. Hennion, *J. Chromatogr. B*, 745 (2000) 15.
34 P.L. Ferguson, C.R. Iden, A.E. McElroy and B.J. Brownawell, *Anal. Chem.*, 73 (2001) 3890.
35 O. Brüggemann, K. Haupt, L. Ye, E. Yilmaz and K. Mosbach, *J. Chromatogr. A*, 889 (2000) 15.
36 K. Haupt, *Analyst*, 126 (2001) 747.
37 N. Masqué, R.M. Marcé and F. Borull, *Trends Anal. Chem.*, 20 (2001) 477.
38 M.T. Muldoon and L.H. Stanker, *Anal. Chem.*, 69 (1997) 803.
39 K. Haupt, A. Dzgoen and K. Mosbach, *Anal. Chem.*, 70 (1998) 628.
40 I. Ferrer and D. Barceló, *Trends Anal. Chem.*, 18 (1999) 180.
41 E. Hogendoorn and P. van Zoonen, *J. Chromatogr. A*, 892 (2000) 435.
42 R. Koeber, C. Fleischer, F. Lanza, K.-S. Boos, B. Sellergren and D. Barceló, *Anal. Chem.*, 73 (2001) 2437.
43 C. Baggiani, C. Giovannoli, L. Anfossi and C. Tozzi, *J. Chromatogr. A*, 938 (2001) 35.
44 C.P. Desilets, M.A. Rounds and F.E. Regnier, *J. Chromatogr.*, 544 (1991) 25.
45 K.S. Boos and C.H. Grimm, *Trends Anal. Chem.*, 18 (1999) 175.
46 K.S. Boos and A. Rudolphi, *LC-GC*, 15 (1997) 602.
47 C. Schafer and D. Lubda, *J. Chromatogr. A*, 909 (2001) 73.
48 A. Rudolphi and K.S. Boos, *LC-GC*, 15 (1997) 814.
49 E.A. Hogendoorn, E. Dijkman, B. Baumann, C. Hidalgo, J.V. Sancho and F. Hernandez, *Anal. Chem.*, 71 (1999) 1111.
50 P. Parilla Vazquez, J.L. Marinez Vidal and J. Marinez Fernández, *Analyst*, 125 (2000) 1549.
51 P. Önnerfjord, D. Barceló, J. Emnéus, L. Gorton and G. Marko-Varga, *J. Chromatogr. A*, 737 (1996) 35.

52 S. Lacorte and D. Barceló, *Anal. Chem.*, 68 (1996) 2464.
53 I. Ferrer, M.-C. Hennion and D. Barcelo, *Anal. Chem.*, 69 (1997) 4508.
54 I. Ferrer, V. Pichon, M.-C. Hennion and D. Barcelo, *J. Chromatogr. A*, 777 (1997) 91.
55 I. Ferrer and D. Barceló, *J. Chromatogr. A*, 854 (1999) 197.
56 T. Renner, D. Baumgarten and K.K. Unger, *Chromatographia*, 45 (1997) 199.
57 C. Aguilar, I. Ferrer, F. Borrull, R.M. Marcé and D. Barceló, *J. Chromatogr. A*, 794 (1998) 147.
58 A.C. Hogenboom, M.P. Hofman, D.A. Jolly, W.M.A. Niessen and U.A.Th. Brinkman, *J. Chromatogr. A*, 885 (2000) 377.
59 J. Slobodník, A.C. Hogenboom, A.J.H. Louter and U.A.Th. Brinkman, *J. Chromatogr. A*, 730 (1996) 353.
60 J. Slobodník, S. Ramalho, B.L. van Baar, A.J. Louter and U.A.Th. Brinkman, *Chemosphere*, 41 (2000) 1469.
61 M.J. López de Alda and D. Barceló, *J. Chromatogr. A*, 911 (2001) 203.
62 M.J. López de Alda and D. Barceló, *J. Chromatogr. A*, 938 (2001) 145.
63 J. Chen and J. Pawliszyn, *Anal. Chem.*, 67 (1995) 2530.
64 R. Eisert and J. Pawliszyn, *Anal. Chem.*, 69 (1997) 3145.
65 Y. Gou, R. Eisert and J. Pawliszyn, *J. Chromatogr. A*, 873 (2000) 137.
66 J. Pawliszyn, *Solid Phase Microextraction: Theory and Practice*, Wiley-VCH, New York, 1997.
67 J. Pawliszyn, *Applications of Solid Phase Microextraction*, Royal Society of Chemistry, Cambridge, 1999.
68 S.A. Scheppers-Wercinski (Ed.), *Solid Phase Microextraction: A Practical Guide*, Dekker, New York, 1999.
69 A. Peñalver, E. Pocurull, F. Borull and R.M. Marcé, *Trends Anal. Chem.*, 18 (1999) 557.
70 M.F. Alpendurada, *J. Chromatogr. A*, 889 (2000) 3.
71 D.Y. Shang, M.G. Ikonomou and R.W. Macdonald, *J. Chromatogr. A*, 849 (1999) 467.
72 M. Petrovic and D. Barceló, *Chromatographia*, 56 (2002) 535.
73 V. Camel, *Anal. Bioanal. Chem.*, 372 (2002) 39.
74 J.A. Field and R.L. Reed, *Environ. Sci. Technol.*, 33 (1999) 2782.
75 S.B. Hawthorne, Y. Yang and D.J. Miller, *Anal. Chem.*, 66 (1994) 2912.
76 Y. Yang, S.B. Hawthorne and D.J. Miller, *Environ. Sci. Technol.*, 31 (1997) 430.
77 V. Fernandez-Perez and M.D. Luque de Castro, *J. Chromatogr. A*, 902 (2000) 357.
78 S. Morales-Muñoz, J.L. Luque-García and M.D. Luque de Castro, *Anal. Chem.*, 74 (2002) 4213.
79 T. Hyötyläinen, T. Andersson, K. Hartonen, K. Kuosmanen and M.L. Riekkola, *Anal. Chem.*, 72 (2000) 3070.
80 K. Hartonen, S. Bowadt, S.B. Hawthorne and M.-L. Riekkola, *J. Chromatogr. A*, 774 (1997) 229.
81 B. van Bavel, K. Hartonen, C. Rappe and M.-L. Riekkola, *Analyst*, 124 (1999) 1351.
82 K. Kuosmanen, T. Hyötyläinen, K. Hartonen and M.-L. Riekkola, *J. Chromatogr. A*, 943 (2002) 113.
83 C. Molins, E.A. Hogendoorn, H.A.G. Heusinkveld, D.C. van Harten, P. van Zoonen and R.A. Baumann, *Chromatographia*, 43 (1996) 527.
84 Z. Vryzas, E.N. Papadakis and E. Papadopoulou-Mourkidou, *J. Agric. Food Chem.*, 50 (2002) 5026.

85 S.J. Stout, A.R. daCunha and D.G. Allardice, *Anal. Chem.*, 68 (1996) 653.
86 I. Silgoner, R. Krska, O. Gans, E. Rosenberg and M. Grassebauer, *Fresenius J. Anal. Chem.*, 362 (1998) 120.
87 O. Zuloaga, N. Etxebarria, L.A. Fernadez and J.M. Madariaga, *Fresenius J. Anal. Chem.*, 367 (2000) 733.
88 S.J. Stout, A.R. daCunha, G.L. Picard and M.M.J. Sfarpour, *J. Agric. Food Chem.*, 44 (1996) 3548.
89 A. Bouaid, A. Martin-Esteban, P. Fernadez and C. Camara, *Fresenius J. Anal. Chem.*, 367 (2000) 291.
90 Z. Vryzas, E.N. Papadakis and E. Papadopoulou-Mourkidou, *J. Agric. Food Chem.*, 50 (2002) 2220.
91 C. Blasco, G. Font and Y. Pico, *J. Chromatogr. A*, 1 (2002) 1.
92 M. Petrovic, S. Tavazzi and D. Barceló, *J. Chromatogr. A*, 971 (2002) 37.
93 T.L. Keith, S.A. Snyder, C.G. Naylor, C.A. Staples, C. Summer, K. Kannan and J.P. Giesy, *Environ. Sci. Technol.*, 35 (2001) 10.
94 W.L. Shelver, G.L. Larsen and J.K. Huwe, *J. Chromatogr. B*, 705 (1998) 261.
95 M.A. Concejero, R. Galve, B. Herradón, M.J. González and M. de Frutos, *Anal. Chem.*, 73 (2001) 3119.
96 W.L. Shelver, J.K. Huwe, L.H. Stanker, D.G. Patterson, Jr. and W.E. Turner, *Organohalogen Compd.*, 45 (2000) 33.
97 S.A. Snyder, T.L. Keith, S.L. Pierens, E.M. Snyder and J.P. Giesy, *Chemosphere*, 44 (2001) 1697.
98 S.A. Snyder, T.L. Keith, C.G. Naylor, C.A. Staples and J.P. Giesy, *Environ. Toxicol. Chem.*, 20 (2001) 1870.
99 S.A. Barker, A.R. Long and M.E. Hines, *J. Chromatogr.*, 629 (1993) 23.
100 S.A. Barker, *LC-GC*, 11 (1998) 719.
101 S.A. Barker, *J. Chromatogr. A*, 885 (2000) 115.
102 G.J. Reimer and A. Suarez, *J. Chromatogr.*, 555 (1991) 315.
103 G.J. Reimer and A. Suarez, *J. AOAC Int.*, 75 (1992) 979.
104 C.C. Walker, H.M. Lott and S.A. Barker, *J. Chromatogr.*, 642 (1993) 225.
105 J. Tolls, M. Haller and D.T. Sijm, *J. Chromatogr. A*, 839 (1999) 109.
106 J. Tolls, M. Haller and D.T. Sijm, *Anal. Chem.*, 71 (1999) 5242.
107 P. de Voogt, O. Kwast, R. Hendriks and N. Jonkers, *Analusis*, 28 (2000) 776.
108 M. Zhao, F. van der Wielen and P. de Voogt, *J. Chromatogr. A*, 837 (1999) 129.
109 Y.-C. Ling, M.-Y. Chang and I.-P. Huang, *J. Chromatogr. A*, 669 (1994) 119.
110 Y.-C. Ling and I.-P. Huang, *Chromatographia*, 40 (1995) 259.
111 M.D. Crouch and S.A. Barker, *J. Chromatogr. A*, 774 (1997) 287.
112 A.C. Hogenboom, J. Slobodník, J.J. Vreuls, J.A. Rontree, B.L.M. van Baar, W.M.A. Niessen and U.A.Th. Brinkman, *Chromatographia*, 42 (1996) 506.
113 W.A. Minnaard, A.C. Hogenboom, U.K. Malmqvist, P. Manini, W.M.A. Niessen and U.A.Th. Brinkman, *Rapid Commun. Mass Spectrom.*, 10 (1996) 1569.
114 A.C. Hogenboom, W.M.A. Niessen and U.A.Th. Brinkman, *J. Chromatogr. A*, 794 (1998) 201.
115 A.C. Hogenboom, U.K. Malmqvist, K. Nolkrantz, J.J. Vreuls and U.A.Th. Brinkman, *J. Chromatogr. A*, 759 (1997) 55.
116 A. Asperger, J. Efer, T. Koal and W. Engewald, *J. Chromatogr. A*, 960 (2002) 109.
117 K. Cabrera, D. Lubda, H.-M. Eggenweiler, H. Minakuchi and K. Nakanishi, *J. High Resolut. Chromatogr.*, 23 (2000) 93.

118 N. Tanaka, H. Nagayama, H. Kobayashi, T. Ikegami, K. Hosoya, N. Ishizuka, H. Minakuchi, K. Nakanishi, K. Cabrera and D. Lubda, *J. High Resolut. Chromatogr.*, 23 (2000) 111.
119 L.S. de Jager and A.R. Andrews, *Analyst*, 125 (2000) 1943.
120 J.A. Syage, B.J. Nies, M.D. Evans and K.A. Hanold, *J. Am. Soc. Mass Spectrom.*, 12 (2001) 648.
121 J.W. Cochran, *J. Chromatogr. Sci.*, 40 (2002) 254.
122 M. Hada, M. Takino, T. Yamagami, S. Daishima and K. Yamaguchi, *J. Chromatogr. A*, 874 (2000) 81.
123 F.J. Santos and M.T. Galceran, *Trends Anal. Chem.*, 21 (2002) 672.
124 G.S. Frysinger, R.B. Gaines and C.M. Reddy, *Environ. Forensics*, 3 (2002) 27.
125 J. Phillips and J. Beens, *J. Chromatogr. A*, 856 (1999) 331.
126 R.B. Gaines, E.B. Ledford and J.D. Stuart, *J. Microcol. Sep.*, 10 (1998) 597.
127 G.S. Frysinger and R.B. Gaines, *J. Sep. Sci.*, 24 (2001) 87.
128 L. Xu, C.M. Reddy, J.W. Farrington, G.S. Frysinger, R.B. Gaines, C.G. Johnson, R.K. Nelson and T.I. Eglinton, *Org. Geochem.*, 32 (2001) 633.
129 E. Ledford and C. Billesbach, *J. High Resolut. Chromatogr.*, 23 (2000) 202.
130 J. Beens, M. Adahchour, R.J.J. Vreuls, K. van Altena and U.A.Th. Brinkman, *J. Chromatogr. A*, 919 (2001) 127.
131 H.J. de Geus, A. Schelvis, J. de Boer and U.A.Th. Brinkman, *J. High Resolut. Chromatogr.*, 23 (2000) 189.
132 R. Vreuls, M. Kristenson, U.A.Th. Brinkman, C. Danielson, P. Haglund, P. Korytar, P.E.G. Leonards and J. de Boer, *Organohalogen Compd.*, 55 (2002) 127.
133 A.C. Hogenboom, W.M.A. Niessen, D. Little and U.A.Th. Brinkman, *Rapid Commun. Mass Spectrom.*, 13 (1999) 125.
134 A.C. Hogenboom, W.M.A. Niessen and U.A.Th. Brinkman, *Rapid. Commun. Mass Spectrom.*, 14 (2000) 1914.
135 R. Morris, T. Paxton, A. Dell, J. Langhorne, M. Berg, R.S. Bordoli, J. Hoyes and R.H. Bateman, *Rapid Commun. Mass Spectrom.*, 10 (1996) 889.
136 I. Bobeldijk, P.G.M. Stoks, J.P.C. Vissers, E. Emke, J.A. van Leerdam, B. Muilwijk, R. Berbee and Th.H.M. Noij, *J. Chromatogr. A*, 970 (2002) 167.
137 I. Bobeldijk, J.P. Vissers, G. Kearney, H. Major and J.A. Van Leerdam, *J. Chromatogr. A*, 929 (2001) 63.
138 M. van der Berg, L. Birnbaum, A.T.C. Bosveld, B. Brunstrom, P. Cook, M. Feeley, J.P. Giesy, A. Hanberg, R. Hasegawa, S.W. Kennedy, T. Kubiak, J.C. Larsen, F.X.R. Leeuwen, A.K.D. Liem, C. Nolt, R.E. Peterson, L. Poellinger, S. Safe, D. Schrenk, D. Tillitt, M. Tysklind, M. Younes, F. Waern and T. Zacharewski, *Environ. Health Perspect.*, 106 (1998) 775.
139 U.S. EPA Method 1613A, Tetra- through Octa-Chlorinated Dioxins and Furans by Isotope Dilution HRGC/HRMS, 1994.
140 U.S. EPA Method 8290A, Polychlorinated Dibenzodioxins (PCDDs) and Polychlorinated Dibenzofurans (PCDFs) by High Resolution Gas Chromatography/High Resolution Mass Spectrometry (HRGC/HRMS), 1998.
141 U.S. EPA Method 1668, Toxic Polychlorinated Biphenyls by Isotope Dilution High Resolution Gas Chromatography/High Resolution Mass Spectrometry, 1997.
142 R. Bacher and K. Ballschmiter, *Chromatographia*, 34 (1992) 137.
143 E. Abad, E.J. Caixach and J. Rivera, *J. Chromatogr. A*, 786 (1997) 125.
144 T. Matsumura, H. Tsubota, Y. Ikeda, Y. Chisaki, H. Ito and M. Morita, *Organohalogen Compd.*, 31 (1997) 14.
145 T. Matsumura, H. Tsubota, Y. Ikeda, Y. Chisaki, H. Ito and M. Morita, *Organohalogen Compd.*, 35 (1998) 141.

146 P. Korytár, U.A.Th. Brinkman, P.E.G. Leonards and J. de Boer, *Organohalogen Compd.*, 50 (2001) 21.
147 J. de Boer, H.J. de Geus and U.A.Th. Brinkman, *Organohalogen Compd.*, 45 (2000) 1.
148 E. Eljarrat and D. Barceló, *J. Mass Spectrom.*, 37 (2002), 1105.
149 E.J. Reiner, D.H. Schellenberg, V.Y. Taguchi, R.S. Mercer, J.A. Townsend, T.S. Thompson and R.E. Clement, *Chemosphere*, 20 (1990) 1385.
150 R. Malisch, B. Baumann, P.A. Behnisch, R. Canady, D. Fraisse, P. Fürst, D. Hayward, R. Hoogenboom, R. Hoogerbrugge, D. Liem, O. Päpke, W. Traag and T. Wiesmüller, *Organohalogen Compd.*, 50 (2001) 53.
151 G. Eppe, J.F. Focant, C. Pirard and E. Pauw, *Organohalogen Compd.*, 50 (2001) 186.
152 Y. Kemmochi, K. Tsutsumi and K. Futami, *Chemosphere*, 46 (2002) 1451.
153 F. Guidugli, *J. Mass Spectrom.*, 34 (1999) 1389.
154 B. van Bavel, J. Hughes, S. Davis, H. Wingfors and G. Lindstrom, *Organohalogen Compd.*, 40 (1999) 293.
155 L. Hardell, B. van Bavel, G. Lindström, M. Fredrikson, H. Hagberg, M. Nordström, G. Liljegren and B. Johansson, *Int. J. Oncol.*, 9 (1996) 603.
156 J.M. Dimandja, J. Grainger and D.G. Patterson, Jr., *Organohalogen Compd.*, 40 (1999) 23.
157 J.F. Focant, E. de Pauw, J. Grainger, D.G. Patterson, Jr. and J.M.D. Dimandja, *Organohalogen Compd.*, 50 (2001) 25.
158 J. Dallüge, P. Roose and U.A.Th. Brinkman, *J. Chromatogr. A*, 965 (2002) 207.
159 A. Sjödin, E. Jakobsson, A. Kierkegaard, G. Marsh and U. Sellström, *J. Chromatogr. A*, 822 (1998) 83.
160 J. de Boer, K. de Boer and J.P. Boon, *Handbook of Environmental Chemistry, Vol. 3, Part K, New Types of Halogenated Compounds*, 2000, p. 61.
161 C. Robinson, M. Alaee, I. D'Sa, A. Newton, R. Rao, K. McPherson and E. Reiner, *Organohalogen Compd.*, 55 (2002) 119.
162 E. Eljarrat, S. Lacorte and D. Barceló, *J. Mass Spectrom.*, 37 (2002) 76.
163 C. Pirard, C. Douny, G. Eppe and E. de Pauw, *Organohalogen Compd.*, 55 (2002) 29.
164 J. de Boer and W.P. Cofino, *Chemosphere*, 46 (2002) 625.
165 J. de Boer, D.E. Wells and K. Norén, *Organohalogen Compd.*, 58 (2002) 197.
166 S. Lacorte, C. Molina and D. Barcelò, *Anal. Chim. Acta*, 281 (1993) 71.
167 V. Leoni, *AOAC Int.*, 75 (1992) 511.
168 F.E. Ahmed, *Trends Anal. Chem.*, 20 (2001) 649.
169 R.B. Geerdink, W.M.A. Niessen and U.A.Th. Brinkman, *J. Chromatogr. A*, 970 (2002) 65.
170 O. Nuñez, E. Moyano and M.T. Galceran, *J. Chromatogr. A*, 974 (2002) 243.
171 P. Skladal, M. Pavlik and M. Fiala, *Anal. Lett.*, 27 (1994) 29.
172 G.S. Nunes, M.P. Marco, M.L. Ribeiro and D. Barceló, *J. Chromatogr. A*, 823 (1998) 109.
173 G.S. Nunes, D. Barceló, B.S. Grabaric, J.M. Díaz-Cruz and M.L. Ribeiro, *Anal. Chim. Acta*, 300 (1999) 37.
174 G.S. Nunes, P. Skladal, H. Yamanaka and D. Barceló, *Anal. Chim. Acta*, 362 (1998) 59.
175 G.R. van der Hoff and P. van Zoonen, *J. Chromatogr. A*, 843 (1999) 301.
176 R. Ong, P. Marriott, P. Morrison and P. Haglund, *J. Chromatogr. A*, 962 (2002) 135.
177 R. Tauler, D. de Almeira Azevedo, S. Lacorte, R. Céspedes, P. Viana and D. Barceló, *Environ. Technol.*, 22 (2001) 1043.
178 E. Pocurull, C. Aguilar, F. Borrull and R.M. Marcè, *J. Chromatogr. A*, 818 (1998) 85.
179 F. van Hoof, P. van Wiele, A. Bruchet, I. Schmitz, I. Bodeldiji, F. Sacher, F. Ventura, I. Marti, M.H. Marecos do Monte and M. sa da Costa, *J. AOAC Int.*, 84 (2001) 1420.
180 M. Kochman, A. Gordin, P. Goldshlag, S.J. Lehotay and A. Amirav, *J. Chromatogr. A*, 974 (2002) 185.

181 L. Amendola, F. Botrè, A.S. Carollo, D. Longo and L. Zoccolill, *Anal. Chim. Acta*, 461 (2002) 97.
182 M.M. van Deursen, J. Beens, H.-G. Janssen, P.A. Leclercq and C.A. Cramers, *J. Chromatogr. A*, 878 (2000) 205.
183 S.J. Lehotay and J. Hajšlová, *Trends Anal. Chem.*, 21 (2002) 686.
184 J. Dalluge, P. Roose and U.A.Th. Brinkman, *J. Chromatogr. A*, 970 (2000) 213.
185 J. Quintana, I. Marti and F. Ventura, *J. Chromatogr. A*, 938 (2001) 3.
186 D. de Almeida Azevedo, S. Lacorte, T. Vinhas, P. Viana and D. Barceló, *J. Chromatogr. A*, 879 (2000) 13.
187 L. Brossa, R.M. Marce, F. Borrull and E. Pocurull, *J. Chromatogr. A*, 963 (2002) 287.
188 J.J. Vreuls, A.J.H. Louter and U.A.Th. Brinkman, *J. Chromatogr. A*, 856 (1999) 279.
189 K.A. Hostetler and E.M. Thurman, *Sci. Total Environ.*, 248 (2000) 147.
190 F. van Hoof, P. van Wiele, F. Acobas, J.-L. Guinamant, A. Bruchet, I. Schmitz, I. Bodeldijk, F. Sacher, F. Ventura and R. Boleda, *J. AOAC Int.*, 84 (2002) 375.
191 T.C.R. Santos, J.C. Rocha and D. Barceló, *J. Environ. Anal. Chem.*, 70 (1998) 19.
192 T.C.R. Santos, J.C. Rocha and D. Barceló, *J. Chromatogr. A*, 879 (2000) 3.
193 K. Vandecasteele, I. Gaus, W. Debreuck and K. Walraevens, *Anal. Chem.*, 72 (2000) 3093.
194 E.M. Thurman, I. Ferrer and D. Barcelò, *Anal. Chem.*, 73 (2001) 5441.
195 R. Bossi, K.V. Vejrup, B.B. Mogensen and W.A.H. Asman, *J. Chromatogr. A*, 957 (2002) 27.
196 F. Hernández, J.V. Sancho, O. Pozo, A. Lara and E. Pitarch, *J. Chromatogr. A*, 939 (2001) 1.
197 A. Asperger, J. Efer, T. Koal and W. Engewald, *J. Chromatogr. A*, 937 (2001) 65.
198 R.A. Yokley, L.C. Mayer, S.-B. Huang and J.D. Vargo, *Anal. Chem.*, 74 (2002) 3754.
199 L. Grey, B. Nguyen and P. Yang, *J. Chromatogr. A*, 958 (2002) 25.
200 I. Bobeldijk, J.P.C. Vissers, G. Kearney, H. Major and J.A. van Leerdam, *J. Chromatogr. A*, 929 (2001) 63.
201 E.M. Thurman, I. Ferrer and D. Barcelo, *J. Chromatogr. A*, 957 (2002) 3.
202 T.M. Schmitt, *Analysis of Surfactants*, Dekker, New York, 2001, p. 197.
203 A. Di, *Corcia. J. Chromatogr. A*, 794 (1998) 165.
204 H.Fr. Schröder and F. Ventura, in D. Barceló (Ed.), *Sample Handling and Trace Analysis of Pollutants – Techniques, Applications and Quality Assurance*, Elsevier, Amsterdam, 2000, p. 828.
205 H.B. Lee, *Water Qual. Res. J. Canada*, 34 (1999) 3.
206 M. Petrovic and D. Barcelo, *J. Mass Spectrom.*, 36 (2001) 1173.
207 J.A. Field, Th.M. Field, Th. Ploguer and W. Giger, *Environ. Sci. Technol.*, 28 (1994) 497.
208 J. Riu, I. Schonsee and D. Barceló, *J. Mass Spectrom.*, 33 (1998) 653.
209 E. Gonzalez-Mazo and J.M. Foria, *Gomez-Parra. Environ. Sci. Technol.*, 32 (1998) 1636.
210 M. Castillo, E. Martinez, A. Ginebreda, L. Tirapu and D. Barceló, *Analyst*, 125 (2000) 1733.
211 V.M. León, A. Gómez-Parra and E. González-Mazo, *Fresenius J. Anal. Chem.*, 371 (2001) 479.
212 V.M. León, M. Sáez, E. González-Mazo and A. Gómez-Parra, *Sci. Total Environ.*, 288 (2002) 215.
213 M.J.-F. Suter, R. Reiser and W. Giger, *J. Mass Spectrom.*, 31 (1996) 357.
214 W.-H. Ding, J.-H. Lo and S.-H. Tzing, *J. Chromatogr. A*, 818 (1998) 270.
215 T. Reemtsma, *J. Chromatogr. A*, 733 (1996) 473.
216 P. Eichhorn and Th.P. Knepper, *Environ. Toxicol. Chem.*, 21 (2002) 1.
217 A. Marcomini, A. Giacometti and G. Pojana, *Ann. Chim.*, 92 (2002) 27.
218 T.P. Knepper and M. Kruse, *Tenside Surf. Deter.*, 37 (2000) 41.
219 J. Riu, E. Martinez, D. Barcelo, A. Ginebreda and L. Tirapu, *Fresenius J. Anal. Chem.*, 371 (2001) 448.

220 D. Temesi and B. Law, *LC-GC*, 3 (1999) 175.
221 A. Di Corcia, F. Casassa, C. Crescenzi, A. Marcomini and R. Samperi, *Environ. Sci. Technol.*, 33 (1999) 4112.
222 M. Ahel, W. Giger and M. Koch, *Water Res.*, 28 (1994) 1131.
223 N. Jonkers, T.P. Knepper and P. de Voogt, *Environ. Sci. Technol.*, 35 (2001) 335.
224 A. Di Corcia, A. Constantino, C. Crescenzi, E. Marinoni and R. Samperi, *Environ. Sci. Technol.*, 32 (1998) 2401.
225 A. Di Corcia, R. Cavallo, C. Crescenzi and M. Nazzari, *Environ. Sci. Technol.*, 34 (2000) 3914.
226 M. Reinhard, N. Goodman and K.E. Mortelmans, *Environ. Sci. Technol.*, 16 (1982) 351.
227 F. Ventura, A. Figueras, J. Caixach, I. Espadaler, J. Romero, J. Guardiola and J. Rivera, *Water Res.*, 22 (1988) 1211.
228 M. Petrovic, A. Diaz, F. Ventura and D. Barcelo, *Anal. Chem.*, 73 (2001) 5886.
229 H.B. Lee and T.E. Peart, *Anal. Chem.*, 67 (1995) 1976.
230 E.R. Bennet and C.D. Metcalfe, *Environ. Toxicol. Chem.*, 17 (1998) 1230.
231 D.T. Bennie, C.A. Sullivan, H.B. Lee, T.E. Peart and R.J. Maguire, *Sci. Total Environ.*, 193 (1997) 263.
232 W.H. Ding and C.T. Chen, *J. Chromatogr. A*, 862 (1999) 113.
233 M. Ahel, T. Conrad and W. Giger, *Environ. Sci. Technol.*, 21 (1987) 697.
234 E. Stephanou, M. Reinhard and H.A. Ball, *Biomed. Environ. Mass Spectrom.*, 15 (1988) 275.
235 C. Hao, T.R. Croley, R.E. March, B.G. Koenig and C.D. Metcalfe, *J. Mass Spectrom.*, 35 (2000) 818.
236 J.A. Field and R.L. Reed, *Environ. Sci. Technol.*, 30 (1996) 3544.
237 C. Wahlberg, L. Renberg and U. Wideqvist, *Chemosphere*, 20 (1990) 179.
238 H.M. Kuch and K. Ballschmiter, *Environ. Sci. Technol.*, 35 (2001) 3201.
239 W.H. Ding and C.H. Tzing, *J. Chromatogr. A*, 824 (1998) 79.
240 M. Petrovic, E. Eljarrat, M.J. Lopez de Alda and D. Barcelo, *J. Chromatogr. A*, 974 (2002) 23.
241 D.Y. Shang, M.G. Ikonomou and R.W. Macdonald, *J. Chromatogr. A*, 849 (1999) 467.
242 M. Takino, S. Daishima and K. Yamaguchi, *J. Chromatogr. A*, 904 (2000) 65.
243 D.Y. Shang, R.W. Macdonald and M.G. Ikonomou, *Environ. Sci. Technol.*, 33 (1999) 1366.
244 C. Crescenzi, A. Di Corcia, R. Samperi and A. Marcomini, *Anal. Chem.*, 67 (1995) 1797.
245 P.L. Ferguson, C.R. Iden and B.J. Brownawell, *Anal. Chem.*, 72 (2000) 4322.
246 A. Di Corcia, C. Crescenzi, A. Marcomini and R. Samperi, *Environ. Sci. Technol.*, 32 (1998) 711.
247 P.L. Ferguson, C.R. Iden and B.J. Brownawell, *J. Chromatogr. A*, 938 (2001) 79.
248 M. Petrovic and D. Barceló, *J. AOAC Int.*, 84 (2001) 1074.
249 S.N. Pedersen and C. Lindholst, *J. Chromatogr. A*, 864 (1999) 17.
250 F. Houde, C. DeBlois and D. Berryman, *J. Chromatogr. A*, 961 (2002) 245.
251 H.F. Schröder, *J. Chromatogr. A*, 926 (2001) 127.
252 M. Petrovic, A. Diaz, F. Ventura and D. Barcelo, *J. Am. Soc. Mass Spectrom.*, 14 (2003) 516.
253 C. Pelissero, G. Flouriot, J.L. Foucher, B. Bennetau, J. Dunoguès, F. Le Gac and J.P. Sumpter, *J. Steroid Biochem. Mol. Biol.*, 44 (1993) 263.
254 C.E. Purdom, P.A. Hardiman, V.J. Bye, N.C. Eno, C.R. Tyler and J.P. Sumpter, *Chem. Ecol.*, 8 (1994) 275.
255 P.-D. Hansen, H. Dizer, B. Hock, A. Marx, J. Sherry, M. McMaster and Ch. Blaise, *Trends Anal. Chem.*, 17 (1998) 448.
256 T. Colborn, F.S. vom Saal and A.M. Soto, *Environ. Health Perspect.*, 101 (1993) 378.

257 R. Stone, *Science*, 265 (1994) 308.
258 Environment Agency. The Identification and Assessment of Estrogenic Substances in Sewage Treatment Effluents. R&D Publication, Nov. 1996, The Environment Agency, Bristol, UK, 1996
259 M.J. López de Alda and D. Barceló, *Fresenius J. Anal. Chem.*, 371 (2001) 437.
260 M. Petrovic, E. Eljarrat, M.J. López de Alda and D. Barceló, *Trends Anal. Chem.*, 20 (2001) 637.
261 S. Diaz-Cruz, M.J. López de Alda and D. Barceló, *Trends Anal. Chem.*, 22 (2003) 340.
262 K. Shimada, K. Mitamura and T. Higashi, *J. Chromatogr. A*, 935 (2001) 141.
263 M. Seifert, G. Brenner-Weiß, S. Haindl, M. Nusser, U. Obst and B. Hock, *Fresenius J. Anal. Chem.*, 363 (1999) 767.
264 J. Rose, H. Holbech, C. Lindholst, U. Norum, A. Povlsen, B. Korsgaard and P. Bjerregaard, *Comp. Biochem. Physiol. C*, 131 (2002) 531.
265 T.R. Croley, R.J. Hughes, B.G. Koenig, C.D. Metcalfe and R.E. March, *Rapid Commun. Mass Spectrom.*, 14 (2000) 1087.
266 A. Gentili, D. Perret, S. Marchese, R. Mastropasqua, R. Curini and A. Di Corcia, *Chromatographia*, 56 (2002) 25.
267 T. Benijts, R. Dams, W. Günther, W. Lambert and A. De Leenheer, *Rapid Commun. Mass Spectrom.*, 16 (2002) 1358.
268 M. Lopez de Alda and D. Barceló, *J. Chromatogr. A*, 892 (2000) 391.
269 A. Laganà, A. Bacaloni, G. Fago and A. Marino, *Rapid Commun. Mass Spectrom.*, 14 (2000) 401.
270 M. Petrovic, M. Solé, M.J. López de Alda and D. Barceló, *Environ. Toxicol. Chem.*, 21 (2002) 2146.
271 H.M. Kuch and K. Ballschmiter, *Fresenius J. Anal. Chem.*, 366 (2000) 392.
272 L.B. Barber, G.K. Brown and S.D. Zaugg, in L.H. Keith, T.L. Jones and L.L. Needham (Eds.), *Analysis of Environmental Endocrine Disruptors*, American Chemical Society, Washington, DC, 2000, p. 97.
273 C. Kelly, *J. Chromatogr. A*, 872 (2000) 309.
274 H.G.J. Mol, S. Sunarto and O.M. Steijger, *J. Chromatogr. A*, 879 (2000) 97.
275 C.-H. Huang and D.L. Sedlak, *Environ. Toxicol. Chem.*, 20 (2001) 133.
276 A.H. Grange and G.W. Sovocool, in L.H. Keith, T.L. Jones and L.L. Needham (Eds.), *Analysis of Environmental Endocrine Disruptors*, American Chemical Society, Washington, DC, 2000, p. 133.
277 B. Halling-Sorensen, S.N. Nielsen, P.F. Lanzky, F. Ingerslev, H.C. Lutzhoft and S.E. Jorgensen, *Chemosphere*, 36 (1998) 357.
278 A.D. Russel and F.R.C. Path, *Am. J. Infect. Control*, 30 (2002) 495.
279 S.E. Jorgensen and B. Halling-Sorensen, *Chemosphere*, 40 (2000) 691.
280 E. Zuccato, D. Calamari, M. Natangelo and R. Fanelli, *Lancet*, 355 (2000) 1789.
281 K.P. Henschel, A. Wenzel, M. Diedrich and A. Fliedner, *Regul. Toxicol. Pharmacol.*, 25 (1997) 220.
282 F.M. Christensen, *Regul. Toxicol. Pharmacol.*, 28 (1998) 212.
283 J. Schneider, *Quím. Anal.*, 13 (1994) S34.
284 J. Tolls, *Environ. Sci. Technol.*, 35 (2001) 3397.
285 T.A. Ternes, *Trends Anal. Chem.*, 20 (2001) 419.
286 D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber and H.T. Bastón, *Environ. Sci. Technol.*, 36 (2002) 1202.
287 F. Sacher, F.T. Lange, H.J. Brauch and I. Blankenhorn, *J. Chromatogr. A*, 938 (2001) 199.
288 R. Hirsch, T. Ternes, K. Haberer and K.L. Kratz, *Sci. Total Environ.*, 225 (1999) 109.

289 R. Hirsch, T.A. Ternes, K. Haberer, A. Mehlich, F. Ballwanz and K.L. Kratz, *J. Chromatogr. A*, 815 (1998) 213.
290 W. Arher, E. Scherwenk and W. Buchberger, *J. Chromatogr. A*, 910 (2001) 69.
291 T.A. Ternes, R. Hirsch, J. Mueller and K. Haberer, *Fresenius J. Anal. Chem.*, 362 (1998) 329.
292 G.G. Rimkus, R. Gatermann and H. Hühnerfuss, *Toxicol. Lett.*, 111 (1999) 5.
293 G.G. Rimkus, *Toxicol. Lett.*, 111 (1999) 37.
294 D. Herren and J.D. Berset, *Chemosphere*, 40 (2000) 565.
295 L.I. Osemwengie and S. Steinberg, *J. Chromatogr. A*, 932 (2001) 107.
296 M. Winkler, J.V. Headley and K.M. Peru, *J. Chromatogr. A*, 903 (2000) 203.
297 C. García-Jares, M. Llompart, M. Polo, C. Salgado, S. Macías and R. Cela, *J. Chromatogr. A*, 963 (2002) 277.
298 S. Scalia, *J. Chromatogr. A*, 870 (2000) 199.
299 A. Chisvert, M.C. Pascual-Martí and A. Salvador, *J. Chromatogr. A*, 921 (2001) 207.
300 A. Chisvert, A. Salvador and M.C. Pascual-Martí, *Anal. Chim. Acta*, 428 (2001) 183.
301 D.A. Lambropoulou, D.L. Giokas, V.A. Sakkas, T.A. Albanis and M.I. Karayannis, *J. Chromatogr. A*, 967 (2002) 243.
302 M. Graovac, M. Todorovic, M.I. Trtanj, M.M. Kopecni and J.J. Comor, *J. Chromatogr. A*, 705 (1995) 313.
303 T. Okamura and Y. Nishikawa, *Anal. Chim. Acta*, 325 (1996) 175.
304 D.C. McAvoy, B. Schatowitz, M. Jacob, A. Hauk and W.S. Eckhoff, *Environ. Toxicol. Chem.*, 21 (2002) 1323.
305 A. Lindrström, I.J. Buerge, T. Poiger, P.A. Bergqvist, M.D. Müller and H.R. Buser, *Environ. Sci. Technol.*, 36 (2002) 2322.
306 W. Kalsch, *Sci. Total Environ.*, 225 (1999) 143.
307 R. Hirsch, T.A. Ternes, A. Lindart, K. Haberer and R.-D. Wilken, *Fresenius J. Anal. Chem.*, 366 (2000) 835.
308 M.J. Baurer and R. Herman, *Sci. Total Environ.*, 208 (1997) 49.
309 D. Berset and R. Etter-Holzer, *J. AOAC Int.*, 84 (2001) 383.
310 W.C. Brumley, M.E. Shafter and P.E. Tillander, *J. AOAC Int.*, 77 (1994) 1230.
311 M. Petrovic and D. Barceló, *Anal. Chem.*, 72 (2000) 4560.
312 H. Jing, Z. Lin, M.G. Ikonomou and F.A.P.C. Gobas, ASMS Conference on Mass Spectrometry, Chicago, 2001, p. 77
313 R. Kumar, *Am. Lab.*, 31 (1999) 32.
314 M. Castillo, M.F. Alpendurada and D. Barceló, *J. Mass Spectrom.*, 32 (1997) 1100.
315 T. Hyötyläinen, K. Grob, M. Biedermann and M.L. Riekkola, *J. High Resolut. Chromatogr.*, 20 (1997) 410.
316 C.A. Staples, P.B. Dorn, G.M. Klecka, S.T. O'Block and L.R. Harris, *Chemosphere*, 36 (1998) 2149.
317 M. del Olmo, A. Gonzalez-Casado, N.A. Navas and J.L. Vilchez, *Anal. Chim. Acta*, 346 (1997) 87.
318 D.A. Markham, D.A. Mcnett, J.H. Birk, G.M. Klecka, M.J. Bartels and C.A. Staples, *Int. J. Environ. Anal. Chem.*, 69 (1998) 83.
319 A. Gonzalez-Casado, N. Navas, M. Del Olmo and J.L. Vilchez, *J. Chromatogr. Sci.*, 36 (1998) 565.
320 P. Spengler, W. Körner and J.W. Merzger, *Environ. Toxicol. Chem.*, 20 (2001) 2133.
321 R.A. Rudel, S.J. Melly, P.W. Geno, G. Sun and J.G. Brody, *Environ. Sci. Technol.*, 32 (1998) 861.
322 H. Fromme, T. Küchler, T. Otto, K. Pilz, J. Müller and A. Wenzel, *Water Res.*, 36 (2002) 1429.
323 T. Reemtsma and M. Jekel, *Water Res.*, 31 (1997) 1035.

324 M. Castillo, D. Barceló, A.S. Pereira and F.R. Aquino Neto, *Trends Anal. Chem.*, 18 (1999) 26.
325 M. Castillo, M.C. Alonso, J. Riu and D. Barceló, *Environ. Sci. Technol.*, 33 (1999) 1300.
326 A. Houben, E. Meulenberg, T. Noij, C. Gronert and P. Stoks, *Anal. Chem. Acta*, 399 (1999) 69.
327 P. Bou Carrasco, R. Escolà, M.P. Marco and J.M. Bayona, *J. Chromatogr. A*, 909 (2001) 61.
328 J. Dallüge, T. Hankemeier, R.J. Vreuls and U.A. Brinkman, *J. Chromatogr. A*, 830 (1999) 377.
329 A. Martin-Esteban, P. Fernandez, D. Stevenson and C. Camara, *Analyst*, 122 (1997) 1113.
330 C. Miege, M. Bouzige, S. Nicol, J. Dugay, V. Pichon and M.C. Hennion, *J. Chromatogr. A*, 859 (1999) 29.
331 S. Perez, I. Ferrer, M.C. Hennion and D. Barceló, *Anal. Chem.*, 70 (1998) 4996.
332 S. Perez and D. Barceló, *Analyst*, 125 (2000) 1273.
333 M. Bouzige, G. Machtalère, P. Legeay, V. Pichon and M.-C. Hennion, *Waste Manage.*, 19 (1999) 171.
334 C. Miège, M. Bouzige, S. Nicol, J. Dugay, V. Pichon and M.C. Hennion, *J. Chromatogr. A*, 859 (1999) 29.
335 S. Ouyang, Y. Xu and Y.H. Chen, *Anal. Chem.*, 70 (1998) 931.
336 E. Millan and J. Pawliszyn, *J. Chromatogr. A*, 873 (2000) 63.
337 J. Gui-bin, Z. Qun-fang, L. Ji-yan and W. Di-Jing, *Environ. Pollut.*, 115 (2001) 81.
338 M. Abalos, J.M. Bayona and J. Pawliszyn, *J. Chromatogr. A*, 873 (2000) 107.
339 Y. He, Y. Wang and H.K. Lee, *J. Chromatogr. A*, 874 (2000) 149.
340 M.A. Stack, G. Fitzgerald, S. O'Connell and K.J. James, *Chemosphere*, 41 (2000) 1821.
341 D.A. Lambropoulou and T.A. Albanis, *J. Chromatogr. A*, 922 (2001) 243.
342 T. Henriksen, B. Svensmark, B. Lindhardt and R.K. Juhler, *Chemosphere*, 44 (2001) 1531.
343 M. Takino, S. Daishima and T. Nakahara, *Analyst*, 126 (2001) 602.
344 J.P. Perez-Trujillo, S. Frias, J.E. Conde and M.A. Rodríguez-Delgado, *J. Chromatogr. A*, 963 (2002) 95.
345 D.A. Lambrooulou, V.A. Sakkas, D.G. Hela and T.A. Albanis, *J. Chromatogr. A*, 963 (2002) 107.
346 Y. Gou and J. Pawliszyn, *Anal. Chem.*, 72 (2000) 2774.
347 W.R. Barrionuevo and F.M. Lanças, *Bull. Environ. Contam. Toxicol.*, 69 (2002) 123.
348 D.A. Lambrooulou, I.K. Konstantinou and T.A. Albanis, *J. Chromatogr. A*, 893 (2000) 143.
349 K. Luks-Betlej, P. Popp, B. Janoszka and H. Paschke, *J. Chromatogr. A*, 938 (2001) 93.
350 Y. Saito, Y. Nakao, M. Imaizumi, Y. Morishima, Y. Kiso and K. Jinno, *Anal. Bioanal. Chem.*, 373 (2002) 81.
351 A. Peñalver, E. Pocurull, F. Borull and R.M. Marcé, *J. Chromatogr. A*, 872 (2000) 191.
352 B. Cancho, F. Ventura and M.T. Galceran, *J. Chromatogr. A*, 943 (2002) 1.
353 R.A. Doong, S.M. Chang and Y.C. Sun, *J. Chromatogr. A*, 879 (2000) 177.
354 Y. Yang, D.J. Miller and S.B. Hawthorne, *J. Chromatogr. A*, 800 (1998) 257.
355 S.D. Huang, C.Y. Ting and C.S. Lin, *J. Chromatogr. A*, 796 (1997) 239.
356 A. Peñalver, E. Pocurull, F. Borull and R.M. Marcé, *J. Chromatogr. A*, 953 (2002) 79.
357 M.N. Sarrion, F.J. Santos and M.T. Galceran, *J. Chromatogr. A*, 947 (2002) 155.
358 M. Moeder, S. Schrader, M. Winkler and P. Popp, *J. Chromatogr. A*, 873 (2000) 95.
359 C. Achten, A. Kolb and W. Püttmann, *Fresenius J. Anal. Chem.*, 371 (2001) 519.
360 J. Dron, R. Garcia and E. Millan, *J. Chromatogr. A*, 963 (2002) 259.
361 D.A. Lambrooulou, V.A. Sacas and T.A. Albanis, *Anal. Chim. Acta*, 468 (2002) 171.
362 A. Peñalver, E. Pocurull, F. Borull and R.M. Marcé, *J. Chromatogr. A*, 964 (2002) 153.

363 A. Diaz, F. Ventura and M.T. Galceran, *Anal. Chem.*, 74 (2002) 3869.
364 A. Diaz, F. Ventura and M.T. Galceran, *J. Chromatogr. A*, 963 (2002) 159.
365 C. Garcia-Jares, M. Llompart, M. Polo, C. Salgado, S. Macias and R. Cela, *J. Chromatogr. A*, 963 (2002) 277.
366 E.R. Bennet and C.D. Metcalfe, *Environ. Toxicol. Chem.*, 19 (2000) 784.
367 S. Valsecchi, S. Polesello and S. Cavalli, *J. Chromatogr. A*, 925 (2001) 297.
368 R.C. Hale, C.L. Smith, P.O. de Fur, E. Harvey, E.O. Bush, M.J. La Guardia and G.G. Vadas, *Environ. Toxicol. Chem.*, 19 (2000) 946.
369 M. Petrovic, S. Lacorte, P. Viana and D. Barceló, *J. Chromatogr. A*, 959 (2002) 15.
370 A.J. Sweetman, *Water Res.*, 28 (1994) 343.
371 V.M. León, E. González-Mazo and A. Gómez-Parra, *J. Chromatogr. A*, 889 (2000) 211.
372 M.L. Trehy, W.E. Gledhill and R.G. Orth, *Anal. Chem.*, 62 (1990) 2581.
373 M. Rabolle and N.H. Spliid, *Chemosphere*, 40 (2000) 715.
374 G. Hamscher, S. Sczesny, H. Höper and H. Nau, *Anal. Chem.*, 74 (2002) 1509.
375 R. Delépée, D. Maume, B. Le Bizec and H. Pouliken, *J. Chromatogr. B*, 748 (2000) 369.
376 T.A. Ternes, H. Andersen, D. Gilberg and M. Bonerz, *Anal. Chem.*, 74 (2002) 4398.
377 H. Fromme, T. Otto and K. Pilz, *Water Res.*, 35 (2001) 121.
378 H. Eun, N. Seike, K. Baba, R. Uegaki, Y. Ishii, M. Kuwahara and M. Ueji, *Organohalogen Compd.*, 55 (2002) 163.
379 R.A. Düring and St. Gäth, *Fresenius J. Anal. Chem.*, 368 (2000) 684.
380 E. Eljarrat, J. Caixach and J. Rivera, *Chemosphere*, 36 (1998) 2359.
381 E. Björklund, S. Bowadt, T. Nilsson and L. Mathiasson, *J. Chromatogr. A*, 836 (1999) 285.
382 H. Bautz, J. Polzer and L. Stieglitz, *J. Chromatogr. A*, 815 (1998) 231.
383 J.W. Choi, S. Hashimoto, N. Suzuki, J. Onodera, H. Ito and M. Morita, *Organohalogen Compd.*, 52 (2001) 53.
384 S. Lacorte, E. Martínez, M. Guillamón, M. Mantilla, P. Viana and D. Barceló, *Organohalogen Compd.*, 59 (2002) 175.
385 K. Hartonen, S. Bowadt, S.B. Hawthorne and M.L. Riekkola, *J. Chromatogr. A*, 774 (1997) 229.
386 C. Crescenzi, G. Di Ascenzo, A. Di Corcia, M. Nazzari, S. Marchese and R. Samperi, *Anal. Chem.*, 71 (1999) 2157.
387 K.A. Barnes, R.J. Frussell, J.R. Startin, M.K. Pegg, S.A. Thorpe and S.L. Rynolds, *Mass Spectrom.*, 11 (1997) 117.
388 X. Lou, D.J. Miller and S.B. Hawthorne, *Anal. Chem.*, 72 (2000) 481.
389 D. Baglio, D. Kotzias and B.R. Larsen, *J. Chromatogr. A*, 854 (1999) 207.
390 E.M. Thurman, I. Ferrer and R. Parry, *J. Chromatogr. A*, 957 (2002) 3.
391 H. Kataoka, S. Narimatsu, H.L. Lord and J. Pawliszyn, *Anal. Chem.*, 71 (1999) 4237.
392 J. Dallüge, M. van Rijn, J. Beens, R.J.J. Vreuls and U.A.Th. Brinkman, *J. Chromatogr. A*, 965 (2002) 207.

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 22

Phytochemical analysis

G. STECHER and G.K. BONN

CONTENTS

22.1 INTRODUCTION

The vast array of natural organic compounds in plants produced by their primary and secondary metabolism can be arranged in six large groups: proteins (Chap. 16), lipids (Chap. 17), carbohydrates (Chap. 18), alkaloids, phenolics, and terpenoids [1]. The first three are covered in other chapters of this book. Alkaloids, phenolics and terpenoids,

which will be dealt with in this chapter, are of special interest on account of their pharmacological activity [2]. The alkaloids are characterized by the presence of a heterocyclic nitrogen in their structure, their basic nature, and some of their physiological effects on man and animals [3,4]. Drugs, such as the analgesic morphine in *Papaver somniferum* and the very toxic aconitin in *Aconitum napellus* are two examples of this heterogeneous class of substances. Phenolics, especially flavonoids, have therapeutic uses, e.g., diuretics in *Betula* sp., *Equisetum arvense*, and *Solidago virgaurea*, vulneraries in *Calendula officinalis* and *Juglans regia*, nostrums against colds in *Sambucus nigra* and *Tilia* sp., secretagogs in *Primula officinalis*, and vasodilators in *Gingko biloba* [2]. Finally, the terpenoids include the carotenoids, like β-carotene (a precursor of vitamin A), and vitamin E [5].

Analogous to proteomics [6], phytomics [7] may be defined as the study of the primary and secondary metabolism of plants. Although drugs from plants are not as strictly regulated as synthetic pharmaceuticals, their analysis is important, not only from the pharmacological point of view, but also in the emerging field of phytomics [8,9]. In view of their complex composition, chromatography occupies a central role in phytochemical analysis. Not only thin-layer-chromatography (TLC) (Chap. 6) but also high-performance liquid chromatography (HPLC) (Chap. 2), gas chromatography (GC) (Chap. 8), and electrochemical techniques, such as capillary electrophoresis (CE) (Chap. 9), and electrochromatography (CEC) (Chap. 7), and combinations with spectroscopic methods (Chap. 10), like mass spectroscopy (MS), nuclear magnetic resonance (NMR), ultraviolet (UV), and infrared (IR) spectroscopy are widely used for the analysis of plant constituents [10–13]. Miniaturized separation systems (Sec. 22.4, Chap. 11) are fast and economic analytical tools commanding increasing interest [14].

22.2 SAMPLE PREPARATION AND PURIFICATION

22.2.1 Extraction

The principal objectives of sample preparation for chromatographic and electrophoretic analysis are dissolution of the analytes in a suitable solvent and removal from the solution of as much interfering material as possible and necessary. Classical techniques of great utility include selective extraction, filtration, precipitation, dialysis, centrifugation, and simple open-column chromatography [15–20]. The most frequently used extraction procedures in phytoanalysis are solid/liquid extraction (SLE) [21–25] with additional stirring or mechanical agitation, forced-flow SLE [13], percolation, solid-phase extraction (SPE) [26–37], liquid/liquid extraction (LLE) [38], supercritical-fluid extraction (SFE) [39–42], Soxhlet extraction [43], microwave extraction [43–45], ultrasonic extraction [45–47], and on-line dialysis [48]. Nowadays, the method of choice for pre-concentration and clean-up of analytical samples is SPE. It offers a large variety of sorbents, based on silica, organic [*e.g.*, polystyrene/divinylbenzene (PS/DVB), ring-opening metathesis polymerization (ROMP)-based polymers, and

(sulfonated) divinylbenzene/*n*-vinylpyrrolidone copolymer] [49], ion-exchange materials, graphitized carbon [50,51], and even immuno-adsorbents [52] for optimizing the extraction of the substances of interest from a complex matrix [5,53]. SPE can be performed either off-line, by use of membrane-extraction disks [54,55] or cartridges, packed with an adsorbent, or the cartridge can be coupled on-line with the chromatographic system [56,57]. Pre-packed cartridges operate on the principle of liquid/solid extraction and may be used to retain either the interfering matrix compounds or the components of interest. Solid-phase extraction lends itself particularly well to automation and is especially helpful when a large number of samples must be routinely purified for chromatographic analysis [15]. Not only the stationary phase, but also the solvent should be tailored to the analytical problem. Low-polarity solvents elute the more lipophilic components, while alcohol-containing solvents produce a larger spectrum of apolar and polar materials. For LLE two immiscible phases, like ethyl acetate/water or hexane/water are used. In solvent partitioning, multiple LLE with an increasing or decreasing hydrophobicity of solvents yields fractions enriched with analytes of varying polarity [58,59]. Solvent partitioning methods remove a large proportion of extraneous material and, especially when used in conjunction with a bioassay, can serve for screening of anti-tumor and anti-HIV agents from plant sources [15,60–62]. Filtration is the easiest and most obvious method of preparing samples for LC separation techniques [15]. In addition to the use of syringe filters or filter paper, samples may be purified by filtering the solution through a short column of silica gel or other suitable packing material [63].

22.2.2 Planar chromatography

Paper and thin-layer chromatography, along with several variants, are generically referred to as planar chromatography (Chap. 6) [64]. These techniques can be used for the qualitative and quantitative determination of almost any kind of analytes. In phytochemical analysis planar chromatography plays a central role, not only for the preparative isolation of analytes [19], but also for the screening of plant extracts [65] and for quality assurance [66]. As detailed by Adamovics [64] and Nyiredy [13], the separation of analytes in planar chromatography is influenced by both the sorbent and the composition of the mobile phase. For paper chromatography, the sorbent used is α-cellulose; for TLC, silica gel is by far the most widely used. Alumina serves as a stationary phase for the separation of fat-soluble vitamins, alkaloids, and antibiotics [64]. High-performance (HP)TLC plates with adsorbent layers of fine particles yield higher separation efficiency. Nyiredy and Glowniak [67] have reviewed the applications of planar chromatography in phytochemical analysis with a flow-chart of procedures for the identification of key compounds in medicinal and aromatic plants. In the same context, they reported the use of specialized TLC techniques, such as stepwise-gradient TLC, originally described by Matysik and Soczewinski [68], and forced-flow planar chromatography (FFPC), in which pressure is used to drive the mobile phase through the stationary phase [69].

22.2.3 Preparative chromatography

Preparative chromatographic techniques for use in plant analysis have been summarized by Hostettmann [15] and by Nyiredy and Glowniak [67]. Therefore, we will restrict ourselves to some illustrative examples in this chapter. Preparative procedures can be divided into several steps, beginning with the extraction, fractionation, isolation, and, finally, structure elucidation. Between these steps identification tests must be performed to monitor the progress of purification. To make up for losses during purification, an excess quantity of plant material must be extracted. For instance, Huck *et al.* [70] described the extraction of flavonones from *Primula veris*, where 12 mg of pure substance was obtained from 11 kg of plant material. Generally, fractionation of extracts and isolation of analytes are first performed by open-tubular low-pressure chromatography, subsequently by medium-pressure chromatography, and finally by HPLC. Not only normal-phase (NP) and reversed-phase (RP) material is used [71,72], but size-exclusion material is also valuable [73]. NP planar chromatography was used by Huck *et al.* [70] for the selective isolation of flavones and by Hajnos *et al.* [74] for the isolation of taxoids. In both cases PC was found to be most useful for the final purification step, yielding material of high quality for pharmacological investigations. The structure of isolated compounds needs to be elucidated by means of UV/vis spectroscopy, IR, MS, ^{1}H-NMR, and ^{13}C-NMR, and the identity must be validated by the analytical data of a synthesized sample.

22.3 EXAMPLES OF ANALYSES

22.3.1 Terpenoids

Terpenoids comprise the largest group of natural plant products, and over twenty thousand such structures from plant sources have been described [1,75]. Structures can be classified into mono-, sesqui-, di-, tri-, tetra-, and polyterpenes according to their constituent isoprene (2-methylbutadiene) units [76]. In addition, there are numerous other secondary metabolites of mixed biosynthetic origin which either contain terpenoid substituents or which are largely of isoprenoid origin. The terpenoid literature is very extensive. Their chemistry is regularly reviewed by the journal *Natural Product Reports*, the biochemistry and function of plant terpenoids by the Phytochemical Society of Europe [77], and the methodology by Charlwood and Banthrope [76].

22.3.1.1 Monoterpenes

Monoterpenoids are the simplest class of isoprenoids with a characteristic C_{10} structure, arising, at least formally, from the head to tail linkage of two biogenetic isoprene units. More than 1000 naturally occurring monoterpenoids are presently known, most of which have been isolated from higher plants, but also many from marine organisms, insects, and even from some vertebrate animals. These natural products are of interest, aside from their utility as perfumes and food flavors, in the pharmaceutical industry as anti-bacterial, anti-fungal and anti-cancer agents [76,78]. Based on the work of Dev *et al.* [79],

Charlwood and Banthorpe [76] have presented a classification of monoterpenes according to 38 different skeletal types. The combination of such biodiversity together with optical isomerization (+ and −) of monoterpenes and glycoside derivatives make qualitative and quantitative analysis a challenge. New compounds are discovered and described daily [80].

Analysis of monoterpenoids involves in most cases the extraction and purification of substances of interest from plants. For this purpose, different techniques like steam distillation, solvent extraction, and supercritical-fluid extraction can be employed [81]. In special cases, gas chromatography allows direct analysis *via* headspace sampling or direct sampling from secretory structures. For steam distillation, extraction time, temperature, and pH must be optimized to avoid isomerization, polymerization, saponification, hydrolysis, and rearrangements of monoterpenes [76]. Extraction of 15 to 3 g fresh plant material may yield 1 mL to 100 μL of essential oil, respectively [82–84]. Artifact formation may be reduced by solvent extraction of the essential oil directly from the plant material. The exact choice of extraction solvent depends on the nature of the analyte and on the chromatographic and analytical tool that will be subsequently employed. The main disadvantage is that non-volatile lipids and phenolics may also be extracted together with the monoterpenoids, and these interfering compounds can cause difficulties during subsequent analysis by gas chromatography [85]. Monoterpenoid glycosides are only sparingly soluble in organic solvent and must be extracted with water, ethanol, or methanol. The extracted glycosides may be separated by TLC, open-column chromatography, HPLC, or countercurrent distribution [76].

The extraction of essential oils can be accomplished with supercritical carbon dioxide without the disadvantages associated with normal solvent extraction or distillation [86–89]. This method finds application in the commercial preparation or downstream processing, *e.g.*, the selective removal of toxic thujone from alcoholic preparations of wormwood (*Artemisia absinthium*) [76,90]. Direct coupling to GC has also been reported [91]. Nowadays, essential oils produced by tropical plants are investigated by headspace analysis directly, as they are emitted from the plant organ [80]. The headspace technique offers a number of advantages, such as simple and rapid sample pretreatment and stable composition of the volatile fraction. For the subsequent GC analysis two different techniques can be used: equilibrium-headspace GC, where plant material is ground up for a limited time in a blender, equipped with a gas-tight valve; and dynamic-headspace GC, where volatiles are continuously stripped from the plant sample in a stream of inert gas, allowing the preconcentration on an adsorbent [76].

For the chromatographic separation of monoterpenes, TLC, HPLC, and GC are used. TLC has been largely superseded by GC, but its use for the purification of single components and subsequent spectroscopic analysis and for small-scale pre-fractionation of samples prior to GC has been reported [76]. TLC is also used for routine analyses, such as quality control of plant material used in drug preparation. The use of newer techniques, such as rotation planar chromatography and over-pressured layer chromatography (Chap. 6) is described in conjunction with the preparative isolation of analytes. Column chromatography, especially HPLC, in most cases does not yield sufficient resolution of the components of complex essential oils [92,93], but is used for the preparative pre-fractionation of samples, allowing combination with HR-GC, as shown in several

publications [94–96]. HPLC is an alternative for the separation of monoterpene derivatives, *e.g.*, monoterpenoid indol alkaloids [97], phosphorylated terpenes [98], and 2,4-dinitrophenylhydrazine terpenes [99]. The separation of enantiomers by complexation with α-cyclodextrins in RP-HPLC has been reported [100–103]. Supercritical-fluid chromatography was touted in 1991 as a technique with much potential for essential oil analysis [76], but only few papers concerning this topic can be found [104]. Gas chromatography is the most widely used technique for the analysis of monoterpenoids in essential oils [76], owing to many advantages offered by this method, such as the extremely high resolving power, the facile on-line attachment to a variety of spectroscopic instruments, and the reliability of the results. Fig. 22.1 shows an example of the potential of GC in separating 127 components of essential oils in 50 min. Packed and coated columns can be used for separation, as summarized by Charlwood and Banthorpe [76]: wall-coated open tubular (WCOT), in which the liquid stationary phase is directly deposited on the glass surface; whiskered wall-coated open-tubular (WWCOT), in which the stationary phase is supported on an HF-etched layer on the inner wall; support-coated open-tubular (SCOT), and porous-layer open-tubular (PLOT), in which the stationary phase is held on a neutral porous support within the capillary. Packed columns, which have rather restricted theoretical plate numbers, rely on a large variety of stationary phases to provide the selectivity required for adequate separation of complex samples. For routine essential oil analysis, the apolar silicon phases such as DB-1 (100% dimethylpolysiloxane; similar phases: HP-1, HP-101, Ultra-1, SPB-1, CP-Sil 5 CB Low Bleed/MS, Rtx-1, BP-1, OV-1, O07-1(MS), SP-2100, SE-30, CP-Sil 5 CB MS, ZB-1, AT-1, MDN-1) [105–107] and DB-5 (5%-Phenyl-methylpolysiloxane; similar phases: HP-5-MS; HP-5; Ultra-2, SPB-5, CP-Sil 8 CB, Rtx-5, BP-5, OV-5, 0007-2(MPS-5), SE-52, SE-54, XTI-5, PTE-5, ZB-5, AT-5, MDN-5) [108–110] are recommended. For analyzing more polar analytes, high-molecular-weight polyethylene glycols, such as Superoxes or Carbowaxes and their nitroterephthalic esters, must be used as stationary phases [84,107].

The first experiments with comprehensive two-dimensional gas chromatography (Chap. 8) were published in 1991 by Liu and Phillips [111,112]. GC × GC offers the opportunity to combine different selectivities in gas chromatography, *i.e.* the entire sample is subjected to analysis in two separation dimensions, and thus, it is potentially many times more powerful than other multi-dimensional gas-chromatographic techniques. The widely reported advantages of superior resolution [113] and improved sensitivity [114] and the unique opportunity to generate structured retentions, allowing facile identification and interpretations of the 2D separation [115] are acknowledged [116].

22.3.1.2 Sesquiterpenes

Sesquiterpenes occur in higher plants, liverworts and mosses, fungi, and algae. They have also been isolated from arthropods, marine invertebrates, and in a few cases from bacteria [76]. Biological properties of this class of substances range from the antifeedant sesquiterpenoids to the insect juvenile hormones and insect pheromones, phytoalexins, plant growth hormones, toxins, and antibiotics. The main sesquiterpenoid mycotoxins are the trichothecenes [117–123], which may serve as an example of sample preparation.

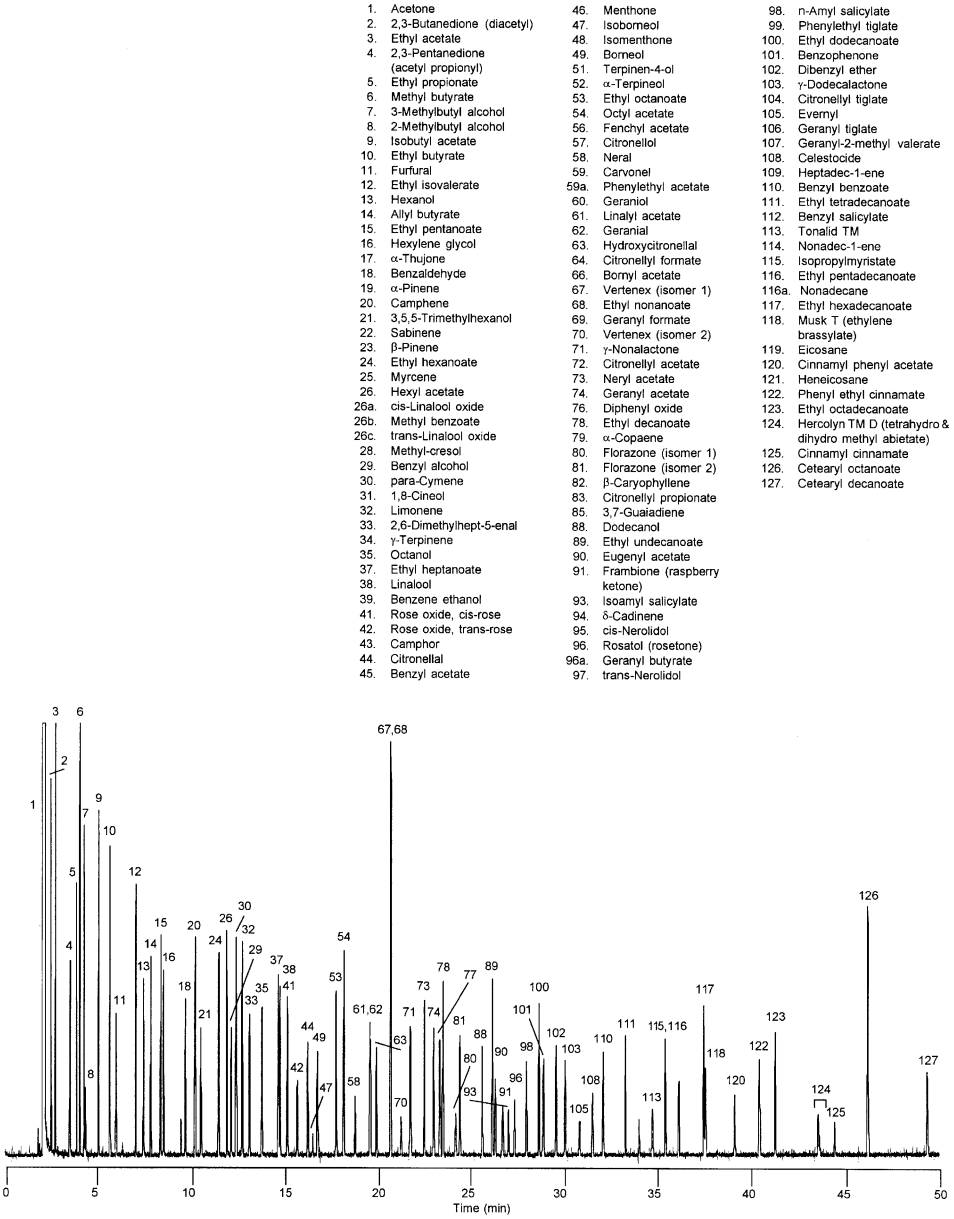


Fig. 22.1. Separation of 106 compounds in essential oils by gas chromatography. Column, DB-1 (30 m × 0.25 mm I.D., 0.25 μm); carrier, He at 25 cm/sec (measured at 150°C); oven, 40°C for 1 min, 40–290°C at 5°/min; injector, split 1:50, 250°C, 1 μL of a 1:20 dilution of neat sample in acetone; detector, MSD, 300°C transfer line. (Courtesy of Agilent Technologies.)

Since they vary in polarity, a compromise is necessary for optimal clean-up. Solid-phase extraction (SPE) with combined polar and non-polar materials proved to be selective and time-saving, enabling almost complete removal of interfering materials [119–137], next to liquid/liquid partitioning, immuno-affinity chromatography, and size-exclusion chromatography [31,118,138–145]. Jarukamjorn *et al.* [146] investigated the effect of different extraction procedures on the determination of trichothecenes. Six purification procedures were evaluated (Table 22.1): (a) solid-phase extraction (SPE) with Mycosep 227 clean-up columns, (b) SPE with C-8 cartridges, (c) SPE with C-18 cartridges, (d) SPE with OASIS™ HLB cartridges, (e) liquid/liquid extraction (LLE) with ethyl acetate, and (f) LLE with diethyl ether for B- and macrocyclic trichothecenes. The recovery of type B trichothecenes by the Mycosep clean-up method ranged from 60.08 to 94.52%, whereas its effectiveness for macrocyclic trichothecenes was very low. The recovery of nivalenol by the Mycosep method could not be improved, owing to its extremely high polarity. The effectiveness of all SPE cartridges was high for macrocyclic trichothecenes, while only OASIS™ HLB provided superior recovery for B-trichothecenes. As the recovery for B-trichothecenes was enhanced from C-18 to C-8, the purification by C-4 cartridge, which is less hydrophobic than C-8 and C-18 cartridges, was additionally examined. However, the recovery was inferior for both B- and macrocyclic trichothecenes (data not shown). Both liquid/liquid partition methods, with either ethyl acetate or diethyl ether, gave varying recoveries for B- and macrocyclic trichothecenes with high CV values for three replicates (Table 22.1).

A variety of chromatographic methods for the determination of trichothecenes have been published by TLC [147–151], by high-resolution gas chromatography (HR-GC) [152–157] with electron-capture (ECD) [31,118,119,123,125,126,133,135,141] or mass-spectrometric (MS) detection [119,128,129,135,136,158], and by HPLC with UV [119, 131,134,139,141,144,145], fluorescence [31,119,125,126,132,137,140], MS or tandem mass-spectrometric (MS/MS) [119–121,128,135,136,139,142,143] detection. Other techniques, such as SFC/MS and enzyme-linked immunosorbent assay (ELISA) were also used [138,159–161]. In TLC and GC methods, derivatization is required for separation or identification. In particular, owing to the lack of a UV chromophore in type A trichothecenes, derivatization enables their determination by HPLC/FLD. The use of MS allows characterization and identification of trichothecenes as well as time saving in sample preparation with an enhanced sensitivity of the analysis [146,162].

22.3.1.3 Diterpenes

Diterpenes are a large and ubiquitous family of isoprenoids, derived from 2*E*, 6*E*, 10*E*-geranylgeranyl pyrophosphate [76]. They are classified according to their structure as acyclic diterpenoids, bicyclic, tricyclic, tetracyclic (including gibberellins) terpenoids, macrocyclic, and miscellaneous diterpenoids. In this chapter two examples of their extraction and chromatographic separation will be given, *i.e.* the macrocyclic diterpene, taxol, and the tetracyclic diterpenoid acids, gibberellins.

Taxol has attracted attention as a cytotoxic and anti-leukemic constituent of *Taxus baccata*. The fact that *Taxus* trees are quite rare and taxol occurs in the bark of the plant in

TABLE 22.1

EFFECTIVENESS OF THE CLEAN-UP PROCEDURES FOR WHEAT SAMPLE SPIKED WITH 2 PPM OF TYPE B AND MACROCYCLIC TRICHOTHECENES DETERMINED BY HPLC/UV

Trichothecenes		Percent recovery*					
Types		Solid-phase extraction				Liquid/liquid extraction	
		Mycosep 227	Supelclean LC-18	Isolute C-8	OASIS™ HLB	Ethyl acetate	Diethyl ether
B	Nivalenol	60.08 ± 2.72	ND**	47.87 ± 2.69	32.52 ± 0.68	14.95 ± 1.32	5.33 ± 0.57
B	Deoxynivalenol	93.84 ± 1.37	ND**	46.45 ± 3.29	85.81 ± 5.42	88.01 ± 8.20	41.94 ± 4.42
B	Fusarenone-X	94.52 ± 1.39	ND**	64.53 ± 3.21	81.90 ± 4.30	106.39 ± 9.53	64.07 ± 4.38
Macrocyclic	Verrucarin-A	1.24 ± 0.12	87.95 ± 1.48	89.97 ± 0.95	97.35 ± 0.96	134.15 ± 9.04	127.55 ± 10.05
Macrocyclic	Roridin-A	8.77 ± 0.83	90.36 ± 0.92	88.56 ± 0.95	97.76 ± 1.28	119.50 ± 5.08	139.79 ± 7.02

* Results are expressed as mean ± coefficient of variation [%] (n = 3).
** Compounds not adequately separated from the matrix; recovery cannot be determined.

very low concentrations makes extraction and analysis challenging. Although the total and partial synthesis as well as tissue culture of taxol have been reported, the yew bark and leaves are still the best sources of taxol and related compounds, especially cephalomannine and 7-epi-taxol, which are difficult to separate from taxol. Crude extracts of different yew species are rich in lipophilic and highly polar components. Because the complicated LLE procedure for the purification of yew extracts [163] suffers from some practical problems, including emulsion formation, SPE on octadecylsilane (C-18) cartridges and TLC are preferable alternatives [37,164–170]. A comparison between these two alternatives was published by Glowniak *et al.* in 1996 and is reproduced in Table 22.2 [37]. Bioassays have also been reported, but they were prone to cross-reactivity

TABLE 22.2

QUANTITATIVE ANALYSIS (MEAN ± S.D.; n = 5) OF TAXOL (t) AND CEPHALOMANNINE (c) IN *TAXUS* spp. (μg/g OF DRY WT.) BY SPE/HPLC AND TLC/HPLC; REPRODUCED FROM REF. 37 WITH PERMISSION

No.	Specimen	SPE/HPLC		TLC/HPLC	
		t	c	t	c
1	*T. baccata* needles	25.1 ± 1.9	13.9 ± 0.6	194 ± 0.9	10.2 ± 0.6
2	*T. baccata* twigs	16.0 ± 1.9	4.0 ± 0.5	18.7 ± 0.9	5.5 ± 1.6
3	*T. baccata var. elegant.* needles	29.9 ± 1.6	27.1 ± 2.3	24.4 ± 2.5	20.0 ± 1.9
4	*T. baccata var. elegant.* twigs	8.6 ± 1.2	3.9 ± 0.8	6.3 ± 1.0	3.5 ± 1.3
5	*T. media var. Hicksii* needles	65.8 ± 1.8	47.0 ± 1.8	54.0 ± 1.7	40.3 ± 1.5
6	*T. media var. Hicksii* twigs	23.6 ± 0.8	22.0 ± 1.8	18.3 ± 1.9	16.2 ± 1.4
7	*T. media var. Hatfieldii* needles	12.8 ± 0.9	4.3 ± 0.6	13.0 ± 0.8	4.8 ± 0.8
8	*T. media var. Hatfieldii* twigs	20.1 ± 1.7	4.5 ± 0.5	21.1 ± 0.6	5.6 ± 1.0
9	*T. cuspidata* needles	181.0 ± 9.8	30.9 ± 2.8	128.4 ± 12.6	28.6 ± 9.8
10	*T. cuspidata* twigs	3.6 ± 0.9	1.9 ± 0.4	2.7 ± 0.5	2.4 ± 1.0

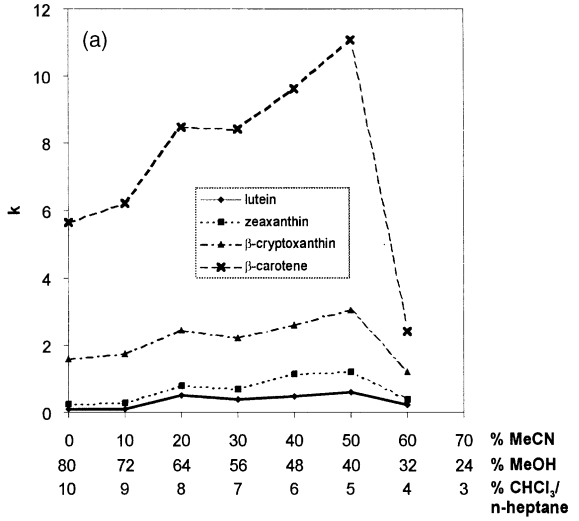
against paclitaxel analogs and especially cephalomannine [171–176]. ELISA and related methods have thus preferentially been used for the semi-quantitative determination of paclitaxel and 10 DAB III in biological fluids and plant extracts, enabling the rapid and sensitive screening for taxanes in various samples [29,173–175]. The use of immunoaffinity chromatography for the purification of *Taxus* plant and cell extracts prior to HPLC has been reported [29]. Separation and determination methods for taxoids include HPLC on different stationary phases, such as pentafluorophenyl packing material [177], octadecylsilane [37,178,179], octylsilane [22,180], and cyano- and phenyl-bonded silica [181] with UV or MS detection [182–184]. Special stationary phases for the determination of taxol-like compounds have also been developed [170,185].

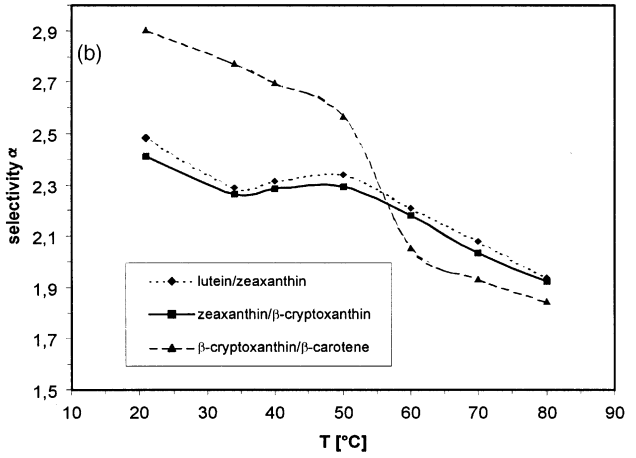
Gibberellins are a group of compounds essential for normal growth and development of plants. They are produced in minute quantities by the plants, where they act as regulators or hormones of various developmental processes, such as stem elongation, bolting, fruit

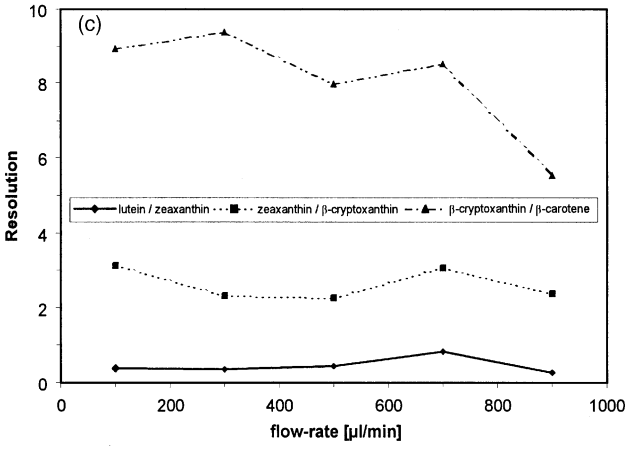
and seed development, ending dormancy, and seed germination [76]. Almost all of the methods that have been developed for natural-product isolation have been applied to the gibberellins at one time or another [186–188]. As all gibberellins contain at least one carboxylic acid group, the partition of extracts into acidic and non-acidic fractions is usually the first step in any purification process. Attention must be paid to the pH, as pH extremes can cause rearrangement and degradation of some gibberellins. In mineral acids, the exo-16,17 double bond of gibberellins rearranges to the endo-15,16 position [188]. Wagner-Meerwein rearrangement, reversible retro-aldol reaction, and lactone formation are possible reactions described in the literature. Thus, it is advisable to keep the pH of aqueous solutions of gibberellins between 2.5 and 8.5 during extraction. For the chromatographic determination, procedure chosen depends upon the size and type of the extract in question. For small-scale work, HPLC on a reversed-phase (C_{18}) column, followed by GC/MS of individual fractions, is often adequate for the identification of the gibberellins present. For larger-scale extracts further group separations may be necessary.

22.3.1.4 Triterpenes

The triterpenes constitute a large and diverse group of natural products, biosynthetically derived from squalene. More than 4000 triterpenes have been isolated so far, and more than 40 skeletal types have been identified [189,190]. They may be classified into acyclic and cyclic compounds [191]. Further classification may be based on the occurrence of sugar side-chains as it is in the cardenolides and saponins. The techniques for the isolation of free triterpenoids from plant material involve mainly solvent extraction in combination with flash chromatography or column chromatography on silica gel or alumina. For the final purification, preparative TLC or HPLC are frequently used. Special purification steps may be required, *e.g.*, hydrolysis of saponins to sapogenins by the use of acids or enzymes, crystallization of certain triterpenoids [190], or silver-ion chromatography for certain sterols [192]. The formation of a persistent foam during extraction and concentration of a plant extract is a reliable indication that saponins are present. During the extraction of saponins, care should be taken to monitor the individual steps by TLC, because they can undergo enzymatic hydrolysis during water extraction [193,194], esterification of acidic saponins during alcohol treatment, hydrolysis of labile ester groups, or transacylation. Before 1991, most separations or at least preliminary fractionations reported in the literature were still carried out by conventional open-column chromatography [193], although modern techniques, such as centrifugal TLC, flash chromatography, medium-pressure LC, and droplet counter-current chromatography (DCCC) are of special importance in this group. Several papers concerning these new techniques have been published since then [195–199]. The separation, purification, and assay of triterpenes by TLC, HPLC, GC and GC/MS have been reviewed [200]. Special tools for the qualitative determination of saponins depend on their physical and pharmacological properties: The ability of saponins to rupture erythrocyte membranes has been used for decades as a detection and quantitation method [193]. The separation of cardenolides can be facilitated by acetylation of crude extracts. For the small amounts of material in insects, HPLC, medium-pressure and RP flash chromatography, and DCCC


(a)
k
lutein
zeaxanthin
β-cryptoxanthin
β-carotene
% MeCN
% MeOH
% CHCl3/ n-heptane


(b)
selectivity α
lutein/zeaxanthin
zeaxanthin/β-cryptoxanthin
β-cryptoxanthin/β-carotene
T [°C]


(c)
Resolution
lutein / zeaxanthin
zeaxanthin / β-cryptoxanthin
β-cryptoxanthin / β-carotene
flow-rate [µl/min]

have been employed. Identification of the sugar residues relies mainly on ^{1}H-NMR spectroscopy. Acid hydrolysis of the glycoside, followed by silylation and GC analysis of the derivatized monosaccharides, has also been used effectively [190,201].

22.3.1.5 Tetraterpenes

Tetraterpenes are biosynthesized from two molecules of the C_{20} intermediate geranylgeranyl diphosphate. Carotenoid hydrocarbons are collectively called carotenes, and derivatives containing oxygen functions are called xanthophylls. Carotenoids are extensively used as natural, nontoxic colorants in food, beverages, confectionery, cosmetics, and animal feeds. Generally, many of the procedures used for the extraction, purification and analysis of carotenoids are similar to those used for other classes of isoprenoid compounds, but special care is needed, as the conjugated polyene chromophore of carotenoids renders them sensitive to oxygen, light, and heat. Therefore, extractions should be performed as rapidly as possible after the material is obtained, in order to minimize oxidative or enzymatic degradation. The extraction, purification, and separation of carotenoids have been reviewed by Britton [202]. For the separation on C_{18} stationary phases, a number of variables have been tested, such as column length, particle size, and endcapping of free silanol groups, as well as the composition of the mobile phase, column temperature, and flow-rate [32,203–206]. Fig. 22.2a shows the dependence of the capacity factor on the mobile-phase composition, Fig. 22.2b the influence of temperature on the selectivity factors of the mobile phase, and Fig. 22.2c the connection between resolution and flow-rate. As can be deduced from Fig. 22.2a, the best separation of the analytes was achieved with 50% MeCN/40% MeOH/5% chloroform/5% *n*-heptane. The best selectivity was obtained at 21°C (Fig. 22.2b) with diminishing values at increasing temperature [207]. The optimized mobile-phase system was used to evaluate different RP-HPLC columns for the separation of the standard carotenoid mixture. The best capacity factors were obtained with a Phenomenex RP Si C_{18} column (5 μm, 100 Å, 250 × 2 mm ID).

22.3.1.6 Polyterpenes

Hydrocarbons of high molecular mass, built up of isoprene units, are widely distributed in the plant kingdom. X-ray diffraction studies show that the isoprene units are in the *cis*-configuration in natural rubber and in the *trans*-configuration in gutta percha, which occurs

---

Fig. 22.2. Development and evaluation of a method for the determination of selected carotenoids. (a) Dependence of capacity factor (k) on mobile phase composition. Column, Phenomenex Luna C_{18} (250 mm × 2 mm, 5 μm, 100 Å); mobile phase, MeCN (0.1% butylated hydroxytoluene (BHT))/MeOH (0.05 *M* NH_4OAc, 0.05% triethylamine)/$CHCl_3$ (0.1% BHT)/*n*-heptane (0.1% BHT); flow-rate, 0.3 mL/min; ambient temperature; detection, 450 nm; sample volume, 20 μL. (b) Dependence of selectivity (α) on column temperature. For conditions see Fig. 22.2a. (c) Dependence of resolution between standard carotenoids on flow-rate. For conditions see Fig. 22.2a. (Reproduced from Ref. 7 with permission.)

as a resin at ordinary temperatures but as a rubber at high temperatures [208]. Information about structure, extraction and purification can be found in the reviews of Tanaka [208] and Britton [202].

22.3.2 Phenolic compounds

Phenolic compounds or polyphenols are plant substances with an aromatic ring, bearing one or more hydroxyl groups. There are about eight thousand naturally occurring compounds and about half of them are flavonoids [209]. Harborne *et al.* [209] classify phenolics into several groups according to structural complexity and biosynthetic origin: The simpler classes are the phenols themselves, phenolic acids, and phenolic ketones. The phenylpropanoids, based on a C_6-C_3 nucleus, are a large group, and there are various derived phenylpropanoids, including the coumarins, chromones and chromenes, the benzofurans and the dimeric lignans. Three further classes of phenolics considered here are the xanthones (with a $C_6-C_1-C_6$ skeleton), the stilbenoids (with a $C_6-C_2-C_6$ skeleton), and the quinines. The last-mentioned are pigments with a quinine nucleus, which are further subdivided into benzoquinones, naphthoquinones, and anthraquinones. This review will focus on flavonoids, phenylpropanoids, stilbenes, and chromane-isoprene derivatives. Several monographs on methodology and biochemistry and a dictionary are available for this field [209–213].

22.3.2.1 Flavonoids

The name flavonoid is derived from the yellow color of the first identified compounds, *i.e.* quercetin from *Quercus tinctoria*, luteolin from *Reseda luteola*, and morin from *Morus tinctoria*, three plants used for dyeing wool and cotton [2]. Being phenolics, flavonoids change color when treated with base, and this readily enables detection on thin-layer chromatograms and in solution. The conjugated aromatic system of flavonoids produces analytically useful absorption in the UV and visible regions of the spectrum (Table 22.3) [214].

Most flavonoids are water-soluble and can easily be extracted with alcoholic solutions. A general procedure for surveying plant tissue for flavonoids has been described by Harborne [214]: After hydrolysis of a small amount of plant tissues in 2 *M* HCl at 100°C for 30–40 min, the cooled extract is filtered and extracted with ethyl acetate. If the aqueous solution is colored, it is further heated to remove traces of ethyl acetate and then extracted with a small volume of amyl alcohol. The ethyl acetate phase is concentrated to dryness, taken up in 1–2 drops of ethanol, and aliquots are subjected to TLC in one dimension, alongside authentic markers, in Forestal solvent (acetic acid/conc. HCl/water, 30:3:10). The result of this chromatogram is supplemented by chromatograms in 50% aq. acetic acid, BAW (*n*-butanol/acetic acid/water, 4:1:5), water-saturated phenol, and water. The amyl alcohol extract, which should be colored, is concentrated to dryness, taken up in a few drops of 1% methanolic HCl, and aliquots are chromatographed in two dimensions: (1) Forestal solvent and (2) formic acid/conc. HCl/water, 5:2:3). Fig. 22.3 shows a thin-layer chromatogram of the common flavonoid aglycones of plants. Two-dimensional TLC

TABLE 22.3

SPECTRAL CHARACTERISTICS OF FLAVONOID PIGMENTS; REPRODUCED FROM REF. 214 WITH PERMISSION

Principal maxima [nm]	Subsidiary maxima [nm] (with relative intensities)	Flavonoid class
475–560	*ca.* 275 (55%)	Anthocyanins
390–430	240–270 (32%)	Aurones
365–390	240–260 (30%)	Chalcones
350–390 250–270	*ca.* 300 (40%)	Flavonols
330–350 250–270	Absent	Flavones and biflavonyls
275–290 *ca.* 225	310–330 (30%)	Flavanones and flavanonols
255–265	310–330 (25%)	Isoflavones

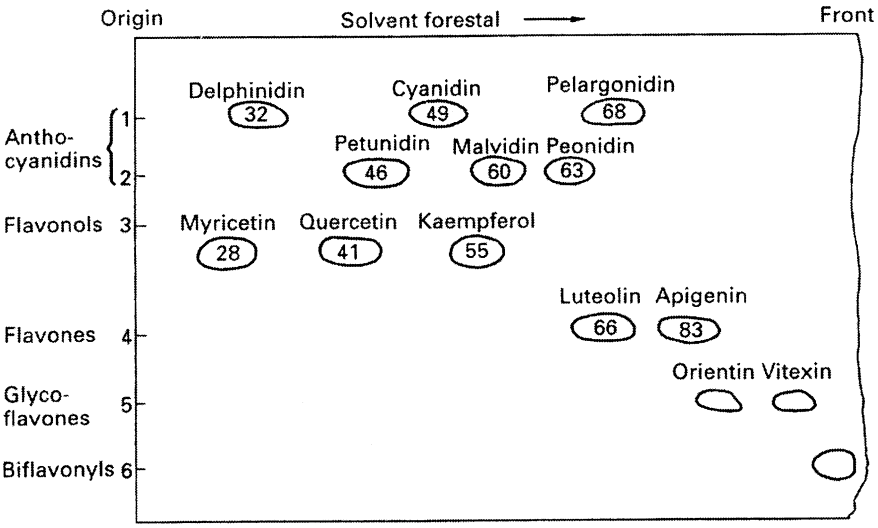


Fig. 22.3. Thin-layer chromatogram of the common flavonoid aglycones of plants. Forestal solvent; color key: 1 (see left axis), mauve (spot 32), red (49) and orange (68), respectively, in visible light; 2, mauve (46), mauve (60) and red (63) in visible light; 3 (spots 28, 41, 55), bright yellow in UV light; 4, 5 (spots 66, 83, 2 spots without number), dull-brown in UV light changing to bright yellow-green with NH_4OH; 6 (spot without number), dull-brown unchanged by NH_4OH. (Reproduced from Ref. 214 with permission.)

with BAW and 5% acetic acid is also used for routine screening of plant extracts. Some spots are detected directly in visible light and most of them in UV light with or without additional exposure of the chromatogram to ammonia. After the classification of samples into the several subdivisions of flavonoids, further investigations, such as separation, isolation and structural identification can be performed [215–225].

22.3.2.2 Phenylpropanes

Phenylpropanoids are naturally occurring compounds with an aromatic ring to which a three-carbon side-chain is attached. They include the coumarins, phenylpropenes, and lignans. The most commonly occurring phenylpropanoids are hydroxycinnamic acids, such as ferulic, sinapic, caffeic and *p*-coumaric acids. Hydroxycinnamic acids are usually extracted with ether or ethyl acetate after acid or alkaline hydrolysis of the plant extract. After washing, drying, and re-dissolving the extract in an appropriate solvent, they can be chromatographed one-dimensionally on paper or two-dimensionally on plates of microcrystalline cellulose. Hydroxycinnamic acids, which fluoresce (Table 22.4), can be detected in UV light. Treatment with ammonia vapor intensifies the fluorescence. UV and fluorescence detection facilitate the analysis of phenylpropanes by HPLC. Gas chromatography is used in combination with mass spectrometry (MS) [226–230]. The separation of phenylpropanes by HPLC on a column of Lichrospher 100 RP18 with a water/methanol gradient system has been described [231,232].

22.3.2.3 Stilbenes

Stilbenes are biogenetically related to the chalcones, but have one less carbon atom in their basic skeleton, which is $C_6–C_2–C_6$ [233]. The best-known stilbene is now resveratrol, an antioxidant occurring in red wine in free, oligomeric, and glycosidic form. It is regarded as a medicinally useful for prevention of coronary disease and cancer. Since the first report on the presence of resveratrol in red wine in 1992 [234], the quantification of phenolics, especially resveratrol has been performed by various analytical methods. Organic solvent extraction [234–239], SPE [236,240–245], and direct injection [242,244, 246] have been used in the analysis of stilbenes by GC [237,242,245,247–252], SFC [253], HPLC [235,239–243,246,254–258], and CE [259,260]. Most of the GC methods require the conversion of resveratrol to its volatile silyl ether, which is detected with FID [237,247,248] or MS [242,245,249,251]. A number of RP-HPLC methods with UV-absorbance [73,235,239,242,243,246,256], electrochemical [257,261], and fluorometric detection [257,262] have been described. The use of MS for the identification of resveratrol and its isomers in wine has also been reported [240,241,254,255,263].

22.3.2.4 Flavonoids and stilbenes in human body fluids

Polyphenolic compounds, like flavonoids and stilbenes occur in low concentrations in human body fluids, like blood and urine. Before questions about bioavailability can be addressed, ultrasensitive methods are necessary to assay the low concentrations of

TABLE 22.4

R_F, COLOR AND SPECTRAL DATA FOR HYDROXYCINNAMIC ACIDS, REPRINTED FROM REF. 214 WITH PERMISSION

Cinnamic acid	R_F ($\times 100$) in*				Color		EtOH λ_{max}	EtOH–NaOH λ_{max}
	BAW	BN	BEW	Water	UV	UV + ammonia		
p-Coumaric	92	16	88	42, 85	None	Mauve	227, 310	335
Caffeic	79	04	79	26, 69	Blue	Light blue	243, 326	Decomposition
Ferulic	88	12	82	33, 75	Blue	Bright blue	235, 324	344
Sinapic	84	04	88	62	Blue	Blue-green	239, 325	350
o-Coumaric	93	21	85	82	Yellow	Yellow-green	227, 275, 325	390
p-Methoxycinnamic	95	17	87	23	Dark absorbing		274, 310	298
Isoferulic	89	12	67	37	Mauve	Yellow	295, 323	345
3,4,5-Trimethoxycinnamic	95	18	87	75	Mauve	Dark	232, 303	293

* *Key*: BAW = *n*-BuOH–HOAc–H_2O (4:1:5, top layer) BN = *n*-BuOH–2 mNH_4OH (1:1, top layer) BEW = *n*-BuOH–ethanol–water (4:1:2.2).

polyphenols. Approximately 30 years ago, the absorption and urinary excretion of catechin in human subjects was indirectly demonstrated by measurement of total phenols and tracer studies with [^{14}C] catechin; newer publications have concentrated on the analysis of quercetin in blood and urine [264]. Hollman *et al.* [265–267] and Stumpf *et al.* [268] demonstrated that after oral administration of quercetin, quercetin glucoside, and quercetin rutinoside, these compounds could be detected in the blood, where most of the quercetin is adsorbed on serum albumin [269].

Bertelli and colleagues [270–273] were the first to demonstrate the occurrence of *trans*-resveratrol in rat blood and its excretion in the urine. Kuhnle and coworkers [274] showed that most of resveratrol is coupled to glucuronic acid during the transfer through the biomembrane. Table 22.5 gives an overview of extraction, separation and detection methods for stilbenes and flavonoids in blood and urine. Most procedures employ SPE on silica C-18 cartridges and LLE with ethyl acetate. Chromatographic methods include HPLC and GC.

22.3.3 Tocopherols

The vitamin E group includes the α-, β-, γ-, and δ-tocopherols, trienols, and the α-tocopheryl acetate and nicotinate [275]. Several analytical techniques have been applied to their qualitative and quantitative analysis: TLC [276], GC [277], NP- [278,279] and RP-HPLC [280–283] with UV and MS [284–290]. In this section we present a comparison between NP- and RP-HPLC systems, discuss the influence of the stationary phase on the separation of the vitamin E analytes, and show examples of the HPLC/MS combination. Some of the disadvantages of NP-HPLC are the need for solvents as mobile phases, long equilibration times, and the often unstable pump back-pressure caused by differences in compressibility between these liquids. Nevertheless, the use of NP-HPLC in the determination of vitamin E is justified by the fact that tocopherols differ only by the substituents in the chromanol ring, while their terpenoid side-chain is the same. In particular, β- and γ-tocopherol differ only in the position of one methyl group, and that makes their separation critical. Using silica as the stationary phase, different mobile phases, such as *n*-hexane with diisopropylether, isooctane, and 1,4-dioxane were tested. *n*-Hexane/diisopropylether caused unstable pump back-pressure, but isooctane and 1,4-dioxane gave satisfactory results. In changing from the analytical column (4.6 mm ID) to a narrow-bore column (2 mm ID) diisopropylether was added because of its higher selectivity. Fig. 22.4 illustrates the determination of tocopherols by NP- (Fig. 22.4a) and RP- (Fig. 22.4b) HPLC, MS with APCI being used as detection method. Fig. 22.4b shows the reverse elution order of tocopherols compared with Fig. 22.4a. Using a RP-C_{18} stationary phase β- and γ-tocopherol could not be separated, but the use of a RP-C_{30} column (Fig. 22.4c) allowed the partial separation of these two substances. Thus, vitamin E is an illustration of the analytical uses of different stationary phases. Although NP-HPLC involves some problems in the handling of the system, it gave the best resolution for the substances of interest. The RP-system showed the influence of the chain-length of the stationary phase. While no separation was achieved with a RP-C_{18} column, the RP-C_{30} column yielded a resolution factor of 0.9 for β- and γ-tocopherol.

TABLE 22.5

DETERMINATION OF STILBENES AND FLAVONOIDS IN BLOOD AND URINE

1 Analyte	Method	Detection	Extraction	Application	Ref.
t-Resveratrol	HPLC	DAD	SPE-C18	Rat plasma	[310]
t-Resveratrol	HPLC	UV	LLE	Human plasma spiked	[311]
t-Resveratrol, c-Resveratrol	HPLC	UV, FLD	SPE	Human plasma spiked	[312]
t-Resveratrol, c-Resveratrol	GC	FID, MS	Acetone	Rat plasma, Human plasma spiked	[313]
t-Resveratrol	HPLC	MS	SPE-C18, freeze	Rat serum and tissues	[270–273]
t-Resveratrol	HPLC	UV	LLE (ethyl acetate)	Blood	[314]
t-Resveratrol, c-Resveratrol	HPLC	DAD, MS	LLE (ethyl acetate)	Human plasma and urine	Unpublished
t-Resveratrol, Catechin, Quercetin	GC	MS	LLE (ethyl acetate)	Human plasma and urine	[264]
Quercetin, Rutin	HPLC	Electro-chemical	SPE-C18	Human plasma	[315,316]
Quercetin	HPLC	UV, FLD	SPE-C18	Human plasma	[317]
Quercetin, Kaempferol, Isorhamnetin	HPLC	FLD with Al^{3+}		Human plasma and urine	[266,318,319]
Flavonol glycosides	HPLC	MS	LLE (ethyl acetate)	Human plasma	[320]
Flavonoids	HPLC	MS		Human urine	[321]
Rutin	HPLC	UV	SPE mixed-mode RP AEX	Human plasma	[322]
Flavone	HPLC	UV	Absolute EtOH	Human plasma	[323]

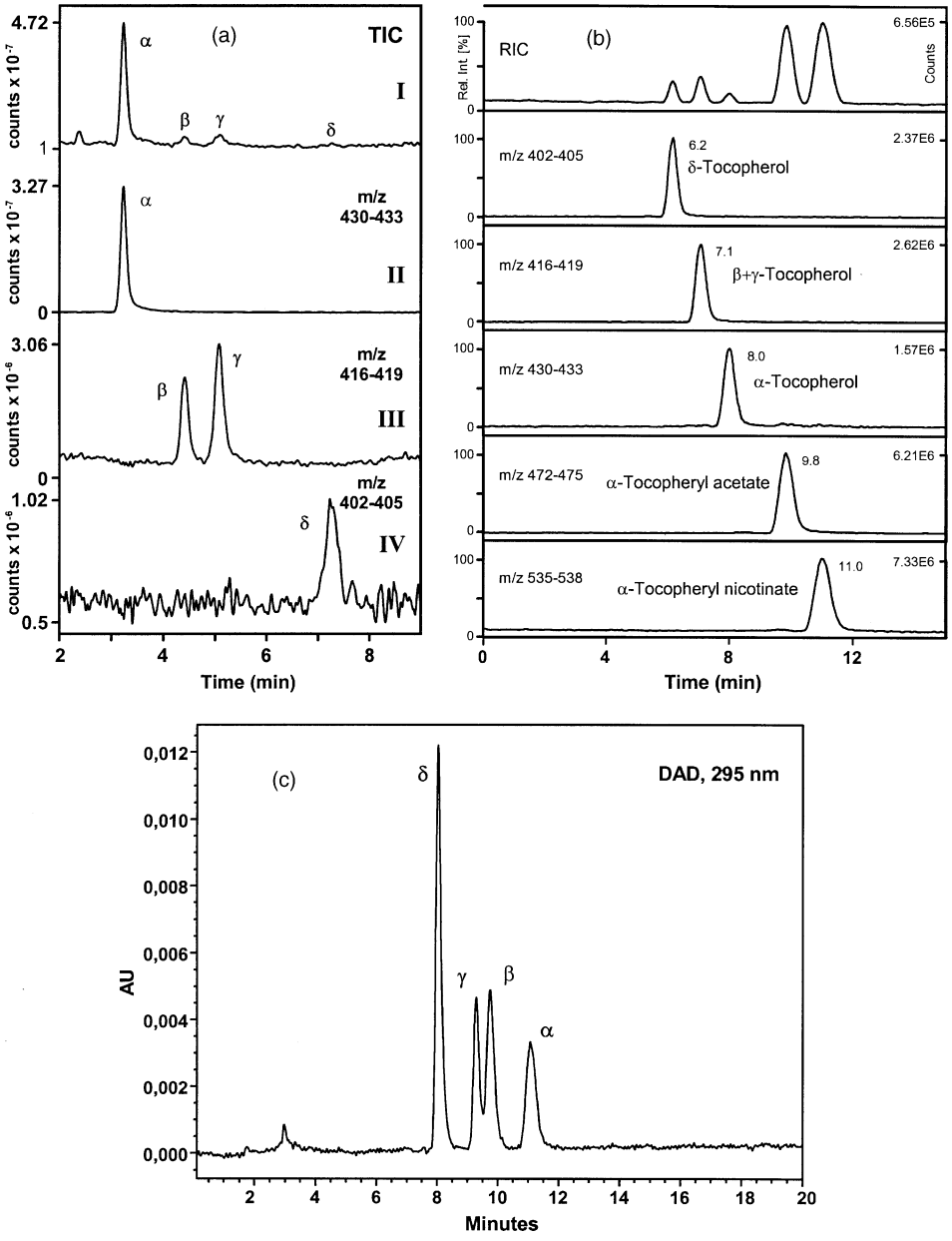


Fig. 22.4. NP-HPLC/APCI-MS of α-, β-, γ- and δ-tocopherol standard mixture. Stationary phase, Hypersil Silica 120-5 (5 μm, 120 Å, 100 mm × 2.1 mm); mobile phase, isooctane/diisopropylether (93/7); flow-rate, 0.2 mL/min; full-scan detection, 390–440; sample volume, 5 μL. (b) RP-HPLC/APCI-MS of a tocopherol standard mixture. Stationary phase, Hypersil BDS C_{18} (3 μm, 130 Å, 250 mm × 1 mm); mobile phase, MeOH; flow rate, 80 μL/min; full-scan detection, 390–550; sample volume, 5 μL. (c) RP-HPLC/DAD of a tocopherol standard mixture. Stationary

22.3.4 Alkaloids

The alkaloids are characterized by the presence of a heterocyclic nitrogen, their basic reactivity and their physiological effects [3,4,291]. Analgesics, like morphine (*Papaver somniferum*), and toxins, like aconitine (*Aconitum napellus*), are two examples of this heterogeneous class of substances. Alkaloids were thought to occur only in the plant kingdom; however, they are now known to occur also in some animals, *e.g.*, in the toxic secretions of fire ants, ladybugs, and toads [1]. Classification is usually based on their chemical structure, *e.g.*, purine alkaloids, tryptophan, ornithin and lysine derivatives. Subclasses may be based on the type of ring system present [3,4,292]. The alkaloid literature is extensive. Harborne and coworkers [1] have written an overview of publications on this topic: The most comprehensive and up-to-date single reference work is the dictionary of Southon and Buckingham [293]. The best modern account of the chemistry, biosynthesis, and pharmacology is the monograph by Cordell [294]. Two books which concentrate on alkaloid biochemistry and biology are the works of Robinson [295] and of Waller and Nowacki [296]. Three series of review publications on alkaloids should also be mentioned [297–300].

The detection of alkaloids in plant extracts is easily accomplished with general (Dragendorff, Mayer) or specific (Ehrlich) color reagents. These tests reveal only the presence of alkaloids, but cannot distinguish between particular compounds or subclasses. The extraction of alkaloids takes advantage of their basic character, with the use of acidulated (1 *M* HCl, 10% acetic acid) alcoholic solvents and subsequent precipitation by concentrated ammonia. Subsequent purification by paper chromatography and TLC is necessary, and further analysis of the spots enables the classification of substances of interest. Detection is performed by fluorescence in UV light and application of various sprays (Table 22.6). Volatile alkaloids are best separated by GLC, whereas higher-molecular-weight alkaloids are best examined by TLC [209]. An overview of the preparative isolation of alkaloids by preparative centrifugal TLC, flash chromatography, and medium-pressure LC, HPLC, countercurrent chromatography and centrifugal partition chromatography has been presented by Hostettmann [15]. Table 22.7 is a summary of methods for the preparative isolation of alkaloids, based on the work of Hostettmann [15] and Harborne [214].

22.4 MICRO-SEPARATION SYSTEMS

Micro-separation systems, especially mico-LC (μLC) and capillary electrochromatography (CEC) or chip-technology [301,302] are new and promising fields (Chap. 11). These methods have the advantage of economic running conditions, due to low flow-rates (2 μL/min or less) and low injection volumes (0.5 μL or less). For μLC, fused-silica capillary columns with 200-μm ID are used, for CEC capillaries with 75-μm ID, and for

---

phase, Bischoff Prontosil 200-3-C_{30} (3 μm, 200 Å, 250 mm × 4.6 mm); mobile phase, MeOH; flow rate, 1 mL/min; temperature, 25°C; sample volume, 20 μL. (Reproduced from Ref. 7 with permission.)

TABLE 22.6

R_F AND COLOR PROPERTIES OF SOME ALKALOIDS; REPRODUCED FROM REF. 214 WITH PERMISSION

Alkaloid*	R_F ($\times 100$)** on		Behavior in UV light	Recommended reagent for detection***	Spectral max [nm] in 0.1 M H_2SO_4
	Paper	TLC			
Cytisine	03	32	Blue	Dragendorff	303
Nicotine	07	57	Absorbs		260
Tomatine	08	62	Invisible	Iodoplatinate	–
Morphine	14	34	Absorbs		284
Solanine	15	52	Invisible	Marquis	–
Codeine	16	35	Absorbs		284
Berberine	25	07	Fluorescent yellow		228
Strychnine	30	22			254
Thebaine	32	41	Absorbs	Iodoplatinate	284
Atropine	37	18			258
Quinine	46	52	Bright blue		250
Coniine	56	26	Invisible		268

* Alkaloids are in order of R_F on paper. Data from Clarke (1970), who gives similar data for over 150 common alkaloids.

** Solvent on paper (previously buffered with 5% sodium dihydrogen citrate): *n*-BuOH/aqueous citric acid (870 mL/4.8 g citric acid in 130 mL H_2O); solvent on silica gel: MeOH/NH_4OH (200/3).

*** Dragendorff: orange-brown spots on a yellow background; Marquis: yellow to purple spots; iodoplatinate: range of colors.

microchip technology channels with 50-μm ID. The stationary phase is held in place in the capillary by fritted filters (frits) [303], by a second column with smaller inner diameter [304], or by *in situ* polymerization (monoliths) [14,305]. Fritless stationary phases are used partly to avoid problems of bubble formation. The field of plant proteomics is just starting out and lags several years behind proteomics of unicellular prokaryotes and eukaryotes [306]. Most published studies are limited to the comparison of expression levels without actual identification of the proteins. In only a few cases have limited sets of proteins been identified through Edman sequencing. These studies focused mainly on the use of patterns to identify possible markers for different genotypes and phenotypes or phylogenetic relationships [307]. Van Wijk's review [306] covers mainly recent studies on *Arabidopsis*, root proteomics of maize, chloroplast proteomics, post-translational modifications, and the limitations and possibilities of plant proteomics with non-sequenced plant species.

TABLE 22.7

SUMMARY OF CHROMATOGRAPHIC METHODS FOR THE PREPARATIVE ISOLATION OF ALKALOIDS; DATA TAKEN FROM REFS. 15 AND 214

Class	Method	2 Details	Ref.
Tropane	PC	EtOAc/25% HCO_2H (4/3); tank presatd. with aq. phase for 14 h	[214]
Tropane	TLC	Silica Gel G in MeOH/$CHCl_3$ (3/17)	[214]
Tropane	GLC	5.6% polyethylene glycol support (2 m × 5 mm column) temp. 170–200°C	[214]
Norditerpene alkaloids from *Delphinium ajacis*	Preparative centrifugal TLC	Al_2O_3 PF_{254} (1 mm) with *n*-C_6H_{14}/Et_2O (75/25, 1/1)	[324]
Alkaloids from *Zanthoxylum budrunga*	Preparative centrifugal TLC	Silica Gel PF_{254} with *n*-C_6H_{14}/Et_2O, different mixtures	[325]
Pyiperidine alkaloids from tunicate (*Pseudodistoma kanoko*)	Flash chromatography	45 × 2.2 cm; $CHCl_3$/*n*-BuOH–HOAc/H_2O (1.5/6/1/1)	[326]
Indole	Low pressure LC	RP-8; MeOH/0.02 *M* NH_4OAc (3:2)	[327]
Alkaloid glycosides	Medium pressure LC	100 × 10 mm, RP-18 (40–63 μm); CH_3CN/H_2O (24/76)	[328]
Alkaloids from *Ancistrocladus korupensis*	Preparative HPLC	Dynamax NH_2, 250 × 41.4 mm; CH_2Cl_2/0.1% $(NH_4)_2CO_3$ in MeOH (22/3)	[329]
Benzyl-aporphine dimers from *Thalictrum fauriei*	RLCC*	$CHCl_3$/MeOH/pH 3.0 acetate buffer (5/5/3), upper phase	[330]
Indole	Centrifugal partition chromatography on MLCCC**	Me_2CO/*n*-BuOH/H_2O (1/8/10)	[331]

* Rotation locular countercurrent chromatography.
** Multilayer countercurrent chromatograph (PC Inc.).

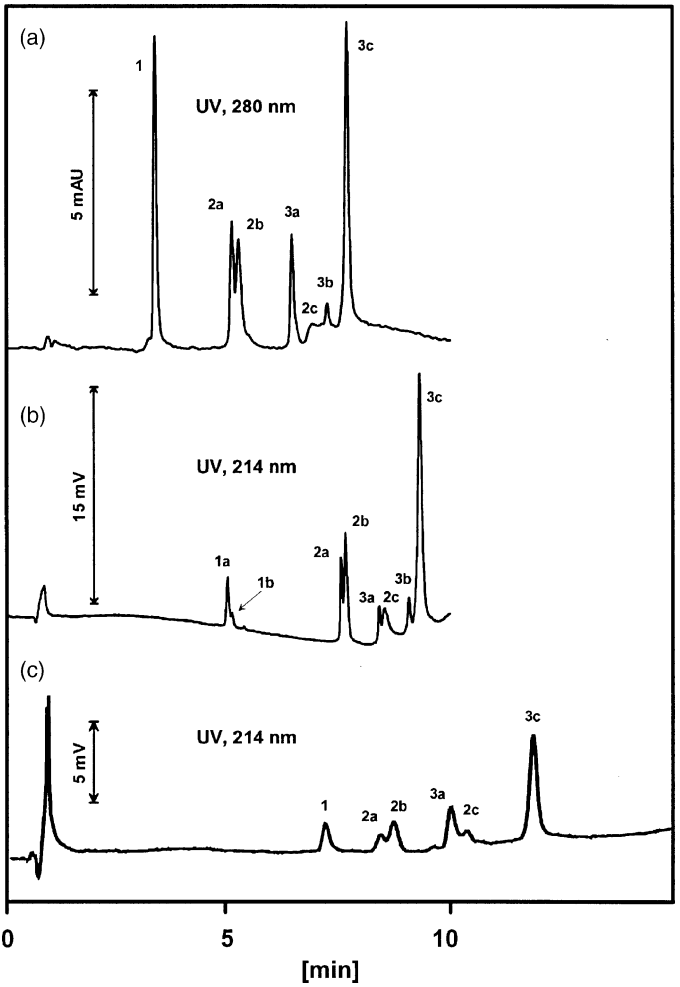


Fig. 22.5. LC/UV of a lectin standard mixture. Column, non-porous PS-DVB-C_{18} (2.1 μm, 50 mm × 4.6 mm); mobile phase, (A) 0.1% TFA in water, (B) 0.1% TFA in MeCN; gradient, 0 min, 10% B; 0.5 min, 33% B; 2.5 min 33% B; 6 min, 50% B; flow-rate, 1.0 mL/min; detection, 280 nm; temperature, 25°C; sample volume, 20 μL. (b) LC/UV of a lectin mixture. Column, monolithic PS-DVB (7 cm × 200 μm); for mobile phases see Fig. 22.5a; gradient, 0 min, 90% A; 1 min, 70% A; 10 min, 0% A; flow-rate, 2.3 μL/min; detection, 214 nm; sample size, 0.5 μL. (c) LC/UV of a lectin standard mixture. Column, encapsulated silica (ProntoSIL, 3 μm, 200 Å) – PS-DVB (70 mm × 200 μm); for mobile phases see Fig. 22.5b; flow-rate 2 μL/min; detection, 214 nm. Peak assignments: 1a: wheat germ isolectin 2 and 3; 1b: wheat germ isolectin 1; 2a: lentil isolectin B – α subunit; 2b: lentil isolectin A – α subunit, 3a: concanavalin A, proteolytic fragment 1–118; 2c: lentil isolectins A + B – β subunit; 3b: concanavalin A, proteolytic fragment 119–237; 3c: concanavalin A. (Reproduced from Ref. 7 with permission.)

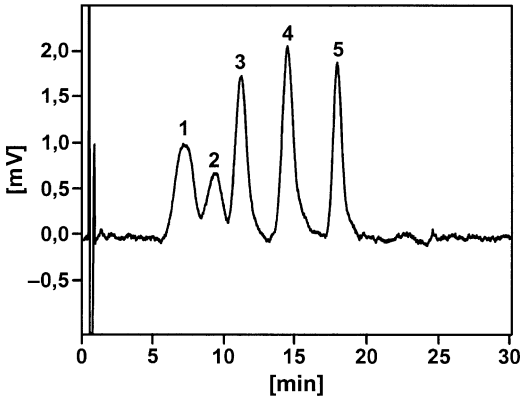


Fig. 22.6. Separation of flavonoid and stilbene standard mixture by μ-LC. Column: Si-C_{18} PS-DVB (60 Å, 3 μm, 81 mm × 200 μm); mobile phase, (A) H_2O/MeOH/TFA (900 g/79 g/0.2%), (B) H_2O/MeOH/THF/TFA (600 g/79 g/267 g/0.2%); gradient: 10% B to 70% B in 20 min, to 10% B at 22 min; flow-rate, 150 μL/min with split, 2.2 μL/min in capillary; detection, UV absorbance: 210 nm; temperature, 50°C; sample volume, 0.5 μL. Peak assignments: 1 *trans*-resveratrol, 2 myricetin, 3 *cis*-resveratrol, 4 quercetin, 5 kaempferol. (Reproduced from Ref. 7 with permission.)

Bonn's group [308,309] has used polymer-based monolithic, particularly non-porous octadecylated polystyrene/divinylbenzene (PS/DVB) and PS/DVB-encapsulated C_{18} silica for the simple and rapid separation and characterization of plant isolectins from lentil (*Lens culinaris*), wheat germ (*Triticum vulgaris*), and jack bean (*Canavalia ensiformis*) as well as for the determination of smaller analytes, such as flavonoids and stilbenes. In comparison with the particulate non-porous octadecylated PS/DVB packing, the monolithic PS/DVB phase showed higher efficiency in the separation of the isoforms of wheat germ lectin and the proteolytic fragments of concanavalin A. Using UV detection at 214 nm, multiple peaks were observed, corresponding to fragments or isoforms of each individual lectin (Figs. 22.5a and b). Fig. 22.5c shows the separation of the isolectins on a column of encapsulated silica with a pore size of 200 Å. The analysis of flavonoids and stilbenes showed good separation on a PS/DVB-encapsulated C_{18}-silica with 60 Å pore size (Fig. 22.6) [308], but less resolution on a particulate PS/DVB-C_{18} column.

REFERENCES

1 J.B. Harborne, H. Baxter and G.P. Moss, *Phytochemical Dictionary – A Handbook of Bioactive Compounds From Plants*, Taylor & Francis, London, 1999.
2 R. Hänsel, O. Sticher and E. Steinegger, *Pharmakognosie – Phytopharmazie*, Springer, Berlin, 1999.
3 P.G. Waterman, *Alkaloids*, 50 (1998) 537.
4 P.G. Waterman, *Phytochemistry*, 49 (1998) 1175.

5 V. Pichon, L. Chen, N.L.F. Durand and M.C. Hennion, *J. Chromatogr. A*, 725 (1996) 107.
6 A. Shimizu, T. Nakanishi, R. Koyama and T. Ikeda, *Rinsho Byori*, 50 (2002) 169.
7 G. Stecher, C.W. Huck, W.M. Stöggl and G.K. Bonn, *Trends Anal. Chem.*, 22 (2003) 1.
8 G. Stecher, F. Schwienbacher, S. Mayr and H. Bauer, *Phyton*, 39 (1998) 205.
9 P.G. Waterman, *Phytochemistry*, 58 (2001) 186.
10 J.C. Touchstone, *Handbook of Analytical Techniques*, Wiley, New York, 2001, p. 327.
11 J. Sherma, *J. Chromatogr. A*, 880 (2000) 129.
12 H. Kalasz and M. Bathori, in K. Valko (Ed.), *Handbook of Analytical Separations*, Elsevier, Amsterdam, 2000, p. 439.
13 Sz. Nyiredy, *Planar Chromatography*, Springer, Budapest, 2001.
14 Cs. Horvath, G. Choudhary and X. Huang, in *Proceedings of the 19th Int. Symp. on Capillary Chromatography and Electrophoresis*, Wintergreen, VA, USA, 1997.
15 K. Hostettmann, A. Marston and M. Hostettmann, *Preparative Chromatographic Techniques – Applications in Natural Product Isolation*, Springer, Berlin, 1998.
16 J. Tekel and S. Hatrík, *J. Chromatogr. A*, 754 (1996) 397.
17 Y. Gaillard and G. Pepin, *J. Chromatogr. B*, 733 (1999) 181.
18 C.W. Huie, *Anal. Bioanal. Chem.*, 373 (2002) 23.
19 E. Stahl and W. Schild, *Isolierung und Charakterisierung von Naturstoffen*, G. Fischer Verlag, Stuttgart, 1986.
20 J.A. Jönsson and L. Mathiasson, *Trends Anal. Chem.*, 18 (1999) 318.
21 R. Chizzola, *J. Chromatogr. A*, 668 (1994) 427.
22 D.R. Lauren, D.J. Jensen and J.A. Douglas, *J. Chromatogr. A*, 712 (1995) 303.
23 B. Bjerg, O. Olsen, K. Wedel Rasmussen and H. Sorensen, *J. Liq. Chromatogr.*, 7 (1984) 691.
24 A. Romani, A. Baldi, M. Tattini and F.F. Vinicieri, *Chromatographia*, 39 (1994) 35.
25 C.C.T. Wörth, M. Wießler and O.J. Schmitz, *Electrophoresis*, 21 (2000) 3634.
26 V. Mani and C. Woolley, *LC-GC*, 13 (1995) 734.
27 T. Mroczek and K. Glowniak, *J. Pharm. Biol. Anal.*, 26 (2001) 89.
28 A. Steffen and J. Pawliszyn, *J. Agric. Food Chem.*, 44 (1996) 2187.
29 G. Theodoridis, W. Haasnoot, G. Cazemier, R. Schilt, M. Jaziri, B. Diallo, I.N. Papadoyannis and G.J. de Jong, *J. Chromatogr. A*, 948 (2002) 177.
30 M. de Fátima Alpendurada, *J. Chromatogr. A*, 889 (2000) 3.
31 R. Krska, *J. Chromatogr. A*, 815 (1998) 49.
32 M.B. Müller, C. Zwiener and F.H. Frimmel, *J. Chromatogr. A*, 862 (1999) 137.
33 M. Shimoni and R. Reuveni, *J. Chromatogr.*, 646 (1993) 99.
34 J. Toivo, V. Piironen, P. Kalo and P. Varo, *Chromatographia*, 48 (1998) 745.
35 D.A. Martens and D.A. Frankenberger, Jr., *Chromatographia*, 32 (1991) 417.
36 E.O. Hochleitner and H. Malissa, Jr., *Fresenius J. Anal. Chem.*, 369 (2001) 516.
37 K. Glowniak, G. Zgórka, A. Józefczyk and M. Furmanowa, *J. Pharm. Biomed. Anal.*, 14 (1996) 1215.
38 P.J. Hernes and J.I. Hedges, *Anal. Chem.*, 72 (2000) 5115.
39 M. Bártlová, L. Opletal, V. Chobot and H. Sovová, *J. Chromatogr. B*, 770 (2002) 283.
40 T. Veress, *J. Chromatogr. A*, 668 (1994) 285.
41 C.A. Thomson and D.J. Chesney, *Anal. Chem.*, 64 (1992) 848.
42 W.N. Moore and L.T. Taylor, *J. Nat. Prod.*, 59 (1996) 690.
43 K. Ganzler, A. Salgó and K. Valkó, *J. Chromatogr.*, 371 (1986) 299.
44 H.M. Kingston and L.B. Jassie, *Introduction to Microwave Sample Preparation*, American Chemical Society, Washington, 1988.
45 A.V. Filgueiras, J.L. Capelo, I. Lavilla and C. Bendicho, *Talanta*, 53 (2000) 433.
46 S.S. Yang, I. Smetena and A.I. Goldsmith, *J. Chromatogr. A*, 746 (1996) 131.

47 S. Glasl, U. Kastner, J. Jurenitsch and W. Kubelka, *J. Chromatogr. B*, 729 (1999) 361.
48 E. Vérette, F. Qian and F. Mangani, *J. Chromatogr. A*, 705 (1995) 195.
49 C.W. Huck and G.K. Bonn, *J. Chromatogr. A*, 885 (2000) 51.
50 S. Hatrik and H. Tekel, *J. Chromatogr. A*, 733 (1996) 217.
51 G. Font, J. Manes, J.C. Molto and Y. Pico, *J. Chromatogr. A*, 642 (1993) 135.
52 A. Martin-Esteban, P. Kwasowski and D. Stevenson, *Chromatographia*, 45 (1997) 364.
53 V. Pichon, L. Chen and M.C. Hennion, *Anal. Chim. Acta*, 311 (1995) 429.
54 V. Pichon, M. Charpak and M.C. Hennion, *J. Chromatogr. A*, 795 (1998) 83.
55 G. Durand, V. Chiron, V. Boouvot and D. Barcelo, *Int. J. Environ. Anal. Chem.*, 49 (1992) 31.
56 S. Sennert, D. Volmer, K. Levsen and G. Wünsch, *Fresenius J. Anal. Chem.*, 351 (1995) 642.
57 S. Guenu and M.C. Hennion, *J. Chromatogr. A*, 737 (1996) 15.
58 S.Y. Veselov, G.R. Kudoyarova, N.L. Egutkin, V.Z. Gyuli-Zade, A.R. Mustafina and E.M. Kof, *Physiol. Plant.*, 86 (1992) 93.
59 J.K. Whitehead and K. Rothwell, *Br. J. Cancer*, 23 (1969) 840.
60 K.R. Gustafson, J.H. Cardellina, J.B. McMahon, R.J. Gulakowski, J. Ishitoya, Z. Szallasi, N.E. Lewin, P.M. Blumberg, O.S. Weislow, J.A. Beutler, R.W. Buckheit, G.M. Cragg, P.S. Cox, J.P. Bader and Mr. Boyd, *J. Med. Chem.*, 35 (1992) 1978.
61 G.R. Pettit, S.B. Singh, M.R. Boyd, E. Hamel, R.K. Pettit, J.M. Schmidt and F. Hogan, *J. Med. Chem.*, 38 (1995) 1666.
62 M.E. Wall, M.C. Wani and H. Taylor, *Cancer Treat. Rep.*, 60 (1976) 1011.
63 K. Hostettmann, M.J. Pettei, I. Kubo and K. Nakanishi, *Helv. Chim. Acta*, 60 (1977) 670.
64 J.A. Adamovics, *Chromatographic Analysis of Pharmaceuticals*, Dekker, New York, 1990.
65 T. Mroczek, K. Glowniak and M. Hajnos, *Biomed. Chromatogr.*, 14 (2000) 516.
66 T. Wawrzynowicz, M. Hajnos, K. Glowniak and M. Furmanowa, *Acta Pol. Pharm.*, 56 (1999) 48.
67 S. Nyiredy and K. Glowniak, in S. Nyiredy (Ed.), *Planar Chromatography*, Springer, Budapest, 2001.
68 G. Matysik and E. Soczewinski, *J. Planar Chromatogr.*, 9 (1996) 404.
69 M. Mazurek and Z. Witkiewicz, *Chem. Anal. (Warsaw)*, 42 (1997) 767.
70 C.W. Huck, C.G. Huber, K.H. Ongania and G.K. Bonn, *J. Chromatogr. A*, 870 (2000) 453.
71 J. Ito and M. Niwa, *Tetrahedron*, 52 (1996) 9991.
72 K.S. Huang, Y.H. Wang, R. Li and M. Lin, *J. Nat. Prod.*, 63 (2000) 86.
73 M.T. Ribeiro de Lima, P. Waffo-Teguo, P.L. Teissedre, A. Pujolas, J. Vercauteren, J.C. Cabanis and J.M. Merillon, *J. Agric. Food Chem.*, 47 (1999) 2666.
74 M.L. Hajnos, K. Glowniak, M. Waksmundzka-Hajnos and S. Piasecka, *Chromatographia*, 56 (2002) 91.
75 A. Zamponi, G. Viola and W. Knöss, *Pharmazie*, 53 (1998) 39.
76 B.V. Charlwood and D.V. Banthorpe, *Methods in Plant Biochemistry – The Terpenoids*, Academic Press, London, 1991.
77 J.B. Harborne and F.A.T. Barberan, *Ecological Chemistry and Biochemistry of Plant Terpenoids*, Chapman & Hall, London, 1991.
78 J.F. Jackson and H.F. Linskens, *Analysis of Taste and Aroma*, Springer, Berlin, 2002.
79 S. Dev, A.P.S. Narula and J.S. Yadav, *CRC Handbook of Terpenoids Vol 1 and 2*, CRC Press, Boca Raton, 1982.
80 R. Kaiser and P. Kraft, *Chem. Unserer Zeit*, 35 (2001) 8.
81 A. Koedam, P. Sandra and C. Bicchi, *Capillary Gas Chromatography in Essential Oil Analysis*, Huethig Verlag, Heidelberg, 1987.
82 M. Godefroot, P. Sandra and M. Verzele, *J. Chromatogr. A*, 203 (1981) 325.

83 C. Bicchi, A. D'Amato, G.M. Nano and C. Frattini, *J. Chromatogr.*, 279 (1983) 409.
84 V.K. Rai, S.C. Gupta and B. Singh, *Biol. Plant.*, 46 (2003) 121.
85 S. Shuenn-Jyi and L. Kwei-Lan, *J. High Resolut. Chromatogr.*, 21 (1998) 569.
86 A. Caredda, B. Marongiu, S. Porcedda and C. Soro, *J. Agric. Food Chem.*, 50 (2002) 1492.
87 P. Chatzopoulou, A. de Haan and S.T. Katsiotis, *Planta Med.*, 68 (2002) 827.
88 M. Ondarza and A. Sanchez, *Chromatographia*, 30 (1990) 16.
89 E.E. Stashenko, M.A. Puerta and M.Y. Combariza, *J. Chromatogr. A*, 752 (1996) 223.
90 E. Stahl and D. Gerard, *Z. Lebensm. Unters. Forsch.*, 176 (1983) 1.
91 S.B. Hawthorne, M.S. Krieger and D.J. Miller, *Anal. Chem.*, 60 (1988) 472.
92 L. Tao and M.A. Pereira, *J. Chromatogr. A*, 793 (1998) 71.
93 P. Morin, M. Caude, H. Richard and R. Rosset, *Analyst*, 13 (1985) 196.
94 P. Morin, M. Caude, H. Richard and R. Rosset, *J. Chromatogr.*, 363 (1986) 57.
95 L. Mondello, K.D. Bartle, G. Dugo and P. Dugo, *J. High Resolut. Chromatogr.*, 17 (1994) 312.
96 G. Dugo, A. Verzera, A. Trozzi, A. Cotroneo, L. Mondello and K.D. Bartle, *Essenze, Derivati Agrumari*, 64 (1994) 35.
97 I. Gerasimenko, Y. Sheludko, M. Unger and J. Stockigt, *Phytochem. Anal.*, 12 (2001) 96.
98 I. Ney, E. Jaeger, M. Herderich, P. Schreier and W. Schwab, *Phytochem. Anal.*, 7 (1996) 233.
99 V. Van den Bergh, H. Coeckelberghs, I. Vanhees, R. De Boer, F. Compernolle and C. Vinckier, *Anal. Bioanal. Chem.*, 372 (2002) 630.
100 J. Zukowski and M. Pawlowska, *J. High Resolut. Chromatogr.*, 16 (1993) 505.
101 D.W. Armstrong and J. Zukowski, *J. Chromatogr. A*, 666 (1994) 445.
102 A.K. Chatjigakis, I. Clarot, P.J.P. Cardot, R. Nowakowski and A. Coleman, *J. Liq. Chromatogr. Rel. Technol.*, 22 (1999) 1267.
103 J. Mookdasanit and H. Tamura, *Food Sci. Technol. Res.*, 8 (2002) 367.
104 P. Manninen, M.L. Riekkola, Y. Holm and R. Hiltunen, *J. High Resolut. Chromatogr.*, 13 (1990) 167.
105 O. Lopes, E.N. Barata, H. Mustaparta and J. Araújo, *Arthropod Struct. Dev.*, 31 (2002) 1.
106 I. Jerkovic and J. Mastelic, *Phytochemistry*, 63 (2003) 109.
107 A.P. Martins, L.R. Salgueiro, M.J. Goncalves, A. Proenca da Cunha, R. Vila and S. Canigueral, *Planta Med.*, 69 (2003) 77.
108 D.J. Daferera, B.N. Ziogas and M.G. Polissiou, *Crop Prot.*, 22 (2003) 39.
109 C. Gherman, M. Culea and O. Cozar, *Talanta*, 53 (2000) 253.
110 I. Schwob, J.M. Bessiére and J. Viano, *C.R. Biologies*, 325 (2002) 781.
111 Z. Liu and J.B. Phillips, *J. Chromatogr. Sci.*, 29 (1991) 227.
112 K.D. Bartle and P. Myers, *Trends Anal. Chem.*, 21 (2002) 547.
113 P. Marriott, R. Shellie, J. Fergeus, R. Ong and P. Morrison, *Flavour Fragr. J.*, 15 (2000) 225.
114 A.L. Lee, K.D. Bartle and A.C. Lewis, *Anal. Chem.*, 73 (2001) 1330.
115 J. Beens, J. Blomberg and J.P. Schoenmakers, *J. High Resolut. Chromatogr.*, 23 (2000) 182.
116 R. Shellie and P.J. Marriott, *Anal. Chem.*, 74 (2002) 5426.
117 Y. Ueno, *Trichothecenes: Chemical, Biological, and Toxicological Aspects*, Kondansha, Tokyo, 1983.
118 F. Kotal, K. Hoadova, J. Hajslova, J. Poustka and Z. Radova, *J. Chromatogr. A*, 830 (1999) 219.
119 W. Langseth and T. Rundberget, *J. Chromatogr. A*, 815 (1998) 103.
120 U. Berger, M. Oehme and F. Kuhn, *J. Agric. Food Chem.*, 47 (1999) 4240.
121 T. Tuomi, L. Saarinen and K. Reijula, *Analyst*, 123 (1998) 1835.

122 H. Kurata and T. Ueno, *Toxicogenic Fungi. Their Toxic and Health Hazards*, Elsevier, Amsterdam, 1984, p. 118.
123 Z. Radova, K. Holadova and J. Hajslova, *J. Chromatogr. A*, 829 (1998) 259.
124 R. Krska, S. Baumgartner and R. Josephs, *Fresenius J. Anal. Chem.*, 371 (2001) 285.
125 T. Yoshisawa, J.E. Smith and R.S. Henderson, *Mycotoxins and Animal Foods*, CRC Press, Boca Raton, 1991, p. 423.
126 J.D. Miller and H.L. Trenholm, *Mycotoxins in Grains, Compounds Other Than Aflatoxins*, Eagan Press, St. Paul, 1994, p. 135.
127 D.B. Prelusky, B.A. Rotter, R.G. Rotter, J.D. Miller and H.L. Trenholm, *Mycotoxins in Grains, Compounds Other Than Aflatoxins*, Eagan Press, St. Paul, 1994, p. 359.
128 G.A. Lombaert, *Adv. Exp. Med. Biol.*, 504 (2002) 141.
129 C.J. Mirocha, E. Kolaczkowski, W. Xie, H. Yu and H. Jelen, *J. Agric. Food Chem.*, 46 (1998) 1414.
130 R. Schmidt and K. Dose, *J. Anal. Toxicol.*, 8 (1984) 43.
131 D.R. Lauren and R. Greenhalgh, *J. Assoc. Off. Anal. Chem.*, 70 (1987) 479.
132 A. Sano, S. Matsutani, M. Suzuki and S. Takitani, *J. Chromatogr.*, 410 (1987) 427.
133 J. Weingärtner, R. Krska, W. Praznik, M. Grasserbauer and H. Lew, *Fresenius J. Anal. Chem.*, 357 (1997) 1206.
134 R.D. Josephs, R. Krska, M. Grasserbauer and J.A.C. Broekaert, *J. Chromatogr. A*, 795 (1998) 297.
135 M. Schollenberger, U. Lauber, H. Terry Jara, S. Suchy, W. Drochner and H.M. Müller, *J. Chromatogr. A*, 815 (1998) 123.
136 T. Tanaka, A. Yoneda, S. Inoue, Y. Sugiura and Y. Ueno, *J. Chromatogr. A*, 882 (2000) 23.
137 M. Jimenez, J.J. Mateo and R. Mateo, *J. Chromatogr. A*, 870 (2000) 473.
138 J.J. Park and F.S. Chu, *J. Assoc. Off. Anal. Chem. Int.*, 79 (1996) 465.
139 A. Visconti and A. Bottalico, *Chromatographia*, 17 (1983) 97.
140 H. Cohen and B. Boutin-Muma, *J. Chromatogr. A*, 595 (1992) 143.
141 A. Visconti, J. Chelkowski and A. Bottalico, *Mycotoxin Res.*, 2 (1986) 59.
142 T. Krishnamurthy, D.J. Beck and R.K. Isensee, *J. Chromatogr.*, 469 (1989) 209.
143 E. Rajakylä, K. Laasasenaho and P.J.D. Sakkers, *J. Chromatogr.*, 384 (1987) 391.
144 L.M. Cahill, S.C. Kruger, B.T. McAlice, C.S. Ramsey, R. Prioli and B. Kohn, *J. Chromatogr. A*, 859 (1999) 23.
145 R.P. Huopalahti, J. Ebel and J.D. Henion, Jr., *J. Liq. Chromatogr. Rel. Technol.*, 20 (1997) 537.
146 K. Jarukamjorn, G. Stecher, C. W. Huck and G. K. Bonn, *J. Sep. Sci.* (submitted).
147 L. Czerwiecki and H. Giryn, *Pol. J. Food Nutr. Sci.*, 44 (1994) 111.
148 P.M. Scott, *J. Assoc. Off. Anal. Chem.*, 65 (1982) 878.
149 A.E. Pohland, C. Thorpe, M.W. Trucksess, R.M. Eppley, J.L. Richard and J.R. Thurston, *Diagnosis of Mycotoxicoses*, Martinus Nijhoff, Dordrecht, 1986.
150 C. Fernandez, M.E. Stack and S.M. Musser, *J. Assoc. Off. Anal. Chem.*, 77 (1994) 253.
151 M.W. Trucksess, M.T. Flood, M.M. Mossobu and S.W. Page, *J. Agric. Food Chem.*, 35 (1987) 445.
152 P.M. Scott, S.R. Kanhere and E.J. Tarter, *J. Assoc. Off. Anal. Chem. Int.*, 69 (1989) 889.
153 P.M. Scott, G.A. Lombaert, P. Pellaers, S. Bacler, S.R. Kanhere, W.F. Sun, P.Y. Lau and D. Weber, *Food Addit. Contam.*, 6 (1989) 489.
154 H.D. Rood, S.P. Swanson and W.B. Buck, *J. Chromatogr. Biomed. Appl.*, 378 (1986) 375.
155 H.D. Rood, W.B. Buck and S.P. Swanson, *J. Agric. Food Chem.*, 36 (1988) 74.
156 H.D. Rood, W.B. Buck and S.P. Swanson, *J. Assoc. Off. Anal. Chem. Int.*, 71 (1988) 493.

157 R.M. Black, R.J. Clarke and R.W. Read, *J. Chromatogr.*, 388 (1987) 365.
158 K.F. Neilsen and U. Thrane, *J. Chromatogr. A*, 929 (2001) 75.
159 R.D. Smith, H.R. Udseth and B.W. Wright, *J. Chromatogr. Sci.*, 23 (1985) 192.
160 E. Usleber, E. Märtlbauer, R. Dietrich and G. Terplan, *J. Agric. Food Chem.*, 39 (1991) 2091.
161 E. Schneider, R. Dietrich, E. Märtlbauer and E.T.G. Usleber, *Food Agric. Immunol.*, 3 (1991) 185.
162 J.P. Swanson, W.G. Jennings and J.G. Nikelly, *Capillary Chromatography – The Applications*, Hüthig, Heidelberg, 1991, p. 39.
163 K. Glowniak and T. Mroczek, *J. Liq. Chromatogr. Rel. Technol.*, 22 (1999) 2483.
164 K. Glowniak, T. Wawrzynowicz, M. Hajnos and T. Mroczek, *J. Planar Chromatogr.*, 12 (1999) 328.
165 T. Wawrzynowicz, M. Hajnos, K. Glowniak and M. Furmanowa, *Acta Pol. Pharm.*, 56 (1999) 48.
166 M.L. Hajnos, K. Glowniak, M. Waksmundzka-Hajnos and P. Kogut, *J. Planar Chromatogr.*, 14 (2001) 119.
167 N. Vidensek, P. Lim, A. Campbell and C. Carlson, *J. Nat. Prod.*, 53 (1990) 1609.
168 S.O.K. Auriola, A.M. Lepisto, T. Naaranlahti and S.P. Lapinjoki, *J. Chromatogr.*, 594 (1992) 153.
169 E.R.M. Wickremesinhe and R.N. Artec, *J. Liq. Chromatogr.*, 16 (1993) 3263.
170 M.J.I. Mattina and G.J. MacEachern, *J. Chromatogr. A*, 679 (1994) 269.
171 E. Hamel, C.M. Lin and D.C. Jones, *Cancer Treat. Rep.*, 66 (1982) 169.
172 C.M. Lin, Y.Q. Jiang, A.C. Chaudhary, J.M. Rimoldi, B.C.L. Kingston and E. Hamel, *Cancer Chemother. Pharmacol.*, 38 (1996) 136.
173 P.G. Grothaus, T.J.G. Rayhoud, G.S. Bignami, C.B. Lazo and J.B. Byrnes, *J. Immunol. Methods*, 158 (1993) 5.
174 J.-G. Leu, B.X. Chen, P.B. Schiff and B.F. Erlanger, *Cancer Res.*, 53 (1993) 1388.
175 M. Jaziri, B. Diallo, M. Vanhaelen, P. Vanhaelen Fastre, A. Zhiri and J. Homes, *J. Pharm. Bel.*, 46 (1991) 93.
176 S.-I. Suye, S. Tandel and A. Muichandani, *Anal. Chem.*, 69 (1997) 3633.
177 S.L. Richheimer, D.M. Tinnermeier and D.W. Timmons, *Anal. Chem.*, 64 (1992) 2323.
178 W. Fang, Y. Wu, J. Zhou, W. Chen and Q. Fang, *Phytochem. Anal.*, 4 (1993) 115.
179 G. Theodoridis and R. Verpoorte, *Phytochem. Anal.*, 7 (1996) 169.
180 T. Nguyen, J. Eshraghi, G. Gonyea, R. Ream and R. Smith, *J. Chromatogr. A*, 911 (2001) 55.
181 K.M. Witherup, S.A. Look, M.W. Stasko, T.G. McCloud, H.J. Issaq and G.M. Muschik, *J. Liq. Chromatogr.*, 12 (1989) 2117.
182 E.H. Kerns, K.J. Volk and S.E. Hill, *J. Nat. Prod.*, 57 (1994) 1391.
183 F. Bitsch, *J. Chromatogr.*, 615 (1993) 273.
184 A. Zhiri, M. Jaziri, Y. Guo, R. Vanhaelen-Fastre, M. Vanhaelen, J. Homes, K. Yoshimatsu and K. Shimomura, *Biol. Chem. Hoppe-Seyler*, 376 (1995) 583.
185 R.E.B. Ketchum and D.M. Gibson, *J. Liq. Chromatogr.*, 16 (1993) 2519.
186 D.W. Pearce, M. Koshioka and R.P. Pharis, *J. Chromatogr.*, 658 (1994) 91.
187 P. Gaskin and J. Macmillan, *Soc. Exp. Biol. Semin. Ser.*, 4 (1978) 79.
188 M.H. Beale and C.L. Willis, in B.V. Charlwood and D.V. Banthorpe (Eds.), Methods in Plant Biochemistry, Vol. 7, Academic Press, London, 1991.
189 J.D. Connolly and R.A. Hill, *Nat. Prod. Rep.*, 6 (1989) 475.
190 J.D. Connolly and R.A. Hill, in B.V. Charlwood and D.V. Banthorpe (Eds.), Terpenoids, Vol. 7, Academic Press, London, 1991.
191 A. Burger and H. Wachter, *Hunnius Pharmazeutisches Wörterbuch*, 8th Edn. Walter de Gruyter, Berlin, 1998.

192 L.J. Goad, in B.V. Charlwood and D.V. Banthorpe (Eds.), *Terpenoids*, Vol. 7, Academic Press, London, 1991.
193 K. Hostettmann, M. Hostettmann and A. Marston, in B.V. Charlwood and D.V. Banthorpe (Eds.), *Terpenoids*, Vol. 7, Academic Press, London, 1991.
194 B. Domon and K. Hostettmann, *Helv. Chim. Acta*, 67 (1984) 1310.
195 G. Arramon, C. Saucier, D. Colombani and Y. Glories, *Phytochem. Anal.*, 13 (2002) 305.
196 R. Amarowicz, Y. Yoshiki and K. Okubo, *Z. Naturforsch. C: Biosci.*, 53 (1998) 918.
197 B.K. Gosse, J.N. Gnabre, Y. Ito and R.C. Huang, *J. Liq. Chromatogr. Rel. Technol.*, 25 (2002) 3199.
198 S.M. Abdel Wahab, G.M. Wassel, E.A. Aboutabl, N.M. Ammar and M.S. Afifi, *Egypt. J. Pharm. Sci.*, 38 (1997) 209.
199 J.D. Connolly and R.A. Hill, *Nat. Prod. Rep.*, 13 (1996) 151.
200 R.F. Taylor and M. Ikawa, *Methods Enzymol.*, 67 (1980) 233.
201 S. Van Oycked, T. Randoux, J.C. Braekman, D. Daloze and J.M. Pasteels, *Bull. Soc. Chim. Belg.*, 97 (1988) 297.
202 G. Britton, in B.V. Charlwood and D.V. Banthorpe (Eds.), *Terpenoids*, Academic Press, London, 1991.
203 H.J.C.F. Nells and A.P. Leenherr, *Anal. Chem.*, 55 (1983) 270.
204 F. Khachik, G.R. Beecher and N.F. Whittaker, *J. Agric. Food Chem.*, 34 (1986) 603.
205 N.E. Craft, S.A. Wise and J.H. Soares, *J. Chromatogr.*, 589 (1992) 171.
206 R.J. Bushway, *J. Liq. Chromatogr.*, 8 (1985) 1527.
207 L.C. Sander and S.A. Wise, *J. Sep. Sci.*, 24 (2001) 910.
208 Y. Tanaka, in B.V. Charlwood and D.V. Banthorpe (Eds.), *Terpenoids*, Academic Press, London, 1991.
209 J.B. Harborne, H. Baxter and G.P. Moss, *Phytochemical Dictionary – A Handbook of Bioactive Compounds from Plants*, Taylor & Francis, London, 1999.
210 J.B. Harborne, *Phytochemical Analysis*, Chapman & Hall, London, 1988.
211 J.B. Harborne, *The Flavonoids: Advances in Research Since 1986*, Chapman & Hall, Cambridge, 1994.
212 J.B. Harborne, *Phytochemical Methods*, Chapman & Hall, London, 1998.
213 J.B. Harborne, *Phytochemistry*, 55 (2000) 481.
214 J.B. Harborne, *Phytochemical Methods*, Chapman & Hall, London, 1998.
215 E. Heftmann, *Chromatography, Part B*, Elsevier, Amsterdam, 1983.
216 K. Yoshitama and T. Hori, *Ginkgo Biloba – A Global Treasure*, Springer, Tokyo, 1997, p. 287.
217 J.B. Harborne and C.A. Williams, *Nat. Prod. Rep.*, 18 (2001) 310.
218 M.A. Berhow, *Adv. Exp. Med. Biol.*, 505 (2002) 61.
219 H.M. Merken and G.R. Beecher, *J. Agric. Food Chem.*, 48 (2000) 577.
220 H.M. Merken and G.R. Beecher, *ACS Symp. Ser.*, 803 (2002) 21.
221 P. Pietta and P. Mauri, *Methods Enzymol.*, 335 (2001) 26.
222 S.J. Bloor, *Methods Enzymol.*, 335 (2001) 3.
223 F. Cuyckens, Y.L. Ma, G. Pocsfalvi and M. Claeys, *Analusis*, 28 (2001) 888.
224 O. Sticher, B. Meier and A. Hasler, *Medicinal and Aromatic Plants – Industrial Profiles*, 12 (2000) 179.
225 G.E. Rohr, B. Meier and O. Sticher, in Atta-ur-Rahman (Ed.), *Studies in Natural Products Chemistry*, Vol. 21, 2000, p. 497
226 S.F. Palmeira, L.M. Conserva, E.H.DeA. Andrade and G.M.S.P. Guilhon, *Flavour Fragr. J.*, 16 (2001) 85.
227 R. Oprean, L. Oprean, M. Tamas, R. Sandulescu and L. Roman, *J. Pharm. Biomed. Anal.*, 24 (2001) 1163.

228 D. Solecka, A.M. Boudet and A. Kacperska, *Plant Physiol. Biochem.*, 37 (1999) 491.
229 M.M. Al-Azizi, M.M. El-Olemy, A.M. El-Sayed and M.A. Al-Yahya, *J. Pharm. Sci.*, 16 (1995) 10.
230 G.I.C. Simpson and Y.A. Jackson, *J. Ess. Oil Res.*, 14 (2002) 6.
231 L. Chevolot, R. Dubois-Dauphin, J.C. Yvin and Y. Lienart, *Phytochem. Anal.*, 8 (1997) 22.
232 D. Deliorman, I. Calis, F. Ergun and U. Tamer, *J. Liq. Chromatogr. Rel. Technol.*, 22 (1999) 3101.
233 O.A. Neumüller, *Römpps Chemie-Lexikon Band 5*, Franckh'sche Verlagshandlung, Stuttgart, 1987.
234 E.H. Siemann and L.L. Creasy, *Am. J. Enol. Vitic.*, 43 (1992) 49.
235 E. Celotti, R. Ferrarini, R. Zironi and L.S. Conte, *J. Chromatogr. A*, 730 (1996) 47.
236 S. Malovana, F.J. Garcia Montelongo, J.P. Perez and M.A. Rodriguez-Delgado, *Anal. Chim. Acta*, 428 (2001) 245.
237 P. Jeandet, R. Bessis and B. Gautheron, *Am. J. Enol. Vitic.*, 42 (1991) 41.
238 P. Jeandet, R. Bessis, B.R. Maume, P. Meunier, D. Peyron and P. Trollat, *J. Agric. Food Chem.*, 43 (1995) 316.
239 D. Stahl, B. Lippmann, V. Leinhos, A. Mäser, J. Dettendorfer, S. Gunstheimer, S. Ernst, H. Bergmann and H. Hilscher, *Deutsche Gesellschaft Für Qualitätsforschung E.V.*, (1996) 203, Sonderband.
240 F. Mattivi, F. Reniero and S. Korhammer, *J. Agric. Food Chem.*, 43 (1995) 1820.
241 K. Gamoh and K. Nakashima, *Rapid Commun. Mass Spectrom.*, 13 (1999) 1112.
242 G.J. Soleas, D.M. Goldberg, E. Ng, A. Karumanchiri, E. Tsang and E.P. Diamandis, *Am. J. Enol. Vitic.*, 48 (1997) 169.
243 F. Mattivi, *Z. Lebensm. Unters. Forsch.*, 196 (1993) 522.
244 D.M. Goldberg, A. Karumanchiri, E. Ng et al. *Am. J. Enol. Vitic.*, 44 (1993) 344.
245 D.M. Goldberg, A. Karumanchiri, E. Ng, J. Yan, E.P. Diamandis and G.J. Soleas, *J. Agric. Food Chem.*, 43 (1995) 1245.
246 A.I. Romero-Perez, R.M. Lamuela-Raventós, A.L. Waterhouse and C. de la Torre-Boronat, *J. Agric. Food Chem.*, 44 (1996) 2124.
247 P. Langcake and R.J. Pryce, *Physiol. Plant Pathol.*, 9 (1976) 77.
248 P. Jeandet, R. Bessis, B.F. Maume and M. Sbaghi, *J. Wine Res.*, 4 (1993) 79.
249 G.J. Soleas, D.M. Goldberg, E.P. Diamandis, A. Karumanchiri, J. Yan and E. Ng, *Am. J. Enol. Vitic.*, 46 (1995) 346.
250 A. Antonelli, C. Fabbri and G. Lercker, *Chromatographia*, 42 (1996) 469.
251 D.M. Goldberg, J. Yan, E. Ng et al. *Anal. Chem.*, 66 (1994) 3959.
252 D.M. Goldberg, J. Yan, E. Ng et al. *Am. J. Enol. Vitic.*, 46 (1995) 159.
253 M.T. Tena, A. Rios and M. Valcarcel, *Fresenius J. Anal. Chem.*, 361 (1998) 143.
254 G. Stecher, C.W. Huck, M. Popp and G.K. Bonn, *Fresenius J. Anal. Chem.*, 371 (2001) 73.
255 E. Cantos, C. Garcia-Viguera, S. de Pascual-Teresa and F.A. Tomas-Barberan, *J. Agric. Food Chem.*, 48 (2000) 4606.
256 A. Rechner, C.D. Patz and H. Dietrich, *Deutsche Lebensmittel-Rundschau*, 11 (1998) 363.
257 R. Pezet, V. Pont and P. Cuenat, *J. Chromatogr. A*, 663 (1994) 191.
258 P. Jeandet, R. Bessis, M. Sbaghi, P. Meunier and P. Trollat, *Am. J. Enol. Vitic.*, 46 (1995) 1.
259 U. Seitz, P.J. Oefner, S. Nathakarnikitkool, M. Popp and G.K. Bonn, *Electrophoresis*, 13 (1992) 35.
260 X. Gu, Q. Chu, M. O'Dwyer and M. Zeece, *J. Chromatogr. A*, 881 (2000) 471.
261 K.D. McMurtrey, J. Minn, K. Pobanz and T.P. Schultz, *J. Agric. Food Chem.*, 42 (1994) 2077.
262 R.M. Lamuela-Raventos, A.I. Romero-Perez, A.L. Waterhouse and M.C. de la Torre-Boronat, *J. Agric. Food Chem.*, 43 (1995) 281.

263 B. Baderschneider and P. Winterhalter, *J. Agric. Food Chem.*, 48 (2000) 2681.
264 G.J. Soleas, J. Yan and D.M. Goldberg, *Methods Enzymol.*, 335 (2001) 130.
265 P.C. Hollman and M.B. Katan, *Free Radic. Res.*, 31 (1999) 75.
266 P.C.H. Hollman, J.M.P. van Trijp and M.N.C.P. Buysman, *Anal. Chem.*, 68 (1996) 3511.
267 P.C.H. Hollman, J.H.M. de Vries, S.D. van Leeuwen, M.J.B. Mengelers and M.B. Katan, *Am. J. Clin. Nutr.*, 62 (1995) 1276.
268 C. Stumpf, W. Andlauer and P. Fürst, *Lebensmittelchemie*, 55 (2001) 9.
269 D.W. Boulton, U.K. Walle and T. Walle, *J. Pharm. Pharmacol.*, 50 (1998) 243.
270 A.A.E. Bertelli, L. Giovannini, R. Stradi, A. Bertelli and J.P. Tillement, *Int. J. Tissue React.*, 18 (1996) 67.
271 A.A.E. Bertelli, L. Giovannini, R. Stradi, S. Urien, J.-P. Tillement and A. Bertelli, *Int. J. Clin. Pharm. Res.*, 16 (1996) 77.
272 A.A.E. Bertelli, L. Giovannini, R. Stradi, S. Urien, J.P. Tillement and A. Bertelli, *Drugs Exp. Clin. Res.*, 24 (1998) 51.
273 A. Bertelli, A.A.E. Bertelli, A. Gozzini and L. Giovannini, *Drugs Exp. Clin. Res.*, 24 (1998) 133.
274 G. Kuhnle, J.P.E. Spencer, G. Chowrimootoo, H. Schroeter, E.S. Debnam, K.S. Srai, C. Rice-Evans and U. Hahn, *Biochem. Biophys. Res. Commun.*, 272 (2000) 212.
275 H.E. Evans, *Vitam. Horm.*, 20 (1963) 379.
276 R.H. Bunnell, P. György, W.N. Pearson, The Vitamins, Vol. 6, Academic Press, New York, 1967, p. 261.
277 I.D. Desai, L.J. Machlin, J. Augustin and B.P. Klein, *Methods of Vitamin Assays 4/E*, Wiley, New York, 1985, p. 255.
278 S.L. Abidi and T.L. Mounts, *J. Chromatogr. A*, 670 (1994) 67.
279 T. Ando, Y. Nakayama and S. Hara, *J. Liq. Chromatogr.*, 12 (1989) 739.
280 P.J. Van Niekerk and R. Macrae, *HPLC in Food Analysis*, Academic press, London, 1988, p. 144.
281 Y. Satomura, M. Kimura and Y. Itokawa, *J. Chromatogr.*, 625 (1992) 372.
282 S.L. Abidi and T.L. Mounts, *J. Chromatogr. A*, 782 (1997) 25.
283 S. Strohschein, M. Pursch, D. Lubda and K. Albert, *Anal. Chem.*, 70 (1998) 13.
284 W.M. Stöggl, C.W. Huck, H. Scherz, M. Popp and G.K. Bonn, *Chromatographia*, 54 (2001) 179.
285 A.P. Bruins, *Trends Anal. Chem.*, 13 (1994) 37.
286 A.P. Bruins, *Trends Anal. Chem.*, 13 (1994) 81.
287 A.P. Bruins, T.R. Covey and J.D. Henion, *Anal. Chem.*, 59 (1987) 2642.
288 X. He, L. Lin and L. Lian, *J. Chromatogr. A*, 755 (1996) 127.
289 C.M. Whitehouse, R.N. Dreyer, M. Yamashita and J.B. Fenn, *Anal. Chem.*, 57 (1985) 675.
290 D.J. Carroll, I. Dzidic, R.N. Stillwell, K.D. Haegele and E.C. Hornig, *Anal. Chem.*, 47 (1975) 2369.
291 P.G. Waterman, *Phytochemistry*, 49 (1998) 1175.
292 P.G. Waterman, *Biochem. Syst. Ecol.*, 27 (1999) 395.
293 I.W. Southon and J. Buckingham, *Dictionary of Alkaloids*, Chapman & Hall, London, 1989.
294 G. Cordell, *Introduction to Alkaloids: A Biogenic Approach*, Wiley-Interscience, New York, 1981.
295 T. Robinson, *The Biochemistry of Alkaloids*, Springer, Berlin, 1981.
296 G.R. Waller and E.K. Nowacki, *Alkaloid Biology and Metabolism in Plants*, Plenum Press, New York, 1978.
297 R.H.F. Manske, R. Rodrigo, A. Brossi and G.A. Cordell, *The Alkaloids*, Academic Press, New York, 1950.

298 J.E. Saxton, *The Alkaloids. Specialist Periodical Reports, Volumes 1–5*, Royal Society of Chemistry, London, 1971–1975.
299 M.F. Grundon, *The Alkaloids. Specialist Periodical Reports, Volumes 6–13*, Royal Society of Chemistry, London, 1976–1983.
300 S.W. Pelletier, *Alkaloids: Chemical and Biological Perspectives*, Wiley, New York, 1983.
301 B. Xie, K. Ramanathan and B. Daielsson, *Trends Anal. Chem.*, 19 (2000) 340.
302 J. Khandurina and A. Guttman, *J. Chromatogr. A*, 943 (2002) 159.
303 R.J. Boughtflower, T. Underwood and C.J. Paterson, *Chromatographia*, 40 (1995) 329.
304 E. Rapp and E. Bayer, *J. Chromatogr. A*, 887 (2000) 367.
305 J. Hjerten, *J. Chromatogr.*, 473 (1989) 273.
306 K.J. Van Wijk, *Plant Physiol.*, 126 (2001) 501.
307 H. Thiellement, N. Bahrman, C. Damerval, C.R.M. Plomion, V. Santoni, D. de Vienne and M. Zivy, *Electrophoresis*, 20 (1999) 2013.
308 R. Bakry, W.M. Stöggl, E.O. Hochleitner, G. Stecher, C.W. Huck and G.K. Bonn, *Anal. Chem.*, (in prep.)
309 E.O. Hochleitner, R. Bakry, C.W. Huck, F. Flores, W.M. Stöggl, G. Stecher and G.K. Bonn, *Int. J. Mass Spectrom.*, 223–224 (2002) 519.
310 M.E. Juan, R.M. Lamuela-Raventos, M.C. de la Torre-Boronat and J.M. Planas, *Anal. Chem.*, 71 (1999) 747.
311 Z. Zhu, G. Klironomos, A. Vachereau, L. Neirinck and D.W. Goodman, *J. Chromatogr. B*, 724 (1999) 389.
312 C. Giachetti, C. Tognolo, P. Gnemi and A. Tenconi, *Chromatographia*, 50 (1999) 571.
313 D. Blache, I. Rustan, P. Durand, G. Lesgards and N. Loreau, *J. Chromatogr. B*, 702 (1997) 103.
314 L. Belguendouz, L. Frémont and M.T. Gozzelino, *Biochem. Pharmacol.*, 55 (1998) 811.
315 I. Erlund, G. Alfthan, H. Siren, K. Ariniemi and A. Aro, *J. Chromatogr. B*, 727 (1999) 179.
316 I. Erlund, T. Kosonen, G. Alfthan, J. Mäenpää, K. Perttunen, J. Kenraali, J. Parantainen and A. Aro, *Eur. J. Clin. Pharmacol.*, 56 (2000) 545.
317 J.F. Young, S.E. Nielsen, J. Haraldsdottir, B. Daneshvar, S.T. Lauridsen, P. Knuthsen, A. Crozier, B. Sandström and L. O'Dragsted, *Am. J. Clin. Nutr.*, 69 (1999) 87.
318 J.H.M. de Vries, P.C.H. Hollman, S. Meyboom, M.N.C.P. Buysman, P.L. Zock, W.A. van Staveren and M.B. Katan, *Am. J. Clin. Nutr.*, 68 (1998) 60–233.
319 J.H.M. De Vries, P.C.H. Hollman, I. van Amersfoort, M.R. Olthof and M.B. Katan, *J. Nutr.*, 131 (2001) 745.
320 P.L. Mauri, L. Iemoli, C. Gardana, P. Riso, P. Simonetti, M. Porrini and P.G. Pietta, *Rapid Commun. Mass Spectrom.*, 13 (1999) 924.
321 S.E. Nielsen, R. Freese, C. Cornett and L.O. Dragsted, *Anal. Chem.*, 72 (2000) 1503.
322 K. Ishii, T. Furuta and Y. Kasuya, *J. Chromatogr. B*, 759 (2001) 161.
323 P. Morrica, M. Marra, S. Seccia, M. Ventriglia and C.O. Moro, *American Lab.*, 29 (1997) 38.
324 J. Lu, H.K. Deai, S.A. Ross, H.M. Sayed and S.W. Pelletier, *J. Nat. Prod.*, 56 (1993) 2098.
325 B.S. Joshi, K.M. Moore, S.W. Pelletier and M.S. Puar, *Phytochem. Anal.*, 2 (1991) 20.
326 M. Ishibashi, Y. Ohizumi, T. Sasaki, H. Nakamura, Y. Hirata and J. Kobayashi, *J. Org. Chem.*, 52 (1987) 450.
327 L. Angenot, M.L. Belem-Pinheiro, A.F. Imbiriba da Rocha, P. Poukens-Renwart, J. Quetin-Leclercq and R. Warin, *Phytochemistry*, 29 (1990) 2746.
328 A. Pirillo, L. Verotta, P. Gariboldi, E. Torregioni and E. Bombardelli, *J. Chem. Soc. Perkin Trans. I*, (1995) 583.

329 Y.F. Hallock, J. Dai, H.R. Bokesch, K.B. Dillah, K.P. Manfredi, J.H. Cardellina and M.R. Boyd, *J. Chromatogr. A*, 688 (1994) 83.
330 S.S. Lee and R.W. Doskotch, *J. Nat. Prod.*, 59 (1996) 738.
331 J. Quetin-Leclercq, L. Angenot, L. Dupont and N.G. Bisset, *Phytochemistry*, 27 (1988) 4002.

This page is intentionally left blank

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 23

Forensic analysis

MACIEJ J. BOGUSZ and ANGEL CARRACEDO

CONTENTS

23.1 INTRODUCTION

Forensic science serves society in very special way – it is a combination of various scientific disciplines, focused on the clarification of a given bit of reality for the sake of justice. What is expected of the forensic scientist is an unequivocal answer. Conflicting interests in his report have a major influence on quality management in forensic science [1]. Forensic science seldom uses "official" methods or "closed" analytical systems, where nothing can be changed or modified. Since the methods he uses are modified from the literature to fit the physical evidence on hand, it is the responsibility of the toxicologist to provide data of incontrovertible quality. Forensic science seldom deals with large series of similar samples. This differentiates forensic analysis from clinical chemistry or pharmaceutical analysis. In addition, the time factor is not so critical. Therefore, in forensic laboratories high-throughput chromatography, so important in drug discovery studies, is practically non-existent. Forensic analysis very often deals with single samples, which may contain a broad spectrum of unpredictable compounds. The problems of validation in forensic toxicology were recently discussed [2,3]. The following validation parameters are generally accepted: selectivity, sensitivity, accuracy, and stability. Additionally, such parameters as reproducibility and ruggedness are particularly important in forensic toxicology, due to possible control analysis of the same sample in a second laboratory.

23.2 DRUGS OF ABUSE

Substances of abuse form a large group consisting of compounds differing widely in their origin, availability, and chemical nature. Although many drugs, such as alcohol and

tobacco are also abused, this chapter is focused on only illicit drugs. The analysis of illicit drugs belongs to most important challenges of forensic and clinical toxicology. As any other branch of forensic sciences, forensic toxicological analysis must also apply and keep particularly high standards of quality. This is for one reason: analytical results may have a direct and permanent impact on the fate of the persons involved. Consequently, forensic toxicological examinations are subjected to very tight scrutiny and are often performed in several laboratories in the same case. This requirement should apply not only primarily to forensic cases, but also to all kinds of clinical analysis for illicit drugs. It must be kept in mind that each result of drug analysis has potentially forensic relevance, irrespective of the primary purpose of examination.

It has often been said that the interpretation of results is the most important and most difficult part of forensic toxicology. Notwithstanding the value of a correct interpretation, it must be also said that the correct result enables further action. This is particularly true in situations, where the analytical result itself may serve as an evidence of illegal action. The introduction of *per se* laws in traffic legislation may serve as an example. Laws limiting the allowable concentration of alcohol in body fluids of a driver have been in existence for a long time, but only recently have such laws been introduced in various countries concerning other drugs of abuse, like amphetamines, cannabis, cocaine, and opiates [4–6]. In their case, the law forbids any use by drivers, and the legal limits are defined by the quality of analytical methods, practically reflecting the limits of detection. The analysis of drugs of abuse is important not only in enforcement of traffic safety. It also enables the differentiation between chronic and occasional drug users and allows the source of a particular batch of illegal drug to be identified.

23.2.1 Opioid agonists

23.2.1.1 Opiates in plant material and illicit preparations

All natural and semisynthetic opiates originate from opium, a dried, brown juice obtained from green heads of *Papaver somniferum*. The alkaloid composition in the plant depends on many factors, such as climatic conditions, harvesting time, soil composition, and plant variety [7–9]. There are two main methods of clandestine heroin production: the lime method used in Southeast Asia and the ammonia method used in Southwest Asia. Both methods give similar yields of morphine, codeine, and thebaine, but the content of noscapine and papaverine is much higher with the ammonia method [10]. Heroin from Southeast Asia predominates in the drug market of the United States; the Southwest-Asian-type heroin, originating in Turkey, Lebanon, Afghanistan, and Iran is found mainly on the European market [11–14]. The latter heroin contains over 10% of noscapine and over 2% of papaverine. Noscapine may be used as an adulterant of street heroin [15].

Thin-layer chromatography (TLC) may be used for a preliminary examination of illicit heroin samples [16–18]. Generally, the sensitivity and selectivity of TLC is not high enough to separate all relevant compounds and to differentiate between several batches of heroin. For this, gas chromatography (GC) is ideally suited [19]. Besides opiates and adulterants, some volatile substances occluded in illicit heroin may facilitate the positive

identification of a particular batch of drug [20]. High-pressure liquid chromatography (HPLC) has also been applied to heroin profiling. Hays *et al.* [21] used reversed-phase HPLC with multi-wavelength detection for quantification of heroin adulterants. Besides opiates, the following compounds were analyzed: caffeine, methaqualone, nicotinamide, phenobarbital, phenolphthalin, and *N*-phenyl-2-naphtylamine. Capillary electrophoresis (CE) has also found application in the analysis of illicit heroin seizures [22,23]. This method combines two important features of other column chromatographic methods: it assures very high resolution, like capillary GC, and is applicable to polar and thermally unstable compounds, like HPLC. Therefore, CE is particularly useful for the analysis of drug mixtures, like illicit heroin samples. Since the composition of illicit heroin is sometimes very complicated and a single method may hardly detect all relevant constituents, a multi-method approach is usually preferred. Hernandez *et al.* [24] used a combination of HPLC/DAD and GC/NPD for the identification of illicit heroin and cocaine constituents. Chiarotti *et al.* [25] applied headspace GC to the detection of volatiles in heroin, TLC and GC/MS to the opiates and adulterants, and HPLC to the sugar diluents. Illicit drugs have also been analyzed for Fe and Zn content by atomic absorption spectroscopy. Another multi-method approach was applied by Besacier *et al.* [26], who used, first, GC/FID for the identification of opiates and adulterants in heroin samples; secondly, GC/FID for the impurities; and thirdly, the isotope ratios $^{13}C/^{12}C$, calculated with a GC isotope-ratio mass spectrometer. Chromatographic methods for the analysis of heroin seizures have been reviewed by Chiarotti and Fucci [27].

23.2.1.2 Opiates in biological samples of human origin

After administration by any route, heroin is rapidly de-acetylated to 6-monoacetylmorphine (6-MAM) and then to morphine. Morphine is consecutively coupled with glucuronic acid to 3- and 6-glucuronides (M3G and M6G). Since the first de-acetylation step is very rapid (the half-life of heroin has been estimated at 3–8 min), only metabolites of heroin are usually detectable in blood [28–31]. In urine, mainly conjugated morphine is excreted, as well as free morphine, 6-MAM, and a very small fraction of unchanged heroin. It must be stressed that after de-acetylation, heroin follows metabolic routes which are common to morphine and, to some extent, also to codeine. Therefore, only 6-MAM may be regarded as a heroin-specific metabolite and as a marker of heroin use. This analyte is usually detectable in the blood and urine samples of heroin users [32]. TLC can be used for the detection of heroin metabolites in urine extracts [33–35], but this technique is not sensitive enough for examination of blood or serum. GC, usually with mass spectrometric detection, is the most frequently used technique for the detection and quantification of 6-MAM, morphine, and associated opiates in biological material. Several GC/MS methods have been developed for the determination of morphine and 6-MAM in urine [36–41]. The latter compound may also be found in blood samples, taken shortly after heroin abuse [42]. It must be stressed that only acetylcodeine (AC) may be regarded as specific marker of illicit heroin. AC is produced from codeine during acetylation of opium. Its content in illicit heroin ranges from 2 to 7% [10]. O'Neal and Poklis [43,44] developed a GC/MS method for the detection of AC in urine and found this drug in over 30% of

morphine-positive specimens in concentrations ranging from 100 to 4600 μg/L. 6-MAM was found in over 70% of these samples. Codeine, a possible metabolite of AC, was found in all urine samples. Staub *et al.* [45] detected AC in over 85% and 6-MAM in over 94% of urine samples from heroin users. Bogusz *et al.* [46] determined heroin markers in 25 morphine-positive urine samples by liquid chromatography/atmospheric-pressure chemical-ionization mass spectrometry (LC/APCI-MS). Codeine 6-glucuronide was found in all samples, but codeine, noscapine, 6-MAM, papaverine, DAM, and AC in only some of the samples. Heroin metabolites and associated substances in blood samples have usually been determined simultaneously with GC/MS. Chromatographic methods for opiates were recently reviewed by Bogusz [47]. A comparison of analytical conditions and performance of selected GC/MS procedures is shown in Table 23.1.

HPLC has one important advantage over GC in opiate analysis: It allows the determination of polar metabolites, mainly glucuronides, simultaneously with the primary drugs. The hydrolysis step, which must be applied for glucuronide cleavage before GC analysis, can be omitted, and the important differentiation between the active morphine-6-glucuronide (M6G) and inactive morphine 3-glucuronide (M3G) is feasible. HPLC with diode-array detection (DAD) was applied in a quantitative method for heroin, 6-MAM, 3-MAM, morphine, M3G, M6G, normorphine (NM), codeine (C), and codeine 6-glucuronide in human serum, extracted with a C_{18} SPE cartridge [48]. Zuccaro *et al.* [49] developed an electrospray ionization (ESI) LC/MS method for the simultaneous determination of heroin, 6-MAM, morphine, M3G, and M6G in serum. The drugs were extracted with SPE C_2 cartridges and separated on a normal-phase silica column with MeOH/MeCN/formic acid as the mobile phase. The LOD for heroin was 0.5 μg/L and for 6-MAM 4 μg/L. Bogusz *et al.* [50] used LC/APCI-MS for the determination of heroin metabolites (6-MAM, morphine, M3G, and M6G) in body fluids of heroin users. The drugs were extracted with C_{18} cartridges; the LODs ranged from 0.1 to 1 μg/L. Subsequently, Bogusz *et al.* [51] extended the LC/APCI-MS method to the determination of 6-MAM, M3G, M6G, morphine, codeine, and C6G, with a deuterated internal standard for each compound. The detection limits ranged from 0.5 to 2.5 μg/L. This method was applied to routine forensic examinations of blood samples, taken from suspected heroin abusers [52]. Selected liquid-chromatographic methods for opiates are summarized in Table 23.2.

Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) have also been applied to the determination of M3G in urine, with either direct injection or SPE extraction [59]. Tagliaro [60] used capillary electrophoresis with DAD for the determination of morphine in hair samples of drug abusers. Wu [61] used the same method for the detection of morphine and M3G in the urine of heroin addicts. Ion-trap MS was used for morphine, isolated by CZE. The sensitivity was lower than that of LC/MS methods [62].

23.2.1.3 Some opioids in body fluids

Methadone is a synthetic opioid with a very long elimination half-life (15–55 h), which is used mainly as a heroin substitute in the treatment of heroin addicts.

TABLE 23.1

SELECTED GAS-CHROMATOGRAPHIC METHODS FOR HEROIN, 6-MAM, AND OTHER OPIATES

Drug	Sample	Isolation	Derivatization	Column, conditions	Detection	LOD (μg/L)	Ref.
Heroin, 6-MAM	Serum, saliva, urine, hair	SPE	BSTFA/TMCS	HP 1, 70°–250°	EI-MS (SIM)	1	[38]
Heroin, 6-MAM, M	Body fluids, organs	SPE	MBTFA	Rtx-5, 150°–290°	EI-MS (SIM)	1	[39]
M, C, NM	Plasma	l/l pH 9.5	HBFA	HP-1, 100–257°C	NCI-MS (SIM)	1 pg	[40]
M	Plasma	l/l pH 9	PFPA	HP-5, 150–250°C	EI-MS (SIM)	0.2	[41]
6-MAM	Serum, urine, hair	SPE C_{18}	PFPA	n.s.	EI-MS (SIM)	n.s.	[42]
AC, 6-MAM, M, C, NC	Urine	SPE	Propionylation	HP-1, 170°–280°	EI-MS (SIM)	0.5	[43]

M = morphine, 6-MAM = 6-monoacetylmorphine, NM = normorphine, C = codeine, AC = acetylcodeine, NC = norcodeine, n.s. = not stated.

TABLE 23.2

SELECTED LIQUID-CHROMATOGRAPHIC METHODS FOR HEROIN, 6-MAM, MORPHINE, AND OTHER OPIATES

Drug	Sample	Isolation	Column, elution conditions	Detection	LOD (μg/mL)	Ref.
DAM, 6-MAM, M, M3G, M6G, C	Serum	SPE C_2	Silica, MeCN/MeOH/H_2O/HCOOH	ESI-MS	0.5–4	[49]
6-MAM, M, M3G, M6G	Body fluids	SPE C_{18}	ODS, MeCN/$HCOONH_4$	APCI-MS	0.1–1	[50]
6-MAM, M, M3G, M6G, C, C6G	Body fluids	SPE C_{18}	ODS, MeCN/$HCOONH_4$	APCI-MS	0.5–100	[51]
M, M3G, M6G	Plasma	SPE C_2	Phenyl, MeOH/HCOOH	ESI-MS-MS	3.8–12	[53]
M, M3G, M6G, NM	Plasma, urine	SPE C_2	ODS, H_2O/MeCN/THF/HCOOH	ESI-MS-MS	0.5–2.5	[54]
MAM, M, M3G, M6G, C, C6G	Blood	SPE C_8	ODS, MeCN/$HCOONH_4$	ESI-MS	0.5–5	[55]
M, M3G, M6G	Serum	SPE C_{18}	ODS, MeCN/$HCOONH_4$	ESI-MS	1–5	[56]
M, M3G, M6G	Plasma	SPE C_{18}	ODS, H_2O/MeCN/HCOOH	ESI-MS-MS	0.25–0.5	[57]
M, M3G, M6G	Plasma	SPE C_{18}	Silica, H_2O/MeCN/HCOOH	ESI-MS-MS	0.5–1	[58]

DAM = diacetylmorphine, M = morphine, M3G = morphine-3-glucuronide, M6G = morphine-6-glucuronide, C = codeine, C6G = codeine-6-glucuronide, NM = normorphine, Fl = fluorescence detection, EC = electrochemical detection.

Methadone is metabolized to the inactive 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and to a lesser extent to 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP). All of these compounds contain a chiral center, and the enantiomers could be separated. This is of pharmacological relevance, since (R)-(−)-methadone (levomethadone) is 25–50 times more potent than the (S)-(+)-methadone. Commercial methadone preparations may contain either the racemic form or levomethadone. Several GC/MS procedures have been described for the determination of methadone, EDDP, and EMDP in plasma, urine, and hair. Usually, solvent extraction or SPE was applied [63–65]. The detection limits for all substances were 5 μg/L. Sporkert and Pragst [66] applied headspace solid-phase micro-extraction (SPME), coupled with electron ionization (EI) GC/MS to the determination of methadone, EDDP, and EMDP in human hair. Absolute extraction yields were in the range of 10.5 to 17.4% for all compounds examined; the detection limits were 0.03 μg/g for methadone and 0.05 μg/g for metabolites. HPLC with UV detection was used for the separation of methadone and EDDP enantiomers in urine samples obtained from methadone maintenance patients and from patients with chronic pain [67]. The drugs were separated on a RP8 column, coupled serially with a chiral AGP column. The limit of detection (LOD) was 9 μg/L. This column combination improved the separation and prolonged the lifetime of the chiral column. Kintz *et al.* [68] published the first LC/MS method for the enantioselective separation of methadone and EDDP in hair, using deuterated analogs of all compounds for quantification. Ortelli *et al.* [69] applied LC/MS in the enantioselective determination of methadone in saliva and serum.

Buprenorphine (BP) is a synthetic opiate agonist/antagonist, which was initially used as an analgesic and was later introduced for the treatment of heroin addicts [70]. This drug possesses primary addiction potential and is frequently abused. BP as well as its active metabolite, norbuprenorphine (NBP), may be determined in serum or urine with GC/MS, usually after derivatization [71–73]. BP and NBP may be determined without derivatization by HPLC with electrochemical detection [74]. However, the limit of detection reported for this method (5–35 μg/L) is about 10 times higher than for GC/MS. Much better results were obtained with LC/MS or LC/MS/MS. These methods are more sensitive than GC/MS and are also applicable to glucuronides [75–78]. Dihydrocodeine (DHC) is a semisynthetic opiate, which was first used as an analgesic and antitussive drug. In the late 1980s, DHC was uncritically and extensively used in Germany in the treatment of heroin addicts, and a number of cases of fatal poisoning were reported. Like all opiates, DHC possesses a primary addiction potential and may be abused. In the body, DHC undergoes N-de-methylation to nor-DHC and *O*-demethylation to the very toxic dihydromorphine (DHM). All of these drugs are conjugated to form the appropriate glucuronides [79,80]. The excretion of DHC metabolites in urine was studied by Balikova *et al.* [81,82], who applied GC/MS after SPE and cleavage of conjugates. Bogusz [52] demonstrated that all metabolites of DHC (nor-DHC, DHM, DHC-6-G, DHM-3-G, DHM-6-G, and nor-DHM-3-G) can be detected directly in urine extracts by LC/APCI-MS without hydrolysis and derivatization (Fig. 23.1).

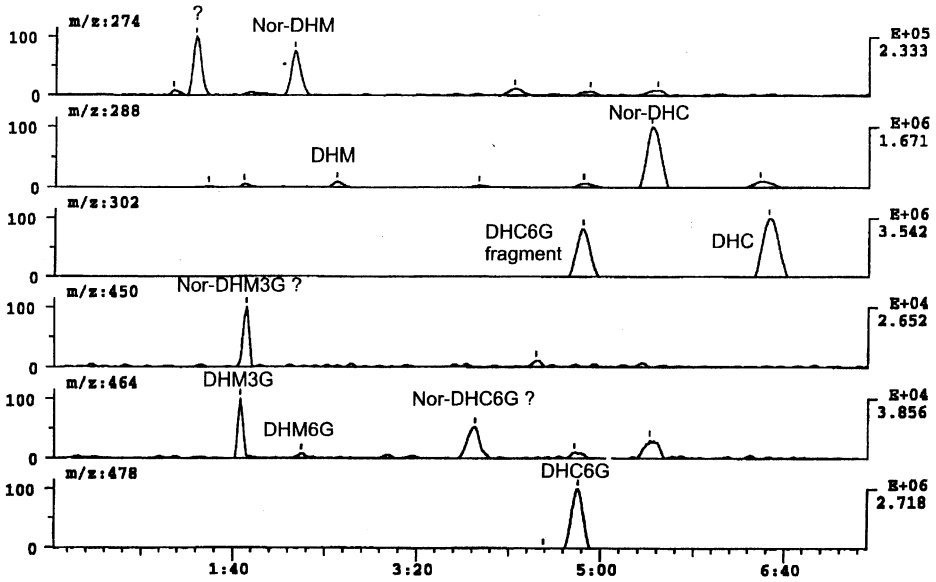


Fig. 23.1. LC/APCI-MS chromatogram of a urine extract from a sample taken 1 h after oral administration of 10 mg dihydrocodeine. DHC, dihydrocodeine; DHM, dihydromorphine; DHM3G, DHM-3-glucuronide; DHM6G, DHM-6-glucuronide; DHC6G, DHC-6-glucuronide. (Reprinted from Ref. 52 with permission.)

23.2.2 Cocaine

Cocaine is one of the most important "hard drugs" of modern society. According to the National Institute of Drug Abuse, in 1997 about 1.5 million Americans were identified as current cocaine users [83]. Although the synthesis of cocaine is possible, essentially all cocaine in illicit traffic is produced by extraction from the leaves of *Erythroxylum coca*. The *Coca* leaves are mixed with water and lime, and extracted with kerosene or gasoline. The kerosene extract is mixed with dilute sulfuric acid, the aqueous layer is collected and made basic with ammonia or lime. The precipitate, known as coca paste, contains 40 to 70% cocaine as a mixture of base and salt, as well as kerosene, sulfuric acid and other impurities. Coca paste, a very dangerous intermediate product, is smoked in South America [84]. To form pure cocaine base, the coca paste is dissolved in dilute sulfuric acid, and potassium permanganate is added to oxidize the cinnamoyl cocaines. After filtration, ammonia is added to the solution. Precipitated cocaine base is collected, washed, and dried. In the last step, dried cocaine base is dissolved in ether or acetone and converted to cocaine hydrochloride by the addition of hydrochloric acid. The resulting white, microcrystalline precipitate ("snow") is dried and packaged for sale. Street cocaine preparations are usually adulterated with some other local anesthetics and diluted with neutral compounds. Cocaine hydrochloride is water-soluble and may be applied intranasally ("snorting") or intravenously. In order to obtain a smokable form of drug,

cocaine salt must be converted again to the volatile base. This procedure, known as "free-basing", involves dissolving cocaine hydrochloride in water, alkalization, and extraction with ether and evaporation. Free-basing created an extremely hazardous situation due to risk of fire and explosion and was replaced by "crack". Crack is produced by heating a cocaine hydrochloride solution with sodium carbonate, cooling, and filtering. The term "crack" refers to the crackling sound when the drug is smoked. Nowadays, crack is the most prevalent and most dangerous form of street cocaine.

23.2.2.1 Cocaine and associated compounds in street samples

Illicit cocaine preparations are a very complex mixtures of various substances, which may exist in different proportions in samples originating from different sources or even from different production batches. This complexity allows the identification of particular samples of the drug through analytical "fingerprinting". Ensing *et al.* [85] measured the relative abundance of six congeners present in illicit cocaine with GC/ NPD. Janzen *et al.* [86] calculated area ratios of four congeners, also using GC/NPD. Headspace GC/MS, combined with microwave irradiation, was applied to the analysis of residual solvents in illicit cocaine hydrochloride samples [87]. Capillary electrophoresis (CE) has been used for the analysis of street cocaine. CE, coupled with ES-TOF-MS was applied to the detection of various drugs of abuse, including cocaine [88]. Cocaine and adulterants were quantified by CE [89]. Chiarotti and Fucci [27] have reviewed chromatographic methods used for the analysis of cocaine seizures.

In recent years, the transport of internally concealed cocaine, so-called "body packing" became a standard smuggling procedure, especially in airline transport [90]. Cocaine containers are usually machine-made and consist of several (up to 7) layers of wax, latex, and foil. One drug courier may swallow more than 100 containers with a total gross weight more than 1000 g [91]. "Body packing" is an extremely hazardous smuggling method due to the possibility of container rupture. In such cases, acute, fatal intoxication occurs, with very high levels of unmetabolized drug [92,93]. Cocaine trafficking, *i.e.* the exchange of illicit drug for money is an everyday occurrence. As a consequence, most American currency has become contaminated with cocaine. Oyler *et al.* [94] and Negrusz *et al.* [95] demonstrated the ubiquitous presence of cocaine on $1 and $20 bills. Analytical aspects of the detection of cocaine and other drugs of abuse on banknotes have recently been addressed by Sleeman *et al.*[96,97], who developed a thermal-desorption APCI-MS/MS procedure for this purpose.

23.2.2.2 Cocaine and metabolites in biological fluids and tissues

After administration to human subjects, cocaine is extensively metabolized. The most prevalent metabolites are benzoylecgonine (BZE) and ecgonine methyl ester (EME). Both of these compounds are then degraded to ecgonine. The biological half-life of cocaine in plasma ranges from 11 to 87 min, for BZE it is 7.5 h, and for EME 3.6 h. Simultaneous ingestion of cocaine and alcoholic beverages results in formation of cocaethylene, ecgonine ethyl ester and nor-cocaethylene [98]. Cocaine metabolism occurs not only

in corpore, but also *in vitro*. Enzymatic hydrolysis of cocaine to EME and, partially, to BZE may be inhibited by the addition of esterase inhibitors, like sodium fluoride [99]. Also, some drugs, which inhibit the activity of serum cholinesterase, like amitriptyline or procainamide, may inhibit the degradation of cocaine or cocaethylene and prolong the half-life of these drugs in serum [100]. The final product of cocaine degradation, ecgonine, may be detected for much longer than BZE and EME, even in unpreserved blood samples [101]. Cocaine is often used together with opiates, particularly heroin. Therefore, several authors have developed GC methods for the simultaneous determination of cocaine, heroin, and metabolites of both compounds in blood, serum [37], or hair [102]. Lately, all analysts have almost exclusively used the mass spectrometer as a detector after GC separation. One exception was the study of Watanabe *et al.* [103], who applied GC with surface-ionization detection (SID) to measure cocaine and cocaethylene in blood extracts with a LOD of 1.5 to 3 μg/L. Since cocaine is rapidly metabolized, all modern GC/MS methods are designed for a broad spectrum of cocaine transformation products. Crouch *et al.* [104] developed a GC/MS method for the determination of cocaine and its metabolites in biological fluids and tissues. *tert*-Butyldimethylsilane afforded stable derivatives with mass spectral ions of higher mass than TMS derivatives. The important problem of detectability of ecgonine – a polar, final metabolite of cocaine – was addressed by Logan *et al.* [105,106], who developed a GC/MS method for the determination of this compound. Extractive propylation, followed by solvent extraction of ecgonine as well as cocaine and its major metabolic products from urine was applied. Among 11 metabolites, BZE, EME, and ecgonine were excreted in the highest concentrations. Ecgonine was detected as the only cocaine metabolite in old, unpreserved blood samples.

Methylecgonidine (anhydroecgonine methyl ester) was identified as a specific marker of cocaine (crack) smoking [107]. Methylecgonidine was determined in blood with GC/single-ion monitoring (SIM) MS after derivatization with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MBDSTFA). Cocaine, BZ, and EME were analyzed simultaneously. The concentrations of methylecgonidine ranged from 3 to 34 μg/L blood [108]. A hydrolytic product of methylecgonidine – ecgonidine – was isolated in acidic medium by SPE and analyzed by GC/MS after butyldimethylsilylation [109]. Cocaine and its metabolites have frequently been determined in hair or nails of autopsy specimens, where the detectability is much higher than in post-mortem blood or urine samples [110, 111].The problem of intrauterine exposure to cocaine has been addressed from an analytical point of view. Cocaine metabolites, predominantly BZE, were detected in meconium [112,113], in vernix caseosa [114], and in amniotic fluid and umbilical-cord tissue [115]. Table 23.3 lists selected gas-chromatographic methods used recently for cocaine analysis.

The advent of LC/MS has brought substantial progress to liquid chromatography of cocaine metabolites, which may be detected without derivatization by this technique. Bogusz *et al.* [52,116] have used LC/APCI-MS for the examination of various drugs of abuse, among them cocaine, BZE, and EME in biological fluids. The LOD ranged from 0.2 to 0.5 μg/L. Tandem LC/ESI-MS was used for the simultaneous determination of cocaine and opiate metabolites in biological fluids with a LOQ of 5 μg/L [117]. Sosnoff *et al.* [118] have detected BZE in dried blood spots with tandem LC/MS. A LOD of 2 μg/L was

TABLE 23.3

SELECTED GAS-CHROMATOGRAPHIC METHODS FOR COCAINE AND METABOLITES

Drug	Sample	Isolation	Derivatization	Column, conditions	Detection	LOD (μg/L)	Ref.
COC, BZE, EME, NCOC, CE	Fluids/tissues	SPE	BSTFA	HP-1, 70°–250°	EI-MS (SIM)	1–5	[37]
COC	US currency	SPE	–	HP-5, 130°–280°	EI-MS (f.sc.)	1 ng	[94]
COC, CE	Blood	SPE	–	DB-17, 150°–280°	GC-SID	1.5–3	[102]
COC, BZE, EME, ECG, CE, NCOC	Blood	L/L	Alkylation	BP-5, 80°–295°	EI-MS (SIM)	<10	[104]
COC, BZE, EME, ECG	Urine	SPE	Propylation	BP-5, 100°–295°	EI-MS (SIM)	100	[105]
COC, BZE, EME, CE	Fluids/tissues	SPE	MBDSTFA	DB-5, 115°–280°	PCI-MS (SIM)	2	[103]
COC, BZE, EME, ECG, AEME	Serum	SPE	MBDSTFA	HP-5, 55°–310°	EI-MS (SIM)	1	[107]
COC, BZE, NCOC, CE	Toenails	SPE	MSTFA	BD-5, 150°–260°	EI-MS (SIM)	0.3 ng	[110]

COC = cocaine, BZE = benzoylecgonine, EME = ecgonine methyl ester, CE = cocaethylene, NCOC = norcocaine, AEME = anhydroecgonine methyl ester, f.sc. = full scan, MSSID = surface-ionization detector.

achieved with a 12-μL sample. This technique was used for automated urinalysis by Jeanville *et al.* [119]. Centrifuged urine samples were injected into a LC/MS/MS system, equipped with an on-line extraction unit. The total analysis time was under 4 min, and the LOD for EME, BZ, and cocaine was 0.5, 2.0, and 0.5 μg/L, respectively. Skopp *et al.* [120] carried out a study on the stability of cocaine and metabolites at different temperatures, using LC/MS. Only ecgonine appeared to be stable at room temperature. Singh *et al.* [121] applied acetonitrile precipitation to the isolation of cocaine and metabolites from plasma. The drugs were determined with LC/APCI-MS/MS. Table 23.4 presents some recently published liquid-chromatographic methods for cocaine analysis.

TABLE 23.4

SELECTED LIQUID-CHROMATOGRAPHIC METHODS FOR COCAINE AND METABOLITES

Drug	Sample	Isolation	Column, elution conditions	Detection	LOD (μg/L)	Ref.
COC, BZE, EME	Fluids, tissues	SPE	ODS, ammonium formate isocratic	APCI-MS	0.2–0.5	[115]
COC, BZE, EME, CE, AEME	Blood, urine	L/L	C8, ammonium formate, isocratic	ESI-MS-MS	5	[116]
BZE	Blood spots	Methanol	ODS, ammonium acetate isocratic	APCI-MS-MS	2	[117]
COC, BZE	Urine	Filtration	C8, ammonium formate, gradient	ESI-MS-MS	2.5	[118]
COC, BZE, EME, NCOC	Plasma	Acetonitrile	ODS, ammonium acetate, gradient	APCI-MS-MS	2–5	[120]

COC = cocaine, BZE = benzoylecgonine, EME = ecgonine methyl ester, CE = cocaethylene, NCOC = norcocaine, AEME = anhydroecgonine methyl ester, ECG = ecgonine.

23.2.3 Amphetamines and their precursors

Amphetamine and methamphetamine are powerful stimulants, and some of their methylenedioxy analogs, such as methylenedioxymethamphetamine (MDMA), methylenedioxyethylamphetamine (MDEA), and methylenedioxyamphetamine (MDA) have been tested in the treatment of psychiatric disorders. These drugs, known under the common name "designer amphetamines" or "Ecstasy", are discussed in Sec. 23.2.4. Generally, psychoactive phenethylamines present a large group of compounds of very different forensic relevance. Some of them, like MDMA or MDA, are illicit drugs with no therapeutic applications, while others, like diethylpropione or methylphenidate are

prescription medicines, and some, like pseudoephedrine or phenylpropanolamine, occur in over-the-counter preparations. In addition, there are some prescription drugs, mainly anorectics, which are metabolized in the body to amphetamine or methamphetamine. Proper interpretation of the results after administration of these drugs is thus of primary importance.

23.2.3.1 Amphetamines in street samples

Since amphetamines and designer amphetamines (Ecstasy) are often present in the same street preparation, the analytical method should be able to differentiate all related phenethylamines in one run. Negative-ion chemical-ionization (NICI)-MS has been applied to the study of the fragmentation of derivatized amphetamine, methamphetamine, MDMA, MDA, MDEA, MBDB, and deuterated analogs [122]. Solvent-free SPME, combined with GC/NPD was applied to impurity profiling of illicit Ecstasy and amphetamine samples [123], and HPLC with UV detection at 200 nm was similarly used. The drugs were separated on a base-deactivated column with a LOD ranging from 0.06 to 0.1 μg/g [124]. Amphetamine, methamphetamine, MDMA, MDEA, MDA, as well as diethylpropione and ephedrine were determined in street samples by HPLC/DAD and CE/DAD. For the latter method, a chiral column was used to separate enantiomers [125]. Backofen *et al.* [126] applied nonaqueous CE with electrochemical detection for the determination of amphetamines, cocaine, and benzoylecgonine in illicit drugs. Amphetamine, methamphetamine, cathinone, methcatinone, ephedrine, norephedrine, and pseudoephedrine were labeled with 4-fluoro-7-nitrobenzofurazane and subjected to MEKC with laser induced fluorescence (LIF) detection. For chiral separations, cyclodextrins with sodium dodecylsulfate (SDS) were applied [127].

23.2.3.2 Amphetamines in biological fluids and tissues

All amphetamines undergo extensive metabolism and are excreted in the urine. Since all of these drugs are strong bases, their elimination depends on the pH of the urine [128]. The last positive-screened urine specimen was observed after 9 day of imprisonment. The group of psychoactive phenethylamines comprises over-the-counter preparations, prescription drugs, as well as illicit drugs, and thus identification of particular compounds becomes necessary. An overview of all chromatographic methods for amphetamines and related drugs was published recently by Cody [129]. Valentine *et al.* [130] studied the methods of identification of phenethylamines intended for emergency ward needs. Licit and illicit phenethylamines were isolated from urine with chloroform/2-propanol, derivatized on-column with heptafluorobutyric acid (HFBA) and subjected to GC/MS. Vorce *et al.* [131] compared the precision and quantitative accuracy of ion-trap and quadrupole mass detectors for the analysis of different classes of abused drugs, extracted from urine. Amphetamine, methamphetamine, 11-nor-Δ^9-tetrahydrocannabinol 9-carboxylic acid (THCCOOH), morphine, codeine, and 6-MAM were analyzed. The sensitivity and accuracy of the ion-trap mass analyzer and of quadrupole-SIM measurements were about the same for all compounds, except 6-MAM. After SPME of

amphetamine and methamphetamine from serum, Lee *et al.* [132] subjected them to headspace derivatization with HFBA vapor, taking advantage of their volatility, and then quantified them by GC/MS. Jurado *et al.* [133] also isolated amphetamine, methamphetamine, MDMA, and MDA from urine with SPME, using the headspace technique. The drugs were derivatized with trifluoroacetic acid (TFA) by exposing the fibers in the headspace of another vial to the vapors, and then desorbed in the injection port of the GC/MS instrument. With SIM, the LOQ ranged from 10 to 20 μg/L. Okajima *et al.* [134] isolated amphetamine and methamphetamine from whole blood by headspace/SPME. The drugs were derivatized with pentafluorobenzoyl (PFB) bromide in a headspace vial and subjected to GC/MS-SIM. The LOD was 0.5 μg/L, and the method was applied to autopsy cases. Hensley and Cody [135] analyzed the enantiomers of amphetamines and Ecstasy. The drugs were extracted from urine with solvent, derivatized with trifluoroacetyl-l-prolyl chloride, and analyzed by GC/MS.

LC has also been applied to psychoactive phenethylamines. Amphetamine and 8 other phenethylamines were analyzed in 250 μL of plasma, using automatic on-line extraction, column switching, and HPLC with UV detection at 210 nm, with a LOD ranging from 50 to 250 μg/L [136]. Much better sensitivities were achieved with LC/MS. Amphetamine, methamphetamine, MDMA, MDA, MDEA, as well as eight other phenethylamines were extracted with ether from serum and urine, derivatized with phenylisothiocyanate and subjected to HPLC with either APCI-MS or UV-DAD detection. LC/APCI-MS provided *ca.* 10 times better detection than UV, with a LOD ranging from 1 to 5 μg/L [137]. In a subsequent study by the same group [138], the derivatization step as well as DAD was abandoned in favor of to LC/APCI-MS-SIM, with a LOD ranging from 1 to 5 μg/L. This method was applied in routine casework [52]. Kataoka *et al.* [139] applied SPME to the isolation of amphetamine, methamphetamine, MDMA, MDEA, and MDA from urine. The drugs were desorbed by mobile-phase flow and detected *via* ESI-MS-SIM, with a LOD below 2 μg/L urine.

Capillary electrophoretic immuno-assay was applied in the detection of amphetamines, designer amphetamines, and other drugs of abuse in urine. The results were validated with GC/MS [140]. Enantiomers of amphetamine, methamphetamine, MDMA, MDA, methadone, and its metabolite, EDDP, were separated using CE with a buffer, containing (2-hydroxypropyl)-β-cyclodextrin [141]. Capillary electrophoresis, coupled with ion-trap mass spectrometry was applied to the determination of amphetamine, methamphetamine, MDA, and MDMA in urine extracts [142]. The sensitivity was comparable to that achieved with UV detection, but the selectivity was much greater, enabling detection of amphetamines even in unresolved peaks. Chromatographic methods applied to amphetamines are summarized in Tables 23.5 and 23.6.

23.2.3.3 Amphetamine precursors in biological fluids and tissues

In addition to illicit amphetamine and its analogs, there are several therapeutic drugs that are metabolized to amphetamine or methamphetamine. This may create difficulties in the interpretation of results. For example, mefenorex is an anorectic, which is partially metabolized to amphetamine. Kraemer *et al.* [143] identified thirteen metabolites in urine

TABLE 23.5

SELECTED GAS-CHROMATOGRAPHIC METHODS FOR AMPHETAMINES

Drug	Sample	Isolation	Derivatization	Column, conditions	Detection	LOD (μg/L)	Ref.
A, MDMA, impurities	Illicit drugs	L/L or SPME	–	SPB-1, 80°–300°	FID		[123]
A	Urine	SPE	HFBA	DB-5, 65°–300°	EI-MS (Q,IT)	–	[130]
MDMA, MDA, MDEA	Urine	L/L	HFBA	HP-1, 80°–280°	EI-MS	–	[129]
A, MA, MDMA, MDA	Urine	SPME	TFA	HP-1, 60°–290°	EI-MS (SIM)	3–6	[132]

A = amphetamine, MA = methamphetamine, MDMA = methylenedioxymethamphetamine, MDEA = methylenedioxyethylamphetamine, MDA = methylenedioxyamphetamine.

TABLE 23.6

SELECTED LIQUID-CHROMATOGRAPHIC METHODS FOR AMPHETAMINES

Drug	Sample	Isolation	Column, elution conditions	Detection	LOD (μg/L)	Ref.
14 amphetamines and rel. drugs	Serum, urine	SPE	ODS, MeCN/ ammonium formate	APCI-MS (SIM)	1–5	[137]
A, MA, MDMA, MDA, MDEA	Urine	SPME	CN, MeCN/ ammonium acetate	ESI-MS (SIM)	2	[138]
MDMA, MDA, MDEA	Blood, urine	L/L	ODS, MeCN/ MeOH/ammonium acetate	Fl, ESI-MS-MS	0.8–2	[165]
MDMA, MDA, MDEA	Blood, serum	L/L	ODS, MeCN/ MeOH/H_2O	ESI-MS-MS (TOF)	1	[166]

A = amphetamine, MA = methamphetamine, MDMA = methylenedioxymethamphetamine, MDEA = methylenedioxyethylamphetamine, MDA = methylenedioxyamphetamine, MBDB = *N*-methylbenzoxodiazolylbutanamine.

after oral administration of this drug to volunteers. In the late phase of elimination, neither mefenorex nor its specific metabolites were detectable, but amphetamine was still present. Therefore, it is not always possible to distinguish between mefenorex and amphetamine intake. Clobenzorex is an another anorectic drug which is metabolized to amphetamine. In order to differentiate between clobenzorex intake and amphetamine abuse, Cody *et al.* [144] analyzed urine samples for the metabolite 4-hydroxyclobenzorex with GC/MS. This compound was always present in urine samples besides amphetamine after administration of clobenzorex, even in cases when the parent drug was not detectable. Benzphetamine and its metabolites benzylamphetamine, hydroxybenzphetamine, hydroxybenzylamphetamine, methamphetamine, and amphetamine were also isolated from urine by SPE, separated with an alkaline mobile phase, and detected by ESI-MS with a LOD from 0.3 to 10 μg/L urine [145]. Dimethylamphetamine and its metabolites, dimethylamine-*N*-oxide, methamphetamine, and amphetamine, were determined in urine, after isolation by SPE, by means of LC/ESI-MS with a LOD of 5–50 μg/L urine [146]. Selegiline, an inhibitor of monoamine oxidase-B, used in the treatment of Parkinson's disease, is metabolized to methamphetamine and amphetamine. Katagi *et al.* [147] determined this drug as well as the specific metabolite, selegiline-*N*-oxide, methamphetamine, and amphetamine in human urine by isolation with SPE C_{18} cartridges, separation on a cation-exchange column, and ESI-MS detection in full-scan acquisition mode. In urine samples, collected from selegiline-treated patients, only metabolites were detected. The method may serve for differentiation between selegiline and methamphetamine intake. Selegiline is metabolized to R(−)-amphetamine and R(−)-methamphetamine but not to S(+)-amphetamine and S(+)-methamphetamine. In order to differentiate between selegiline and illicit amphetamine/methamphetamine use, Heo *et al.* [148] developed a CE method for quantitation of amphetamine and methamphetamine enantiomers in urine extracts. Stereoisomers were separated in phosphate buffer containing carboxymethyl-β-cyclodextrin. The method was used for differentiating between selegiline and illicit amphetamine intake.

23.2.4 Ecstasy, LSD, and other hallucinogens

23.2.4.1 Designer amphetamines in biological samples

In contrast to amphetamine, MDMA did not become so widely abused until the late 1970s. This drug is known under the names "Ecstasy", "XTC", or "Adam". Paradoxically, the widespread abuse of psychoactive phenethylamines was propagated by the book of the eminent pharmacologist Alexander Shulgin, who in his book *Pihkal, A Chemical Love Story* [149] published procedures for synthesizing 179 drugs of this group. In addition, information concerning the recommended dosage and expected symptoms was given. This is extremely interesting for the forensic toxicologist, as the book is freely available and certainly helped in the proliferation of psychoactive phenethylamines in society. Amphetamine derivatives are usually manufactured as tablets and distributed illegally, mainly in discotheques. These drugs, besides having stimulating action, may alter thermoregulation and have caused a growing number of deaths, mainly due to heat stroke

at rave parties [150–153]. Also, chronic abuse of psychoactive phenethylamines causes detectable mental impairment. High-dependency amphetamine users performed worse than controls on attention/concentration and memory tests [154]. Recreational users of MDMA performed worse than the controls in regard to memory and learning tasks and in tasks reflecting general intelligence [155]. It must be stressed that the recreational use of "ecstasy" is very often associated with consumption of other psychotropic drugs or alcohol. Examination of urine samples taken from 64 attendees of rave parties revealed that in the majority of cases MDMA was present in combination with amphetamine, methamphetamine, or other designer amphetamines [156]. This suggests that the majority of the ravers are multi-drug users.

Enslin *et al.* [157] studied the metabolism of racemic MDE after oral administration to healthy volunteers. Urinary metabolites were analyzed by GC/MS and HPLC. It was demonstrated that in the first phase *O*-de-alkylation to 3,4-dihydroxymetabolites occurs with consecutive methylation. The second pathway leads through *N*-de-alkylation to the corresponding primary amines. All Phase 1 hydroxy metabolites are partially conjugated. Maurer *et al.* [158,159] studied the urinary excretion of metabolites of racemic MDMA, MDA, MDEA, *N*-methylbenzoxodiazolylbutanamine (MBDB), and benzodioxoazolylbutanamine (BDB). The metabolites were identified by GC/MS. In the first phase, *N*-de-alkylation and de-methylenation was observed; in the second phase, conjugation with glucuronic and sulfuric acid occurred. The MDMA metabolism and pharmacokinetics in human volunteers, who were given MDMA in doses of 50–150 mg orally, were studied by de la Torre *et al.* [160]. They found that, while the dose of MDMA rose threefold, the area of drug under the curve increased up to 10-fold. The urinary excretion of 3,4-dihydroxymethamphetamine, a de-methylenated MDMA metabolite, was constant for all doses, but the excretion of MDMA increased out of proportion. This indicated that MDMA metabolism might be saturated, even at moderate doses of drug, enhancing its toxicity. All amphetamines have a single asymmetric center and therefore exist as two enantiomers, each of which has different pharmacological activities. Fallon *et al.* [161] determined the enantiomers of MDMA and MDA in plasma and urine after oral administration of 40 mg of racemic MDMA to volunteers. The plasma concentration of (*R*)-MDMA was more than twice as high as that of (*S*)-MDMA. The plasma half-life of the *R*-enantiomer was significantly longer than that of the *S*-enantiomer. The results showed that the more active (*S*)-MDMA has a reduced area under the curve (AUC) and a shorter half-life.

The analytical methods for amphetamine, methamphetamine, and designer amphetamines were described in Sec. 23.2.3. Here, only the methods for the Ecstasy group will be presented. Kunsman *et al.* [162] extracted MDMA and MDA from immunoassay-positive urine samples with butyl chloride and derivatized them with chlorofluoroacetic anhydride. GC/MS-SIM analysis was performed on a temperature-programmed DB-5 column. Helmlin *et al.* [163] analyzed MDMA and its metabolites, MDA, HMMA, and HHA, in urine by HPLC/DAD and GC/MS. The drugs were extracted from plasma and urine with SPE SCX columns and derivatized with HFBA prior to GC/MS. MDMA, MDEA, and MDA were determined in body fluids by HPLC with fluorometric and MS/MS detection, with a LOD for fluorometric detection of 0.8 μg/L in blood and 2 μg/L in urine. Very good correlation between both methods was found [164]. Clauwert *et al.* [165] determined MDMA, MDEA, and MDA in body fluids by HPLC with fluorometric

detection. The results were comparable to those with GC/MS. The same group of authors applied LC/ESI-TOF-MS to the quantification of MDMA in biological fluids [166]. Kintz and Samyn [167] have reviewed the methods used for the determination of Ecstasy in alternative samples, *e.g.*, saliva, sweat, and hair.

23.2.4.2 LSD and other hallucinogens in biological fluids

Lysergic acid diethylamide (LSD) is an extremely potent hallucinogenic drug. Its single dose ("trip") ranges from 30 to 100 μg. The drug is rapidly and extensively metabolized, and only a very small fraction is excreted unchanged in the urine [168]. Webb *et al.* [169] developed an immuno-affinity extraction method for LSD from urine, followed by LC/ESI-MS. Methysergide was used as internal standard for quantitation, and the protonated molecular ion and two fragments were monitored. The LOD was 0.5 μg/L for a 5-mL urine sample. In a subsequent study by the same group, methysergide was replaced by a triply deuterated LSD analog as internal standard [170], yielding much greater accuracy and precision, but the LOD (500 ng/L) was not improved. Kanel *et al.* [171] isolated LSD and its de-methylated metabolite from 1 mL blood, serum, plasma, or urine by automated mixed-phase SPE. The drugs were separated by LC on a phenyl column and detected by ESI-MS/MS with a LOD of 25 ng/L for both substances. In the study of Bodin *et al.* [172], LSD was extracted from urine with an organic solvent, back-extracted into an acetate buffer, and subjected to LC/ESI-MS, achieving a LOD of 20 ng/L.

The window of detection of LSD in urine following its use is not longer than 12–22 h [173]. Poch *et al.* [174] demonstrated the importance of determining 2-oxo-3-hydroxy-LSD, a prevalent metabolite of LSD, excreted in the urine. This metabolite, as well as LSD, nor-LSD, and iso-LSD, were determined in urine by LC/APCI-ion-trap MS. The concentrations of 2-oxo-3-hydroxy-LSD were distinctly higher than those of the parent drug and other metabolites. In a subsequent study, Poch *et al.* [175] compared three analytical methods for LSD and metabolites: LC/APCI-MS, LC/APCI-MS/ion-trap MS, and GC/MS. GC/MS was used only for the parent drug. Very good agreement between both LC/MS methods was found. According to these authors, the determination of the metabolite significantly increases the detection window for LSD. Reuschel *et al.* [176] determined LSD and 2-oxo-3-hydroxy-LSD in urine samples by GC/MS/MS. They demonstrated that the concentration of the metabolite was about ten times higher than of LSD; the LOQ was 10 ng/L. Verstraete *et al.* [177], who used GC/MS, found the ratio of 2-oxo-3-hydroxy-LSD to LSD in urine samples to range from 4 to 41. Sklerov *et al.* [178] determined LSD and 2-oxo-3-hydroxy-LSD in blood and urine. The drugs were isolated from urine by alkaline solvent extraction, while for blood a solvent extraction, followed by SPE, was used. LC/ESI-MS was applied with in-source collision-induced dissociation and monitoring of three ions for each compound. The LOD was 0.1 μg/L for LSD and 0.4 μg/L for the metabolite in urine. In authentic cases, 2-oxo-3-hydroxy-LSD was found in high concentrations in urine but it was not present in blood samples. A LC/ESI-MS/MS technique was applied by Canezin *et al.* [179] to the determination of LSD and iso-LSD in plasma with a LOD of 0.02 μg/L for both compounds. In urine, metabolites were also detected, *e.g.*, 2-oxo-3-hydroxy-LSD, nor-LSD, nor-iso-LSD,

13- and 14-OH-LSD, lysergic acid ethylamide, trioxylated-LSD, and lysergic acid ethyl-2-hydroxyethylamide.

Psilocybin is an indole derivative of tryptamine with hallucinogenic action. This compound is present in various species of mushrooms of the genus *Psilocybe*, *Panaeolus*, *Conocybe*, and *Gymnopilus*. These mushrooms, which are widely distributed in forests, are known in the drug scene as "magic mushrooms" and are collected and cultivated. After administration, psilocybin is rapidly de-phosphorylated to psilocin, which is also biologically active. "Magic mushrooms", mixed with honey and offered in Dutch "coffee shops", were analyzed for psilocin by HPLC/DAD and LC/APCI-MS [180]. Psilocin was determined in urine by GC/MS after mixed-bed SPE and silylation; the LOQ was 10 μg/L. Enzymatic hydrolysis of psilocin-positive urine samples before extraction gave higher results, indicating the presence of psilocin glucuronide in urine [181].

23.2.5 Cannabinoids

Cannabis products – marijuana and hashish – are surpassed only by ethyl alcohol as the most commonly abused drugs. Among the three known cannabis preparations in the illicit drug market – herbal cannabis (marijuana), cannabis resin (hashish), and liquid cannabis (cannabis oil) – marijuana is the most popular one. It is the most widely used illicit drug in the world. Besides the illicit use of cannabis as recreational drug, attempts to use cannabis preparations or its active compound tetrahydrocannabinol (THC) in medicine have an equally long tradition. The discovery of cannabinoid receptors as well as endogenous receptor agonists stimulated research on the role and possible therapeutic use of cannabis. THC as well as its synthetic analogs (nabilone, levonantradol, and marinol) were tried in treatments of several disorders. It was demonstrated that cannabinoid receptor agonists were effective as antiemetics and appetite stimulants, and cannabinoids have been prescribed for HIV patients [182,183]. Among other possible uses, the suppression of muscle spasticity associated with multiple sclerosis, the relief of chronic pain, and the therapy of behavior disorders in Tourette syndrome have been mentioned [184–186]. Martin *et al.* [187] synthesized several analogs of THC, modifying its alkyl side-chain. This manipulation produced high-affinity ligands with antagonist, partial agonist, or full agonist effects on the cannabinoid receptor. It seems that the development of THC analogs, devoid of unwanted psychotropic action, may be of considerable interest for clinical medicine.

23.2.5.1 Cannabinoids in plant material

The determination of cannabinoids in plant material has several purposes. The first and most important one is to identify and quantify the main components, like active THC and other cannabinoids – cannabinol (CBN) and cannabidiol (CBD). On the basis of quantitative determinations it is possible to classify plant material as resin-type (for the illicit drug) and fiber-type (for industrial purposes). The other purpose of analysis is to identify the production site and sometimes a particular batch of the drug. *Cannabis sativa* contains several hundred constituents, the proportions of which may differ in relation to

geography, climate, and cultivation mode. Thin-layer chromatography (TLC) has frequently been applied in the analysis of plant material; the use of overpressured TLC has also been reported [188,189]. Debruyne *et al.* [190] proposed a TLC or HPLC separation of main cannabis constituents (THC, CBN, and CBD) with subsequent measurement of UV spectra as an alternative to GC/MS identification. GC/FID and GC/MS as well as HPLC have been applied to the differentiation of cannabis samples of different origin; over 100 compounds could be identified. The composition as well as peak ratios of particular compounds were used for tracking the origin of confiscated cannabis samples [191]. Lehmann *et al.* [192] used HPLC with DAD for the classification of cannabis chemotypes into three types: drug type, intermediate type, and fiber/industrial type. Supercritical-fluid chromatography (SFC), coupled with APCI-MS was applied to the determination of THC, CBN, and CBD in cannabis products with a LOD (on-column) of 0.55 to 2.1 ng [193]. Lurie *et al.* [194] have used CE/DAD for cannabinoids; seven were separated and identified.

Some cannabis products have found their way to the food market. Thus, hemp oil products are being marketed as a healthy source of essential omega fatty acids. The content of THC in hemp oils, measured with GC/MS, ranged from 11 to 117 μg/g. After administration of 15 g of the oil to volunteers, the level of THCCOOH in urine was always below the cut-off value of 50 μg/L [195]. Zoller *et al.* [196] developed a HPLC method for the determination of THC and Δ^9-tetrahydrocannabinolic acid in food, containing hemp products. GC/MS analysis was applied to the determination of THC in drug- and fiber-type cannabis seeds. Drug-type seeds contained much more THC (35–124 μg/g) than the fiber type (0–12 μg/g). Most of THC was located on the surface and could easily be washed off with chloroform [197].

23.2.5.2 Tetrahydrocannabinol and metabolites in blood and urine

ElSohly and Salem [198] and Staub [199] have reviewed metabolic and analytical studies of cannabinoids. The concentration of THC in the blood reaches a maximum 5 to 15 min after cannabis smoking and afterwards undergoes biphasic kinetics. In the first phase, the THC concentration decreases very rapidly (in *ca.* 2–3 h) from *ca.* 100–150 μg/L to *ca.* 5 μg/L. In the second phase, the terminal half-life of THC is *ca.* 25 h. The concentration of THCCOOH reaches a maximum *ca.* 1 h after smoking and then slowly decreases. This substance may be detected in the blood up to two days and in urine up to 5 days after a single exposure.

GC/EI-MS (positive ions in SIM mode) is a standard method for the determination of cannabinoids in biological fluids. Weller *et al.* [200] applied ion-trap (IT)GC–MS to the analysis of THC, 11-OH-THC, and THCCOOH in serum. The drugs were extracted with SPE cartridges and silylated (TMS). The GC/MS/IT-MS analysis allowed full-scan production spectra to be obtained at concentrations of 0.25–2.5 μg/L of serum. The selectivity of this method was regarded as superior to GC/MS-SIM. Chiarotti *et al.* [201] accomplished the detection of THCCOOH in biological fluids at ng/L-levels with GC/MS/MS analysis. Newer applications of LC to the analysis of cannabinoids deal with the use of LC/MS for this purpose. In the study of Mireault [202], blood or urine samples

were extracted with SPE cartridges and chromatographed on a C_8 column with methanol/ammonium acetate. An ion-trap instrument, equipped with an APCI source was used in MS/MS mode (positive ions). Detection limits of 1 μg/L were achieved for THC, 11-OH-THC, and THCCOOH. In a subsequent study Mireault *et al.* [203] used APCI-MS/MS and achieved a LOQ of 0.25 μg/L for all three compounds. In a direct comparison with the ion trap, the use of a triple-quadrupole instrument improved the limit of detection in blood and urine by a factor of 10–40. Breindahl and Andreasen [204] have determined THCCOOH in urine by LC/ESI-MS following basic hydrolysis and SPE. THCCOOH and its deuterated analog were assayed by HPLC/ESI-MS, using a C_8 reversed-phase column, gradient elution with acetonitrile/formic acid, and SIM detection (positive ions). In-source collision-induced dissociation was applied, and a protonated, quasi-molecular ion as well as two fragment ions were monitored; the LOD was 15 μg/L. Tai and Welch [205] determined THCCOOH by HPLC/ESI-MS (negative ions) after isolation from urine by SPE, using isocratic elution from an ODS column with methanol/ammonium acetate. Only deprotonated quasi-molecular ions of the drug and its deuterated analog were monitored. Weinmann *et al.* [206] used LC/MS/MS for the simultaneous determination of THCCOOH and its glucuronide in urine. In this method, the cleavage of conjugates was omitted. THCCOOH and its glucuronide were extracted from urine with ethyl acetate/ether (1:1) and separated on a C_8 column with a gradient of ammonium formate buffer and acetonitrile (Fig. 23.2). ESI-MS/MS was used for detection with protonated quasi-molecular ions as precursor-ions and two fragment-ions for each drug as product-ions. The specificity of the method was checked by way of enzymatic hydrolysis of the THCCOOH glucuronide. Selected chromatographic methods for cannabinoids are summarized in Tables 23.7 and 23.8.

23.2.5.3 Tetrahydrocannabinol and metabolites in alternative samples

Blood collection is an invasive method, and urine sampling requires co-operation and privacy. Moreover, both blood and urine results have quite a narrow detection window, ranging from a few hours to a few days after drug exposure. Among alternative samples, saliva and sweat have the advantage that they can be collected non-invasively and on the spot, *e.g.*, for roadside testing. Meconium analysis may give evidence of intrauterine exposure, whereas hair or nail analysis can document chronic drug exposure. The analysis of cannabinoids and other drugs of abuse in alternative matrices was reviewed by Kintz and Samyn [207]. After regular abuse of cannabis, THC, CBN, and CBD can easily be detected in hair. The detection of the metabolite THCCOOH is much more difficult and is best performed with GC/NCI-MS/MS. Sachs *et al.* [208] have developed a method for the determination of THCCOOH in hair, following alkaline digestion, solvent extraction, and purification by HPLC. HPLC eluate fractions were extracted with hexane/ethyl acetate, derivatized, and subjected to GC/MS; the LOD was 0.3 ng/g. Fingernail clippings were analyzed for THC and THCCOOH [209]. Blood, urine, saliva, and sweat were simultaneously collected from drivers admitted to an emergency ward. Of the 22 subjects positive for THCCOOH in urine, 14 were positive for THC in saliva and 16 for THC

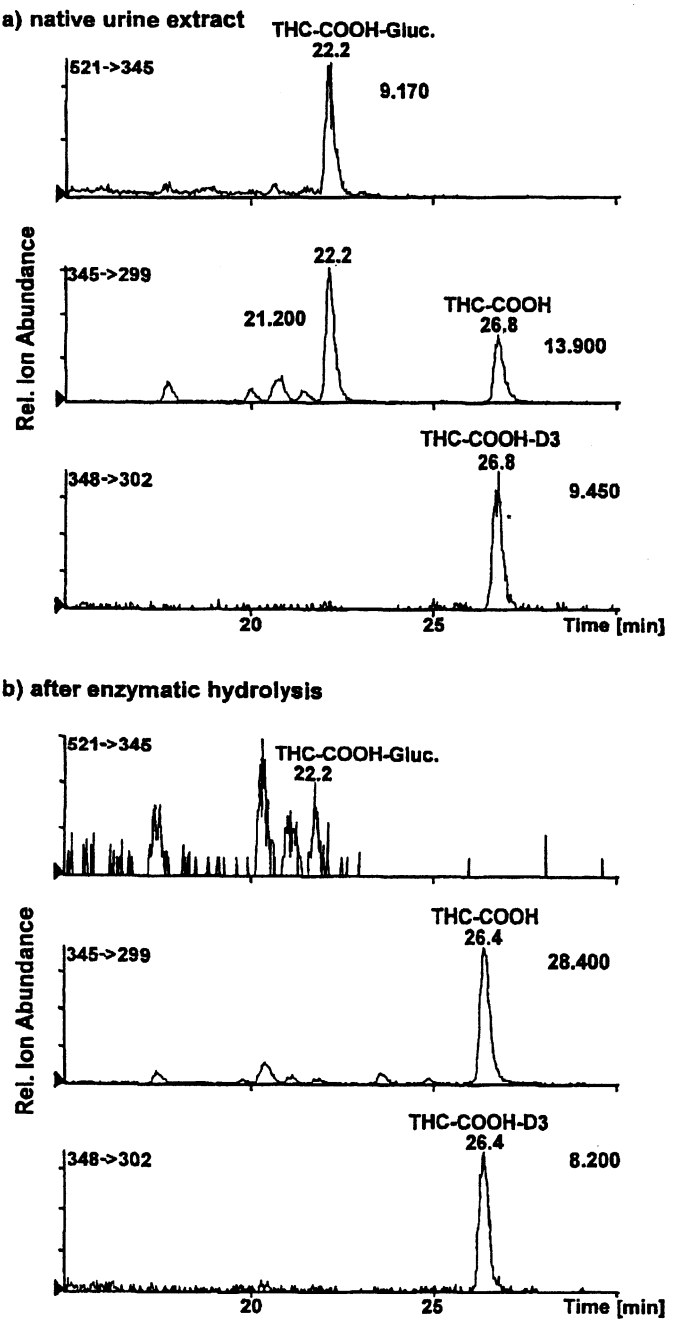


Fig. 23.2. Analysis of THC-COOH by LC/MS-MS. (a), Native urine extract; (b) urine extracted after glucuronidase treatment. (Reprinted from Ref. 206 with permission.)

TABLE 23.7

GAS-CHROMATOGRAPHIC METHODS FOR CANNABINOIDS

Drug	Sample	Isolation	Derivatization	Column, conditions	Detection	LOD (μg/L)	Ref.
THC, 11-OH-THC, THCCOOH	Serum	SPE	MSTFA	DB-1, 120°–310°	EI-MS (IT)	0.2–2.5	[200]
THC	Saliva, sweat	L/L	Methylation	HP-5, 60°–290°	EI-MS (SIM)	1	[209]
THC, 11-OH-THC, THCCOOH	Plasma, meconium	L/L	BSTFA + TMCS	HP-5, 200°–280°	EI-MS (SIM)	1–2.5	[212]

THC = Δ^9-tetrahydrocannabinol, CBN = cannabinol, CBD = cannabidiol, THC-COOH = 11-nor-Δ^9-THC-9-carboxylic acid.

in sweat; THCCOOH and 11-OH-THC were not detected in saliva and sweat [210]. Saliva specimens, collected with the EPITOPE system, was centrifuged, filtered, subjected to SPME, and analyzed by GC/MS [211]. El Sohly *et al.* [212] developed a GC/MS method for the determination of THCCOOH, benzoylecgonine, morphine, and amphetamines in meconium specimens. An immunoaffinity extraction procedure was developed for the isolation of THC and its hydroxymetabolites from urine, plasma, and meconium. After silylation, the drugs were analyzed by GC/MS (LOD for meconium, 1–2.5 ng/g) [213].

Several urine adulterants are commercially available, purportedly capable of making cannabinoids undetectable. They usually contain nitrite ion. An addition of these preparations to urine may affect the original THCCOOH concentrations in GC/MS analysis, particularly when the urine is acidic [214]. In addition, some preparations are available which are supposed to wash out cannabinoids incorporated into hair. Some decrease in drug concentrations was observed after washing, but a single treatment with Ultra Clean shampoo could not remove drugs from hair to the undetectable level [215]. The use of Cannabio shampoo, which is produced from cannabis oil, apparently prevents incorporation of cannabinoids into hair *in vivo* and *in vitro* [216].

23.2.6 Volatile narcotic compounds and toxins

Glue sniffing, endemic mostly among young people, involves mainly aromatic hydrocarbons (benzene, toluene, xylenes) and gasoline [217]. Park *et al.* [218] tested blood and urine samples for toluene by GC/FID and GC/MS. As a rule, values in blood

TABLE 23.8

LIQUID-CHROMATOGRAPHIC METHODS FOR CANNABINOIDS

Drug	Sample	Isolation	Column, elution conditions	Detection	LOD (μg/L)	Ref.
THC, THC-A, CBD, CBN	Cannabis	L/L	ODS, MeCN/ phosphoric acid, grad.	UV-DAD	1 ng	[192]
THC, THC-A	Hemp product	L/L	ODS, MeCN/ phosphoric acid, isocr.	UV + Fl	0.01 ng	[196]
THC, 11-OH-THC, THCCOOH	Blood, urine	SPE	C8, MeOH/ formic acid, isocr.	MS (APCI)	1	[202]
THCCOOH	Urine	SPE	C8, MeCN/ formic acid, grad.	MS (ESI)	15	[204]

THC = Δ^9-tetrahydrocannabinol, THC-A = tetrahydrocannabinolic acid, CBN = cannabinol, CBD = cannabidiol, THC-COOH = 11-nor-Δ^9-THC-9-carboxylic acid.

were higher than in urine, mostly in the range of 0.1 to 5.0 mg/L, and even higher in cases of fatal intoxication. Narcotic solvents may be used not only for recreation, but also as incapacitating agents or murder weapons. Risse *et al.* [219] reported two cases of homicide by chloroform. Chloroform concentrations in blood, measured by headspace GC/MS, ranged from 5 to 115 mg/L. The authors recommended the use of glass containers for blood samples and the storage of specimens in the frozen state. In the case of a homicide, published by Madea *et al.* [220], a halothane-moistened towel was used as a murder weapon. Sampling and sample treatment are dictated by the volatility of solvents. Various modifications of headspace sampling, including headspace-cryogenic trapping, have been used. Generally, samples of whole blood or urine were subjected to headspace analysis, and the headspace vapor was injected into the GC instrument. At first, the oven temperature was set at −30 to 5°C, depending on the analyte. After cryogenic trapping, the GC separation with FID detection was performed. The method was applied to chloroform and dichlormethane [221], to xylenes [222], hexane and cyclohexane [223], and to the anesthetics halothane, isoflurane, sevoflurane, and enflurane [224]. The sensitivity of cryogenic trapping/GC was about ten times greater than for the headspace/SPME/GC, as applied to various aliphatic, aromatic, and halogenated solvents [225].

Headspace solid-phase microextraction (HS-SPME) is now frequently used for the isolation of volatile narcotic solvents. Lee *et al.* [226] have presented a study on the determination of toluene, benzene, *n*-butyl acetate, *n*-butanol, and isoamyl acetate in blood

and urine. The solvents were isolated by SPME, using polydimethylsiloxane-coated fibers. Detection limits ranging from 1.1 to 2.4 ng per 0.5 mL sample were obtained in GC analysis. Tranthim-Fryer *et al.* [227] applied HS-SPME in cases of abuse of volatile compounds (toluene, butane, and halothane). The identification and quantitation were accomplished with GC/MS analysis on a DB-1701 column. Kim and Park [228] compared HS analysis with HS-SPME for toluene determination by GC/MS. For SPME, fibers coated with polydimethylsiloxane were used. The LOD for HS-SPME was 10 times higher than for HS analysis. In a case of autoerotic fatality, a pressurized can of Fix-A-Flat tire repair was found near the body. Analysis by GC/MS identified the components of the inflating preparation (tetrachloroethylene and chlorodifluoromethane) in blood, liver, and lungs [229]. In a fatal case of 1,1,1-trichloroethane inhalation [230] and one of inhalation of cigarette lighter gas [231], containing propane, isobutane, and *n*-butane, HS-GC/MS analysis of the blood was performed on a CP-Select 624 column in SIM mode.

23.3 THERAPEUTIC DRUGS OF FORENSIC RELEVANCE

Almost any therapeutic drug may be used in circumstances of forensic relevance. This is particularly true of drugs showing primary addiction potential, most of which have been treated in Sec. 23.2. However, there are certain groups of drugs which have been used for criminal purposes, like incapacitation, rape, or murder. These drugs will be discussed here.

23.3.1 Incapacitating drugs

A recent monograph, dealing with drugs used for incapacitating purposes treats these substances in detail: ethanol, flunitrazepam and other benzodiazepines, γ-hydroxybutyrate (GHB) and related products, hallucinogens, and opioids [232]. Most of them are determined by chromatographic methods. Benzodiazepines are the most common so-called "date-rape" drugs. Among benzodiazepines, flunitrazepam (Rohypnol), a potent hypnotic drug, is of particular interest, owing to its toxicity in combination with ethyl alcohol and misuse in drug-facilitated sexual assaults. Since flunitrazepam is extensively metabolized, the simultaneous determination of its active metabolites, particularly of 7-aminoflunitrazepam, is very important. Negrusz *et al.* used GC/NCI-MS for the determination of flunitrazepam and 7-aminoflunitrazepam in urine [233] and hair [234,235]. The method included SPE, derivatization with HFBA, and GC/NCI-MS in SIM mode. Two ions were monitored for each drug and for the deuterated internal standard. The LOD was 3 and 50 ng/L for 7-aminoflunitrazepam and flunitrazepam, respectively. It was shown that 7-aminoflunitrazepam is detectable in urine and in hair after a single 2-mg oral dose of flunitrazepam (Fig. 23.3). In the urine, 7-aminoflunitrazepam could be detected only within 3 days. In hair, this metabolite appeared 1 to 21 days after administration of flunitrazepam. Bogusz *et al.* [236] developed a LC/APCI-MS method for the determination of flunitrazepam and its metabolites, 7-aminoflunitrazepam, *N*-desmethylflunitrazepam, and 3-OH-flunitrazepam, in blood. The comparison of ionization

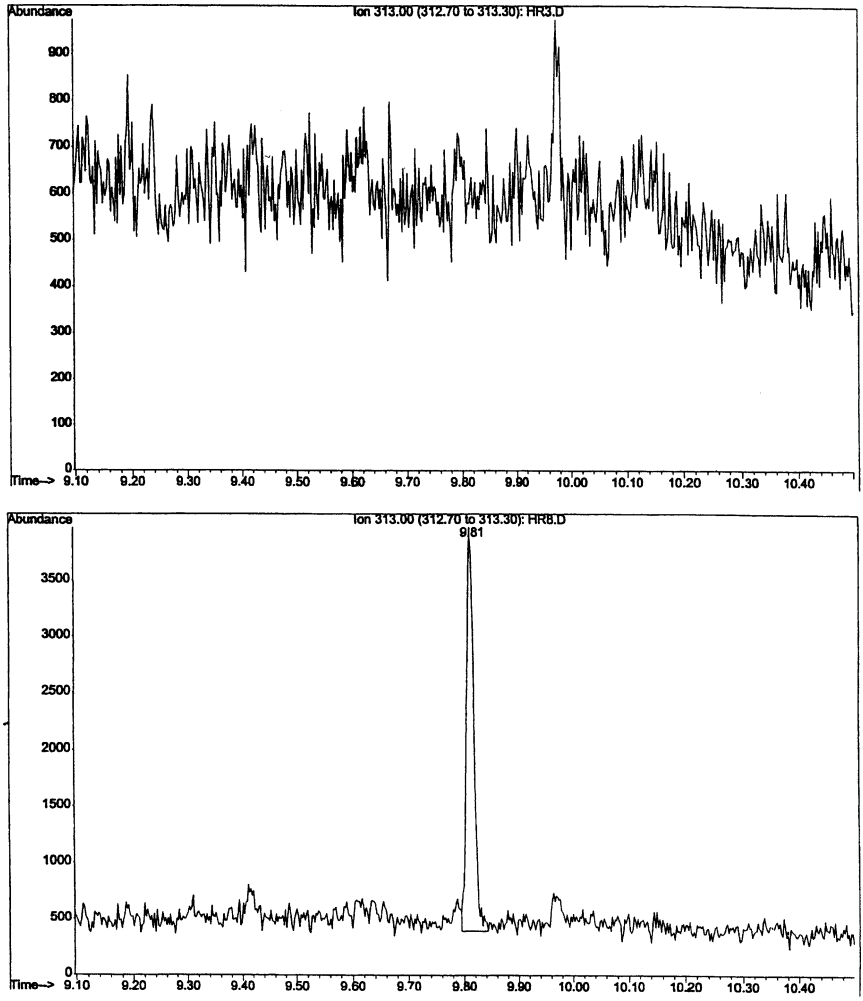


Fig. 23.3. GC/MS-SIM at *m/z* 313 of extracts of hair before flunitrazepam administration (top) and three days after administration of a single dose of the drug (bottom). Flunitrazepam concentration 2.3 pg/mg. (Reprinted from Ref. 235 with permission.)

responses showed that APCI gave a signal 7–30 times stronger than ESI for all compounds but 7-aminoflunitrazepam. After SPE on C_{18} cartridges, the drugs were separated on a C_{18} column with MeCN/ammonium formate buffer (pH 3.0) and detected by APCI-MS. For each compound, a protonated quasi-molecular ion was monitored. The LOD values ranged from 0.2 to 1 μg/L, 7-aminoflunitrazepam being detectable in urine for 60 h after a single 1-mg oral dose. This method has been applied in routine toxicological casework [52]. LeBeau *et al.* [237] determined flunitrazepam, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam in blood and urine after SPE on mixed-phase cartridges by LC/ESI-MS/IT-MS. Protonated quasi-molecular ions of the

drugs involved were monitored and their identity was confirmed by fragmentation. The LOD in blood ranged from 0.5 to 1 μg/L. Darius *et al.* [238] also used ion-trap LC/ESI-MS/MS for the determination of flunitrazepam in serum. The drug was extracted with *tert*-butyl methyl ether and chromatographed on a C_{18} column with aq. MeCN as the mobile phase. Product-ions of flunitrazepam and the internal standard (clonazepam) were monitored; the LOD was 0.2 μg/L. Yuan *et al.* [239] described an automated in-tube SPME, combined with LC/ESI-MS for the determination of diazepam, nordiazepam, temazepam, oxazepam, 7-aminoflunitrazepam, and *N*-desmethylflunitrazepam in serum and urine. The isolation technique used allowed solvent-free, automatic extraction in 15 min. The extraction procedure was optimized, and the drugs were detected with LC/ESI-MS (full-scan *m/z* 100–400) and SIM; a LOD of 0.02 to 2 μg/L was achieved. Two drawbacks of the procedure were the relatively low recovery in serum (below 50%) and peak-broadening, caused by automatic desorption. Midazolam is a short-acting benzodiazepine used for the induction of anesthesia and also misused as a "date-rape" agent. This drug and its active hydroxylated metabolite were extracted from serum with ether/2-propanol (98:2) at high pH and separated on an ODS column (Nucleosil C18, 150 × 1 mm). The drugs were determined by LC/ESI-MS in the SIM mode. Protonated quasi-molecular ions and fragments of both compounds were monitored. The LOQ for both compounds was 0.5 μg/L [240]. In another study, midazolam and hydroxymidazolam as well as triazolam and hydroxytriazolam, used as internal standards, were extracted from plasma with Oasis HLB cartridges and determined by LC/APCI-MS/MS [241]. Matz and Hill [242] applied ion-mobility spectrometry, combined with MS for the rapid, two-dimensional separation of five benzodiazepines. GHB has been used earlier as a general anesthetic without analgesic properties. It is also effective in the treatment of narcolepsy and alcoholism, but its abuse in 1980s caused its sale to be banned in the USA. GHB is abused in discotheques as "liquid Ecstasy" and as an incapacitating drug. After implementation of tighter control of GHB, its precursors – γ-butyrolactone (GBL) and 1,4-butanediol – appeared on the market, predominantly *via* the Internet. Both substances are converted to GBL *in corpore*. Recent overviews of the pharmacokinetics, clinical effects, and toxicity of GHB were published [243,244]. The interpretation of the results for GHB is sometimes very difficult, since this substance is a natural body constituent, being a metabolite of the neurotransmitter γ-aminobutyric acid (GABA). The concentration of endogenous GHB in blood increases rapidly after death, particularly during putrefaction [245,246]. All methods for the detection of GHB are based on GC/MS. Usually, a solvent extraction is followed by silylation and GC/MS in the SIM mode. The LOQ is *ca.* 1 mg/L in blood or urine [247–249].

23.3.2 Anti-diabetic drugs

Anti-diabetic drugs are often misused not only by people who want to simulate an illness, but also for suicides or murder [250,251]. Oral anti-diabetics of the sulfonylurea type are usually determined by LC/MS. Ramos *et al.* [252] published a fast LC/APCI-MS/MS method for the determination of glibenclamide in human plasma after acetonitrile precipitation, with a deuterated analog as internal standard. The retention time of the drug was 3 min and the LOQ 1 μg/L. Sulfonylurea anti-diabetics

(tolbutamide, chlorporopamide, glibenclamide, and glipizide) were detected and quantified in serum by LC/ESI-MS [253]. SPE (with C_{18} cartridges) gave much cleaner extracts than acidic toluene extraction. The drugs were separated with a methanol/acetic acid gradient and detected by ESI-MS in full-scan mode (positive ions, m/z 265–510). For quantitation, protonated quasi-molecular ions were used; the LOQ was 10 μg/L for all drugs. Maurer *et al.* [254] published a LC/APCI-MS procedure for screening and library-assisted quantification of the anti-diabetics glibenclamide, glibornuride, gliclazide, glimepiride, glipizide, gliquidone, glisoxepide, tolazamide, and tolbutamide. The drugs were extracted from plasma with ether/ethyl acetate and separated on a Select B column by gradient elution. Protonated quasi-molecular ions were used for screening and quantification, and full-scan spectra for identification. Limits of detection varied from 2 to 3 mg/L.

Identification, detection, and quantitation of insulin in blood are very important in clinical and forensic toxicology. The presence of bovine or porcine insulin in the blood of non-diabetic person may serve as an evidence of poisoning. Since insulin and C-peptide are released from proinsulin, the determination of both products may be of relevance. Very high levels of human insulin without elevation of the C-peptide indicate exogenous administration [255]. When both insulin and C-peptide are present in high concentrations, the presence of a pancreatic tumor or administration of insulin-releasing drugs, like sulfonylureas, is possible. A LC/ESI-MS method for the determination of human, bovine, and porcine insulin, as well as the C-peptide, was published by Darby *et al.* [256]. The drugs were isolated from acidified plasma with C_{18} SPE cartridges, separated on a C_{18} column, and detected with IT-ESI-MS. Multiply charged molecular ions $(M + 3H)^{+3}$ and $(M + 4H)^{+4}$ were monitored. Both ions were selected for quantitation, since the intensity ratios were variable between runs. The LOQ was 1 μg/L, and comparison with radioimmunoassay showed good agreement of values. The stability of insulin from blood, stored under various conditions, was studied. Zhu *et al.* [257] developed a LC/ESI-MS/MS assay for the quantification of human insulin, its analog, and their catabolites in plasma. The compounds were isolated by precipitation, followed by SPE; the quantitation range was 100–500 μg/L.

23.3.3 Muscle relaxants

Neuromuscular blocking agents constitute a real analytical challenge, due to fast metabolism and difficult detectability. These drugs, normally used for skeletal-muscle relaxation during anesthesia or surgery, are also known to be used as means of euthanasia or murder [258]. Muscle relaxants are usually quaternary ammonium compounds of high polarity and low thermal stability. Therefore, liquid chromatography is the method of choice for their separation. Zecevic *et al.* [259] used HPLC with UV detection at 210 nm for the determination of pancuronium in pharmaceutical preparations. Farenc *et al.* [260] determined atracurium and its major metabolite, laudanosine, in human plasma, using HPLC with fluorometric detection, with a LOQ of 40 μg/L. The same group of authors applied LC/ESI-MS to the determination, in plasma, of rocuronium, a neuromuscular blocking agent widely used during general anesthesia. The drug and the internal standard (verapamil) were extracted from plasma with dichloromethane and separated on an ODS

column by gradient elution with MeCN/0.1% trifluoroacetic acid (TFA). Protonated quasi-molecular ions for both compounds were monitored. The LOQ was 25 μg/L [261]. Ballard *et al.* [262] developed a LC/quadrupole-TOF-MS/MS method for the determination of pancuronium, vecuronium, tubocurarine, rocuronium, and succinylcholine in post-mortem tissues. These drugs were isolated with a combination of solvent extractions, followed by ion-pairing SPE on C_{18} cartridges. The detection was based on exact mass measurement of product-ions. Kerskes *et al.* [263] performed a systematic study on the detectability of pancuronium, rocuronium, vecuronium, gallamine, suxametonium, mivacurium, and atracurium in biological fluids by means of LC/IT-ESI-MS after SPE. The method was successfully applied in a toxicological emergency case. Laudanosine, a metabolite of atracurium, can also be analyzed by GC/MS. The distribution of this drug in the tissues of a suicide victim was reported by Kintz *et al.* [264], who used GC/MS after solvent extraction.

23.4 CHROMATOGRAPHIC SCREENING METHODS

Often, an unexplained death makes it necessary to rule out poisoning, even though there is no indication of any specific substance. Even in the presence of some definite clues, *e.g.*, when some tablets are found near the victim, it is common and reasonable practice not to limit the toxicological examination to the components of the tablets. Such an "undirected search", has often revealed a multitude of relevant substances. The application of chromatographic methods as well as the mathematical aspects of forensic toxicological screening have recently been reviewed and discussed [265–267].

23.4.1 Thin-layer chromatography

TLC has been used extensively as a screening technique. It is useful for the detection and presumptive identification of poisons in preparations (*e.g.*, street drugs), in urine, or in stomach contents. Because of its limited sensitivity and specificity, the use of TLC as a screening procedure is not recommended for blood analysis. Among the collections of TLC data applicable to forensic toxicology, two are most important: A large database of R_F values in eight developing systems for several hundred acidic, neutral, and basic substances was published by de Zeeuw *et al.* [268]. In order to compensate for interlaboratory variations in chromatographic mobility, a correction factor for R_F values was introduced with a standard set of substances. This system is open-ended and may be expanded according to individual needs. The second TLC-based screening system is Toxi-Lab (from Ansys Inc.). It includes standardized solvent extraction procedures, chromatographic development along with standards, provided by the manufacturer, and spraying with standardized reagents, also provided with the system. Toxi-Lab has documented over 700 drugs and metabolites and is being systematically expanded.

23.4.2 Gas chromatography

Capillary GC, in combination with EI-MS is a most important method in modern toxicological screening. It is the "gold standard" of screening analysis [269].

The success of GC/MS in this field is based on such features as: the very high separation efficiency of capillary columns, high interlaboratory reproducibility of retention parameters due to use of the Kováts retention index (RI) scale, and high selectivity and reproducibility of EI-generated mass spectra. As the universal phase, a dimethylsilicone stationary phase (SE-30, OV-1, DB-1, etc.) is used. A large library of RI values for thousands of toxicologically relevant substances was generated using this phase [270]. It should be stressed that the RI value for a particular substance, analyzed on different stationary phases, may be quite different. The most important collection of RI data and mass spectra for several thousands compounds (drugs, pesticides, environmental poisons) was published by Pfleger *et al.* [271]. This five-volume book is also available as an on-line MS library.

23.4.3 Liquid chromatography

The application of HPLC to screening analysis is relatively new and is still in a development stage. Generally, two approaches have been used: HPLC/DAD and LC/MS. HPLC/DAD has a high identification potential, due to the combination of retention and UV spectrum as identification parameters. Several databases, comprising more than a thousand substances, have been established [272]. Establishment of a universally applicable HPLC database was hindered by poor interlaboratory reproducibility of retention time, but this problem has been solved by the introduction of a retention index scale, based on alkyl aryl ketones [273] or 1-nitroalkanes [274]. Later on, 1-nitroalkanes were replaced by acidic and basic standard drugs with known retention index values. This approach assured very good interlaboratory reproducibility [275,276]. Besides the retention index, the relative retention times are also used for HPLC/DAD libraries. Such a system, based on two reference compounds, is commercially available as REMEDi (Bio-Rad). This system includes automatic extraction of urine samples, on-line separation, detection, and identification with a library of over 800 drugs. The database is expandable according to individual needs.

The use of LC/MS for toxicological screening was perceived as an extremely attractive possibility, since this technique has a much broader detection spectrum than GC/MS and is much more sensitive than HPLC/DAD. However, the establishment of a generally applicable LC/MS library, analogous to the GC/MS databases, is hindered by great interlaboratory variability in mass spectra. Mass spectra of identical substances, generated on the same instruments under nominally identical conditions in three different laboratories, showed large differences in the degree of fragmentation [277]. However, controlled changes in the composition of the mobile phase did not have any relevant effect on the mass spectra. The groups of Marquet [278] and Weinmann [279] initially established databases for ESI-generated mass spectra for over one thousand compounds, using positive and negative ionization modes. Weinmann *et al.* [280] combined the LC/ESI-MS library with a library of UV spectra. Rittner *et al.* [281] established a library of mass spectra for 70 psycho-active drugs and metabolites with LC/ESI-MS. In a preliminary study, the efficiency of various SPE methods and various HPLC columns was tested. The best results were obtained with C_{18} SPE cartridges and C_{18} columns.

Chromatographic separation was performed by gradient elution with MeCN/H_2O/MeOH/formic acid. Mass spectra of drugs were recorded at two levels of fragmentation energy in the full-scan mode (*m*/*z* 100–650, positive ions). For many drugs, sodium, potassium, and acetonitrile adducts were observed. The usefulness of the screening procedure was checked on 140 serum samples taken from road traffic offenders. In 9.8% of the cases, various drugs, mostly benzodiazepines, were detected. Lips *et al.* [282] performed a study on the applicability of LC/ESI-MS (positive ions) to the development of a mass-spectral library, based on in-source collision-induced fragmentation. The influence of mobile-phase composition on the reproducibility of the mass spectra of drugs was tested, and the data obtained with two instruments of the same brand, but of different type, were compared. The breakdown curves (*i.e.*, fragmentation profiles related to fragmentation energy) of selected drugs were compared with the data of other authors. The authors stated that the concentration of organic modifier, pH, and the molarity of the buffer had very little influence on the mass spectra. This observation is in agreement with the previous finding of Bogusz *et al.* [277]. In order to obtain reproducible mass spectra, the fragmenter voltage was dynamically ramped, based on the mass of the substance. The efficiency of identification was tested on over 40 extracts from plasma containing various acidic and basic drugs. All drugs, except phenobarbital were correctly recognized. Phenobarbital was not detected at all, because of the positive ionization applied. Acidic drugs were properly detected, when the experiments were performed in negative-ionization mode. In order to overcome variability in fragmentation intensity, Weinmann *et al.* [283] used tuning compounds for standardization of in-source collision-induced dissociation (CID). Four drugs: haloperidol, paracetamol, metronidazole, and metamizole were selected as tune compounds for LC/ESI-MS. Comparative experiments were performed, using two LC-MS instruments with different interface (Sciex API 365 and Agilent 1100 MSD). Very similar fragmentation patterns were observed after adjustment of the fragmentor voltages of both instruments. Therefore, the establishment of a generally applicable library of mass spectra obtained with different LC/MS instruments is possible, when the fragmentation energy is adjusted, using selected tune compounds. Gergov *et al.* [284] presented a straightforward approach for the application of LC/ESI-TOF-MS in toxicological screening. The features of TOF-MS were utilized, *i.e.*, high mass accuracy and high sensitivity over the full spectrum. On this basis, a library was established, containing 433 toxicologically relevant compounds (parent drugs and their metabolites), simply by calculation of their mono-isotopic masses. The mass range for compounds in the library extended from 105 to 734 Da. This library was used for the identification of drugs in urine extracts, by applying gradient elution with MeCN/ammonium acetate buffer at pH 3.2. The retention data of the drugs were stored, but not used for identification in this study. The results of LC/MS-TOF screening agreed with the results of GC and TLC screening, run in parallel. The method is very promising and allows screening for compounds with known formula, even without reference compounds. This is of practical value, particularly for metabolites, because reference standards are often unavailable. An intra-laboratory reproducibility study of mass spectra, obtained with similar or different instruments, is of primary relevance for establishing and using mass-spectral libraries. Weinmann *et al.* [285] compared mass spectra, obtained with two identical and one different LC/MS/MS system in three laboratories. Methadone, benzoylecgonine, and diazepam were used as test substances.

Product-ion spectra of protonated quasi-molecular were similar for all laboratories. Also, in-source CID spectra were very similar to the product-ion spectra. The last point is very important, because in the first step of LC/MS screening, the in-source generated mass spectra are collected and eventually confirmed by MS/MS analysis.

23.5 NATURAL COMPOUNDS

The analysis of natural toxins seems to play a secondary role in forensic toxicology. This is mainly due to the overwhelming pressure of drugs of abuse on one side, and by underreporting and ignorance concerning the social dangers of natural compounds on the other. It should be stressed that acute poisoning with natural compounds occurs mainly in developing countries, where the level of diagnostics and data gathering is low. Nevertheless, the use of herbal medicines in industrialized countries is rapidly growing [286]. Uncritical use of these preparations is associated with risk of acute or chronic poisoning with heavy metals as well as with toxic natural or synthetic compounds [287–289]. Gaillard *et al.* [290] have reviewed the analytical aspects of the forty toxic plants responsible for most fatal poisonings. They also published a LC/MS/MS method for the determination of plant toxins [291]. The samples were prepared by solvent extraction (for oleandrin, taxol, and the alkaloids) and acetone precipitation (for cyanogenic compounds and atractyloside). LC/MS was performed on an ODS column by gradient elution, and positive ionization was used for most compounds.

Lacassie *et al.* [292] reported a case of digitalis poisoning in which acetyldigitoxin, convallatoxin, deslanoside, digitoxigenin, digitoxin, digoxin, gitaloxin, gitoxin, latanoside C, methyldigoxin, oleandrin, proscilardin, and strophantidin were determined in blood and urine by LC/ESI-MS. After acetonitrile precipitation, the drugs were extracted with an organic solvent mixture and separated on an ODS column, using a gradient of MeCN/ammonium formate at pH 3.0. For each analyte, the protonated quasi-molecular ion $(M + H)^+$ and one or two fragment ions were monitored. The recovery was 67.8 to 98.6%, and the LOD 1 to 10 μg/L. This sensitivity was adequate for acute poisoning cases but not for therapeutic drug monitoring. Glycoside levels in blood were monitored from 8 to 100 h after intoxication. In a case reported by Gaillard and Pepin [293], veratridine and cevadine (toxins present in *Veratrum album*) were identified and quantified by LC/ESI-MS. Blood concentrations in two cases were 0.17 and 0.40 μg/L for veratridine and 0.32 and 0.48 μg/L for cevadine. The cardiac glycosides oleandrin, odoroside, neritaloside, and the aglycone oleandrigenin in an extract of *Nerium oleander* were analyzed by Q-TOF-MS. CID mass spectra were obtained with mass accuracy greater than 5 ppm; the LOD for oleandrin was 20 pg (injected) [294]. The method was applied to the determination of oleandrin in plasma and urine at 1.1 μg/L by LC/ESI-MS [295]. The alkaloids of *Aconitum napellus* are extremely toxic substances. Chen *et al.* [296] isolated 12 diterpenoid alkaloids, among them aconitine, by solvent extraction and TLC purification. Identification and structure elucidation was achieved by LC/IT-ESI-MS in MS^n mode. Maurer *et al.* [297] determined α- and β-amanitine in the urine and plasma of people poisoned with *Amanita* mushrooms. The toxins were isolated with immuno-affinity extraction columns and determined by LC/ESI-MS, with a LOD of 2.5 μg/L.

23.6 DOPING SUBSTANCES

Generally, doping agents may be divided into two main classes. The first includes all substances that are prohibited and should not occur in urine at all. The second group includes compounds that are broadly used socially (*e.g.*, caffeine) or for some therapeutic purposes (*e.g.*, ephedrine) as well as physiological substances (*e.g.*, testosterone). For the second group cut-off concentrations have been defined to differentiate between social use and misuse for doping. The International Olympic Committee [298] and The International Equestrian Federation [299] have published lists of prohibited compounds and lists of threshold values for physiological or therapeutic substances. These lists are being permanently reviewed and amended. Segura *et al.* [300] and Maurer [301] have reviewed chromatographic methods used in doping analysis. In this chapter, the methods and procedures published after 1999 will be presented.

Jimenez *et al.* [302] have formulated the following validation criteria for GC/MS, the most important method used in doping laboratories: absence of interfering substances at the retention time of the analyte in the blank sample (signal-to-noise ratio < 1), LOD recommended by international sport authorities, relative standard deviation $< 25\%$ at the low-concentration control and $< 15\%$ at the high-concentration control. No minimum acceptance criteria for recovery were established. As a LOD criterion, a signal-to-noise ratio > 3 for two of the three diagnostic ions in GC/MS analysis was taken. The preferred sample material in doping control is urine, but hair analysis is beginning to find use in the determination of anabolic and steroid hormones or β-adrenergic stimulants [303].

23.6.1 Human sports

Among doping substances used in human sports, the most frequently detected drugs are the anabolic agents (46.8%), followed by stimulants (17.2%), diuretics (4.4%), and narcotics (2.3%) [300]. A high urinary testosterone/epitestosterone ratio (> 6) is indicative of exogenous testosterone administration. Some athletes are therefore taking epitestosterone in order to lower the ratio. Aguilera *et al.* [304] developed an isotope ratio GC/MS method, which allowed them to differentiate between the endogenous and synthetic epitestosterone. The lack of commercially available standards of metabolites of anabolics may preclude the validation of these substances in quantitative doping analysis. Kuuranne *et al.* [305] established a procedure for the glucuronide conjugates of Phase I metabolites of methyltestosterone and nandrolone. The purity of stereoselective glucuronide conjugates was monitored by LC/MS. Kintz *et al.* [306] published a method for the determination of the anabolic steroid methenolone in hair. The drug was isolated after alkaline hydrolysis of hair by solid-phase and pentane extraction. The determination was performed with GC/MS/MS, using multiple reaction monitoring (two product ions). The LOD was 1 pg/mg from a 100-mg sample of hair. Hair analysis detects the abuse of nandrolone, norandrostendione, and norandrostendiol. Because all of these anabolic steroids are metabolized to the same substances, their use cannot be discriminated by urine analysis; in hair, however, the parent compounds can be identified [307].

Clenbuterol, a β-2-adrenergic receptor agonist, which is used as a bronchodilator for the treatment of respiratory diseases, may also be misused by athletes because of its anabolic properties. Several LC/MS [308], GC/MS [309], and GC/MS^n [310] procedures for its determination in urine have been published, with a LOD of 0.1–0.5 μg/L. GC/NPD was applied to the determination of stimulants used for doping. Ephedrine, pseudoephedrine, norephedrine, cathine, and methylephedrine have been isolated from urine by solvent extraction [311]. In another study [312], ephedrines were determined in native urine samples by column-switching HPLC. Both methods were found suitable for doping control. Diuretics are often taken by athletes to dilute the doping substances below the limit of detection. Thieme *et al.* [313] established a method for the screening and quantitation of 32 diuretic compounds and their metabolites in urine. The method, developed for doping control, was based on LC/ESI-MS/MS. The drugs were extracted form urine on a XAD column and separated on a C_8 column with a gradient of MeCN/ammonium acetate. A library of mass spectra was developed with the use of positive or negative ionization and optimized fragmentation conditions for particular compounds. Since diuretics may be either acidic and basic, the authors recommended two successive chromatographic runs (in the positive and negative mode) for the screening procedure. This is better than polarity switching in one run. LC/ESI-MS/MS was also applied to the determination of thiazide diuretics in negative-ionization mode. The structure of the fragments was elucidated [314]. Caslavska and Thormann [315] have determined furosemide in plain or diluted urine samples by CE. Furosemide was detected with LIF or IT-ESI-MS in the negative-ionization mode. The method was claimed to be rapid and simple. A fast screening method for the detection of 32 beta-receptor blocking drugs in hydrolyzed urine samples with LC/APCI-MS/MS has been described [316]. The analysis took only 7 min.

23.6.2 Animal sports

In animal sports, analgesics and anti-inflammatory drugs (*e.g.*, phenylbutazone, oxyphenbutazone, dexamethasone) are used most frequently, followed by stimulants (caffeine, theobromine) [300]. Takeda *et al.* [317] developed a systematic procedure for the detection of 107 doping substances in horse plasma, using mixed-phase SPE and fractional elution. The drugs in the eluates from the acidic and basic fractions were analyzed by GC–MS. Retention indices, based on the diisopropylaminoalkane scale, were calculated and used for identification, together with mass spectra. Detailed mass spectral data (base peaks and intensities of five prominent ions) were listed. Another screening method for the detection of 30 drugs in equine urine by LC/IT-ESI-MS was presented by Skinner *et al.* [318]. After mixed-mode SPE, the urine extracts were analyzed on an ODS column. A 2-μg/mL LOD was reported for all drugs (opiates, anti-depressants, tranquilizers, bronchodilators, and narcotics).

Dehennin *et al.* [319] studied the detection of dehydroepiandrosterone and androstenedione in horse doping by GC/MS in the SIM mode. Since both drugs are converted to testosterone in the body, the urinary testosterone/epitestosterone ratio is not useful. However, androstanediols and androstenediols were detected as markers. In addition, a transient increase in free testosterone was observed. A LC/APCI-MS/MS

method was applied to the determination of 19-nortestosterone and its esters in equine plasma; the LOD was in the 0.1- to 2-μg/L range. Detection of 19-nortestosterone, 23 days after intramuscular injection of the decanoate ester, was demonstrated.

Heavy exercise is a cause of specific disease states among racehorses, which are consequently treated with some drugs. Since gastric ulceration affects around 90% of racehorses, the use of anti-ulcerative drugs is common. Chung *et al.* [320] developed a LC/IT-ESI-MS/MS method for the detection and quantification of eight anti-ulcerative drugs (cimetidine, ranitidine, famotidine, nizatidine, omeprazole, pantoprazole, rabeprazole, and lansoprazole) in horse urine. The metabolites of ranitidine and omeprazole were also identified. The detection time window for ranitidine was more than two weeks after administration. Another common pathological state of racehorses is exercise-induced pulmonary hemorrhage. This may be prevented by administration of phosphodiesterase inhibitors, like sildenafil (Viagra). A LC/ESI-MS/MS method was developed for the identification of sildenafil and its four metabolites in horse plasma and urine after alkaline solvent extraction. A quadrupole-TOF instrument was used [321]. Tang *et al.* [322] applied CE/MS to the analysis of eight quaternary ammonium drugs in equine urine. A CE/MS/MS method was used for confirmation. CE/MS appeared to be superior to LC/MS with regard to sensitivity, separating power, and sample volume.

Clenbuterol is used in both human and animal doping to increase lean muscle mass. Lehner *et al.* [323] developed a LC/MS/MS method for the determination of this drug in horse serum. Clenbuterol was quantitated by an isotope dilution method with a synthetic d-9 analog. The LOD was 4 ng/L, and the detection window was 96 h after oral administration. Endotracheal administration of clenbuterol shortly before the race was detectable within some hours. LC/MS/MS and HPLC/UV methods were applied to the detection of caffeine as a metabolite in horse blood and urine after oral administration of theophylline. On the basis of these experiments, a threshold serum (250 μg/L) and urine (1 mg/L) caffeine concentration was proposed to differentiate between metabolic and administered caffeine [324]. Thiazide diuretics (chlorothiazide, hydrochlorothiazide, and trichlormethiazide) were determined in equine urine by LC/MS with ESI and APCI interfaces. The LOD ranged from 131 to 380 μg/L [325]. Doxepin, used in human medicine as anti-depressant, has been found useful for horse doping, due to its tranquilizing properties. Hagedorn *et al.* [326] have studied the pharmacokinetics of doxepin isomers after intravenous administration to horses by means of GC/MS. In a subsequent study, they applied GC/MS in electron-impact and positive-chemical ionization mode to the determination of the isomers of the nor- and hydroxy-metabolites of doxepin in blood and urine of intravenously treated horses [327].

23.7 ENVIRONMENTAL AND OCCUPATIONAL TOXINS

The analysis of toxic compounds in the environment or workplace is treated in Chap. 21. Here, we will discuss only some recent applications of obviously forensic significance. LC/ESI-MS in combination with GC/MS has been applied to the determination of 61 pesticides in human biological fluids. SPE with polymeric sorbents was used for the isolation and LC/MS for the separation of polar pesticides (carbamates, benzimidazoles).

The method was applied in cases of acute poisoning and in post-mortem casework [328]. Turcant *et al.* [329] reported a case of suicidal poisoning with metobromuron, a phenylurea derivative used as a herbicide. Metobromuron and three metabolites were identified in blood; eight further metabolites were found in the urine. The drugs were extracted with ethyl ether at high pH and determined with HPLC/DAD, LC/ESI-MS, and LC/ESI-MS/MS. Warfarin enantiomers were extracted from plasma with ethyl ether in acidic medium and separated on a β-cyclodextrin column with MeCN/HOAc/Et_3N (1000:3:2.5) in < 10 min. The drugs were determined with NI-ESI-MS/MS, using MRM for enantiomers of warfarin and of the internal standard *p*-chlorowarfarin; the LOQ was 1 μg/L [330].

23.8 CHEMICAL AND BIOLOGICAL WARFARE AGENTS

The spectrum of chemical and biological weapons is very broad and ranges from simple molecules, like nerve gases, to living microorganisms. Chemical weapons were classified by the United Nations in the Chemical Weapons Convention under three schedules, according to their toxicity. Each schedule comprises toxic chemicals and their precursors. A recent, comprehensive review of the analysis of chemical weapons was published by Kostiainen [331]. Most modern methods used for a preliminary detection of chemical and biological weapons do not involve chromatographic separation and are based on direct mass-spectrometric detection. In such a system for detecting 23 agents in the battlefield environment, EI- and IT-CI-MS/MS were used [332]. Nies *et al.* [333] devised a detection system for chemical and biological weapons, based on portable, battery-powered MS in an instrument comprising a photo-ionizer, quadrupole ion trap, and a time-of-flight mass spectrometer. The sample was introduced *via* pyrolysis GC. The total pyrolysis/GC time was 2 min, and the LOD was *ca.* 20 pg. A library of 48 compounds was established. A GC/MS double-focusing magnetic-sector system was used for detecting *Bacillus* and *Clostridium* spores. A detection limit of 5 spores on rice grains was demonstrated [334]. Earlier, the presence of dipicolinic acid – a biomarker for bacterial endospores – was monitored in air, using a portable pyrolysis gas chromatograph, coupled to an ion-mobility mass spectrometer [335]. Zidarov *et al.* [336] demonstrated the potential of pyrolysis MS for fingerprinting and library search of various microorganisms.

LC/MS is the method of choice for the detection of polar metabolites of modern chemical warfare agents. Black and Read [337] separated 19 degradation products of chemical warfare agents on a mixed C_8/C_{18} column. For detection and identification an APCI-MS instrument was used. D'Agostino *et al.* [338] applied capillary LC/ESI-MS to the direct analysis of four organosphosphate chemical warfare agents in aqueous samples with a LOD of 5 ng for full-scan conditions. They also analyzed sarin and 13 related organophosphates on snow with LC/ESI-MS. For the separation they used a packed fused-silica capillary column, filled with ODS material and coupled with ESI-TOF-MS. This method is an attractive alternative to GC/MS methods, because all substances involved can be analyzed directly without additional handling [339]. Hancock and D'Agostino [340] assessed the potential of mass-spectrometric identification of chemical and biological warfare agents, based on three levels of identification: provisional, confirmed, and

unambiguous identification. On each level chromatography/mass spectrometry was involved. LC/ESI-MS was applied in experimental procedures, using ochratoxin A and α-conotoxin as model substances. D'Agostino *et al.* [341] developed a capillary LC method and an ES-MS database for the identification of sixty chemical warfare agents, degradation products, and related compounds. Full mass spectra were collected, using TOF-MS at two dissociation energy levels.

23.9 EXPLOSIVES

Analytical aspects of explosives detection follow a strategy similar to the one in the case of chemical and biological warfare agents, *i.e.*, the methods are divided into preliminary and confirmatory ones. Yinon [342,343] has covered the analytical aspects of explosives up to 1999. In recently published applications of chromatographic methods to this group of compounds, TLC is practically absent. This does not mean that this method has become obsolete or useless. Due to its simplicity and low cost, TLC is widely used. There is, however, no need for further refinements of this proven technique. In recent years, research efforts have focused on application of LC/MS or other mass-spectrometric methods to the analysis of explosives. These methods may be divided into three main groups:

(a) methods used for personal screening (*e.g.*, at airport gates);
(b) methods used in field screening (post-explosion residues, debris); and
(c) confirmatory laboratory methods.

Most of methods used for personal screening are based on mass-spectrometric detection of airborne particles, collected from the examined person with a vacuum sampler. The samples are then subjected to thermal desorption and MS analysis. Chromatographic separation is usually not applied. A MS-based explosives detection portal for passenger screening was developed by Syage *et al.* [344]. The portal works as a clean-air shower, removing particles from the passenger and collecting them in a pre-concentrator. The sample is then thermally desorbed and directed into an analyzer, consisting of a negative-ion source, interfaced with a quadrupole IT-TOF system. The LOD ranges from 1 to 20 pg for various explosives. Sleeman *et al.* [345] constructed a device for the APCI-MS detection of explosives on aircraft boarding passes. The device employs infrared heating of the passes and analysis of the vapors by IT triple-quadrupole or ion-mobility MS, with an APCI interface. A similar system was developed by Takada *et al.* [346], who applied an APCI source, connected with an IT or single-quadrupole mass spectrometer for the identification of explosives in gas samples.

The methods used for field screening of explosives are mainly concerned with the most effective sampling procedures for the subsequent GC/MS or LC/MS analysis. A common procedure for collecting residues of explosives from skin or post-blast debris is to wipe the surface with solvent-saturated cotton swabs and subsequent analysis of the extracted swab. Thompson *et al.* [347] developed a method based on the collection of the explosive residues with cotton swabs soaked with a water/2-propanol mixture. The residues were extracted with water and purified by SPE on different cartridges. Identification was by HPLC/DAD, LC/MS, and GC/MS. The extracts were pure enough

for HPLC/DAD analysis. Furton *et al.* [348] applied SPME to the isolation of explosives from aqueous solution and from post-explosion residues, washed with acetonitrile/water. The extracts were analyzed by GC/ECD and HPLC/UV; 14 standard explosives were separated by both techniques; the LOD was at the ppb level. Miller [349] applied SPME to the field screening of eight common explosives (EGDN, NG, TNT, PETN, RDX, tetryl, and HMX). Samples of water or solid residues, dissolved in 25% aq. NaCl, were subjected to SPME on PDMS/DVB and CA/DVB fibers. The former fibers gave the better recoveries of explosives. SPME extracts were analyzed with GC/ECD and GC/MS. Batlle *et al.* [350] collected airborne traces of explosives by pumping the air through glass-fiber and polyurethane foam. Adsorbed compounds were then eluted and separated on-line by supercritical-fluid extraction/HPLC. The method was applicable to landmine-clearing operations.

The group of confirmatory methods uses gas- or liquid-chromatographic separations, combined, mainly, with mass-spectrometric detection. Yinon [351] applied LC/MS to the identification of various explosives. LC/NI-APCI-MS/IT-MS was applied to the identification of several groups of explosives and their production site. The latter was achieved in the case of trinitrotoluene (TNT) by analyzing the by-products, such as isomers of TNT, dinitrotoluene (DNT), trinitrobenzene, and dinitrobenzene. Nitrate ester explosives were determined by ESI and LC/APCI-MS, using post-column additives. Nitrate ester explosives have different vapor pressures and are difficult to analyze by a single GC method. Zhao and Yinon [352] analyzed pentaerythritol tetranitrate (PETN), nitroglycerin (NG) and ethyleneglycol dinitrate (EGDN) using LC/MS/MS with ESI or NI-APCI. Various post-column additives were tested to enhance sensitivity. The LOD was 5 ppb for PETN and NG, and 1 ppm for EGDN. Davis *et al.* [353] have applied fast-vacuum column chromatography, combined with TOF-MS in a fast-detection method for explosives; 9 compounds were separated in 2 min. Some authors have used other detection methods than mass spectrometry. Wissinger *et al.* [354] applied gradient RP-HPLC/DAD to the identification of various brands of smokeless powder. A library of UV spectra for 16 compounds was established, comprising NG, TNT, DNT isomers, nitrotoluenes, nitrophenylamines, and phtalates. The method allowed different lot samples of the same brand of powder to be distinguished.

Smith *et al.* [355] have demonstrated the usefulness of CE for the detection of smokeless-powder residues. Pipe bombs, filled with known types of smokeless powder, were detonated under controlled conditions. The residues were collected with ethanol-soaked cotton swabs and extracted by SFE. Alternatively, the residues were collected with tape lift and washed with ethanol. The extracts were subjected to MEKC with UV detection at 200 nm. It was possible to identify the type of powder by calculating the peak-height ratio of dinitrotoluene:diphenylamine. The analysis time was 15 min. Bowerbank *et al.* [356] used solvating GC for the analysis of NG, TNT, DNT, and PETN. In this method, separation on packed columns with carbon dioxide as the mobile phase and a chemiluminescence thermal-energy analyzer, specific for nitro functional groups, were used. The analysis of anions, like azide, chlorate, nitrate, nitrite, perchlorate, sulfate, and thiocyanate in post-explosion residues adds important clues for the identification of the compounds involved. Miller *et al.* [357] have critically reviewed the application of ion

chromatography and CE for this purpose and demonstrated the usefulness of both techniques.

Since the detection methods used for explosives are extremely sensitive, the problems of background values and quality assurance are of primary importance. Walker *et al.* [358] have studied the background levels of inorganic anions, cations, and sugars in the general environment by ion chromatography. The samples were taken from clothing, the surfaces of houses, cars, roads, etc. They described a quality assurance testing regime, as applied in the British Forensic Explosives Laboratory. Laboratory contamination with explosives was constantly monitored, using solvent-moistened swabs, which were analyzed by GC/chemiluminescence thermal-energy analyzer. The detection of more than 5 ng of explosive caused a mandatory action [359]. Sigman and Ma [360] studied detection limits for GC/MS analysis of organic explosives, using EI, NI-CI and PI-CI methods. Limits of detection for 9 explosives in GC/EI were from 0.71 to 3.36 ng; in GC/PI-CI, 0.64–2.38 ng; and in GC/NI-CI, 0.10–1.11 ng.

23.10 ARSON ACCELERANTS

The differentiation of arson from unintentional or non-criminal fire is very difficult and cannot be accomplished by chemical analysis alone. However, the analysis of residual ignitable fluids may furnish extremely important information concerning its origin. GC, particularly in combination with MS, is the most important method of analyzing ignitable fluids and their residues in fire debris. An interpretative approach to GC/MS data is based on the fact that the compounds involved may be grouped into defined classes, *e.g.*, paraffins, light aromatics, heavy aromatics, and alkylcyclics. Each of these classes may be characterized by a limited number of ions in the mass spectra. The chromatographic analysis of fire debris for potential accelerants has been thoroughly reviewed by Bertsch and Ren [361]. The first step in the analysis, *i.e.*, the sampling, is based mainly on heated-headspace enrichment. This may be accomplished in a dynamic (purge-and-trap) or static mode (adsorption on carbon strips). Recently, SPME has also been introduced in this area. The mode of sampling may influence the data collected, even within the same sampling technique. This was studied in detail for SPME by Ren and Bertsch [362], who optimized SPME with respect to fiber type, sampling time and temperature, analyte concentration, and the effect of water. In the static adsorption method, where carbon strips are exposed to fire debris, the ratio of lighter compounds (C_7 to C_{10}) to heavier ones (C_{14} to C_{20}) is higher with shorter exposure time [363]. Another source of potential variability of results is the type of fire extinguisher used. McGee and Lang [364] studied the effect of a modern fire suppression agent called a "F-500 Micelle-Encapsulator Fire-Suppression Agent" on debris analysis by GC/FID and GC/IT-MS. The study showed that F-500 generates a characteristic peak pattern and may contribute to background interference, but does not preclude the identification of ignitable fluids. Wallace [365] developed software for the interpretation of GC/MS data collected from the analysis of fire debris. The software discriminates between the main groups of ignitable fluids. The ruggedness of the method, which was applied in forensic casework, has been demonstrated. Two-dimensional GC for the analysis of ignitable fluids was developed by Frysinger and Gaines [366]. The sample,

collected on a carbon strip and eluted with carbon disulfide, was subjected to GC in the first column (4.75 m × 0.10 mm, 0V-1), which was connected to a second column (2.0 m × 0.10 mm, OV-1701). A FID was used and the results were displayed as a multi-color two-dimensional chromatogram, well suited to fingerprinting.

23.11 INKS AND OTHER WRITING OR PRINTING MEDIA

Forensic analysis of writing media serves two main purposes: identification of inks/pigments and determination of the age of the ink on the document. In a recent chapter on the applications of chromatography in the forensic analysis of writing media and documents, Aginski [367] reviewed most of the important developments up to 1998. Specifically, TLC, GC, and GC/MS have been applied to ink dating. Pyrolysis GC has been used for the identification of non-volatile components in inks, photocopy toners, and papers. HPLC and CE have also been applied. Another review of the chromatography of inks was published by Zlotnick and Smith [368]. Intensive use of HPLC for the analysis of inks was predicted. HPLC/DAD, together with principal-component analysis, has been applied to the identification of various ballpoint-pen inks [369]. Andrasko [370] studied the aging of ballpoint-ink writing by HPLC. The exposure of inks to daylight or artificial light is associated with the decomposition of pigments (Fig. 23.4). Therefore, the same ink can give a different chromatogram, depending on the time and history of storage. In a further study, Andrasko [371] observed similar patterns of ink aging on documents stored in darkness. However, the speed of decomposition was much slower. A method for relative dating of documents stored in darkness was proposed. Unfortunately, this method cannot be used when the documents are exposed intermittently to daylight or artificial light. Weyermann *et al.* [372] subjected methyl violet (a common dye used in ballpoint ink) to controlled exposure to heat or light. The compound was then analyzed by MALDI- and LDI-MS. A correlation between the duration of light exposure and degradation of pigment was observed. De Koeijer and de Moel [373] applied pyrolysis-GC/MS and FTIR to the identification of black photocopy toners. The sample was transferred from the document by applying a steel disc heated to 180°C for 2 sec. No visible damage was done to the document, and the sample was used for both methods of analysis.

23.12 DNA TYPING

23.12.1 Introduction

An introduction to nucleic acid analysis is presented in Chap. 19. Here, we will restrict ourselves to forensic applications. DNA typing or DNA profiling, as it is now known, was first described in 1985 by Jeffreys and co-workers [374] and it has had a tremendous impact in forensic genetics. Before that, all forensic genetic casework (paternity testing, criminal casework, individual identification) was performed by classical serological genetic markers. Blood groups, human leukocyte antigen, and polymorphic proteins and

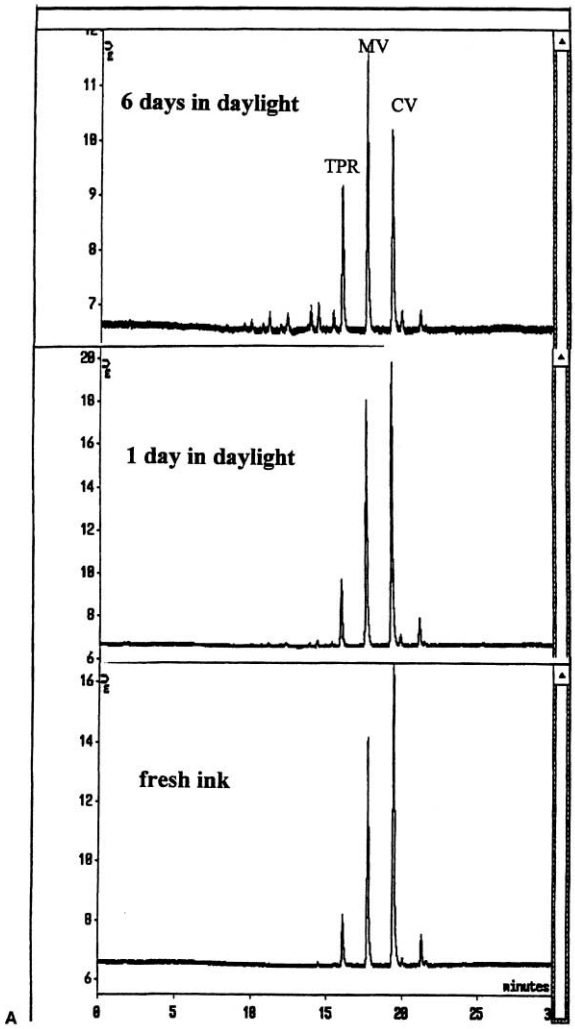


Fig. 23.4. HPLC of a blue ball-point pen ink, showing changes in composition caused by the exposure to daylight. (Reprinted from Ref. 370 with permission.)

enzymes were used for solving forensic genetic casework by means of immunological and electrophoretic methodologies. These genetic markers were nevertheless limited when it was necessary to analyze minimal or degraded material, as is common in forensic cases. It was, in addition, difficult to analyze biological material other than blood, and therefore the information obtained from hair, saliva, and even semen was rather limited. Due to the fact that polymorphic proteins and enzymes are infrequent, it was necessary to obtain as much information as possible. Therefore, sophisticated electrophoretic methods, such as isoelectric focusing, immobilines, or hybrid isoelectric focusing were developed and

applied. In spite of this, the information that the forensic geneticists were able to report was clearly insufficient in many cases.

Since the discovery of polymorphisms in repetitive DNA by Jeffreys *et al.* [374], highly informative and robust DNA typing systems have been developed, which are quite powerful for the individualization of biological materials of human origin. DNA typing has several advantages over traditional protein analysis. First of all, it is more informative and can be applied to minute or degraded material, since DNA is physically much more resistant to degradation than proteins. In addition, the same DNA genotype can be obtained from any tissue (blood, saliva, semen, hair, skin, or bones), whereas the analysis of protein markers is restricted to cells where these proteins are expressed. Nowadays, DNA analysis has become the standard method in forensic genetics, as it is currently used by most of the forensic laboratories and especially in criminal forensic casework (stain and hair analysis).

23.12.2 DNA polymorphisms

Hidden in the *ca.* 3 billion base-pairs of DNA of the haploid human genome are an estimated 35,000 genes [375]. All human genes are encoded in approximately 10% of the human genome. Thus, most (90%) of the human genome represents "non-coding" parts, containing no genetic information directly relevant to protein synthesis. Genetic variation is rather limited in coding DNA, with the exception of the HLA region. This is due to the fact that expressed genes are subjected to selection pressure during evolution to maintain their specific function. In contrast, the non-coding part of the genome is not mainly controlled by selection pressure, and thus mutations in these regions are usually kept and transmitted to the offspring, leading to a tremendous increase in genetic variability. Therefore, these regions are very appropriate for forensic genetics because they are very informative, and at the same time not useful for drawing conclusions about the individual other than for identification purposes. An important percentage of the non-coding DNA (30%) consists of repetitive sequences which can be divided into two classes: tandemly repetitive sequences and interspersed elements. The majority of forensic typing systems in current use are based on genetic loci with tandem repetitive DNA sequences.

Tandemly repeated sequences can be found in satellite DNA, but from the forensic point of view, regions of repetitive DNA, much shorter than satellite DNA, are much more interesting. These regions can be classified into minisatellites [374] and microsatellites or short tandem repeats (STR) [376,377]. Minisatellites, otherwise known as VNTR (variable number of tandem repeats) loci [378] are composed of sequence motifs ranging from *ca.* 15–50 bp in length, reiterated tandemly for a total length of 500 bp–20 Kb. STRs are much shorter. The repeat unit ranges from 2 to 6 bp for a total length between 50 and 500 bp.

In addition, minisatellites and STR have differences in their distribution in the human genome and probably in their biological function. Thus, minisatellites are more common in subtelomeric regions, while STR are widely distributed throughout the human genome, occurring with a frequency of 1 locus every 6–10 kb [379]. The origin of the variability seems to be different as well. While unequal crossing-over and even gene conversion [380] are involved in the variability of minisatellites, replication slippage is mainly involved in

the origin of the variability in microsatellites [381]. The genetic variation between individuals in these minisatellites and STR systems is mainly based on the number of tandemly arranged core repeat elements, but it is also based on differences in the DNA sequence itself, since the repeats can have slight differences in the sequence.

23.12.3 DNA typing methods

Technologies used for DNA typing for forensic purposes differ in their ability to differentiate between two individuals and in the speed and sensitivity with which results can be obtained. The speed of forensic DNA analysis has improved dramatically. DNA testing that previously took more than one week can now be performed in a few hours. Southern blotting with multi-locus and single-locus probe (SLP) DNA probes have been used for paternity testing and forensic stain typing and they are still used (especially SLP) by a few laboratories working in paternity testing. Briefly, the technique is as follows: First, the DNA is extracted and then cut by a specific enzyme (usually Hinf I in Europe and Hae III in the US) into many small fragments. A tiny fraction of those fragments include the particular minisatellite to be analyzed. The fragments are then separated by electrophoresis in agarose gels with Tris/acetate/EDTA (TAE) or Tris/borate/EDTA (TBE) as buffers. After an appropriate length of time, the fragments migrate different distances in the electric field, depending mainly on their sizes, the smaller ones migrating more rapidly. DNA fragments are then chemically treated to separate the double strands into single ones. Because the gels are difficult to work with, the single-stranded fragments are then transferred directly to a nylon membrane, to which they adhere. This process is called Southern blotting, named after its inventor [382]. The next step is to flood the membrane with a single-stranded probe, which will hybridize with the DNA fragments that contain the target minisatellite sequence and adhere to it. The membrane is then washed several times to remove any probe that does not bind to this specific DNA sequence. Probes can be labeled by isotopic or non-isotopic methods. Non-isotopic chemiluminescent methods are now more popular than isotopic ones. Whatever the labeling method is, the nylon membrane is usually placed on an X-ray film, resulting in an autoradiograph with the bands of the minisatellite clearly impressed in the film. The process requires several days for the radioactive decay to produce a visible band on the film.

Originally, multi-locus probes were proposed for forensic genetic analysis. However, this type of probes was not very successful in the forensic field, because, although they are informative, statistical problems of evaluation of the evidence in cases of match and standardization problems arose. Therefore, these probes have been replaced in the forensic field by the analysis of VNTR with SLP under high-stringency conditions. Until the introduction of STR analysis by the polymerase chain reaction (PCR), minisatellite analysis with SLP was very popular in forensic laboratories. Nowadays it is still used in some labs especially for paternity testing analysis. The main advantage of SLP analysis is the enormous variability of some of the minisatellites and the adequate knowledge of the mutation rate in some of them. The main disadvantages are the time needed for the analysis and especially the need for a relatively large amount of non-degraded DNA for SLP typing. Since DNA extracted from forensic specimens is often degraded due to

environmental conditions, these techniques have often failed to produce reliable results. The PCR has overcome these difficulties, and it has strongly enhanced the usefulness of DNA profiling techniques in forensic science.

The PCR is a technique for the *in vitro* amplification of specific DNA sequences by the simultaneous primer extension of complementary strands of DNA. The PCR method was devised and named by Mullis *et al.* [383], although the principle had been described in detail by Khorana *et al.* [384] over a decade earlier. The use of PCR was limited until heat-stable DNA polymerases became available. DNA polymerases carry out the synthesis of a complementary strand of DNA in the 5′- to 3′-direction, using a single-stranded template, but starting from a double-stranded region. The PCR uses the same principle, but employs two primers, each complementary to opposite strands of the region of DNA, which have been denatured by heating. The primers are arranged so that each primer extension reaction directs the synthesis of DNA towards the other. This results in the *de novo* synthesis of the region flanked by the two primers.

Automated temperature cyclers (usually called thermocyclers) allow the exact control of successive steps of denaturation, annealing of the primers, and extension (when the DNA polymerase extends the primer by using a complementary strand as a template). All three steps constitute a cycle, and a normal PCR reaction consists of 20–25 cycles, allowing the formation of 2^{20-25} molecules from a single molecule of template DNA. Most PCR-based typing systems allow alleles to be identified as discrete entities, thus avoiding most of the statistical issues that arise in matching and binning SLP bands, and making standardization easier. Also, apart from the increased sensitivity inherent in any PCR technique, it is more likely to be successful in analyzing old or badly degraded material, mainly because of the small size of some of the DNA polymorphisms [single-nucleotide polymorphism (SNP) and STR] susceptible to analysis by PCR [385,386]. Once PCR has been used to generate a large number of copies of a DNA segment of interest, different approaches may be taken to detect genetic variation within the segment amplified. Because 10^6 or more copies of the target sequence can be produced, it is possible to use non-isotopic methods of detection. A number of imaginative methods for PCR product detection have been described.

The first one was the use of sequence-specific oligonucleotide (SSO) probes [387] to detect variation in HLA-Class II genes, especially in the HLA DQA1 system [388]. A SSO probe is usually a short oligonucleotide (15–30 nucleotides in length), with a sequence exactly matching the sequence of the target allele. The SSO probe is mixed with dissociated strands of PCR reaction product under very stringent hybridization conditions such that the SSO and the PCR product strand will be hybridized if there is a perfect sequence complimentarity, but will not be if the sequences are different. The classical format for the use of SSO probes is to spot dissociated PCR product strands onto a nitrocellulose or nylon membrane and to probe the membranes with labeled SSO; because of the fact that the samples are spotted as a "dot" on the membrane, this format is known as dot blotting. A reverse dot-blot format is much more commonly used [389]. With this format (which has its antecedents in affinity chromatography), the SSO is immobilized on the membrane and is used to capture PCR products containing biotin label in the primers. Several genetic loci can be analyzed by this technology, using commercially available kits. The AmpliType PM PCR amplification kit (Perkin-Elmer) is the most popular one in

forensic laboratories. With this kit, the loci HLA DQA1, LDLR, GYPA, HBGG, D7S8 and GC are amplified in a multiplex fashion. The last five loci listed are typed simultaneously in a single reverse dot-blot strip, containing allele-specific oligonucleotide (ASO) probes; HLADQA1 must be typed in a separate strip. The system was validated for forensic analysis [390], and it is still widely used, although most laboratories now prefer to use more informative STR systems.

The efforts of forensic scientists have mainly addressed the amplification of fragment-length polymorphisms. The minisatellite D1S80 (pMCT118) was the first one to be applied to the forensic routine [391], but these systems have been substituted by STR. Analysis of STR by PCR is the method of choice for forensic identification nowadays. Dinucleotide STR are the most common STR in the human genome and are the genetic markers most commonly used for linkage analysis, although they are not being used in forensic science. The reason for this is that analysis of these STR has been affected by strand slippage during amplification, producing artifactual stutter bands [392]. Nevertheless, tetra- and pentanucleotide repeats appear to be less prone to slippage and are more suitable for forensic purposes. The percent of stutters is very interesting for identifying and selecting ideal STR for forensic purposes, since having a low percentage of stutters is critical for the analysis of mix stains. Some tetranucleotide STR (such as TH01) are known to behave well regarding these characteristics, but pentanucleotides are ideal systems for analyzing mixtures.

According to their structure, STR range from extremely complex to the simplest [393]. Complex STR have the advantages of hypervariability. Simple STRs have the advantages of easy standardization and low mutation rates. Mutation events are more frequent in the male germ line, and the rates of different loci can differ by several orders of magnitude, structure and length being the most influential factors in the rate [394]. In addition to the characteristics already mentioned, the selection of ideal STR for forensic purposes include the analysis of other artifactual bands, the robustness and the size. In general, short sizes are desirable, since the size of the amplified product is critical in degraded samples, and small fragments can be amplified where larger fragments failed [386]. Another important fact is the possibility of amplifying multiple STR loci in a single multiplex reaction. This, coupled with the direct detection of amplified products on polyacrylamide gels, makes STR DNA profiling amenable to automation. Therefore, the ability to be included in multiplexes is another characteristic that should be analyzed for the selection of good STR for forensic purposes. STR were first analyzed with manual electrophoretic systems. Denaturing polyacrylamide gels are recommended for standardization purposes, given that with native gels sequence variation can also be detected, making the typing prone to errors. STR electrophoretic mobility under native and denaturing conditions should also be checked, since some STR (especially AT-rich STR) have been shown to have anomalous mobility in polyacrylamide gels [395].

The introduction of fluorescent-based technology and the use of DNA sequencers have revolutionized the field, allowing the typing of large multiplexes (including up to ten systems) as well as the automation of the typing procedure. Commercially available STR multiplexes for manual electrophoretic systems are available, but the major advantages of the use of sequencers is automation and the possibility of using intelligent systems of interpretation. The use of sequence reference allelic ladders is essential for STR typing.

In general, the reference allelic ladders comprise most of the alleles of the system, but intermediate alleles are always possible, even in the most simple STR. Interpretation guidelines have been produced [396,397] to distinguish these intermediate alleles, and they can be easily implemented in automatic sequencers. Many multiplexes are commercially available. A very popular one is the SGM Plus (Perkin-Elmer) which comprises 10 loci-HUMFIBRA/FGA, HUMVWFA, HUMTH01, D18S51, D21S11, D6S477, D8S1179, D16S539, D19S433, and amelogenine. Promega multiplexes are also very popular, especially in laboratories using manual electrophoretic systems or monochromatic sequencing platforms. But the extremely discriminative 15-plexes are becoming more and more popular. Among them, the Powerplex16 (Promega Corp) and the Identifiler (Perkin-Elmer) are the more commonly used by forensic laboratories. In general, the combined discrimination power of STR is enormous, and the probabilities of two unrelated individuals matching by chance (pM) are lower than 10^{-15} for some of these large multiplexes.

23.12.4 Short tandem repeats in sex chromosomes and mitochondrial DNA

Y-chromosome-specific polymorphisms have proved to be especially useful in forensic work. The applications of Y-polymorphisms include deficiency paternity testing when a male offspring is in question and different applications in criminal casework. Y-polymorphisms are specially interesting for the analysis of male DNA fraction in stains involving male/female mixtures, the most common biological material available in sexual crimes. Especially important is the use of these markers in cases where preferential sperm DNA extractions fails and also in rapes committed by azoospermic individuals. Although the variation in the Y-chromosome is low, the non-pseudo-autosomal region still bears different kinds of polymorphisms, including biallelic markers, STR, and minisatellites. SNP and STR are the most interesting. The most used Y-STR are the trinucleotide repeat DYS392, and the tetranucleotide repeats DYS19, DYS385, DYS389-I, DYS389-II, DYS390, DYS391, and DYS393. This STR comprises the so-called minimum Y-STR haplotype [25], but new STR have recently been described [399,400]. As for mtDNA, statistical interpretation in cases of match is more complicated, and appropriate corrections, taking into account population substructure and sampling errors, need to be performed. Population compilations are therefore very important, and much effort has gone into this [401]. A review and a compilation of recent work in the field can be found in Ref. 402.

STR in the X-chromosome are actually being introduced [403], and they are of interest for some deficiency paternity testing cases. Analysis of the mtDNA control region is an efficient method for the study and comparison of bones, old and degraded DNA and, especially, the analysis of telogenic hairs. In these cases, samples of mtDNA variation can be analyzed, using a variety of strategies. The combination of PCR amplification with direct DNA sequencing is usually the ultimate choice for identification, and it has proved to be a reliable and reproducible method in forensic casework [404]. Analysis of mtDNA is a valid method to be applied in forensic genetics, and it is accepted in courts all over the world. However, problems such as mutation rate, heteroplasmy, and the statistical approach, make the interpretation sometimes difficult. A good review of mtDNA analysis

in forensics can be found in Ref. 405. The ISFG DNA Commission recommendations and EDNAP recommendations on the use of mtDNA, including nomenclature, prevention of contamination (an aspect that it is crucial in mtDNA analysis), and statistical interpretation have been published recently [406,407].

23.12.5 Outlook

The future points definitely to technology and automation, and much progress has been made in this respect over the last few years. Capillary electrophoresis (CE) (Chap. 9) [408] is a powerful technique for DNA sequencing and fragment analysis, especially when it is combined with confocal fluorescence detection, yielding excellent sensitivity, together with high-speed and high-throughput separations. Capillary electrophoresis is replacing the use of gel-based sequencers for both DNA sequencing and fragment analysis. New electrophoretic microdevices (Chap. 11) [409], based on CE strategies promise to revolutionize several aspects of STR typing in forensics through a 10- to 100-fold increase in the rate of analysis and allowing the technique to become both fully automated and sufficiently miniaturized to become portable.

Mass spectrometry (MS) represents another strategy for analyzing STR loci within seconds, with the additional advantage that it is not necessary to use allelic ladders [410]. Substantial improvements have been made in recent years with the development of an effective ionization procedure, known as matrix-assisted laser-desorption ionization (MALDI) (Chap. 10) and the discovery of new matrices, particularly 3-hydroxypicolinic acid. As part of the process, products to be analyzed are co-crystallized with a small organic compound, the matrix. Irradiation with a short laser pulse causes a spontaneous volatilization of matrix molecules. Along with these matrix molecules, DNA molecules are ionized and desorbed into the gas phase, where they are subjected to an electric field. Determination of the molecular weight of the DNA is achieved by measurement of the time of flight (TOF) towards a detector, which is proportional to the mass. The main problem with this technology is the limitation in the size of the amplified product, since only fragments of less than 100 bp can be analyzed.

The use of SNP could be the next step in the continuous evolution of this field. SNP are the simplest polymorphisms (a single base change), and they are usually biallelic markers. Despite their simplicity and the fact that between 50 and 100 SNP are needed to achieve the same discriminating power as with the STR technology in use [411], they have some potential advantages that make them very attractive for forensic scientists. The first is the possibility of analyzing very short fragments, allowing the analysis of degraded material, and, moreover, they have low mutation rates. This is a potential advantage in paternity testing. Finally, different, extremely sensitive technologies can be used for SNP detection. Many technologies have been proposed for SNP typing [412], and in the forensic field the use of real-time PCR [413], MALDI-TOF-MS [414], minisequencing methodologies [415], and the use of DNA micro-arrays [416] have been explored. Concerning the latter, it is now becoming clear that micro-array technology will be a fundamental tool used in forensic genetic analysis. The explosion of interest in array technologies has been sparked by two key innovations. The first was the use of non-porous solid supports, such as glass, which has facilitated miniaturization and fluorescence-based detection [417]. The second

was the development of methods for high-density spatial synthesis of oligonucleotides [418] and the advances in spotting technology. Most of the laboratories exploring SNP typing and micro-arrays are using 3D slides with SBE-TAG methodology. A new generation of array technology allows negatively charged DNA molecules to be electronically addressed to specific sites on a microchip. These chips, called electronically activated chips [419], allow the simultaneous and precise identification of alleles containing different numbers of repeats (STR), as well as SNP, and have a promising future in the field.

23.13 CONCLUDING REMARKS

It is quite certain that chromatographic methods will keep their most important role in forensic analysis, and a very distinct trend in favor of LC/MS is predictable for the next few years. GC is the most mature technique and shows less room for future improvements. GC/MS will retain its position in general screening and in the analysis of volatile compounds, *e.g.*, in arson analysis. LC is in the process of rapid development with respect to both separation and detection. In the near future, the use of affordable, bench-top tandem LC/MS instruments will be ubiquitous. Application areas of LC/MS will cover practically the whole spectrum of compounds of forensic relevance. The use of LC/MS as a tool for general screening will be common, and libraries of mass spectra will be available with any instrument purchase. Another expected trend is the use of highly selective detectors, like TOF-MS, connected directly to the sampling device. Skipping the use of chromatography, which is already observed, has a future in field applications, such as terrorism prevention or arson analysis.

REFERENCES

1 R. Aderjan, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 489–530.
2 F.T. Peters and H.H. Maurer, *Bull. Int. Assoc. Forensic Toxicol.*, 32 (2002) 16.
3 A.H.B. Wu, D.W. Hill, D. Crouch, C.N. Hodnett and H.H. McCurdy, *J. Forensic Sci.*, 44 (1999) 516.
4 R. Wennig and A. Verstraete, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 439–457.
5 R. Aderjan, W. Bonte, T. Daldrup, H. Kaferstein, G. Kauert, H. Joachim, M.R. Moeller, G. Reinhard, G. Schewe and J. Wilske, *Toxichem. Krimtech.*, 65 (1998) 70.
6 Legal text, 625 *Illinois compiled statutes*, § 11501, USA.
7 B.D. Paul, C. Dreka, E.S. Knight and M.L. Smith, *Planta Med.*, 62 (1996) 544.
8 Z. Fater, Z. Samu, M. Szatmary and S. Nyiredy, *Acta Pharmaceut. Hung.*, 67 (1997) 211.
9 M.G. Pelders and J.J.W. Ros, *J. Forensic Sci.*, 41 (1996) 209.
10 H. Huizer, *Analytical Studies on Illicit Heroin*, PhD Thesis, Technical University Delft, 1988.
11 T.A. Gough, in T.A. Gough (Ed.), *The Analysis of Drugs of Abuse*, Wiley, Chichester, 1991, pp. 511–566.
12 A. Johnston and L.A. King, *Forensic Sci. Int.*, 95 (1998) 47.
13 K. Janhunen and M.D. Cole, *Forensic Sci. Int.*, 102 (1999) 1.

14 E. Kaa, *Forensic Sci. Int.*, 64 (1994) 171.
15 S. Klemenc, *Forensic Sci. Int.*, 108 (2000) 45.
16 H. Kalasz, L. Kerecsen, T. Csermely, H. Gotz, T. Friedmann and S. Hosztafi, *J. Planar Chromatogr.*, 8 (1995) 17.
17 N.K. Nair, V. Navaratnam and V. Rajananda, *J. Chromatogr.*, 366 (1986) 363.
18 E. Della Casa and G. Martone, *Forensic Sci. Int.*, 32 (1986) 117.
19 H. Neumann, *Forensic Sci. Int.*, 69 (1994) 7.
20 J. Cartier, O. Gueniat and M.D. Cole, *Sci. Justice*, 37 (1997) 175.
21 P.A. Hays and I.S. Lurie, *J. Liq. Chromatogr.*, 14 (1991) 3513.
22 I.S. Lurie, K.C. Chan, T.K. Spratley, J.F. Casale and H.J. Isaaq, *J. Chromatogr. B*, 669 (1995) 3.
23 I.S. Lurie, *J. Chromatogr. A*, 780 (1997) 265.
24 A.F. Hernandez, A. Pla, J. Moliz, F. Gil, M.C. Goncalvo and E. Villanueva, *J. Forensic Sci.*, 37 (1992) 1276.
25 M. Chiarotti, N. Fucci and C. Furnari, *Forensic Sci. Int.*, 50 (1991) 47.
26 F. Besacier, H. Chaudron-Thozet, M. Rousseau-Tsangaris, J. Girard and A. Lamotte, *Forensic Sci. Int.*, 85 (1997) 113.
27 M. Chiarotti and N. Fucci, *J. Chromatogr. B*, 733 (1999) 127.
28 E.J. Cone, B.A. Holicky, T.M. Grant, W.D. Darwin and B.A. Goldberger, *J. Anal. Toxicol.*, 17 (1993) 327.
29 A.J. Jenkins, R.M. Keenan, J.E. Henningfield and E.J. Cone, *J. Anal. Toxicol.*, 18 (1994) 317.
30 G. Skopp, B. Ganssmann, E.J. Cone and R. Aderjan, *J. Anal. Toxicol.*, 21 (1997) 105.
31 E. Gyr, R. Brenneisen, D. Bourquin, T. Lehmann, D. Vonlanthen and I. Hug, *Int. J. Clin. Pharmacol. Ther.*, 38 (2000) 468.
32 E.J. Cone, P. Welch, J.M. Mitchell and B.D. Paul, *J. Anal. Toxicol.*, 15 (1991) 1.
33 J. Vecerkova, *Soudni Lek.*, 33 (1997) 32.
34 D.J. Dietzen, J. Koenig and J. Turk, *J. Anal. Toxicol.*, 19 (1995) 299.
35 R. Jain, R. Rav, B.M. Tripathi and C. Singh, *Indian J. Pharmacol.*, 28 (1996) 220.
36 R. Wasels and F. Belleville, *J. Chromatogr. A*, 674 (1994) 225.
37 W.L. Wang, W.D. Darwin and E.J. Cone, *J. Chromatogr. B*, 660 (1994) 279.
38 E.J. Cone and W.D. Darwin, *J. Chromatogr.*, 580 (1992) 43.
39 B.A. Goldberger, E.J. Cone, T.M. Grant, Y.H. Caplan and B.S. Levine, *J. Anal. Toxicol.*, 18 (1994) 22.
40 D.G. Watson, Q. Su, J.M. Midley, E. Doyle and N.S. Morton, *J. Pharm. Biomed. Anal.*, 13 (1995) 27.
41 B. Fryirs, M. Dawson and L.E. Mather, *J. Chromatogr. B*, 693 (1997) 51.
42 M.R. Moeller and C. Muller, *Forensic Sci. Int.*, 70 (1995) 125.
43 C.L. O'Neal and A. Poklis, *J. Anal. Toxicol.*, 21 (1997) 427.
44 C.L. O'Neal and A. Poklis, *Forensic Sci. Int.*, 95 (1998) 1.
45 C. Staub, M. Marset, A. Mino and P. Mangin, *Clin. Chem.*, 47 (2001) 301.
46 M.J. Bogusz, R.D. Maier, M. Erkens and U. Kohls, *J. Anal. Toxicol.*, 25 (2001) 431.
47 M.J. Bogusz, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 3–65.
48 D. Bourquin, T. Lehmann, R. Hammig, M. Buhrer and R. Brenneisen, *J. Chromatogr. B*, 694 (1997) 233.
49 P. Zuccaro, R. Ricciarello, S. Pichini, I. Altieri, M. Pellegrini and G. D'Aszenzo, *J. Anal. Toxicol.*, 21 (1997) 268.
50 M.J. Bogusz, R.D. Maier and S. Driessen, *J. Anal. Toxicol.*, 21 (1997) 346.
51 M.J. Bogusz, R.D. Maier, M. Erkens and S. Driessen, *J. Chromatogr. B*, 703 (1997) 115.

52 M.J. Bogusz, *J. Chromatogr. B*, 748 (2000) 3.
53 M. Zheng, K.M. McErlane and M.C. Ong, *J. Pharmaceut. Biomed. Anal.*, 16 (1998) 971.
54 G. Schanzle, S. Li, G. Mikus and U. Hofmann, *J. Chromatogr. B*, 721 (1999) 55.
55 A. Dienes-Nagy, L. Rivier, C. Giroud, M. Augsburger and P. Mangin, *J. Chromatogr. A*, 854 (1999) 109.
56 M. Blanchet, G. Bru, M. Guerret, M. Bromet-Petit and N. Bromet, *J. Chromatogr. A*, 854 (1999) 93.
57 M.S. Slawson, D.J. Crouch, D.M. Andrenyak, D.E. Rollins, J.K. Lu and P.L. Bailey, *J. Anal. Toxicol.*, 23 (1999) 468.
58 W. Naidong, L.W. Lee, X. Jiang, M. Wehling, J.D. Hulse and P.P. Lin, *J. Chromatogr. B*, 735 (1999) 255.
59 P. Wernly, W. Thormann, D. Bourquin and R. Brenneisen, *J. Chromatogr.*, 616 (1993) 305.
60 F. Tagliaro, C. Poesi, R. Aiello, R. Dorizzi, S. Ghieli and M. Marigo, *J. Chromatogr.*, 638 (1993) 303.
61 W.S. Wu and J.L. Tsai, *Biomed. Chromatogr.*, 13 (1999) 216.
62 J.L. Tsai, W.S. Wu and H.H. Lee, *Electrophoresis*, 21 (2000) 1580.
63 M.E. Alburges, W. Huang, R.L. Foltz and D.E. Moody, *J. Anal. Toxicol.*, 20 (1996) 362.
64 D.G. Wilkins, P.R. Nasagawa, S.P. Gygi, R.L. Foltz and D.E. Rollins, *J. Anal. Toxicol.*, 20 (1996) 355.
65 G.A. Cooper and J.S. Oliver, *J. Anal. Toxicol.*, 22 (1998) 389.
66 F. Sporkert and F. Pragst, *J. Chromatogr. B*, 746 (2000) 255.
67 H.R. Angelo, N. Beck and K. Kristensen, *J. Chromatogr. B*, 724 (1999) 35.
68 P. Kintz, H.P. Eser, A. Tracqui, M. Moeller, V. Cirimele and P. Mangin, *J. Forensic Sci.*, 42 (1997) 291.
69 D. Ortelli, S. Rudaz, A.F. Chevalley, J.J. Deglon, L. Balant and J.L. Veuthey, *J. Chromatogr. A*, 871 (2000) 163.
70 L. Amass, J.B. Kamien and S.K. Mikulich, *Drug Alcohol Depend.*, 58 (2000) 143.
71 A.M. Lisi, R. Kaslauskas and G.J. Trout, *J. Chromatogr. B*, 692 (1997) 67.
72 K.A. Hadidi and J. Oliver, *Int. J. Legal Med.*, 111 (1998) 165.
73 J.J. Kuhlman, J. Magluilo, Jr., E.J. Cone and B. Levine, *J. Anal. Toxicol.*, 20 (1996) 229.
74 A. Salem, T.L. Pierce and W. Hope, *J. Pharmacol. Toxicol. Methods*, 37 (1997) 75.
75 A. Tracqui, P. Kintz and P. Mangin, *J. Forensic Sci.*, 42 (1997) 111.
76 H. Hoja, P. Marquet, B. Verneuil, H. Lofti, J.L. Dupuy and G. Lachaitre, *J. Anal. Toxicol.*, 21 (1997) 160.
77 D.E. Moody, J.D. Laycock, A.C. Spanbauer, D.J. Crouch, R.L. Foltz, J.L. Josephs, L. Amass and W.K. Bickel, *J. Anal. Toxicol.*, 21 (1997) 406.
78 A. Polettin and M.A. Huestis, *J. Chromatogr. B*, 754 (2001) 447.
79 G. Skopp, K. Klinder, L. Potsch, G. Zimmer, R. Lutz, R. Aderjan and R. Mattern, *Forensic Sci. Int.*, 95 (1998) 99.
80 R. Aderjan and G. Skopp, *Ther. Drug Monit.*, 20 (1998) 561.
81 M. Balikova and V. Maresova, *Forensic Sci. Int.*, 94 (1998) 201.
82 M. Balikova, V. Maresova and V. Habrdova, *J. Chromatogr. B*, 752 (2001) 179.
83 NIDA Research Report, *Cocaine Abuse and Addiction*, NIH Publ No. 99-4342, 1999.
84 M.A. ElSohly, R. Brenneisen and A.B. Jones, *J. Forensic Sci.*, 36 (1991) 93.
85 J.G. Ensing, C. Racamy and R.A. de Zeeuw, *J. Forensic Sci.*, 37 (1992) 446.
86 K.E. Janzen, L. Walter and A.R. Fernando, *J. Forensic Sci.*, 37 (1992) 436.
87 D.R. Morello, J.F. Casale, M.L. Stevenson and R.F. Klein, *J. Forensic Sci.*, 45 (2000) 1126.
88 I.M. Lazar, G. Naisbitt and M.L. Le, *Analyst*, 123 (1998) 1449.
89 A.S. Krawczeniak and V.A. Bravenec, *J. Forensic Sci.*, 43 (1998) 738.

90 T.A. Gough and G.F. Phillips, in T.A. Gough (Ed.), *The Analysis of Drugs of Abuse*, Wiley, Chichester, 1991, pp. 477–510.
91 M.J. Bogusz, H. Althoff, M. Erkens, R.D. Maier and R. Hofmann, *J. Forensic Sci.*, 40 (1995) 811.
92 C. Wettli and R. Mittleman, *J. Forensic Sci.*, 26 (1981) 492.
93 Y. Balash, L. Pollak, J. Hiss and M.J. Rabey, *Eur. J. Neurol.*, 7 (2000) 555.
94 J. Oyler, W.D. Darwin and E.J. Cone, *J. Anal. Toxicol.*, 20 (1996) 213.
95 A. Negrusz, J.L. Perry and C.M. Moore, *J. Forensic Sci.*, 43 (1998) 626.
96 R. Sleeman, F. Burton, J. Carter, D. Robert and P. Hulmston, *Anal. Chem.*, 72 (2000) 397A.
97 R. Sleeman, F. Burton, J. Luke, J. Carter, S. Richards, M. Hickson, E. Titterton, N. Ronan, G. Carter and S. Jickells, *Poster on 49th ASMS Conference*, Chicago 2001.
98 R.A. Jufer, W.D. Darwin and E.J. Cone, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 67–106.
99 W.C. Brogan, P.M. Kemp, R.O. Bost, D.B. Glamann, R.A. Lange and L.D. Hillis, *J. Anal. Toxicol.*, 16 (1992) 152.
100 D.N. Bailey, *J. Anal. Toxicol.*, 23 (1999) 99.
101 B.K. Logan, *J. Anal. Toxicol.*, 25 (2001) 219.
102 G.Y. Gaillard and G. Pepin, *Forensic Sci. Int.*, 86 (1997) 49.
103 K. Watanabe, H. Hattori, M. Nishikawa, T. Kumazawa, H. Seno and O. Suzuki, *Chromatographia*, 44 (1997) 55.
104 D.J. Crouch, M.E. Alburges, A.C. Spanbauer, D.E. Rollins and D.E. Moody, *J. Anal. Toxicol.*, 19 (1995) 352.
105 D. Smirnow and B.K. Logan, *J. Anal. Toxicol.*, 20 (1996) 463.
106 K.L. Paterson, B.K. Logan and G.D. Christian, *Forensic Sci. Int.*, 73 (1995) 183.
107 A.J. Jenkins and B.A. Goldberger, *J. Forensic Sci.*, 42 (1997) 824.
108 S.W. Toennes, A.S. Fandino and G. Kauert, *J. Chromatogr. B*, 735 (1999) 127.
109 B.D. Paul, L.K. McWhorter and M.L. Smith, *J. Mass Spectrom.*, 34 (1999) 651.
110 D. Garside, J.D. Ropero-Miller, B.A. Goldberger, W.F. Hamilton and W.R. Maples, *J. Forensic Sci.*, 43 (1998) 974.
111 D.A. Engelhart, E.S. Lavins and C.A. Sutheimer, *J. Anal. Toxicol.*, 22 (1998) 314.
112 J. Oyler, W.D. Darwin, K.L. Preston, P. Suess and E.J. Cone, *J. Anal. Toxicol.*, 20 (1996) 453.
113 M.A. ElSohly, W. Kopycki, S. Feng and T.P. Murphy, *J. Anal. Toxicol.*, 23 (1999) 446.
114 C. Moore, D. Dempsey, D. Deitermann, D. Lewis and J. Leikin, *J. Anal. Toxicol.*, 20 (1996) 501.
115 R.E. Winecker, B.A. Goldberger, I. Tebbett, M. Behnke, F.D. Eyler, M. Conlon, K. Wobie, J. Karlix and R.L. Bertholf, *J. Anal. Toxicol.*, 21 (1997) 97.
116 M.J. Bogusz, R.D. Maier, K.D. Kruger and U. Kohls, *J. Anal. Toxicol.*, 22 (1998) 549.
117 A. Cailleux, A. Le Bouil, B. Auger, G. Bonsergen, A. Turcant and P. Allain, *J. Anal. Toxicol.*, 23 (1999) 620.
118 C.S. Sosnoff, Q. Ann, J.T. Bernert, Jr., M.K. Powell, B.B. Miller, L.O. Henderson, W.H. Hannon, P. Fernhoff and E.J. Sampson, *J. Anal. Toxicol.*, 20 (1996) 179.
119 P.M. Jeanville, E.S. Estape, I. Torres-Negron and A. Marti, *J. Anal. Toxicol.*, 25 (2001) 69.
120 G. Skopp, A. Klingmann, L. Potsch and R. Mattern, *Ther. Drug Monit.*, 23 (2001) 174.
121 G. Singh, V. Arora, P.T. Fenn, B. Mets and I.A. Blair, *Anal. Chem.*, 71 (1999) 2021.
122 M.S. Kaufman, A.C. Hatzis and J.G. Stuart, *J. Mass Spectrom.*, 31 (1996) 913.
123 K.E. Kongshaug, S. Pedersen-Bjergaard, K.E. Rasmussen and M. Krogh, *Chromatographia*, 50 (2000) 247.
124 F. Sadeghipour, C. Giroud, L. Rivier and J.L. Veuthey, *J. Chromatogr. A*, 761 (1997) 71.

125 A.M. Di Pietra, R. Gotti, E. Del Borello, R. Pomponio and V. Cavrini, *J. Anal. Toxicol.*, 25 (2001) 99.
126 U. Backofen, F.M. Matysik, W. Hoffman and C.E. Lunte, *Fresnius J. Anal. Chem.*, 367 (2000) 359.
127 S.R. Wallenborg, I.S. Lurie, D.W. Arnold and C.G. Bailey, *Electrophoresis*, 21 (2000) 3257.
128 A.S. Smith-Kielland, B. Skuterud and J. Morland, *J. Anal. Toxicol.*, 21 (1997) 325.
129 J.T. Cody, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 107–141.
130 J.L. Valentine and R. Middleton, *J. Anal. Toxicol.*, 24 (2000) 211.
131 S.P. Vorce, J.H. Sklerov and K.S. Kalasinsky, *J. Anal. Toxicol.*, 24 (2000) 595.
132 M.R. Lee, Y.S. Song, B.H. Hwang and C.C. Chou, *J. Chromatogr. A*, 896 (2000) 256.
133 C. Jurado, M.P. Gimenez, T. Soriano, M. Menendez and M. Repetto, *J. Anal. Toxicol.*, 24 (2000) 11.
134 K. Okajima, A. Namera, M. Yashiki, I. Tsukue and T. Kojima, *Forensic Sci. Int.*, 116 (2001) 15.
135 D. Hensley and J.T. Cody, *J. Anal. Toxicol.*, 23 (1999) 518.
136 R. Herraez-Hernandez and P. Campins-Falco, *Analyst*, 124 (1999) 239.
137 M.J. Bogusz, M. Kala and R.D. Maier, *J. Anal. Toxicol.*, 21 (1996) 59.
138 M.J. Bogusz, K.D. Kruger and R.D. Maier, *J. Anal. Toxicol.*, 24 (2000) 77.
139 H. Kataoka, H.L. Lord and J. Pawliszyn, *J. Anal. Toxicol.*, 24 (2000) 257.
140 L. Geiser, S. Cherkaoui and J.L. Veuthe, *J. Chromatogr. A*, 895 (2000) 111.
141 A. Ramseier, J. Caslavska and W. Thormann, *Electrophoresis*, 20 (1999) 2726.
142 A. Ramseier, C. Siethoff, J. Caslavska and W. Thormann, *Electrophoresis*, 21 (2000) 380.
143 T. Kraemer, I. Vernaleken and H.H. Maurer, *J. Chromatogr. B*, 702 (1997) 93.
144 J.T. Cody and S. Valtier, *J. Anal. Toxicol.*, 25 (2001) 158.
145 M. Sato, T. Mitsui and H. Nagase, *J. Chromatogr. B*, 751 (2001) 277.
146 M. Katagi, M. Tatsuno, A. Miki, M. Nishikawa and H. Tsushihashi, *J. Anal. Toxicol.*, 24 (2000) 354.
147 M. Katagi, M. Tatsuno, A. Miki, M. Nishikawa, K. Nakajima and H. Tsuchihashi, *J. Chromatogr. B*, 759 (2001) 125.
148 Y.J. Heo, Y.S. Whang, M.K. In and K.J. Lee, *J. Chromatogr. B*, 741 (2000) 221.
149 A. Shulgin and A. Shulgin, *Pihkal, A Chemical Love Story*, Transform Press, Berkeley, 1995.
150 A.S. Henry, K.J. Jeffrys and S. Dawling, *Lancet*, 340 (1992) 384.
151 C. Lora-Tamayo, T. Tena and A. Rodriguez, *Forensic Sci. Int.*, 85 (1997) 149.
152 C.M. Milroy, J.C. Clark and A.R.W. Forrest, *J. Clin. Pathol.*, 49 (1996) 149.
153 N. Carter, G.N. Rutty, C.M. Milroy and A.R.W. Forrest, *Int. J. Legal Med.*, 113 (2000) 168.
154 R. McKetin and R.P. Mattick, *Drug Alcohol Depend.*, 50 (1998) 181.
155 E. Gouzoulis-Mayfrank, J. Daumann, F. Tuchtenhagen, S. Pelz, S. Becker, H.-J. Kunert, B. Fimm and H. Sass, *Neurol. Neurosurg. Psychiatry*, 68 (2000) 719.
156 H. Zhao, R. Brenneisen, A. Scholer, A.J. McNally, M.A. ElSohly, T.P. Murphy and S.J. Salamone, *J. Anal. Toxicol.*, 25 (2001) 258.
157 H.K. Ensslin, H.H. Maurer, E. Gouzoulis, L. Hermle and K.A. Kovar, *Drug Metab. Disp.*, 24 (1996) 813.
158 H.H. Maurer, *Ther. Drug Monit.*, 18 (1996) 465.
159 H.H. Maurer, J. Bickeboeller-Friedrich, T. Kraemer and F.T. Peters, *Toxicol. Lett.*, 112 (2000) 133.
160 R. de la Torre, M. Farre, J. Ortuno, M. Mas, R. Brenneisen, P.N. Roset, S. Segura and J. Cami, *J. Clin. Pharmacol.*, 49 (2000) 104.

161 J.K. Fallon, A.T. Kicman, J.A. Henry, P.J. Milligan, D.A. Cowan and A.J. Hutt, *Clin. Chem.*, 45 (1999) 1058.
162 G.W. Kunsman, B. Levine, J.J. Kuhlman, R.L. Jones, R.O. Hughes, C.I. Fujiyama and M.L. Smith, *J. Anal. Toxicol.*, 20 (1996) 517.
163 H.J. Helmlin, K. Bracher, D. Bourquin, D. Vonlanthen, R. Brenneisen and J. Styk, *J. Anal. Toxicol.*, 20 (1996) 432.
164 F. Sadeghipour and J.L. Veuthey, *J. Chromatogr. A*, 787 (1997) 137.
165 K.M. Clauwert, J.F. VanBocxlaer, E.A. De Letter, S. Van Calenbergh, W.E. Lambert and A.P. De Leenheer, *Clin. Chem.*, 46 (2000) 1968.
166 K.M. Clauwaert, L.F. Van Bocxlaer, H.J. Major, J.A. Claereboudt, W.E. Lambert, E. Van den Eeckhout, C.H. Van Peteghem and A.P. De Leenheer, *Rapid Commun. Mass Spectrom.*, 15 (1999) 1540.
167 P. Kintz and N. Samyn, *J. Chromatogr. B*, 733 (1999) 137.
168 R.C. Baselt, *Disposition of Toxic Drugs and Chemicals in Man*, Biomedical Publications, Foster City, 2000, pp. 486–489.
169 K.S. Webb, P.B. Baker, N.P. Cassells, J.M. Francis, D.E. Johnson, S.H. Lancaster, P.S. Minty, G.D. Reed and S.A. White, *J. Forensic Sci.*, 41 (1996) 938.
170 S.A. White, A.S. Kidd and K.S. Webb, *J. Forensic Sci.*, 44 (1999) 375.
171 J. de Kanel, W.E. Vickery, B. Waldner, R.M. Monahan and F.X. Diamond, *J. Forensic Sci.*, 43 (1998) 622.
172 K. Bodin and J.O. Svensson, *Ther. Drug Monit.*, 23 (2001) 389.
173 S.A. Reuschel, D. Eades and R.L. Foltz, *J. Chromatogr. B*, 733 (1999) 145.
174 G.K. Poch, K.L. Klette, D.A. Hallare, M.G. Manglicmot, R.J. Czarny, L.K. McWhorter and C.J. Anderson, *J. Chromatogr. B*, 724 (1999) 23.
175 G.K. Poch, K.L. Klette and C. Anderson, *J. Anal. Toxicol.*, 24 (2000) 170.
176 S.A. Reuschel, S.E. Percey, S. Liu, D.M. Eades and R.L. Foltz, *J. Anal. Toxicol.*, 23 (1999) 306.
177 A.G. Verstraete and E.J. van de Velde, *Acta Clin. Belg.*, Suppl. 1 (1999) 94.
178 J.H. Sklerov, J. Magluilo, K.K. Shannon and M.L. Smith, *J. Anal. Toxicol.*, 24 (2000) 543.
179 J. Canezin, A. Cailleux, A. Turcant, A. LeBouil, P. Harry and P. Allain, *J. Chromatogr. B*, 765 (2001) 15.
180 M.J. Bogusz, R.D. Maier, A.T. Schaefer and M. Erkens, *Int. J. Legal Med.*, 111 (1998) 147.
181 A.F. Grieshaber, K.A. Moore and B. Levine, *J. Forensic Sci.*, 46 (2001) 627.
182 M.R. Tramer, D. Carroll, F.A. Campbell, D.J.M. Reynolds, R.A. Moore and H.J. McQuay, *Br. Med. J.*, 323 (2001) 16.
183 N.A. Darmani, *Neuropsychopharmacology*, 24 (2001) 198.
184 F.A. Campbell, M.R. Tramer, D. Carroll, D.J.M. Reynolds, R.A. Moore and H.J. McQuay, *Br. Med. J.*, 323 (2001) 13.
185 E.M. Williamson and F.J. Evans, *Drugs*, 60 (2000) 1303.
186 K.R. Muller-Vahl, A. Koblenz, M. Jobges, H. Kolbe, H.M. Emrich and U. Schneider, *Pharmacopsychiatry*, 34 (2001) 19.
187 B.R. Martin, R. Jefferson, R. Winckler, J.L. Wiley, J.W. Huffmann, P.J. Crocker, B. Saha and R.K. Razdan, *J. Pharmacol. Exp. Ther.*, 290 (1999) 1065.
188 K. Lavanya and T.R. Baggi, *Forensic Sci. Int.*, 47 (1990) 165.
189 P. Oroszlan, G. Verzar-Perti, E. Mincovics and T. Szekely, *J. Chromatogr.*, 38 (1987) 217.
190 D. Debruyne, F. Albessard, M.C. Bigot and M. Moulin, *Bull. Narc.*, 46 (1994) 109.
191 R. Brenneisen and M.A. ElSohly, *J. Forensic Sci.*, 33 (1988) 1385.
192 T. Lehmann and R. Brenneisen, *J. Liq. Chromatogr.*, 18 (1995) 689.

193 B. Backstrom, M.D. Cole, M.J. Carrott, D.C. Jones, G. Davidson and K. Coleman, *Sci. Justice*, 37 (1997) 91.
194 I.S. Lurie, R.P. Meyers and T.S. Conver, *Anal. Chem.*, 70 (1998) 3255.
195 T.Z. Bosy and K.A. Cole, *J. Anal. Toxicol.*, 24 (2000) 562.
196 O. Zoller, P. Rhyn and B. Zimmerli, *J. Chromatogr. A*, 872 (2000) 101.
197 S.A. Ross, Z. Mehmedic, T.P. Murphy and M.A. ElSohly, *J. Anal. Toxicol.*, 24 (2000) 715.
198 M.A. ElSohly and M. Salem, in M.J. Bogusz (Ed.), Forensic Science, Vol. 2, Elsevier, Amsterdam, 2000, pp. 189–193.
199 C. Staub, *J. Chromatogr. B*, 733 (1999) 119.
200 J.P. Weller, M. Wolf and S. Szidat, *J. Anal. Toxicol.*, 24 (2000) 359.
201 M. Chiarotti and L. Costamagna, *Forensic Sci. Int.*, 114 (2000) 1.
202 P. Mireault, Poster at the 46th ASMS Conference, Orlando, 1998.
203 P. Picotte, P. Mireault and G. Nolin, Poster at the 48th ASMS conference, Long Beach, 2000.
204 T. Breindahl and K. Andreasen, *J. Chromatogr. B*, 732 (1999) 155.
205 S.S. Tai and M.J. Welch, *J. Anal. Toxicol.*, 24 (2000) 385.
206 W. Weinmann, S. Vogt, R. Goerke, C. Muller and A. Bromberger, *Forensic Sci. Int.*, 113 (2000) 381.
207 P. Kintz and N. Samyn, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 459–488.
208 H. Sachs and U. Dressler, *Forensic Sci. Int.*, 107 (2000) 239.
209 N.P. Lemos, R.A. Anderson and J.R. Robertson, *J. Anal. Toxicol.*, 23 (1999) 147.
210 P. Kintz, V. Cirimele and B. Ludes, *J. Anal. Toxicol.*, 24 (2000) 557.
211 N. Fucci, N. De Giovanni, M. Chiarotti and S. Scarlata, *Forensic Sci. Int.*, 119 (2001) 318.
212 M.A. ElSohly, D.F. Stanford, T.P. Murphy, B.M. Lester, L.L. Wright, V.L. Smeroglio, J. Verter, C.R. Bauer, S. Shankaran, H.S. Bada and H.C. Walls, *J. Anal. Toxicol.*, 23 (1999) 436.
213 S. Feng, M.A. ElSohly, S. Salamone and M.Y. Salem, *J. Anal. Toxicol.*, 24 (2000) 395.
214 L.S. Tsai, M.S. ElSohly, S.F. Tsai, T.P. Murphy, B. Twarowska and S.J. Salamone, *J. Anal. Toxicol.*, 24 (2000) 708.
215 J. Rohrich, S. Zorntlein, L. Potsch, G. Skopp and J. Becker, *Int. J. Legal Med.*, 113 (2000) 102.
216 V. Cirimele, P. Kintz, C. Jamey and B. Ludes, *J. Anal. Toxicol.*, 23 (1999) 349.
217 T.C. Chao, D.S. Lo, J. Koh, T.C. Ting, L.M. Quek, T.H. Koh, C.Y. Koh-Tan and A. Zubaidah, *Med. Sci. Law*, 33 (1993) 253.
218 S.W. Park, N.Y. Kim, Y.G. Yang, B. Seo and K.J. Paeng, *J. Forensic Sci.*, 43 (1998) 888.
219 M. Risse, F. Erdmann, H. Schutz and G. Weiler, *Forensic Sci. Int.*, 124 (2001) 209.
220 B. Madea and F. Musshof, *Int. J. Legal Med.*, 113 (1999) 47.
221 K. Watanabe, H. Seno, A. Ishii, O. Suzuki and T. Kumazawa, *Anal. Chem.*, 69 (1997) 5178.
222 H. Hattori, M. Iwai, S. Kurino, T. Yamada, K. Watanabe-Suzuki, A. Ishii, H. Seno and O. Suzuki, *J. Chromatogr. B*, 718 (1998) 285.
223 K. Kondo, X.P. Lee, T. Kumazawa, K. Sato, K. Watanabe-Suzuki, H. Seno and O. Suzuki, *J. Am. Off. Anal. Chem. Int.*, 84 (2002) 19.
224 T. Kojima, A. Ishii, K. Watanabe-Suzuki, R. Kurihara, H. Seno, T. Kumazawa, O. Suzuki and Y. Katsumata, *J. Chromatogr. B*, 762 (2001) 103.
225 K. Watanabe-Suzuki, A. Ishii and O. Suzuki, *Anal. Bioanal. Chem.*, 373 (2002) 75.
226 X.P. Lee, T. Kumazawa and K. Sato, *Int. J. Legal Med.*, 107 (1995) 310.
227 D.J. Tranthim-Fryer, R.C. Hansson and K.W. Norman, *J. Forensic Sci.*, 46 (2001) 934.
228 N.Y. Kim and S.W. Park, *J. Forensic Sci.*, 5 (2000) 702.
229 D.S. Isenschmid, B.J. Cassin, B.R. Hepler and S. Kanluen, *J. Forensic Sci.*, 43 (1998) 231.
230 C.L. Winek, W.W. Wahba, R. Huston and L. Rozin, *Forensic Sci. Int.*, 87 (1997) 161.

231 M.P. Bouche, W. Lambert, J. Van Bocxlaer, M. Miette and A.P. De Leenheer, in I. Rasanen (Ed.), *Proceedings of 38th International Meeting TIAFT 2000*, Helsinki 2000, pp. 199–204.
232 M.A. LeBeau and A. Mozayani (Eds.), *Drug-Facilitated Sexual Assault*, Academic Press, San Diego, 2001.
233 A. Negrusz, C.M. Moore, T.L. Stockham, K.R. Poiser, J.L. Kern, R. Palaparthy, N.L. Le, P.G. Janicak and N.A. Levy, *J. Forensic Sci.*, 45 (2000) 1031.
234 A. Negrusz, C. Moore, D. Deitermann, D. Lewis, K. Kaleciak, R. Kronstrand, B. Feley and R.S. Niedbala, *J. Anal. Toxicol.*, 23 (1999) 429.
235 A. Negrusz, C.M. Moore, K.B. Hinkel, T.L. Stockham, M. Verma, M.J. Strong and P.G. Janicak, *J. Forensic Sci.*, 46 (2001) 1143.
236 M.J. Bogusz, R.D. Maier, K.D. Kruger and W. Fruchtnicht, *J. Chromatogr. B*, 713 (1998) 361.
237 M.A. LeBeau, M.A. Montgomery, J.R. Wagner and M.L. Miller, *J. Forensic Sci.*, 45 (2000) 1133.
238 J. Darius and P. Banditt, *J. Chromatogr. B*, 738 (2000) 437.
239 H. Yuang, Z. Mester, H. Lord and J. Pawliszyn, *J. Anal. Toxicol.*, 24 (2000) 718.
240 P. Marquet, O. Baudin, J.M. Gaulier, E. Lacassie, J.L. Dupuy, B. Francois and G. Lachatre, *J. Chromatogr. B*, 734 (1999) 137.
241 M.L. Ware and B.J. Hidy, Poster at the 49th ASMS Conference, Chicago, 2001.
242 L.M. Matz and H.H. Hill, *Anal. Chim. Acta*, 457 (2002) 235.
243 G.P. Galloway, S.L. Frederick-Osborne, R. Seymour, S.E. Contini and D.E. Smith, *Alcohol*, 20 (2000) 263.
244 C.S. Hornfeldt, K. Lothridge and L.C. Upshaw Downs, *Forensic Sci. Commun.*, 4 (2002), Number 1, www.fbi.gov/lab/fsc/
245 E.L. Fieler, D.E. Coleman and R.C. Baselt, *Clin. Chem.*, 44 (1998) 692.
246 S. Elliott, *J. Anal. Toxicol.*, 25 (2001) 152.
247 R.R. McCusker, H. Paget-Wilkes, C.W. Chronister and B.A. Goldberger, *J. Anal. Toxicol.*, 23 (1999) 301.
248 A.A. Elian, *Forensic Sci. Int.*, 109 (2000) 183.
249 F.J. Couper and B.K. Logan, *J. Anal. Toxicol.*, 24 (2000) 1.
250 R.C. Baselt, *Disposition of Toxic Drugs and Chemicals in Man*, Biomedical Publications, Foster City, 2000, pp. 441–443.
251 R. Fernando, *Med. Sci. Law*, 39 (1999) 354.
252 L. Ramos, R. Bakhtiar and F. Tse, *Rapid Commun. Mass Spectrom.*, 13 (1999) 2439.
253 F. Magni, L. Marazzini, S. Pereira, L. Monti and M. Galli Kienle, *Anal. Biochem.*, 282 (2000) 136.
254 H.H. Maurer, C. Kratzsch, T. Kraemer, F.T. Peters and A.A. Weber, *J. Chromatogr. B*, 773 (2002) 62.
255 H. Iwase, M. Kobayashi, M. Nakajima and T. Ytakatori, *Forensic Sci. Int.*, 115 (2001) 123.
256 S.M. Darby, M.L. Miller, R.O. Allen and M. LeBeau, *J. Anal. Toxicol.*, 25 (2001) 8.
257 Y. Zhu, D. Meyer, Z. Lam and B. Chien, Poster presented on 49th ASMS Conference, Chicago, 2001.
258 H. Maeda, M. Fujita, B.L. Zhu, K. Ishidam, S. Oritani, H. Tsushihashi, M. Nishikawa, M. Izumi and F. Matsumoto, *Med. Sci. Law*, 40 (2000) 169.
259 M. Zecevic, L. Zivanovic and A. Stojkovic, *J. Chromatogr. A*, 949 (2002) 61.
260 C. Farenc, M. Audran, J.Y. Lefrant, I. Mazerm and F. Bressolle, *J. Chromatogr. B*, 724 (1999) 117.
261 C. Farenc, C. Enjalbal, P. Sanchez, F. Bressolle, M. Audran, J. Martinez and J.L. Aubagnac, *J. Chromatogr. A*, 910 (2001) 61.

262 K.D. Ballard, W.E. Vickery, R.D. Citrino, F.X. Diamond and F. Rieders, Poster presented at 49th ASMS Conference, Chicago, 2001.
263 C.H. Kerskes, K.J. Lusthof, P.G. Zweipfenning and J.P. Franke, *J. Anal. Toxicol.*, 26 (2002) 29.
264 P. Kintz, A. Tracqui and B. Ludes, *Int. J. Legal Med.*, 114 (2000) 93.
265 R.A. de Zeeuw and J.P. Franke, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 567–599.
266 O. Drummer, *J. Chromatogr. B*, 733 (1999) 27.
267 A. Polettini, *J. Chromatogr. B*, 733 (1999) 47.
268 R.A. de Zeeuw, J.P. Franke, F. Degel, G. Machbert, H. Schütz and J. Wijsbeek, *Thin Layer Chromatographic f Values for Toxicologically Relevant Substances on Standardized Systems*, VCH, Weinheim, 1992.
269 H.H. Maurer, *J. Chromatogr. B*, 733 (1999) 3.
270 R.A. de Zeeuw, J.P. Franke, H.H. Maurer and K. Pfleger, *Gas Chromatographic Retention Indices of Toxicologically Relevant Substances on Packed or Capillary Columns with Dimethylsilicone Stationary Phases*, VCH, Weinheim, 1992.
271 K. Pfleger, H.H. Maurer and A. Weber, *Mass Spectral, and GC Data of Drugs, Poisons, Pesticides, Pollutants and Their Metabolites, Part 1–5*, VCH, Weinheim, 1992–2000.
272 M.J. Bogusz, *J. Chromatogr. B*, 733 (1999) 65.
273 R.M. Smith, in R.M. Smith (Ed.), *Retention and Selectivity in Liquid Chromatography*, Elsevier, Amsterdam, 1995, pp. 93.
274 M.J. Bogusz, in R.M. Smith (Ed.), *Retention and Selectivity in Liquid Chromatography*, Elsevier, Amsterdam, 1995, pp. 171.
275 M. Bogusz, D.W. Hill and A. Rehorek, *J. Liq. Chromatogr.*, 19 (1996) 1291.
276 S.P. Elliott and K.A. Hale, *J. Anal. Toxicol.*, 22 (1998) 279.
277 M.J. Bogusz, R.D. Maier, K.D. Kruger, K.S. Webb, J. Romeril and M.L. Miller, *J. Chromatogr. B*, 844 (1999) 409.
278 B.I. Shushan, P. Marquet, J.L. Dupuy, J. Anacleto, G. Lachaitre and E. Duchoslav, *Ther. Drug Monit.*, 21 (1999) 467.
279 W. Weinmann, A. Wiedemann, B. Eppinger, M. Renz and M. Svoboda, *J. Am. Soc. Mass Spectrom.*, 10 (1999) 1028.
280 C. Mueller, P. Schaefer, S. Vogt and W. Weinmann, Poster at 50th ASMS Conference, Orlando, 2002.
281 M. Rittner, F. Pragst, W.R. Bork and J. Neumann, *J. Anal. Toxicol.*, 25 (2001) 115.
282 A.G. Lips, W. Lameijer, R.H. Fokkens and N.M. Nibbering, *J. Chromatogr. B*, 759 (2001) 191.
283 W. Weinmann, M. Stoertzel, S. Vogt and J. Wendt, *J. Chromatogr. A*, 926 (2001) 199.
284 M. Gergov, B. Boucher, I. Ojanpera and E. Vuori, *Rapid Commun. Mass Spectrom.*, 15 (2001) 521.
285 W. Weinmann, M. Gergov and M. Goerner, *Analusis*, 28 (2000) 934.
286 D.M. Eisenberg, R.B. Davis, S.L. Ettner, S. Appel, S. Wilkey, M. Van Rompay and R.C. Kessler, *J. Am. Med. Assoc.*, 280 (1998) 1569.
287 E. Ernst, *Trends Pharmacol. Sci.*, 23 (2002) 136.
288 W.F. Huang, K.C. Wen and M.L. Hsiao, *J. Clin. Pharmacol.*, 37 (1997) 344.
289 H.L. Koh and S.O. Woo, *Drug Saf.*, 23 (2000) 351.
290 Y. Gaillard, M. Cheze and G. Pepin, *Ann. Biol. Clin.*, 59 (2001) 764.
291 Y. Gaillard and G. Pepin, *J. Chromatogr. B*, 733 (1999) 181.
292 E. Lacassie, P. Marquet, S. Martin-Dupont, J.M. Gaulier and G. Lachatre, *J. Forensic Sci.*, 45 (2000) 1154.

293 Y. Gaillard and G. Pepin, *J. Anal. Toxicol.*, 25 (2001) 481.
294 X. Wang, J.B. Plomley, R.A. Newman and A. Cisneros, *Anal. Chem.*, 72 (2000) 3547.
295 A. Tracqui, P. Kintz, F. Branche and B. Ludes, *Int. J. Legal Med.*, 111 (1998) 32.
296 Y. Chen, S. Koelliker, M. Oehme and A. Katz, *J. Nat. Prod.*, 62 (1999) 701.
297 H.H. Maurer, C.J. Schmitt, A.A. Weber and T. Kraemer, *J. Chromatogr. B*, 748 (2000) 125.
298 *International Olympic Committee, Olympic Movement Antidoping Code*, Lausanne, 2001.
299 *Veterinary Regulations, Federation Equestre Internationale*, 1997.
300 J. Segura, R. Ventura, M. Gonzales and C. Jimenez, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 531–566.
301 H.H. Maurer, *Comb. Chem. High Throughput Screen.*, 3 (2000) 467.
302 C. Jimenez, R. Ventura and J. Segura, *J. Chromatogr. B*, 767 (2002) 341.
303 P. Kintz and N. Samyn, *Ther. Drug Monit.*, 24 (2002) 239.
304 R. Aguilera, C.K. Hatton and D.H. Catlin, *Clin. Chem.*, 48 (2002) 629.
305 T. Kuuranne, O. Aitoi, M. Vahermo, E. Elovaara and R. Kostiainen, *Bioconjug. Chem.*, 13 (2002) 194.
306 P. Kintz, V. Cirimele, V. Dumestre-Toulet, M. Villain and B. Ludes, *J. Chromatogr. B*, 766 (2002) 161.
307 P. Kintz, V. Cirimele and B. Ludes, *Clin. Chem.*, 476 (2000) 2020.
308 M.W.J. van Hout, C.M. Hofland, H.A.G. Niederlander and G.J. de Jong, *Rapid Commun. Mass Spectrom.*, 14 (2000) 2103.
309 R. Ventura, L. Damasceno, M. Farre, J. Cardoseo and J. Segura, *Anal. Chim. Acta*, 418 (2000) 79.
310 L. Amendola, C. Colamonici, F. Rossi and F. Botre, *J. Chromatogr. B*, 773 (2002) 7.
311 P. Van Eenoo, F.T. Delbeke, K. Roels and P. De Backer, *J. Chromatogr. B*, 760 (2001) 255.
312 G. Gmeiner, T. Geisendorfer, J. Kainzbauer, M. Nikolajevic and H. Tausch, *J. Chromatogr. B*, 768 (2002) 215.
313 D. Thieme, J. Grosse, R. Lang, R.K. Mueller and A. Wahl, *J. Chromatogr. B*, 757 (2001) 49.
314 M. Thevis, G. Opfermann and W. Schanzer, Poster at 50th ASMS Conference, Orlando, 2002.
315 J. Caslavska and W. Thormann, *J. Chromatogr. B*, 770 (2002) 207.
316 M. Thevis, G. Opfermann and W. Schanzer, *Biomed. Chromatogr.*, 15 (2001) 393.
317 A. Takeda, H. Tanaka, T. Shinohara and I. Ohtake, *J. Chromatogr. B*, 758 (2001) 235.
318 W.S. Skinner, D.M. McKernie and S.D. Stanley, Poster at 50th ASMS Conference, Orlando, 2002.
319 L. Dehennin, Y. Bonnaire and P. Plou, *J. Anal. Toxicol.*, 25 (2001) 685.
320 E. Chung, R.C. Cheng, A.S. Fan, E.N. Ho, M.Y. Lau, G.N. Leung, F.W. Tang, T.S. Wan and K.C. Yiu, Poster at the 49th ASMS Conference, Chicago, 2001.
321 J.A. Rudy, C.E. Ubo, L. Soma, F. Guan, E. Birks, M. Kahler, D. Tsang, A. Watson and D. Teleis, Poster at the 49th ASMS Conference, Chicago, 2001.
322 F.P. Tang, G.N. Leung and T.S. Wan, *Electrophoresis*, 22 (2001) 2201.
323 A.F. Lehner, J.D. Harkins, W. Karpiesiuk, W.E. Woods, N. Robinson, L. Dirikolu, M. Fisher and T. Tobin, *J. Anal. Toxicol.*, 25 (2001) 280.
324 F. Todi, M. Mendonca, M. Ryan and P. Herskovits, *J. Vet. Pharmacol. Ther.*, 22 (1999) 333.
325 S.D. Garbis, L. Hanley and S. Kalita, *J. Am. Off. Anal. Chem. Int.*, 81 (1998) 948.
326 H.W. Hagedorn, H. Meiser, H. Zankl and R. Schulz, *J. Vet. Pharmacol.*, 24 (2001) 283.
327 H.W. Hagedorn, H. Meiser, H. Zankl and R. Schulz, *J. Pharm. Biomed. Anal.*, 29 (2002) 317.
328 E. Lacassie, P. Marquet, J. Gaulier, M. Dreyfuss and G. Lachatre, *Forensic Sci. Int.*, 121 (2001) 116.
329 A. Turcant, A. Cailleux, A. LeBouil, P. Allain, P. Harry and A. Renault, *J. Anal. Toxicol.*, 24 (2000) 157.

330 W. Naidong, P.R. Ring, C. Midtlien and X. Jiang, *J. Pharm. Biomed. Anal.*, 25 (2001) 219.
331 O. Kostainen, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 405–435.
332 K.J. Hart, I.F. Robbins, M.B. Wise, S.A. Lammert and W.H. Griest, Poster at 50th ASMS Conference, Orlando, 2002.
333 B.J. Nies, M.D. Evans, J.A. Syage, A.P. Snyder, W. Maswadeh and A. Tripathi, Poster at 50th ASMS Conference, Orlando, 2002.
334 R.B. Cody, J.A. Laramee, G.L. Samuelson and O. Edward, Poster at 50th ASMS Conference, Orlando, 2002.
335 A.P. Snyder; W.M. Maswadeh, A.Tripathi, J. Ho and M. Spence, Poster at 50th ASMS Conference, Orlando, 2002.
336 D. Zidarov, S. Letarte, M. Ethier and M.J. Bertrand, Poster at 50th ASMS Conference, Orlando, 2002.
337 R.M. Black and R.W. Read, *J. Chromatogr. A*, 759 (1997) 79.
338 P.A. D'Agostino, J.R. Hancock and L.R. Provost, *J. Chromatogr. A*, 840 (1999) 289.
339 P.A. D'Agostinho, C.L. Chenier and J.R. Hancock, *J. Chromatogr. A*, 950 (2002) 149.
340 J.R. Hancock and P.A. D'Agostino, *Anal. Chim. Acta*, 457 (2002) 71.
341 P.A. D'Agostino, C.L. Chenier and J.R. Hancock, Poster at 50th ASMS Conference, Orlando, 2002.
342 J. Yinon and S. Zitrin, *Modern Methods and Applications of Analysis of Explosives*, Wiley, Chichester, 1993.
343 J. Yinon, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 603–616.
344 J.A. Syage, K.A. Hanold, C. Rhykerd, F. Bouchier and K. Linker, Poster at 50th ASMS Conference, Orlando, 2002.
345 R. Sleeman, S.L. Richards, W.R. Scott, W.R. Davidson, J.G. Luke, B.J. Kelly and F.A. Burton, Poster at 50th ASMS Conference, Orlando, 2002.
346 Y. Takada, H. Nagano, I. Waki, K. Kusumoto, T. Ohta and J. Nakamura, Poster at 50th ASMS Conference, Orlando, 2002.
347 R.Q. Thompson, D.D. Fetterolf, M.L. Miller and R.F. Mothershead, *J. Forensic Sci.*, 44 (1999) 795.
348 K.G. Furton, L. Wu and J.R. Almirall, *J. Forensic Sci.*, 45 (2000) 857.
349 M.L. Miller, Poster at 50th ASMS Conference, Orlando, 2002.
350 R. Batlle, H. Carlsson, E. Holmgen, A. Colmsjo and C. Creszenzi, *J. Chromatogr. A*, 963 (2002) 73.
351 J. Yinon and X. Zhao, Poster at 50th ASMS Conference, Orlando, 2002.
352 X. Zhao and Y. Yinon, Poster at 50th ASMS Conference, Orlando, 2002.
353 S. Davis, D. Elks, J.H. Moncur, M. Hardman and J. Hughes, Poster at 50th ASMS Conference, Orlando, 2002.
354 C.E. Wissinger and B.R. McCord, *J. Forensic Sci.*, 47 (2002) 168.
355 K.D. Smith, B.R. McCord, W.A. MacCrehan, K. Mount and W.F. Rowe, *J. Forensic Sci.*, 44 (1999) 789.
356 C.R. Bowerbank, P.A. Smith, D.D. Fetterolf and M.L. Lee, *J. Chromatogr. A*, 902 (2000) 413.
357 M.L. Miller, J.M. Doyle, R.A. Lee and R. Gillette, *Forensic Sci. Commun.*, 3 (2002) Number 2, www.fbi.gov/hq/ lab/fsc.
358 C. Walker, H. Cullum and R. Hiley, *J. Forensic Sci.*, 46 (2001) 254.
359 A. Cowson, R.W. Hiley and C.C. Todd, *J. Forensic Sci.*, 46 (2001) 53.
360 M.E. Sigman and C.Y. Ma, *J. Forensic Sci.*, 46 (2001) 6.
361 W. Bertsch and Q. Ren, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 617–678.

362 Q. Ren and W. Bertsch, *J. Forensic Sci.*, 44 (1999) 504.
363 R. Newman, M. Gilbert and K. Lothridge, *GC–MS Guide to Ignitable Fluids*, CRC Press, Boca Raton, FL, 1998.
364 E. McGee and T.L. Lang, *J. Forensic Sci.*, 47 (2002) 267.
365 J.R. Wallace, *J. Forensic Sci.*, 44 (1999) 996.
366 G.S. Frysinger and R.B. Gaines, *J. Forensic Sci.*, 47 (2002) 471.
367 V.N. Aginski, in M.J. Bogusz (Ed.), *Forensic Science*, Elsevier, Amsterdam, 2000, pp. 679–691.
368 J.A. Zlotnick and F.P. Smith, *J. Chromatogr. B*, 733 (1999) 265.
369 A. Kher, E.V. Green and M.I. Mulholland, *J. Forensic Sci.*, 46 (2001) 878.
370 J. Andrasko, *J. Forensic Sci.*, 46 (2001) 21.
371 J. Andrasko, *J. Forensic Sci.*, 47 (2002) 324.
372 C. Weyermann, D. Kirsch, T. Andermann and B. Spengler, Poster at 50th ASMS Conference, Orlando, 2002.
373 J.A. de Koeijer and J.J.M. de Moel, *Problems Forensic Sci.*, 46 (2001) 413.
374 A.J. Jeffreys, V. Wilson and S.L. Thein, *Nature*, 316 (1985) 76.
375 The International Human Genome Mapping Consortium, *Nature*, 409 (2001) 934.
376 M. Litt and J.A. Luty, *Am. J. Hum. Genet.*, 44 (1989) 397.
377 D. Tautz, *Nucleic Acids Res.*, 17 (1989) 6463.
378 Y. Nakamura, M. Carlson, K. Krapcho, M. Kanamori and R. White, *Am. J. Hum. Genet.*, 43 (1988) 854.
379 J.S. Beckman and J.L. Weber, *Genomics*, 12 (1992) 627.
380 A.J. Jeffreys, K. Tamaki, A. MacLeod, D.G. Monckton, D.L. Neil and J.A.L. Armour, *Nat. Genet.*, 6 (1994) 136.
381 A. Di Rienzo, A.C. Peterson, J.C. Garza, A.M. Valdes, M. Slatkin and N.B. Freimer, *Proc. Natl Acad. Sci. USA*, 91 (1994) 3166.
382 E.M. Southern, *J. Mol. Biol.*, 98 (1975) 503.
383 K. Mullis and F. Faloona, in R. Wu (Ed.), *Methods in Enzymology*, Academic Press, New York, 1987, pp. 335.
384 K. Kleppe, E. Ohstuka, R. Kleppe, L. Molineux and H.G. Khorana, *J. Mol. Biol.*, 56 (1971) 341.
385 E. Hagelberg, B. Sykes and R. Hedges, *Nature*, 342 (1990) 485.
386 A. Alvarez-García, I. Muñoz, C. Pestoni, M.V. Lareu, M.S. Rodríguez-Calvo and A. Carracedo, *Int. J. Legal Med.*, 109 (1996) 125.
387 B.J. Connor, A.A. Reyes, C. Morin, K. Itakura, R.L. Teplitz and R.B. Wallace, *Proc. Natl Acad. Sci. USA*, 80 (1983) 278.
388 R. Saiki, T.L. Bugawan, G.T. Horn, K.B. Mullis and H.A. Erlich, *Nature*, 324 (1986) 163.
389 R.K. Saiki, P.S. Walsh, C.H. Levenson and H.A. Erlich, *Proc. Natl Acad. Sci. USA*, 86 (1989) 6230.
390 G. Herrin, N. Fildes and R. Reynolds, *J. Forensic Sci.*, 39 (1994) 1247.
391 B. Budowle, A.M. Giusti and R.C. Allen, in H.F. Polesky and W.R. Mayr (Eds.), *Advances in Forensic Haemogenetics*, Springer, Berlin, 1990, pp. 148–150.
392 X.Y. Hauge and M. Litt, *Hum. Mol. Genet.*, 2 (1993) 411.
393 A. Urquhart, C. Kimpton, T.J. Downes and P. Gill, *Int. J. Legal Med.*, 107 (1994) 13.
394 B. Brinkmann, M. Klintschar, F. Neuhuber, J. Hühne and B. Rolf, *Am. J. Hum. Genet.*, 62 (1998) 1408.
395 M.V. Lareu, C. Pestoni, C. Phillips, F. Barros, D. SynderCombe-Court, P. Lincoln and A. Carracedo, *Electrophoresis*, 19 (1998) 1566.
396 P. Gill, R. Sparkes and C. Kimpton, *Forensic Sci. Int.*, 89 (1997) 185.

397 P. Gill, J. Whitaker, C. Flaxman, N. Brown and J. Buckleton, *Forensic Sci. Int.*, 112 (2000) 17.
398 M. Kayser, A. Cagliá, D. Corach, N. Fretwell, C. Gehrig, G. Graziosi, F. Heidorn and S. Hermann, *Int. J. Legal Med.*, 110 (1997) 125.
399 P.S. White, O.L. Tatum, L.L. Deaven and J.L. Longmire, *Genomics*, 57 (1999) 433.
400 Q. Ayub, A. Mohyuddin, R. Qamar, K. Mazhar, T. Zerjal, S. Mehdi and C. Tyler-Smith, *Nucleic Acids Res.*, 28 (2000) c8.
401 L. Roewer, M. Krawczak, S. Willuweit, M. Nagy, C. Alves, A. Amorim, K. Anslinger, C. Agustin, A. Betz, E. Bosch, A. Caglià, A. Carracedo, D. Corach, A. Dekairelle, T. Dobosz, B.M. Dupuy, S. Furedi, C. Gehrig, L. Gusmao, J. Henke, L. Henke, M. Hiddinng, C. Hohoff, B. Hoste, M.A. Jobling, H.J. Kargel, P. de Knijff, R. Lessig, E. Liebeherr, M. Lorente, B. Martínez-Jarreta, P. Nievas, M. Nowak, W. Parson, V.L. Pascali, G. Penacino, R. Ploski, B. Rolf, A. Sala, U. Schmidt, C. Schmitt, P.M. Schneider, R. Szibor, J. Teifel-Gredding and M. Kayser, *Forensic Sci. Int.*, 118 (2001) 106.
402 L. Roewer (Ed.), *Forensic Sci. Int.*, 118 (2001) Vol. 2–3.
403 S. Hering and R. Szibor, *J. Forensic Sci.*, 45 (2000) 929.
404 A. Carracedo, E. D'Aloja, B. Dupuy, A. Jangblad, M. Karjalainen, C. Lambert, W. Parson, H. Pfeiffer, H. Pfitzinger, M. Sabatier, D. Syndercombe Court and C. Vide, *Forensic Sci. Int.*, 97 (1998) 165.
405 M.M. Holland and T.J. Parsons, *Forensic Sci. Rev.*, 11 (1999) 1.
406 A. Carracedo, W. Bär, P.J. Lincoln, W. Mayr, N. Morling, B. Olaisen, P. Schneider, B. Budowle, B. Brinkmann, P. Gill, M. Holland, G. Tully and M. Wilson, *Forensic Sci. Int.*, 110 (2000) 79.
407 G. Tully, W. Bär, B. Brinkmann, A. Carracedo, P. Gill, N. Morling, W. Parson and P. Schneider, *Forensic Sci. Int.*, 124 (2001) 83.
408 P.C. Simpson, D. Roach and A.T. Woolley, *Proc. Natl Acad. Sci. USA*, 95 (1998) 2256.
409 D. Schmalzing, A. Adourian, L. Koutny, L. Ziaugra, P. Matsudaira and D. Ehrlich, *Anal. Chem.*, 70 (1998) 2303.
410 J.M. Butler, J. Li, T.A. Shaler, J.A. Monforte and C.H. Becker, *Int. J. Legal Med.*, 112 (1999) 45.
411 P. Gill, *Int. J. Legal Med.*, 114 (2001) 204.
412 A.C. Syvanen, *Nat. Rev.*, 2 (2001) 930.
413 M.V. Lareu, B. Sobrino, J. Puente, M. Brión and A. Carracedo, *Forensic Sci. Int.*, 118 (2001) 163.
414 G. Tully, K.M. Sullivan, P. Nixon, R.E. Stones and P. Gill, *Genomics*, 15 (1996) 107.
415 J.N. Hirschorn, P. Sklar, K. Lindblad-Tod, Y. Lim, M. Ruiz-Gutiérrez, S. Bolk and E. Lander, *Proc. Natl Acad. Sci. USA*, 97 (2000) 12164.
416 V.G. Cheung, M. Morley, F. Aguilar, A. Massimi, R. Kucherlapati and G. Childs, *Nat. Genet.*, 21 (1999) 15.
417 R.J. Lipshutz, S. Fodor, T.R. Gingeras and D.J. Lockhart, *Nat. Genet.*, 21 (1999) 20.
418 The International Human Genome Mapping Consortium, *Nature*, 409 (2001) 934.
419 L. Feng and M. Neremberg, *Gene Ther. Mol. Biol.*, 4 (1999) 183.

This page is intentionally left blank

Erich Heftmann (Editor)
Chromatography, 6th edition
Journal of Chromatography Library, Vol. 69B

Chapter 24

Computer resources

AGNIESZKA KRAJ and JERZY SILBERRING

CONTENTS

24.1 INTRODUCTION

This chapter is intended to bridge the gap between the book of the past and the book of the future – the Internet. More than any of the other chapters it will soon be out of date.

However, it may still serve the uninitiated as an introduction to the resources that can be mustered from the computer. It provides a broad overview of the www sites that are or might be found useful in the laboratory, such as software for chromatography, electrophoresis, and mass spectrometry, literature databases, tutorials, instrumentation, vendors, journals, software for proteomics/genomics, etc. The web addresses are divided into sections and followed by a short description of their contents. To cope with obsolescence, each section contains some of the keywords which we used for searches. Obviously, these lists are not complete, but it is hoped that they will form a starting point for individual, more targeted searches.

24.2 PUBLICATIONS

This section describes important addresses for the publishing companies and commonly read periodicals.

24.2.1 Publishers

Key words: publishers, biomedical publisher, science publisher.

Internet address	Description
http://www.elsevier.com/homepage/	Link to Elsevier Science publisher
http://www.wiley-vch.de/publish/en/	Wiley-VCH homepage
http://pubs.acs.org	The American Chemical Society Publications Division
http://www.wspc.com/	World Scientific publisher of science and technology information
http://www.karger.com/	The Karger publishing house. Biomedical science publisher
http://www.humanapress.com/Index.pasp	Humana Press – publisher of medical and scientific books and journals in both print and electronic media
http://www.springer.de/	Springer-Verlag – international scientific publisher (medicine, life sciences, mathematics and engineering)
http://www.blacksci.co.uk/	Blackwell Publishing home page
http://www.bios.co.uk/	BIOS Scientific Publishers Ltd – publisher in biology and medicine
http://www.interpharm.com/	Technical and regulatory information for manufacturers of biotechnology, bulk pharmaceutical chemicals, medical devices, diagnostic products, and pharmaceuticals worldwide

Continued from previous page

Internet address	Description
http://www.dekker.com/index.jsp	International publisher in scientific, technical, and medical fields; includes reference books, textbooks, encyclopedias, journals, and newsletters for students, researchers, and practitioners
http://www.wkap.nl/	Kluwer home page

24.2.2 Periodicals

Key words: science magazines, biology magazines, mass spectrometry journals, chromatography journals, bioscience journals.

Internet address	Description
http://www.sciencedirect.com	One of the world's largest provider of scientific, technical and medical journals, with access to some over 1800 on-line journals
http://www.lcgcmag.com/lcgc/	The LC-GC Magazine on-line. Publication for anyone using, or thinking of using, separation science techniques
http://www.lcgceurope.com/lcgceurope/	An obligatory lecture for those working with various aspects of chromatography. Don't miss the troubleshooting articles by Dolan
http://www.the-scientist.com/	The Scientist – The News Journal for the Life Scientist
http://gnn.tigr.org/main.shtml	Genome News Network – on-line magazine about genomic research
http://www.newscientist.com/news/	New Scientist magazine
http://nextwave.sciencemag.org/	Science's Next Wave is a publication that covers scientific training, career development, and the science job market
http://www.spectroscopymag.com/spectroscopy/	Spectroscopy magazine
http://www.latrobe.edu.au/www/anzsms/Journals.html	Mass spectrometry journals
http://www.liv.ac.uk/Chemistry/Links/journals.html	The Virtual Library, links to biosciences, chemistry, medical and health journals
http://www.interscience.wiley.com/jpages/1076-5174/	Journal of Mass Spectrometry
http://pubs.acs.org/journals/ancham/	Analytical Chemistry Homepage

(*Continued on next page*)

Continued from previous page

Internet address	Description
http://www.impub.co.uk/ems.html	European Journal of Mass Spectrometry – review journal
http://www.elsevier.com/homepage/saa/webjam/	Journal of The American Society for Mass Spectrometry
http://www.interscience.wiley.com/jpages/0277-7037/	Mass Spectrometry Reviews
http://www.wiley-vch.de/publish/en/journals/alphabeticIndex/2120/	Proteomics – source of information about protein separation and characterization, advances in bioinformatics, and novel applications of proteomics in all areas of the life sciences and industry
http://pubs.acs.org/journals/jprobs/	Journal of Proteome Research
http://www.wiley-vch.de/publish/en/journals/alphabeticIndex/2027/	Electrophoresis
http://www.biomedcentral.com/	Database of biomedical journals

24.3 INSTRUMENTATION

This section contains a list of companies/manufacturers of scientific and analytical instrumentation, chemicals, software, as well as representatives, who may be contacted for further details.

24.3.1 Manufacturers and vendors

Key words: science companies, pharmaceutical companies, mass spectrometry companies, chromatography companies.

Internet address	Description
http://www.chromatography.co.uk/default.htm	VWR Chromatography page, containing application, products and introduction to chromatography
http://www.jasco.co.uk/gpc.htm	The Jasco page, containing products, standards, instrumentation, and software
http://www.spotfire.com/	Spotfire Inc., containing analytic application and analytical tools for functional genomics
http://www.bruker.com/	Bruker Company's homepage
http://www.bdal.com/	Bruker Daltonics page, containing application and instrumentation for mass spectrometry. (Fourier transform mass spectrometry, time-of-flight mass spectrometers)

Continued from previous page

Internet address	Description
http://www.gcmsservice.com/	GenTech Scientific, Inc., a GC/MS service company
http://www.jeol.com/ms/ms.html	Magnetic-sector and time-of-flight mass spectrometer manufacturer; also contains applications and tutorials
http://www.micromass.co.uk/	Company dedicated to mass spectrometry
http://www.worthington-biochem.com/	The Worthington Biochemical Corporation web site contains useful information, enzyme manual, tutorials on enzymes and biochemistry with extensive literature references
http://www.amershambiosciences.com	Amersham Biosciences (disease research, drug discovery, drug development, and drug manufacture featuring chromatography, proteomics, and genomics)
http://home.appliedbiosystems.com/	Applied Biosystems: Proteomics and genomics instrument systems, accessories, reagents, application notes (PerSeptive Biosystems, MALDI-TOF mass spectrometers)
http://www.perkin-elmer.com/	Perkin Elmer: Life Sciences, opto-electronics and analytical instruments (LC/mass spectrometers)
http://www.shimadzu.com/	Shimadzu product information: Liquid chromatographs, gas chromatographs, mass spectrometry systems, spectroscopy products, application notes
http://www.chem.agilent.com/scripts/PHome.asp	Agilent Technologies homepage
http://www.thermo.com/eThermo/CDA/BU_Home/BU_Homepage/0,,113,00.html	ThermoFinnigan page: Mass spectrometry and chromatography equipment manufacturer (ion-trap, sector, GC, FT, and ICP mass spectrometers)
http://www.kratos.com/flindex.html	Kratos Analytical: Surface analysis, time-of-flight mass spectrometers
http://www.proteome.co.uk/home.html	Proteome Sciences company homepage. Site containing information on identification of new protein markers for diagnostic purposes and of novel molecular targets for drug discovery
http://www.lcpackings.nl/	LC Packings – A Dionex Company: Capillary/Nano LC systems, microcolumns, LC/MS tools)

(*Continued on next page*)

Continued from previous page

Internet address	Description
http://www.chrompack.com/	High-performance capillary gas and liquid chromatography columns
http://www.waters.com/	Waters Corporation: HPLC, thermal analysis, and mass spectrometry
http://www.celera.com/	Source for proteomics, bioinformatics, and genomics
http://www.ciphergen.com/	Ciphergen Biosystems develops, manufactures, sells, and services ProteinChip® Systems and related products that discover, characterize and assay proteins from native biological samples

24.4 CHROMATOGRAPHY

This section, divided into several subheadings, provides a list of tutorials, instrumentation, software, and applications, for various types of chromatography.

24.4.1 Gas chromatography

Key words: Gas chromatography, GC, GC/MS, gas chromatography mass spectrometry, GC detectors.

Internet address	Description
http://home.att.net/~GCresource/GCmain.html	Excellent GC site (tutorial, instrument manufacturers, applications, etc.)
http://www.registech.com/gc/index.htm	Review of GC derivatization procedures
http://gc.discussing.info/gs/a_introduction/index.html	"Start GC" – comprehensive GC page
http://www.hrgc.com/	High-resolution gas chromatography page (discussion group, troubleshooting, links)
http://www.shsu.edu/~chm_tgc/sounds/sound.html	Chemistry-based QuickTime, shockwave flash GIF animations and streaming audio
http://www.shsu.edu/~chemistry/primers/gcms.html	Coupling gas chromatography to mass spectrometry
http://depts.washington.edu/spectral/massspec/GCMSintro/	Introduction to gas chromatography/mass spectrometry

24.4.2 Liquid chromatography

Key words: LC–MS, chromatography on-line, HPLC/MS, liquid chromatography mass spectrometry.

Internet address	Description
http://ionsource.com/tutorial/chromatography/rphplc.htm#The%20HPLC	Reversed-phase HPLC for proteomics
http://www.lcresources.com/resources/reslinks.html	LC resources page with HPLC-related links
http://groups.yahoo.com/group/chrom-l/	Chromatography discussion group at Yahoo!
http://members.aol.com/chromgroup/links.htm	Chromatography-related Web sites. Page contains tutorials, links to discussion groups, etc.
http://www.acdlabs.com/educators/links/lnkchrom.html#LC	Advanced Chemistry Development page provides chromatography links, including the "HPLC-doctor"
http://www.chem.sc.edu/analytical/chem723/links.html	Links to chromatography resources
http://osoon.ut.ee/~lulla/HPLC/HPLC.html	HPLC links, containing an open forum for chromatography discussion along with job opportunities, gas chromatography and liquid chromatography tips, vendor links, and much more
http://elchem.kaist.ac.kr/vt/chem-ed/analytic/ac-meths.htm	Analytical chemistry and instrumentation site
http://www.instrumentalchemistry.com/	Animated explanations of various concepts in analytical chemistry
http://www.registech.com/chiral/applications/	Chiral Application Guide by Regis Technologies, Inc.
http://www.hplc1.com/	Web site with many links to various producers of HPLC columns and equipment. Includes troubleshooting guide for chromatographers
http://www.netaccess.on.ca/~dbc/cic_hamilton/chrom.html	Chromatography resources, containing theory, useful links to journals, societies, organizations, software
http://home4u.de/niven/rn_hplc.htm	Niven's chromatography site, in several languages
http://www.eng.rpi.edu/dept/chem-eng/WWW/faculty/cramer/prep/slide1.html	Presentation based on Preparative Chromatography in Biotechnology by Steven M. Cramer and Guhan Jayaraman
http://www.raell.demon.co.uk/chem/CHIbook/Chiral.htm	On-line guide to chiral HPLC

(*Continued on next page*)

Continued from previous page

Internet address	Description
http://www.chromatographyforum.com/	Chromatography forum is a public discussion group of chromatographers
http://www.forumsci.co.il/HPLC/index.html	S. Levin's homepage of HPLC and LC/MS including tutorials, courses, etc.
http://www.shu.ac.uk/schools/sci/chem/tutorials/	HPLC introductory theory
http://www.mac-mod.com/cc/cc-toc.html	Mac – Mod HPLC Column Companion. Troubleshooting page
http://chrom.tutms.tut.ac.jp/JINNO/ENGLISH/RESEARCH/research.html	Research information on a wide range of compounds for HPLC

24.4.3 Size-exclusion chromatography

Key words: SEC, size exclusion chromatography, gel permeation chromatography.

Internet address	Description
http://ntri.tamuk.edu/fplc/siz.html	The basis of size-exclusion chromatography, including many useful figures
http://www.ionsource.com/tutorial/capillary/captoc.htm	Introduction to capillary chromatography
http://www.chem.vt.edu/chem-ed/sep/lc/size-exc.html	The Chemistry Hypermedia Project – resources help students learn chemistry. Introduction to size-exclusion chromatography
http://gc.discussing.info/gs/b_theory/classification.html	The classification of chromatographic processes. Good starting point for all sorts of separations
http://ull.chemistry.uakron.edu/analytical/Chromatography/:	A general approach to chromatography, including an overview of plate and rate theory
http://kerouac.pharm.uky.edu/ASRG/HPLC/hplcmytry.html:	History, application, theory of operation, troubleshooting guide and other useful information on HPLC
http://www.forumsci.co.il/HPLC/gpc_handouts.pdf	A good tutorial on SEC/GPC

24.5 ELECTROPHORESIS

This section is divided into two subsections: One collects links to the most popular sites for polyacrylamide gel electrophoresis and the other web sites for capillary electrophoresis.

24.5.1 Gel electrophoresis

Key words: electrophoresis, 2D page, SDS page, 2D gel electrophoresis, gel electrophoresis.

Internet address	Description
http://ca.expasy.org/ch2d/	SWISS-2DPAGE contains data on proteins identified on various 2-D PAGE and SDS-PAGE reference maps
http://www-lecb.ncifcrf.gov/2dwgDB/	The 2DWG meta-database of 2D-PAGE protein gel images found on the Web
http://www.ruf.rice.edu/~bioslabs/studies/sds-page/gellab2.html	Introduction to SDS-PAGE
http://www-lecb.ncifcrf.gov/EP/table2Ddatabases.html	Partial List of Web 2D Electrophoretic Gel Databases
http://www.rit.edu/~pac8612/electro/E_Sim.html	Electrophoresis of protein and DNA simulations site
http://www.bergen.org/AAST/Projects/Gel/	Theoretical basis of gel electrophoresis
http://www4.amershambiosciences.com/APTRIX/upp00919.nsf/content/Elpho_HomePage	Amersham Biosciences electrophoresis site
http://www.aber.ac.uk/parasitology/Proteome/Tut_2D.html	2D gel electrophoresis for proteomics – a tutorial
http://www.separationsnow.com/basehtml/SepH/1,3921,1-4-0-0-0-directories_hot-0-0,00.html	SeparationsNOW electrophoresis page (tutorials, journals, research group, software)
http://www.weihenstephan.de/blm/deg/manual/manfrm.htm	2D gel electrophoresis of proteins in immobilized pH gradients
http://www.orbigen.com/protocols/Protein_Electrophoresis.html	Electrophoresis procedures
http://www.chemsoc.org/exemplarchem/entries/2002/proteomics/proteomics.htm	Strategy for 2D electrophoresis
www.uct.ac.za/microbiology/sdspage.html	SDS gel electrophoresis procedures
http://www.aber.ac.uk/%7Empgwww/Proteome/Tut_2D.html	2D gel electrophoresis for proteomics – a tutorial
http://www.protocol-online.org/prot/Molecular_Biology/Protein/Protein_Electrophoresis/Two-Demensional_SDS-PAGE	2D SDS gel electrophoresis procedures

24.5.2 Capillary electrophoresis

Key words: capillary electrophoresis, CE theory, capillary zone electrophoresis.

Internet address	Description
http://ntri.tamuk.edu/ce/ce.html	Capillary electrophoresis site
http://www.chemsoc.org/chembytes/ezine/2000/altria_nov00.htm	Capillary electrophoresis theory and explanations
http://ull.chemistry.uakron.edu/chemsep/electrophoresis/	Capillary electrophoresis theory
http://www.chem.agilent.com/Scripts/PCol.asp?lPage = 387	Agilent Technologies: Capillary electrophoresis site
http://www.separationsnow.com/basehtml/SepH.html	The Key Resource for capillary and gel electrophoresis and capillary electrochromatography
http://www.microsolvtech.com/ce.asp	Capillary Electrophoresis Primer (theory, helpful hints, books, and links)
http://www.eutechsci.com/ce/cesearch.asp	Capillary Electrophoresis Knowledge Base; over 5000 references, starting from 1981
http://www.dpo.uab.edu/~yangzw/capelec.html	Capillary electrophoresis links
http://www.rsc.org/pdf/books/capelectrosc.pdf	Background theory and principles of capillary electrophoresis
http://www.pharmacy.umaryland.edu/courses/PHAR628/Documents/basicprinciples.ppt	Basic principles of capillary electrophoresis

24.6 MASS SPECTROMETRY

This section deals with various aspects of mass spectrometry, coupling with commonly used chromatographic/electrophoretic techniques, tutorials, procedures, methods for spectral interpretation, troubleshooting, newsgroups, and other important links.

Key words: mass spectrometry, LC–MS, chromatography on-line, HPLC/MS, liquid chromatography mass spectrometry.

Internet address	Description
http://www.sisweb.com/mslinks.htm	Mass spectrometry links, including LC/MS
http://proteome.nih.gov/may2001/links.html	Mass spectrometry links
http://www.lcms.com/	The LC/MS page and the LC/MS book store. Information about books, tutorials, conferences, troubleshooting
http://www.forumsci.co.il/HPLC/lcms_page.html	LC/MS information
http://www.spectroscopynow.com/Spy/basehtml/SpyH	A very useful site provided by Wiley, including the Base Peak resources for mass spectrometry

Continued from previous page

Internet address	Description
http://masspec.scripps.edu/massspec.html	Good site, including a history of mass spectrometry
http://www.i-mass.com/	A web site dedicated to mass spectrometry, containing links to mass spectrometry journals and books, conferences, history, guides, software, and laboratories
http://www.mslinks.com/	Mass spectrometry resources (companies, research groups, application notes, journals, books, products, databases, societies)
http://www.chemistry.gatech.edu/stms/links.html	Mass spectrometry links
http://www.bmss.org.uk/links/resource.html	Page from ETH Zurich AMS labs with many important links
http://www.ionsource.com/	Mass spectrometry and biotechnology resource
http://ionsource.com/tutorial/spectut/spec1.htm	Interpretation of electrospray mass spectra – a tutorial
http://ionsource.com/tutorial/isotopes/contents.htm	Short tutorial concerning isotopes
http://www.chem.purdue.edu/cooks/link.html	Links to many useful mass spectrometry-related pages
http://www.chemistry.gatech.edu/stms/	The Usenet Mass Spectrometry Newsgroup
http://web.mit.edu/speclab/www/ms_links.html	Mass spectrometry resources
http://www.organicworldwide.net/mass.html	Mass spectrometry: Theory, tips, troubleshooting, spectral interpretation, databases, etc.
http://biobenchelper.hypermart.net/tech/mass_spectrometry.htm	MS links: theory, applications, organizations, facilities, and vendors

24.7 APPLICATIONS

This section has been divided into three subsections: proteomics, genomics, and informatics. Each subsection contains information concerning manufacturers, resources, databases, tutorials, tools for identification, available software, and news.

24.7.1 Proteomics

Key words: proteomics, proteome, proteome identification, proteome analysis, protein identification, protein database, database, biological database.

Internet address	Description
http://proteomicssurf.com/	Proteomics surfer. References, application notes, emerging topics, and news
http://www.iacr.bbsrc.ac.uk/notebook/courses/guide/	Basic information about cell biology, DNA, RNA, protein structure and diversity, amino acids and other topics
http://www.proteinscience.com/index.htm	The proteomics portal (links to companies, laboratories, products, research, and literature)
http://www.bioinform.com/index.htm	News about developments in biology and proteomics
http://www.ncbi.nlm.nih.gov/	This site is sponsored by the National Institutes of Health. Comprehensive information about proteomics and genomics
http://www.hip.harvard.edu/	Harvard Institute of Proteomics homepage
http://www.spectroscopynow.com/Spy/basehtml/SpyH/1,2466,10-0-0-0-0-home-0-0,00.html	A very useful site, provided by Wiley, which includes the Base Peak resources for proteomics
http://proteome.nih.gov:8000/home.html	ProtIG Proteomics Interest Group, which organizes monthly seminars in relevant areas of proteomics, including talks on separation and protein identification methods, determination of post-translational modifications, protein/protein interactions, bioinformatics, and data management
http://proteome.nih.gov/links.html	Proteome-related links
http://www.bio.com/	Biotechnology industry news, protocols, and tools
http://www.edv.agrar.tu-muenchen.de/blm/deg/	Two-Dimensional Electrophoresis of Proteins using Immobilized pH Gradients. A Laboratory Manual
http://gimr.garvan.unsw.edu.au/public/corthals/	Biological Mass Spectrometry and Protein Analysis Laboratory (useful protein tools, proteome related resources, databases, etc.)
http://proteomics.uchsc.edu/	The proteomics facility at the University of Colorado Health Sciences Center

24.7.2 Genomics

Key words: genomics, genome, genome sequencing, human genome.

Internet address	Description
http://www.genomeweb.com/	GenomWeb provides news and information covering the business and technology of genomics and bioinformatics
http://klab.agsci.colostate.edu/	The Mosquito Genomics www Server provides access to mosquito genomics databases and links to genomics and other www servers around the world
http://genomics.phrma.org/	Genomics Global Resource from PhRMA (Pharmaceutical Research and Manufacturers of America)
http://www.genomicglossaries.com/	Glossaries and taxonomies related to genomics
http://lgsun.grc.nia.nih.gov/	NIA/NIH Mouse Genomics home page
http://www.genomics.cornell.edu/	Cornell Genomics Initiative (computational, statistical, evolutionary, mammalian, microbial, plant genomics)
http://www-genome.wi.mit.edu/	Whitehead Institute for Biomedical Research/ MIT Center for Genome Research: information on map and sequence releases and software
http://cmgm.stanford.edu/pbrown/mguide/index.html	Pat Brown's lab at Stanford. The complete guide to microarrays: protocols, software, arrayer construction instructions
http://www.tigr.org/	The Institute for Genomic Research databases and software
http://www-hto.usc.edu/	Center for Computational and Experimental Genomics (Journal of Computational Biology, books, research, databases)
http://www.genomicsnews.com/public/genresou.cfm	Independent publication covering a range of topics related to human genome sequencing, gene therapy, etc.
http://www.kumc.edu/gec/	Genetics Education Center – The Human Genome Project, genetic education resources, networking, genetic programs
http://www.dnaftb.org/dnaftb/	Josiah Macy Foundation: Multimedia primer on the basics of genetics and heredity
http://www.informatics.jax.org/mgihome/	Mouse Genome Informatics
http://www.humgen.umontreal.ca/en	HumGen, a website that gives access to a comprehensive international database on the legal, social, and ethical aspects of human genetics
http://gslc.genetics.utah.edu/	The Genetic Science Learning Centre helps people understand how genetics affects their lives and society

24.7.3 Informatics

24.7.3.1 Bioinformatics

Key words: bioinformatics, molecular biology, biochemistry tools, and biotechnology.

Internet address	Description
http://bioinformatics.org/	Sites providing free and open resources for all bioinformatics scientists
http://www.bioinformatics.ca/	Information about bioinformatics resources in Canada
http://cellbiol.com/	The Bio-Web: Resources for molecular and cell biologists – a biology-related site
http://www.nwfsc.noaa.gov/protocols/bioinformatics.html	Useful links to molecular biology resources
http://www.public.iastate.edu/~pedro/research_tools.html	Pedro's Biomolecular Research Tools (a collection of links to information and services useful to molecular biologists, biochemists, protein chemists, and mass spectrometrists)
http://www.sonoma.edu/users/t/thatcher/biol518/links.htm	Biotechnology links
http://www.bioplanet.com/links.htm	Links to bioinformatics resources
http://www.bioinformatik.de/	Data on software, companies, literature, research, education, etc. on bioinformatics
http://life.anu.edu.au/	Bioinformatics Group Web site
http://www.genet.sickkids.on.ca/bioinfo_resources/	Bioinformatics links
http://www.mblab.gla.ac.uk/~julian/Dict.html	The Dictionary of Cell and Molecular Biology
http://www.hum-molgen.de/bioinformatics/	Bioinformatics tools
http://www.ncbi.nlm.nih.gov/About/primer/bioinformatics.html	Basic introduction to the science from the National Center for Biotechnology Information
http://www.cbs.dtu.dk/services/SignalP/	The SignalP World Wide Web server predicts secretory signal peptides and their cleavage sites in eukaryotes and prokaryotes
http://www.bmrb.wisc.edu/	Data from NMR spectroscopy on proteins, peptides, and nucleic acids
http://bioinformatics.weizmann.ac.il/cards/bioinfo_intro.html	Weizmann Institute of Science – Introduction to bioinformatics
http://www.bioinfoinstitute.com/	National Bioinformatics Institute – national organization of bioinformaticians across the United States, working for the advancement of bioinformatics education and certification

Continued from previous page

Internet address	Description
http://biobenchelper.hypermart.net/index.html	A comprehensive collection of internet resources for bio-lab researchers
http://iprotocol.mit.edu/	A free service, containing procedures used in bioscience

24.7.3.2 Databases

Key words: databases, protein databases, proteome databases, genome databases, mass spectrometry databases, biomedical databases.

Internet address	Description
http://www.ludwig.edu.au/jpsl/Databases.asp	JPSL (Joint ProteomicS Laboratory) proteomic databases
http://prowl.rockefeller.edu/recipes/contents.htm	A collection of procedures for protein mass spectrometry
http://www.ebi.ac.uk/swissprot/hpi/hpi.html	Human Proteomics Initiative (HPI) is an initiative, by SIB and the EBI, to annotate all known human sequences according to the quality standards of Swiss-Prot
http://www.ebi.ac.uk/newt/index.html	NEWT is the taxonomy database maintained by the Swiss-Prot group
http://www.harefield.nthames.nhs.uk/nhli/protein/	2-DE gel protein databases at Harefield Hospital
http://www.ncbi.nlm.nih.gov/BLAST/blast_overview.html	BLAST – search similarity program, designed to explore all of the available sequence databases, regardless of whether the query is protein or DNA. Also contains links to free MEDLINE
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = PubMed	The biomedical literature database
http://www.sciencekomm.at/index.html	Medbioworld is a medical reference site, including all medical journals and medical associations, and similar resources in the biological sciences, containing free articles
http://www.cheminfo.org/	This web site contains the Jcamp database (NMR, IR, mass spectra), CCD (database about chemicals) and ChemCalc (a calculator allowing quick calculation of the molecular formula, molecular weight, and isotopic distribution)

(*Continued on next page*)

Continued from previous page

Internet address	Description
http://195.41.108.38/PepSeaIntro.html	PepSea – an advanced tool for protein database searching by mass spectrometric data, such as peptide mass maps or (partial) amino acid sequences
http://www.expasy.ch/tools/aacomp/	AACompIdent identifies a protein from its amino acid composition
http://www.expasy.ch/tools/pi_tool.html	Compute pI/Mw is a tool for calculating the theoretical isoelectric point and molecular weight
http://www.expasy.ch/tools/findmod/	FindMod predicts potential protein post-translational modifications (PTM) and finds potential single-amino-acid substitutions in peptides
http://www.expasy.ch/tools/peptide-mass.html	Peptide Mass cleaves a protein sequence from the SWISS-PROT and/or TrEMBL databases or a user-entered protein sequence with a chosen enzyme, and computes the masses of the generated peptides
http://www.expasy.ch/tools/peptident.html	PeptIdent identifies proteins on the basis of pI, mw and peptide mass fingerprint data
http://www.expasy.ch/tools/tagident.html	TagIdent identifies a protein by pI and molecular weight, or by a sequence tag
http://www.incyte.com/sequence/proteome/index.shtml	The Proteome BioKnowledge Library – a collection of databases focused on the compilation of available protein information
http://www.ebi.ac.uk/proteome/	The Proteome Analysis database. Good collection of protein databases; human, yeast, *Drosophila*, and many more
http://www.abrf.org/index.cfm/dm.home?AvgMass = all	Delta Mass, a database of protein post-translational modifications from the Association of Biomolecular Resource Facilities
http://www.ncbi.nlm.nih.gov/	Molecular biology information resource, containing public databases, software tools for analyzing genome data, and biomedical information
http://falcon.ludwig.ucl.ac.uk/mshome3.2.htm	Protein Prospector is a proteomics tool for mining sequence databases in conjunction with mass-spectrometry experiments
http://www-nbrf.georgetown.edu/pirwww/search/fasta.html	The Protein Information Resource (PIR) FASTA database searches the sequence similarity of your query protein sequence against a non-redundant sequence database

Continued from previous page

Internet address	Description
http://www.expasy.ch/	ExPASy Molecular Biology Server (databases and tools for protein/peptide identification. Many important links)
http://www.expasy.ch/sprot/	Swiss-Prot, a complete, annotated protein sequence database
http://www.rcsb.org/pdb/	Protein Data Bank – macromolecular structure data on proteins, nucleic acids, protein/nucleic acid complexes, and viruses
http://prowl.rockefeller.edu/	PROWL – a resource for protein chemistry and mass spectrometry.
http://bioinformatics.weizmann.ac.il/cards/	GeneCard is a database of human genes, their products and their involvement in diseases
http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html	Human Gene Mutation Database at the Institute of Medical Genetics
http://www.ncbi.nlm.nih.gov/omim/	Database of human genes and genetic disorders
http://www.gdb.org/	The Genome Database (GDB) is the official central repository for genomic mapping data resulting from the Human Genome Initiative
http://www-ls.lanl.gov/dbqueries.html	Chromosome 16 database query

24.7.3.3 Mass spectrometry software

Key words: mass spectrometry software, MS freeware, MS program.

Internet address	Description
http://www.chemistry-software.com/masspec.htm	Software for chemistry and biochemistry (including MS, GC, and LC)
http://www.protana.com/solutions/aminoclc/default.asp	AminoCalc – freeware program for finding consecutive peaks in fragment-ion series, differing by one amino acid
http://www.epa.gov/ttn/emc/ftir/convert.html	File Conversion Program converting SpectraCalc, LabCalc, or GRAMS files (not multifiles) to ASCII. (Freeware)
http://members.aol.com/msmssoft/	MS/MS software. Isotope simulators for Windows. (Freeware)
http://www.enovatia.com/stories/storyReader$156	GC-Calc calculating optimum conditions for GC and GC/MS operation (inlet pressure, linear velocity, and flow-rate). (Freeware)

(*Continued on next page*)

Continued from previous page

Internet address	Description
http://www.kuleuven.ac.be/rega/medchem/pepseq.htm	Pepseq for interpreting collision-induced mass spectra (MS/MS or linked scan) of oligopeptides
http://frl.lptc.u-bordeaux.fr/website-frl/massxpert/massxpert-main.html	MassXpert program for predicting and analyzing mass spectrometric data, obtained on proteins and peptides. (Freeware)
http://www.bruker-daltonik.de/biotools.html	MS Biotools is a specialized post-processing software for protein MS data
http://chemdata.nist.gov/mass-spc/amdis/	The Automated Mass Spectral Deconvolution and Identification System (AMDIS) – a program that extracts spectra for individual components in a GC/MS data file and identifies target compounds by matching these spectra against a reference library. (Freeware)
http://www.highchem.com/mf.htm	Mass Frontier™: Software for the management and interpretation of mass-spectral data. Mass Frontier™ is based on the following ten modules: Structure Editor, Spectra Manager, Fragments and Mechanisms, GC/LC/MS Processor, Spectra Classifier, Spectra Projector, Isotope Pattern, Periodic Table of Isotopes, Fragments Comparator, and Neural Networks
http://www.chemsw.com/12148.htm	ChromView imports GC and GC–MS data into other Windows applications, such as MS Word, Excel, or Powerpoint, as high-resolution images
http://www.massspec.com/CombiLab.html	CombiLab – Software for Combinatorial Chemistry Library Design and Analysis
http://www.chemsw.com/12140.htm	GC and GC/MS File Translator can interconvert various GC, GC/MS data formats, including Hewlett-Packard (Agilent) GC ChemStation, Hewlett-Packard (Agilent) MS ChemStation, Finnigan (ThermoFinnigan) ITDS, Finnigan (ThermoFinnigan) Magnum, Shimadzu MS, EasyChrom, and ASCII
http://welcome.to/gpmaw/	General Protein/Mass Analysis for Windows (GPMAW) is excellent software for interpretating peptide and protein MS data. It provides now the entire SWISS-Prot and EMBL databases, with information on more than 800,000 proteins

Continued from previous page

Internet address	Description
http://www.chemie.uni-hamburg.de/oc/koenig/massfinder.html	MassFinder 2.1 – a software tool for GC/MS analysis
http://www.oraxcel.com/projects/masssim/index.htm	MasSim program simulates the functioning of a real mass spectrometer
http://www.matrixscience.com/cgi/index.pl?page = ../home.html	Mascot – a search engine from Matrix Science for the identification of proteins and peptides. The manufacturers of mass spectrometers commonly apply this software
http://www.sisweb.com/cgi-bin/mass10.pl	Isotope Distribution Calculator and Mass Spec Plotter
http://www.hgmp.mrc.ac.uk/Bioinformatics/Webapp/mowse/	Mowse identifies proteins from peptide digest masses

24.7.3.4 Proteomics and genomics software

Key words: proteomics software, genomics software, bioinformatics software.

Internet address	Description
http://www.matrixscience.com/products.html	Mascot Cluster, the complete software and hardware solution for high-throughput protein identification. This page contains links to other software sites
http://www.molbiol.ox.ac.uk/help/bioinformatics_software_page.html	Bioinformatics software page
http://www.mann.embl-heidelberg.de/GroupPages/PageLink/Peptidesearchpage.html	PeptideSearch – tool for database searching by mass-spectrometric data, such as peptide mass maps or (partial) amino acid sequences
http://www.chemsw.com/16016.htm	The Protein Tools Software Package was designed for biochemists working with proteins and peptides. The package consists of a sequence editor, amino acid analysis software, and application that can help predict the isoelectric point of a designed protein
http://proteome.nih.gov:8000/may2001/software.html	Proteomics software links available at the NIH
http://genes.mit.edu/GENSCAN.html	GENSCAN is a program designed to predict complete gene structures, including exons, introns, promoter and poly-adenylation signals in genomic sequences

(*Continued on next page*)

Continued from previous page

Internet address	Description
http://beagle.colorado.edu/~eesnyder/GeneParser.html	GeneParser is a program for the identification of protein-coding regions in genomic DNA sequences
http://www.itba.mi.cnr.it/webgene/	Tools for prediction and analysis of protein-coding gene structure. (GeneBuilder – protein-coding gene prediction, ORF Gene – gene structure prediction, EST Mapping, etc.)
http://www.cbs.dtu.dk/services/	Prediction servers. Center for Biological Sequence Analysis (glycosylation sites, cleavage sites in plant proteins, serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins, etc.)
http://www.lecb.ncifcrf.gov/flicker/	Program comparing images from different Internet sources on your Web browser (e.g., 2D protein electrophoretic gel images)

24.7.3.5 Size-exclusion chromatography software

Key words: SEC software, chromatography software, chromatography optimization software, chromatography analysis software.

Internet address	Description
http://www.justiceinnovations.com/products/applicsoftware/secgpc.pdf	Software available for SEC/GPC
http://www.ent.ohiou.edu/~guting/CHROM/	Tingyue Gu's Chromatography Simulation Home Page. Chromatography simulation software (free for noncommercial use)
http://www.sisweb.com/software/csw/hplcopt/hplcopt.html	Chromatography Optimization – optimizes isocratic and gradient HPLC conditions
http://www.chemistry-software.com/chromat/10310.htm	Chrom Perfect – Supports one chromatograph with up to two detectors, or two chromatographs with a single detector each. Software for background data acquisition and real-time plots
http://www.chemsw.com/12013.htm	Chromatography Equations: Calculations for volumetric to linear flow, resolution based on peak-width at base-line or at half-height, effective plate counts, and capacity factor

Continued from previous page

Internet address	Description
http://www.adinstruments.com/products/software/pchrom.html	PowerChrom software records and analyses output from GC, HPLC, IC, SEC, and preparative chromatography
http://www.scisw.com/products/elite/index.htm	EZChrom Elite designs custom reports, displays calibration curves, acquires and processes data, creates methods
http://www.acdlabs.com/products/chrom_lab/chrom_manager/	ACD/ChromManager processes chromatographic data (HPLC, GC, CE) and provides database management for chromatograms
http://www.acdlabs.com/products/chrom_lab/lc_simulator/	ACD/LC Simulator predicts elution order and retention times for LC separation of organic chemical mixtures. It optimizes the analysis of experimental chromatograms
http://www.dionex.com/app/tree.taf?asset_id = 279046	Chromeleon provides full control and detailed audit trails for over 150 instruments from more than 20 suppliers

24.8 SCIENTIFIC ORGANIZATIONS

This section collects addresses of scientific organizations/associations and discussion groups that promote the exchange of ideas, organize conferences and courses, and, sometimes, edit newsletters, or provide analytical services.

Key words: scientific organization, scientific society, scientific laboratories, institute.

Internet address	Description
http://www.abrf.org/	Association of Biomolecular Resource Facilities (ABRF) Association that promotes communication and cooperation among core facilities and research laboratories that service various institutions, universities, and companies. Excellent discussion group where you can get help on any question. You can subscribe to this by emailing: abrf-request@aecom.yu.edu
http://www.hupo.org/	The Human Proteome Organization
http://www.ebi.ac.uk/	European Bioinformatics Institute – center for research and services in bioinformatics

(*Continued on next page*)

Continued from previous page

Internet address	Description
http://www.casss.org/	California Separation Science Society is a non-profit chromatography discussion group serving separation scientists in California
http://www.asms.org/	American Society for Mass Spectrometry (discussion groups, positions, links, etc.)
http://www.bmb.leeds.ac.uk/esms/	European Society for Mass Spectrometry
http://www.iscb.org/	The International Society for Computational Biology homepage
http://www.embo.org/	The European Molecular Biology Organization (EMBO) promotes biological sciences in Europe, provides fellowships for research scientists, organizes and (financially) supports scientific courses, lectures, workshops etc.
http://www.bmss.org.uk/	British Mass Spectrometry website
http://www.proteome.org/	Proteome Society
http://www.narrador.embl-heidelberg.de/GroupPages/homepage.html	Protein and Peptide Group EMBL, Heidelberg
http://www.embl-heidelberg.de/	The European Molecular Biology Laboratory
http://www.gene.ucl.ac.uk/hugo/	The Human Genome Organization
http://www.who.int/en/	World Health Organization
http://www.eshg.org/	European Society of Human Genetics
http://www.ifhgs.org/	The International Federation of Human Genetics Societies – a forum for organized groups, dedicated to all aspects of human genetics
http://www.gene.ucl.ac.uk/hugo/	The Human Genome Organisation
http://www.aesociety.org/	The Electrophoresis Society promotes scientific advancement in electrophoretic theory and applications

Subject Index

B

C

D

E

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

JOURNAL OF CHROMATOGRAPHY LIBRARY

A Series of Books Devoted to Chromatographic and Electrophoretic Techniques and their Applications

Although complementary to the Journal of Chromatography, each volume in the library series is an important and independent contribution in the field of chromatography and electrophoresis. The library contains no material reprinted from the journal itself.

Recent volumes in this series

Volume 60 **Advanced Chromatographic and Electromigration Methods in BioSciences**
edited by Z. Deyl, I. Mikšík, F. Tagliaro and E. Tesařová

Volume 61 **Protein Liquid Chromatography**
edited by M. Kastner

Volume 62 **Capillary Electrochromatography**
edited by Z. Deyl and F. Svec

Volume 63 **The Proteome Revisited: theory and practice of all relevant electrophoretic steps**
by P.G. Righetti, A. Stoyanov and M.Y. Zhukov

Volume 64 **Chromatography - a Century of Discovery 1900-2000: the bridge to the sciences/technology**
edited by C.W. Gehrke, R.L. Wixom and E. Bayer

Volume 65 **Sample Preparation in Chromatography**
by S.C. Moldoveanu and V. David

Volume 66 **Carbohydrate Analysis by Modern Chromatography and Electrophoresis**
edited by Z. El Rassi

Volume 67 **Monolithic Materials: Preparation, Properties and Applications**
by F. Svec, T. Tennikova and Z. Deyl

Volume 68 **Emerging Technologies in Protein and Genomic Material Analysis**
by G. Marko-Varga and P. Oroszlan