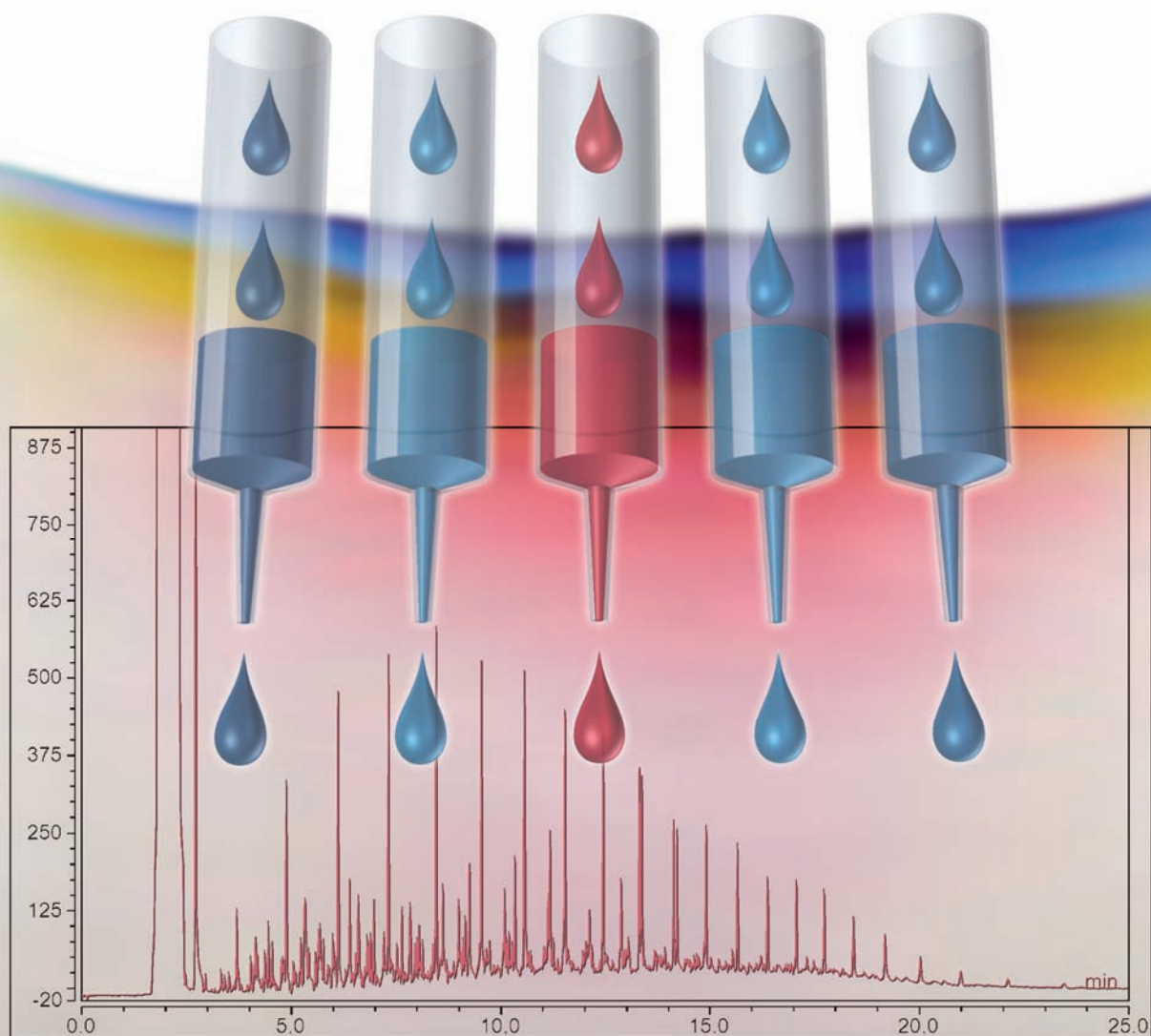


Elsa Lundanes, Léon Reubsaet and Tyge Greibrokk

Chromatography

Basic Principles, Sample Preparations
and Related Methods



Elsa Lundanes

Léon Reubsaet

Tyge Greibrokk

Chromatography

Related Titles

Snyder, L.R., Kirkland, J.J., Dolan, J.W.

Introduction to Modern Liquid Chromatography, 3rd Edition

2010

Print ISBN: 978-0-470-16754-0, also available in digital formats

Wixom, R.L., Gehrke, C.W. (eds.)

Chromatography A Science of Discovery

2010

Print ISBN: 978-0-470-28345-5, also available in digital formats

Meyer, V.R.

Practical High-performance Liquid Chromatography, 5th Edition

2010

Print ISBN: 978-0-470-68217-3, also available in digital formats

Xu, Q. (ed.)

Ultra-High Performance Liquid Chromatography and Its Applications

2013

Print ISBN: 978-0-470-93842-3, also available in digital formats

Striegel, A., Yau, W.W., Kirkland, J.J., Bly, D.D.

Modern Size-Exclusion Liquid Chromatography, 2nd Edition Practice of Gel Permeation and GelFiltration Chromatography

2009

Print ISBN: 978-0-471-20172-4, also available in digital formats

Olsen, B.A., Pack, B.W. (eds.)

Hydrophilic Interaction Chromatography A Guide for Practitioners

2013

Print ISBN: 978-1-118-05417-8, also available in digital formats

Carta, G., Jungbauer, A

Protein Chromatography Process Development and Scale-Up

2010

Print ISBN: 978-3-527-31819-3, also available in digital formats

Schmidt-Traub, H., Schulte, M., Seidel-Morgenstern, A. (eds.)

Preparative Chromatography, 2nd Edition

2012

Print ISBN: 978-3-527-32898-7, also available in digital formats

Journal of Separation Science

www.jss-journal.com

Electrophoresis

www.electrophoresis-journal.com

Biotechnology Journal

www.biotechnology-journal.com

Elsa Lundanes, Léon Reubsaet and Tyge Greibrokk

Chromatography

Basic Principles, Sample Preparations and Related Methods

WILEY-VCH
Verlag GmbH & Co. KGaA

Authors

Elsa Lundanes

University of Oslo
Department of Chemistry
PO Box 1033 Blindern
0315 Oslo
Norway

Léon Reubsæet

University of Oslo
School of Pharmacy
PO Box 1068 Blindern
0316 Oslo
Norway

Tyge Greibrokk

University of Oslo
Department of Chemistry
PO Box 1033 Blindern
0315 Oslo
Norway

Limit of Liability/Disclaimer of Warranty: While the publisher and author have used their best efforts in preparing this book, they make no representations or warranties with respect to the accuracy or completeness of the contents of this book and specifically disclaim any implied warranties of merchantability or fitness for a particular purpose. No warranty can be created or extended by sales representatives or written sales materials. The Advice and strategies contained herein may not be suitable for your situation. You should consult with a professional where appropriate. Neither the publisher nor authors shall be liable for any loss of profit or any other commercial damages, including but not limited to special, incidental, consequential, or other damages

Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library.

Bibliographic information published by the Deutsche Nationalbibliothek

The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at <<http://dnb.d-nb.de>>.

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA,
Boschstr. 12, 69469 Weinheim, Germany

All rights reserved (including those of translation into other languages). No part of this book may be reproduced in any form – by photoprinting, microfilm, or any other means – nor transmitted or translated into a machine language without written permission from the publishers. Registered names, trademarks, etc. used in this book, even when not specifically marked as such, are not to be considered unprotected by law.

Print ISBN: 978-3-527-33620-3

ePDF ISBN: 978-3-527-67520-3

ePub ISBN: 978-3-527-67522-7

Mobi ISBN: 978-3-527-67521-0

Cover Design Grafik-Design Schulz, Germany

Typesetting Thomson Digital, Noida, India

Printing and Binding Markono Print Media Pte Ltd,
Singapore

Printed on acid-free paper

Contents

Preface *XIII*

1	General Concepts	1
1.1	Introduction	1
1.2	Migration and Retention	2
1.2.1	General	2
1.2.2	Mobile and Stationary Phases	3
1.2.3	Chromatograms	3
1.2.4	Retention Factor	3
1.3	Band Broadening	5
1.3.1	Eddy Diffusion	6
1.3.2	Longitudinal Diffusion	6
1.3.3	Resistance to Mass Transfer	7
1.3.4	Combined Band Broadening in a Column	8
1.3.5	Band Broadening outside the Column	9
1.4	Measuring Column Efficiency	9
1.4.1	Plate Numbers	9
1.4.2	Coupling Columns	10
1.4.3	Plate Height	10
1.4.3.1	Reduced Plate Height	11
1.4.4	Effective Plate Number	11
1.4.5	Asymmetry	11
1.5	Resolution	11
1.5.1	Increasing the Resolution	13
1.6	Peak Capacity	13
1.7	Two-Dimensional Systems	13
1.8	Increased Performance	14
	References	15
2	Gas Chromatography	17
2.1	Introduction	17
2.2	Mobile Phase/Carrier Gas	17
2.3	Injection Systems	19

2.3.1	Packed Column Injector (Evaporation Injector)	20
2.3.2	Injection Systems for Capillary Columns	21
2.3.2.1	Split Injection	21
2.3.2.2	Splitless Injection	22
2.3.2.3	On-Column Injection	22
2.3.2.4	Large-Volume Injectors	23
2.3.2.5	Headspace Techniques	23
2.4	Columns	24
2.4.1	Packed Columns	25
2.4.2	Open Tubular Columns	25
2.5	Detectors	26
2.5.1	Introduction	26
2.5.2	Thermal Conductivity Detector	28
2.5.3	Flame Ionization Detector	28
2.5.4	Nitrogen–Phosphorus Detector	30
2.5.5	Electron Capture Detector	31
2.5.6	Mass Spectrometry	32
2.5.6.1	Positive Ionization	33
2.5.6.2	Negative Ionization	33
2.5.6.3	Gas Chromatography–Mass Spectrometry (GC–MS) Interfacing	33
2.5.7	Other Detectors	35
2.5.7.1	The Flame Photometric Detector	35
2.5.7.2	The Chemiluminescent Detector	35
2.5.7.3	The Electrolytic Conductivity Detector	35
2.5.7.4	The Photoionization Detector	35
2.5.7.5	The Atomic Emission Detector	36
2.5.7.6	Fourier Transform Infrared Detector	36
2.6	Stationary Phases	36
2.6.1	GSC – Adsorption Chromatography	36
2.6.2	GLC – Partition Chromatography	37
2.6.2.1	Matrix	37
2.6.2.2	Choosing the Stationary Phase	37
2.6.2.3	Types of Stationary Phases in GLC	38
2.6.2.4	Stationary Phase (Film) Thickness	40
2.6.2.5	Temperature	41
2.7	Two-Dimensional Separations	42
2.8	Qualitative and Quantitative Analyses	43
2.9	Derivatization	44
	References	46

3	High-Performance Liquid Chromatography (HPLC)	47
3.1	Introduction	47
3.2	Solvents and Solvent Delivery	47
3.2.1	Maintenance	49
3.2.2	Automation	50

3.3	Injection	50
3.3.1	Techniques	50
3.3.1.1	Constant Volume Injection	50
3.3.1.2	Variable Volume Injection	51
3.3.1.3	Volumes and Precision	51
3.3.2	Dilution and Refocusing	51
3.3.2.1	Injection Volume Related to Solvent Elution Strength	51
3.3.2.2	Timed Injection	52
3.3.2.3	Carryover	52
3.3.2.4	Combination with Solid-Phase Extractors	52
3.3.3	Calculation of Maximum Injection Volumes	53
3.3.4	Calculating the Dilution of the Analyte in the Column	54
3.4	Columns	54
3.4.1	Packed Columns	54
3.4.1.1	Column Dimensions and Materials	54
3.4.1.2	Effect on Detection	55
3.4.1.3	Solvent Saving	55
3.4.1.4	Column Efficiency	56
3.4.1.5	Column Lifetime	57
3.4.1.6	Peak Shapes	57
3.4.1.7	Flow and Backpressure	58
3.4.1.8	Conventional Totally Porous Particles	58
3.4.1.9	Core–Shell Particles	58
3.4.1.10	Ultrahigh-Pressure LC (UHPLC or UPLC)	59
3.4.2	Monolithic Columns	59
3.4.3	Microchip Columns	60
3.4.4	Open Tubular Columns	61
3.4.5	Temperature Control	61
3.4.6	Preparative LC and Flash Chromatography	63
3.5	Stationary Phases and Their Properties in HPLC	64
3.5.1	Normal-Phase Materials for Adsorption Chromatography	64
3.5.1.1	Separation Principles	64
3.5.1.2	Silica	65
3.5.1.3	Alumina, Titania, and Zirconia	65
3.5.1.4	Silica with Bonded Polar Functional Groups	66
3.5.1.5	Hydrophilic Interaction Liquid Chromatography (HILIC)	67
3.5.1.6	Carbon Materials	68
3.5.2	Reversed-phase Materials	68
3.5.2.1	Separation Principles	68
3.5.2.2	Retention	69
3.5.2.3	The Solvation Parameter Model	70
3.5.2.4	Silica-based Reversed-phase Materials	71
3.5.2.5	Hybrid Materials and Hydrosilated Materials	72
3.5.2.6	Organic Polymer-based Materials	72
3.5.2.7	Ion Pair Chromatography on Reversed-Phase Columns	72

3.5.2.8	Hydrophobic Interaction Chromatography	73
3.5.3	Ion Exchange Materials	73
3.5.3.1	Elution	74
3.5.3.2	Retention	74
3.5.4	Chromatofocusing	74
3.5.4.1	Ion Chromatography for Inorganic Ions	75
3.5.5	Size Exclusion Materials	76
3.5.5.1	Separation Principles	76
3.5.5.2	Materials	76
3.5.5.3	Mobile Phases	77
3.5.6	Materials for Chiral Separations	77
3.5.6.1	Separation Principle	77
3.5.6.2	Materials	78
3.5.7	Affinity Materials	78
3.5.7.1	Separation Principle	78
3.5.7.2	Affinity Materials for Chromatography and Microarrays	79
3.6	Detectors	80
3.6.1	UV Detection	81
3.6.1.1	Some Common Chromophores	82
3.6.1.2	Choosing the Right Wavelength	82
3.6.1.3	Flow Cells	82
3.6.1.4	Filter Photometric Detection	83
3.6.1.5	Spectrophotometric Detection	83
3.6.1.6	Diode Array Detectors	83
3.6.2	Mass Spectrometric Detection	85
3.6.2.1	Electrospray Ionization	86
3.6.2.2	Atmospheric Pressure Chemical Ionization	88
3.6.2.3	Atmospheric Pressure Photoionization	89
3.6.2.4	Inductively Coupled Plasma Ionization	90
3.6.2.5	Mass Analysis	91
3.6.2.6	The Quadrupole Mass Analyzers	91
3.6.2.7	The Ion Trap Analyzers	92
3.6.2.8	The Time-of-Flight Analyzers	92
3.6.2.9	The FTMS Analyzers	93
3.6.2.10	Fragmentation in Mass Spectrometry	94
3.6.3	Fluorescence Detection	95
3.6.3.1	Filter Fluorimeters	97
3.6.3.2	Spectrofluorimeters	97
3.6.3.3	Chemiluminescence Detection	97
3.6.4	Electrochemical Detection	98
3.6.4.1	Amperometric Detection	98
3.6.4.2	Coulometric Detector	99
3.6.5	Light Scattering Detection	100
3.6.6	Refractive Index Detection	100
3.6.7	Other Detectors	102

3.6.7.1	The Conductivity Detector	102
3.6.7.2	The Corona Discharge Detector	102
3.6.7.3	Radioactivity Detectors	102
3.6.7.4	Ion Mobility Spectrometry	103
3.6.7.5	Chemiluminescent Nitrogen Detector	103
3.6.7.6	Chirality Detection	103
3.7	Increased Performance	103
3.7.1	Speed	103
3.7.2	Efficiency	103
3.7.3	Resolution	103
3.7.4	Detection	103
3.7.5	Column Lifetime	104
	References	104
4	Thin Layer Chromatography (TLC)	105
4.1	Introduction	105
4.2	Sample Application	105
4.3	Stationary Phases	106
4.3.1	TLC versus HPTLC	106
4.3.2	Adsorbents	107
4.3.3	Chemically Bonded Phases	107
4.4	Mobile Phases	107
4.5	Elution and Development	108
4.5.1	Vertical Linear Development	108
4.5.2	Horizontal Development	109
4.5.3	Two-Dimensional Development	110
4.5.4	Gradient Development	111
4.5.5	Overpressured Layer Chromatography (OPLC)	111
4.6	R _f Value	111
4.7	Detection	112
4.7.1	Instrumental Detection	113
4.7.2	TLC-MS	114
5	Supercritical Fluid Chromatography	115
5.1	Introduction	115
5.2	Mobile Phases	118
5.2.1	CO ₂ as Mobile Phase	118
5.2.2	Mobile Phase Delivery	119
5.3	Gradient Elution	120
5.4	Injection	121
5.5	Columns	122
5.6	Restrictors	124
5.7	Detectors	124
5.8	Current Performance	125
	References	126

6	Electrophoresis and Potential-Driven Chromatography	127
6.1	Introduction	127
6.2	Theory	127
6.2.1	Secondary Effects	128
6.2.2	Electroosmosis	129
6.3	Gel Electrophoresis Techniques	130
6.3.1	Gels	130
6.3.1.1	Polyacrylamide Gels	130
6.3.1.2	Agarose Gels	131
6.3.2	Instrumentation	131
6.3.2.1	Sample Application	131
6.3.2.2	Separation	132
6.3.2.3	Detection	132
6.3.3	Zone Electrophoresis	133
6.3.4	Isoelectric Focusing	134
6.3.5	Two-Dimensional Separations	134
6.3.6	Selected Applications	134
6.3.6.1	Protein Separations	134
6.3.6.2	Separation of DNA/RNA	135
6.4	Capillary Electrophoresis	135
6.4.1	Instrumentation	136
6.4.1.1	High-Voltage Supply	136
6.4.1.2	Capillaries	136
6.4.1.3	Sample Introduction	137
6.4.1.4	Detection	139
6.4.2	CE Zone Electrophoresis	140
6.4.3	Other CE Separation Principles	142
6.4.3.1	Isoelectric Focusing	142
6.4.3.2	Gel Electrophoresis in CE	142
6.4.3.3	Gel-Free Sieving	142
6.4.3.4	Isotachopheresis	143
6.4.4	Micellar Electrokinetic Capillary Chromatography (MEKC)	143
6.5	Potential-Driven Chromatography (Electrochromatography – CEC)	145
6.5.1	Instrumentation	145
6.5.2	Mobile Phases	145
6.5.3	Columns and Stationary Phases	146
6.5.4	CEC in Separation Science	146
	References	147
 7	 Chromatography on a Chip	 149
7.1	Introduction	149
7.2	Sample Introduction	149
7.3	Columns and Stationary Phases	151
7.3.1	Open Channel Columns	152

7.3.2	Packed Columns	152
7.3.3	Monolithic Columns	152
7.3.4	COMOSS	152
7.4	Flow Management	152
7.5	Detection	153
	Reference	154
8	Field-Flow Fractionation	155
8.1	Introduction	155
8.2	Types of FFF	156
8.2.1	Flow FFF	156
8.2.2	Thermal FFF	157
8.2.3	Sedimentation FFF	158
8.3	Applications	158
	Reference	159
9	Sample Preparation	161
9.1	Introduction	161
9.1.1	Recovery	162
9.1.2	Enrichment	162
9.2	Liquid-Liquid Extraction	164
9.2.1	Back Extraction	167
9.3	Solid-Phase Extraction (SPE)	168
9.3.1	Normal Phase	170
9.3.2	Reversed Phase	172
9.3.3	Ion Exchange	172
9.3.4	Mixed-Mode Ion Exchange	175
9.3.5	MIP	175
9.3.6	RAM	176
9.3.7	SPE Hardware	176
9.3.7.1	Disks	177
9.4	SPME	178
9.4.1	Adsorption/Extraction	178
9.4.2	Desorption/Injection	179
9.4.2.1	SPME-GC	180
9.4.2.2	SPME-HPLC	180
9.4.3	SPME Fiber Materials and Extraction Parameters	180
9.4.3.1	pH	181
9.4.3.2	Ionic Strength	181
9.4.3.3	Water and Organic Solvents	181
9.4.3.4	Temperature	181
9.4.3.5	Agitation	181
9.4.3.6	Extraction Time	182
9.5	Protein Precipitation	182
9.6	Membrane-Based Sample Preparation Techniques	183

9.6.1	Microdialysis	183
9.6.1.1	Perfusion Flow Rate	184
9.6.1.2	Diameter and Length	184
9.6.1.3	Cutoff	184
9.6.1.4	Membrane Chemistry	184
9.6.1.5	Application of Microdialysis	185
9.6.1.6	How to Analyze the Dialysate?	185
9.6.2	LPME	185
9.6.2.1	Two-Phase LPME	186
9.6.2.2	Three-Phase LPME	186
9.6.2.3	Enrichment in LPME	186
9.6.2.4	Donor Phase pH	187
9.6.2.5	Acceptor Phase pH	187
9.6.2.6	Composition of the SLM	187
9.6.2.7	Extraction Time	188
	References	188

10	Quantitation	189
10.1	Introduction	189
10.2	Calibration Methods	192
10.2.1	External Standard	192
10.2.2	Internal Standard	193
10.2.3	Standard Addition	194
10.3	Method Validation	196
10.3.1	Validation Parameters	196
10.3.1.1	Linearity and Range	197
10.3.1.2	Repeatability	197
10.3.1.3	Accuracy	197
10.3.1.4	Selectivity	197
10.3.1.5	Robustness	197
10.3.1.6	Stability	198
10.3.2	Validation Procedure: A Simple Example	198
	Reference	199

Index	201
--------------	-----

Preface

Although the basis of chromatography was developed a century ago, new separation methods still continue to appear. Today the technological developments allow identification and determination of compounds at levels not attainable a few years ago. Attomole concentrations of biomarkers can be determined, and for specific compounds even single cells can be analyzed.

This book aims to aid new users of chromatography, independent of background, in understanding the basics, and also can be used as a textbook for courses at the undergraduate and graduate levels.

The major chromatographic techniques have been included. However, the book does not intend to give a comprehensive overview of the historic developments in separation science, and some classical techniques that are not in use today have not been covered. An example is paper chromatography, which was replaced by the more efficient thin layer chromatography a long time ago. Another example is column liquid–liquid partition chromatography, which more or less disappeared after the introduction of chemically bonded phases in HPLC.

Electrophoresis, although basically not a chromatographic technique, is included due to its close relationship to chromatography and since some chromatographic techniques are hybrids of electrophoresis and chromatography. A chapter on field-flow fractionation has also been included, due to the chromatography-like properties and the increasing recent interest in the technique.

A chapter on sample preparation has been considered important, especially for newcomers to chromatography, since preparing the sample is often more time consuming than the analysis itself. In addition, choosing the right or wrong sample preparation may be decisive for the ability to find analytes at low concentration levels. There is some overlap in describing molecular interactions in Chapters 3 and 9, but this is done on purpose allowing the chapters to be read independent of each other.

Trying to look into the crystal bowl is a difficult task, but it is hard to see a reduced need for chromatography in a time where more and more emphasis is placed on determining trace amounts of both known and unknown compounds.

How important the concept of miniaturized systems like lab-on-a-chip will be for analytical chemistry in the future remains to be seen, but miniaturization is definitely a trend of our time.

Elsa Lundanes
Léon Reubsaet
Tyge Greibrokk

1

General Concepts

1.1

Introduction

The concept of separating sample components in a column was first developed in 1903 by Mikhail Tswett, who introduced the term chromatography in 1906. Unfortunately, his contemporaries showed little interest for the idea and almost 30 years went by before scientists in Germany rediscovered the principle of column liquid chromatography (LC). Then, in 1943 Arne Tiselius (in Sweden) classified chromatography into three modes: frontal, elution, and displacement. The elution mode actually became synonymous with almost all chromatography, but in recent years the displacement mode has attracted new interest, particularly in the separation of proteins.

In the years immediately prior to and during the Second World War, the principles of ion exchange chromatography (IEC) and liquid–liquid partition chromatography began to develop into crude technical solutions. Then after the war, in the early 1950s, the new technique of thin layer chromatography (TLC) came to light and gradually improved the partition principles used in paper chromatography. A. Martin and R.L.M. Synge (in the United Kingdom) received the Nobel Prize in 1952 for the invention of partition chromatography. Martin with James had also developed gas–liquid chromatography at this time. Gas chromatography (GC) was readily accepted by research chemists at the major oil companies, who understood the large potential of this technique and participated in developing the new instrumentation.

Size exclusion chromatography (SEC) was developed in Sweden by Porath and Flodin with dextrin materials (1959), by Hjertén with polyacrylamide (1961) and agarose (1964) materials, and by Moore in the United States with polystyrene–divinylbenzene (PS-DVB) materials (1964).

Supercritical fluid chromatography was demonstrated as early as 1962, but it did not receive much interest until the technology was improved more than 20 years later.

The introduction of open tubular columns into gas chromatography revolutionized GC, first with glass capillaries in the 1970s and then with fused silica columns in the 1980s. A similar revolution started with the gradual development of new

Table 1.1 Properties of chromatographic techniques.

Technique	Mobile phase	Driving force	Stationary phase
GC	Gas	Gas pressure/flow	Solids, liquid films
HPLC	Liquid	Pump flow	Solvated solids
SFC	Supercritical fluid	Pump flow	Solids, liquid films
TLC	Liquid	Capillary forces	Solids
EC	Liquid	Electric field	Solids
MEKC	Liquid	Electric field	Micelles

columns and instrumentation in liquid chromatography. With columns filled with small particles, the high-pressure liquid chromatography of the 1970s–1980s was later renamed high-performance liquid chromatography (HPLC).

Gel electrophoresis (GE) was developed in the 1940s, while capillary electrophoresis appeared 40 years later. Then chromatography with electric potential-driven liquid flow also developed into micellar electrokinetic chromatography (MEKC) and electrochromatography (EC), both with capillary columns. Electrophoresis, thus, is not a chromatographic technique, since there is no stationary phase, except in MEKC and EC.

To date, HPLC has become the dominating chromatographic technique, with capillary GC being second only to it (for the more volatile analytes). Both GC and HPLC are mature separation techniques today; however, HPLC is still being developed toward faster and more efficient separations and also partially toward miniaturized columns, particularly for applications in the life science area. The majority of the other techniques already mentioned are niche techniques today, but still important for a relatively smaller number of users compared to HPLC and GC. Electric potential-driven techniques have an added opportunity for new technology on microchips.

Some of the properties of the chromatographic techniques are shown in Table 1.1.

1.2

Migration and Retention

1.2.1

General

In a chromatographic system, the sample is introduced in a small volume at the inlet of a column or another carrier of the stationary phase. The mobile phase transports the sample components in contact with the stationary phase throughout the column.

Due to different interactions between the sample components and the stationary phase, the sample components migrate through the system at different speeds and elute from the column with different retention times.

The retention time is defined as the time between the sample introduction and the elution from the column.

At the end of the column, a detector provides a signal for all eluting components (universal detection) or for a limited number (selective detection).

In a sample with many components, some compounds will coelute, partly or completely, depending on the complexity of the sample and the peak capacity of the column.

With mass spectrometric detection, even coeluting components can be identified.

1.2.2

Mobile and Stationary Phases

The sample components (solutes) can interact directly with components of the mobile phase, except in gas chromatography where there are no such interactions and the mobile phase is simply a carrier gas for the sample components.

When the stationary phase is a solid, often with polar surface groups, and the mobile phase is either a gas (in GC) or an organic solvent (in LC), the separation principle is based on adsorption, and the term adsorption chromatography can be used. Other not so commonly used terms are gas–solid chromatography and liquid–solid chromatography. The adsorption forces include dispersion interactions, dipolar interactions, acid–base interactions, complexation, and so on.

In gas chromatography, the stationary phase can also be a liquid, where the separation principle is based on partition between the two phases. This was also the case formerly in liquid chromatography, but after the introduction of chemically bonded stationary phases into HPLC, the stationary phase cannot be described as a liquid anymore.

1.2.3

Chromatograms

When the sample components are separated and detected by a detector connected to the outlet of the column and the signals from the detector are visualized as a function of time, a chromatogram is obtained, as shown in Figure 1.1.

In a chromatogram, the elution time is found at the x -axis, while the y -axis constitutes the size of the detector signal.

Depending on the conditions, the separation of the sample components as well as the time of analysis can be adjusted, as shown in Figure 1.2.

With isocratic elution (constant composition of the mobile phase), the peak width will increase with increasing elution time. This cannot be seen clearly in Figure 1.2b as the elution mode is gradient elution (changing composition of the mobile phase).

1.2.4

Retention Factor

At any given time during the migration through the system, there is a distribution of molecules of each component between the two phases:

$$n_s/n_m,$$

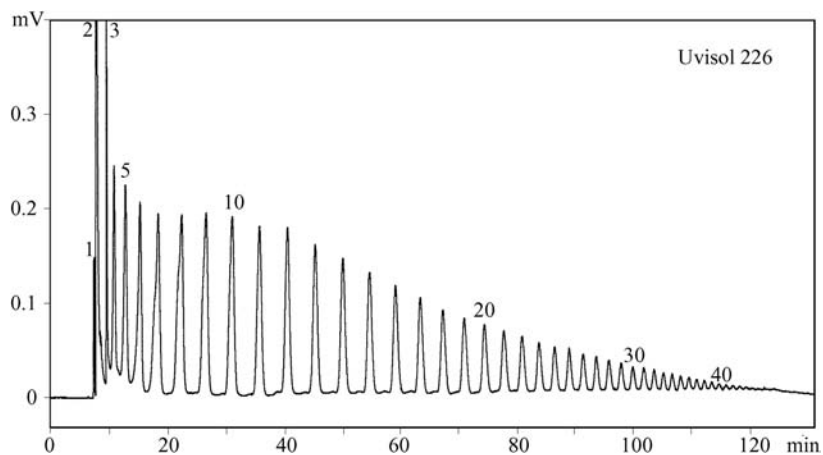


Figure 1.1 Chromatogram of polymeric amines separated by gradient elution in HPLC.

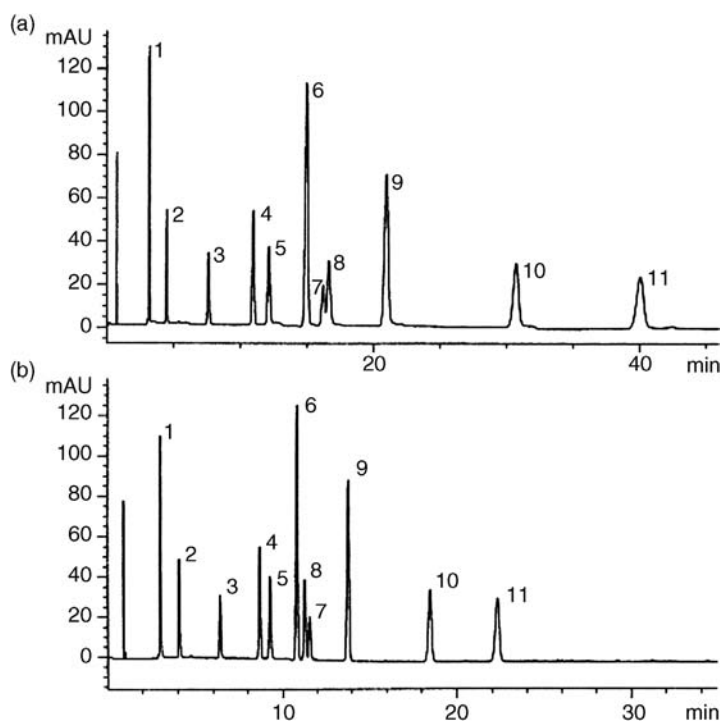


Figure 1.2 Reducing the time of analysis by gradient elution (b) compared to isocratic elution with constant mobile phase composition (a). (From Ref. [7], with permission.)

where n_s and n_m are the number of molecules in the stationary and mobile phases, respectively, at a given time. When n_s is much larger than n_m , the migration is very slow and the analyte elutes with high *retention*. In Figure 1.2, compound **11** has the highest retention:

$k = n_s/n_m$ is called the *retention factor*.

Info-box 1.1

k is the recommended symbol by IUPAC for describing the retention of a compound; it is independent of flow rate, column dimensions, and so on [1].

If one component migrates through the column in the mobile phase only, with no interactions with the stationary phase, the migration time is called t_M . An analyte with interactions with the stationary phase will be retained and will elute at t_R :

$$t_R = t_M + t_M k = t_M(1 + k).$$

The t_M can be determined by injecting a component known to have no interactions with the stationary phase.

From Equation 1.1, we can obtain a method for measuring k :

$$k = (t_R - t_M)/t_M. \quad (1.1)$$

Time units can also be replaced with volume units:

$$V_R = V_M(1 + k).$$

1.3

Band Broadening

A sample is injected in a limited volume at the column inlet. If there were no band broadening, the volume or the width of the band would be exactly the same at the point of detection. Unfortunately, this is not the case. In all chromatographic systems, there is band broadening (Figure 1.2), caused by different physical processes.

In the columns, the following processes can occur:

- Eddy diffusion
- Longitudinal diffusion in the mobile phase
- Resistance to mass transfer: in the mobile phase, stationary phase, and stagnant mobile phase

If the distribution of each band is assumed to be a Gaussian distribution, the extent of band broadening can be expressed by the column efficiency N :

$$N = (t_R/\sigma)^2,$$

where t_R is the retention time and σ^2 is the band variance in time units (σ is the standard deviation of the Gaussian distribution).

Another expression for the band broadening in a column with length L is the plate height H :

$$H = L/N,$$

where H is measured in micrometer.

Since H is a function of the variance, individual contributions to band broadening can be expressed as individual contributions to the plate height.

1.3.1

Eddy Diffusion

Eddy diffusion occurs due to the presence of multiple channels of different widths and lengths in porous structures. Large inhomogeneous particles cause large contributions to band broadening of eddy diffusion. In a packed column, the size of the eddy diffusion is proportional to the particle size. A wide range of particle size also increases the eddy diffusion.

The main contribution of eddy diffusion to the plate height is

$$H = C_e d_p,$$

where d_p is the particle diameter of one-size particles and C_e is a constant.

In open tubular columns, there is no eddy diffusion.

Info-box 1.2

In liquid chromatography, eddy diffusion is responsible for a major part of the band broadening in the column. Since eddy diffusion is a combination of diffusion and convection, the term eddy dispersion might be more correct than eddy diffusion. Contributions to eddy dispersion come from column internal diameter, column length, and column packing efficiency besides particle size and homogeneity [2].

1.3.2

Longitudinal Diffusion

Longitudinal diffusion in the mobile phase is due to the natural tendency of a compound in a concentrated band to diffuse into less concentrated zones. The contribution of longitudinal band broadening is proportional to the diffusion constant. Since the diffusion velocity in gases is about 10^4 times higher than the diffusion in liquids, this contribution to band broadening is much more important in GC than in HPLC.