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# MODERN HPLC FOR PRACTICING SCIENTISTS

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Michael W. Dong

Synomics Pharmaceutical Services, LLC  
Wareham, Massachusetts



A JOHN WILEY & SONS, INC., PUBLICATION



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# CONTENTS

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<b>Preface</b>	<b>xv</b>
<b>1 Introduction</b>	<b>1</b>
1.1 Introduction / 1	
1.1.1 Scope / 1	
1.1.2 What Is HPLC? / 2	
1.1.3 A Brief History / 3	
1.1.4 Advantages and Limitations / 4	
1.2 Modes of HPLC / 5	
1.2.1 Normal-Phase Chromatography (NPC) / 5	
1.2.2 Reversed-Phase Chromatography (RPC) / 7	
1.2.3 Ion-Exchange Chromatography (IEC) / 7	
1.2.4 Size-Exclusion Chromatography (SEC) / 9	
1.2.5 Other Separation Modes / 10	
1.3 Some Common-Sense Corollaries / 11	
1.4 How to Get More Information / 12	
1.5 Summary / 13	
1.6 References / 13	
<b>2 Basic Terms and Concepts</b>	<b>15</b>
2.1 Scope / 16	
2.2 Basic Terms and Concepts / 17	
2.2.1 Retention Time ( $t_R$ ), Void Time ( $t_M$ ), Peak Height (h), and Peak Width ( $w_b$ ) / 17	
2.2.2 Retention Volume ( $V_R$ ), Void Volume ( $V_M$ ), and Peak Volume / 18	
2.2.3 Retention Factor (k) / 19	
2.2.4 Separation Factor ( $\alpha$ ) / 20	
2.2.5 Column Efficiency and Plate Number (N) / 21	
2.2.6 Peak Volume / 22	
2.2.7 Height Equivalent to a Theoretical Plate or Plate Height (HETP or H) / 23	

- 2.2.8 Resolution ( $R_s$ ) / 23
- 2.2.9 Peak Symmetry: Asymmetry Factor ( $A_s$ ) and Tailing Factor ( $T_f$ ) / 24
- 2.3 Mobile Phase / 27
  - 2.3.1 General Requirements / 27
  - 2.3.2 Solvent Strength and Selectivity / 28
  - 2.3.3 Buffers / 31
  - 2.3.4 Acidic Mobile Phases / 32
  - 2.3.5 Ion-Pairing Additives / 32
  - 2.3.6 High pH Mobile Phase / 33
  - 2.3.7 Other Operating Parameters: Flow Rate (F) and Column Temperature (T) / 33
- 2.4 The Resolution Equation / 34
- 2.5 The Van Deemter Equation / 35
- 2.6 Isocratic vs. Gradient Analysis / 39
  - 2.6.1 Peak Capacity (n) / 40
  - 2.6.2 Key Gradient Parameters (Initial and Final Solvent Strength, Gradient Time [ $t_G$ ], and Flow Rate) / 41
  - 2.6.3 The  $0.25\Delta t_G$  Rule: When Is Isocratic Analysis More Appropriate? / 42
- 2.7 Concept of Orthogonality / 42
- 2.8 Sample Capacity / 44
- 2.9 Glossary of HPLC Terms / 44
- 2.10 Summary and Conclusion / 45
- 2.11 References / 46

### 3 HPLC Columns and Trends

47

- 3.1 Scope / 48
- 3.2 General Column Description and Characteristics / 48
  - 3.2.1 Column Hardware—Standard vs. Cartridge Format / 49
- 3.3 Column Types / 50
  - 3.3.1 Types Based on Chromatographic Modes / 50
  - 3.3.2 Types Based on Dimensions / 51
  - 3.3.3 Column Length (L) / 51
- 3.4 Column Packing Characteristics / 52
  - 3.4.1 Support Type / 53
  - 3.4.2 Particle Size ( $d_p$ ) / 54
  - 3.4.3 Surface Area and Pore Size ( $d_{\text{pore}}$ ) / 54
  - 3.4.4 Bonding Chemistries / 54
  - 3.4.5 Some General Guidelines for Bonded Phase Selection / 56
- 3.5 Modern HPLC Column Trends / 57
  - 3.5.1 High-Purity Silica / 58



- 3.5.2 Hybrid Particles / 58
- 3.5.3 Novel Bonding Chemistries / 59
- 3.5.4 Fast LC / 64
- 3.5.5 Micro LC / 66
- 3.5.6 Monoliths / 68
- 3.6 Guard Columns / 69
- 3.7 Specialty Columns / 70
  - 3.7.1 Bioseparation Columns / 70
  - 3.7.2 Chiral Columns / 70
  - 3.7.3 Application-Specific Columns / 70
- 3.8 Column Selection Guides / 70
- 3.9 Summary / 73
- 3.10 References / 74
- 3.11 Internet Resources / 75

## **4 HPLC Instrumentation and Trends**

**77**

- 4.1 Introduction / 78
  - 4.1.1 Scope / 78
  - 4.1.2 HPLC Systems and Modules / 79
- 4.2 HPLC Solvent Delivery Systems / 81
  - 4.2.1 High-Pressure and Low-Pressure Mixing Designs in Multisolute Pumps / 82
  - 4.2.2 System Dwell Volume / 83
  - 4.2.3 Trends / 84
- 4.3 Injectors and Autosamplers / 84
  - 4.3.1 Operating Principles of Autosamplers / 85
  - 4.3.2 Performance Characteristics and Trends / 86
- 4.4 Detectors / 87
- 4.5 UV/VIS Absorbance Detectors / 87
  - 4.5.1 Operating Principles / 87
  - 4.5.2 Performance Characteristics / 88
  - 4.5.3 Trends in Absorbance Detectors / 89
- 4.6 Photodiode Array Detectors / 91
  - 4.6.1 Operating Principles / 91
  - 4.6.2 Trends in PDA Detectors / 93
- 4.7 Other Detectors / 93
  - 4.7.1 Fluorescence Detector (FLD) / 93
  - 4.7.2 Refractive Index Detector (RID) / 94
  - 4.7.3 Evaporative Light Scattering Detector (ELSD) / 94
  - 4.7.4 Corona-Charged Aerosol Detector (CAD) / 94
  - 4.7.5 Chemiluminescence Nitrogen Detector (CLND) / 95
  - 4.7.6 Electrochemical Detector (ECD) / 95

- 4.7.7 Conductivity Detector / 95
- 4.7.8 Radiometric Detector / 95
- 4.8 Hyphenated and Specialized Systems / 96
  - 4.8.1 LC/MS, LC/MS/MS / 96
  - 4.8.2 LC/NMR / 97
  - 4.8.3 Other Hyphenated Systems / 97
  - 4.8.4 Prep LC and Bio-Purification Systems / 97
  - 4.8.5 Proteomics Systems: Capillary LC and Multi-Dimensional LC / 98
  - 4.8.6 High-Throughput Screening (HTS) and Parallel Analysis Systems / 99
  - 4.8.7 Ultra-High-Pressure Liquid Chromatography / 101
  - 4.8.8 Lab-on-a-Chip / 101
  - 4.8.9 Specialized Applications Systems / 101
- 4.9 HPLC Accessories and Data Handling Systems / 102
  - 4.9.1 Solvent Degasser / 102
  - 4.9.2 Column Oven / 102
  - 4.9.3 Column Selector Valve / 103
  - 4.9.4 Data Handling and HPLC Controllers / 103
- 4.10 Instrumental Bandwidth (IBW) / 104
- 4.11 Trends in HPLC Equipment / 107
- 4.12 Manufacturers and Equipment Selection / 108
- 4.13 Summary / 109
- 4.14 References / 109
- 4.15 Internet Resources / 110

## 5 HPLC Operation Guide

111

- 5.1 Scope / 112
- 5.2 Safety and Environmental Concerns / 112
  - 5.2.1 Safety Concerns / 112
  - 5.2.2 Environmental Concerns / 114
- 5.3 Mobile Phase Preparation / 114
  - 5.3.1 Mobile Phase Premixing / 114
  - 5.3.2 Buffers / 114
  - 5.3.3 Filtration / 115
  - 5.3.4 Degassing / 116
- 5.4 Best Practices in HPLC System Operation / 116
  - 5.4.1 Pump Operation / 117
  - 5.4.2 HPLC Column Use, Connection, and Maintenance / 117
    - 5.4.2.1 Column Use / 117
    - 5.4.2.2 Column Precautions / 118

- 5.4.2.3 Column Connection / 118
- 5.4.2.4 Column Maintenance and Regeneration / 118
- 5.4.3 Autosampler Operation / 120
- 5.4.4 Detector Operation / 120
- 5.4.5 System Shutdown / 121
- 5.4.6 Guidelines for Increasing HPLC Precision / 122
  - 5.4.6.1 Guidelines for Improving Retention Time Precision / 122
  - 5.4.6.2 Guidelines for Improving Peak Area Precision / 122
- 5.5 From Chromatograms to Reports / 123
  - 5.5.1 Qualitative Analysis Strategies / 128
  - 5.5.2 Quantitation Analysis Strategies / 128
- 5.6 Summary of HPLC Operation / 129
- 5.7 Guides on Performing Trace Analysis / 129
- 5.8 Summary / 132
- 5.9 References / 132

## **6 Pharmaceutical Analysis**

**135**

- 6.1 Introduction / 136
  - 6.1.1 Scope / 136
  - 6.1.2 Overview: From Drug Discovery to Quality Control / 136
  - 6.1.3 Sample Preparation Perspectives in Drug Product Analysis / 137
  - 6.1.4 High-Throughput LC/MS in Drug Discovery Support / 137
- 6.2 Identification / 138
- 6.3 Assays / 139
  - 6.3.1 Drug Substances / 140
  - 6.3.2 Drug Products / 140
  - 6.3.3 Content Uniformity / 142
  - 6.3.4 Products with Multiple APIs and Natural Products / 142
  - 6.3.5 Assay of Preservatives / 145
- 6.4 Impurity Testing / 145
  - 6.4.1 Trends in Impurity Testing / 148
- 6.5 Dissolution Testing / 148
- 6.6 Cleaning Validation / 148
- 6.7 Bioanalytical Testing / 150
- 6.8 Chiral Analysis / 151
- 6.9 Case Study: HPLC Methods in Early Development / 152
- 6.10 Summary / 153
- 6.11 References / 155

<b>7</b>	<b>Food, Environmental, Chemical, and Life Sciences Applications</b>	<b>157</b>
7.1	Introduction / 158	
7.1.1	Scope / 158	
7.2	Food Applications / 158	
7.2.1	Natural Food Components / 159	
7.2.1.1	Sugars / 159	
7.2.1.2	Fats, Oils, and Triglycerides / 160	
7.2.1.3	Free Fatty Acids and Organic Acids / 162	
7.2.1.4	Proteins and Amino Acids / 162	
7.2.2	Food Additives / 164	
7.2.2.1	Flavors: A Case Study on HPLC Analysis of Capsaicins / 166	
7.2.3	Contaminants / 167	
7.2.3.1	Mycotoxins / 168	
7.2.3.2	Antimicrobial Additives / 168	
7.2.3.3	Pesticide Residues / 169	
7.3	Environmental Applications / 169	
7.3.1	Listing of Important U.S. EPA HPLC Methods / 170	
7.3.2	Pesticides Analysis / 170	
7.3.2.1	Carbamates and Glyphosate / 170	
7.3.3	Polynuclear Aromatic Hydrocarbons (PAH) / 172	
7.3.3.1	Case Study: Quick Turnaround Analysis of PAHs by HPLC in Multimedia Samples / 172	
7.4	Chemical, GPC and Plastics Applications / 173	
7.4.1	Gel-Permeation Chromatography (GPC) and Analysis of Plastics Additives / 175	
7.5	Ion Chromatography / 178	
7.6	Life Sciences Applications / 179	
7.6.1	Proteins, Peptides, and Amino Acids / 179	
7.6.2	Bases, Nucleosides, Nucleotides, Oligonucleotides, Nucleic Acids, and PCR Products / 186	
7.7	Summary / 188	
7.8	References / 190	
<b>8</b>	<b>HPLC Method Development</b>	<b>193</b>
8.1	Introduction / 194	
8.1.1	Scope / 194	
8.1.2	Considerations Before Method Development / 194	
8.1.3	Strategy for Method Development / 195	

- 8.1.4 HPLC Method Development Trends in Pharmaceutical Analysis / 195
- 8.2 Defining Method Types and Goals / 196
  - 8.2.1 Method Goals / 196
- 8.3 Gathering Sample and Analyte Information / 197
  - 8.3.1 Defining Sample Preparation Requirements / 197
- 8.4 Initial HPLC Method Development / 198
  - 8.4.1 Initial Detector Selection / 198
  - 8.4.2 Selection of Chromatographic Mode / 199
  - 8.4.3 Initial Selection of HPLC Column / 199
  - 8.4.4 Generating a First Chromatogram / 200
    - 8.4.4.1 Case Study: Initial Method Development Using a Broad Gradient and Mobile Phase Selection / 200
- 8.5 Method Fine-Tuning / 204
  - 8.5.1 Mobile Phase Parameters (%B, Buffer, pH, Solvent Type) / 205
  - 8.5.2 Operating Parameters (F, T,  $\Delta\phi$ ,  $t_G$ ) / 206
  - 8.5.3 Column Parameters (Bonded Phase Type, L,  $d_p$ ,  $d_c$ ) / 206
  - 8.5.4 Detector Setting and Sample Amount / 206
  - 8.5.5 Summary of Method Development Steps / 207
- 8.6 Phase-Appropriate Method Development / 208
- 8.7 Method Development Software Tools / 210
- 8.8 Case Studies / 210
  - 8.8.1 Composite Assay Method for a Neutral Drug Substance / 210
  - 8.8.2 Composite Drug Substance Method for a Basic Drug Substance / 214
  - 8.8.3 Impurity Method for a Drug Product with Two APIs / 215
- 8.9 Summary and Conclusions / 217
- 8.10 References / 220

## **9 Regulatory Aspects of HPLC Analysis: HPLC System and Method Validation**

221

- 9.1 Introduction / 222
  - 9.1.1 Scope / 222
  - 9.1.2 The Regulatory Environment / 222
- 9.2 HPLC System Qualification / 224
  - 9.2.1 Design Qualification (DQ) / 225
  - 9.2.2 Installation Qualification (IQ) / 225
  - 9.2.3 Operational Qualification (OQ) / 225

- 9.2.4 Performance Qualification (PQ) / 225
- 9.2.5 Documentation / 226
- 9.2.6 System Calibration / 227
- 9.3 Method Validation / 227
  - 9.3.1 Validation Parameters / 230
- 9.4 System Suitability Testing (SST) / 235
- 9.5 Case Study on Method Validation / 237
- 9.6 Cost-Effective Regulatory Compliance / 239
- 9.7 Summary and Conclusions / 240
- 9.8 References / 240
- 9.9 Internet Resources / 241

**10 HPLC Maintenance and Troubleshooting Guide 243**

- 10.1 Scope / 244
- 10.2 HPLC System Maintenance / 244
  - 10.2.1 LC Pump / 244
  - 10.2.2 UV/Vis Detectors / 247
  - 10.2.3 Injector and Autosampler / 248
- 10.3 HPLC Troubleshooting / 248
  - 10.3.1 General Problem Diagnostic and Troubleshooting Guide / 248
  - 10.3.2 Common HPLC Problems / 250
    - 10.3.2.1 Pressure Problems and Causes / 250
    - 10.3.2.2 Baseline Problems (Chromatogram) / 251
    - 10.3.2.3 Peak Problems (Chromatogram) / 253
    - 10.3.2.4 Data Performance Problems / 256
- 10.4 Case Studies / 257
  - 10.4.1 Case Study 1: Reducing Baseline Shift and Noise for Gradient Analysis / 257
  - 10.4.2 Case Study 2: Poor Peak Area Precision Encountered During HPLC System Calibration / 258
  - 10.4.3 Case Study 3: Poor Assay Accuracy Data, an Out-of-Specification Investigation / 260
  - 10.4.4 Case Study 4: Equipment Malfunctioning / 261
- 10.5 Summary and Conclusion / 263
- 10.6 References / 263
- 10.7 Internet Resources / 263

**11 Modern Trends in HPLC 265**

- 11.1 Columns: Shorter and Narrower Packed with Small Particles / 266

11.2	Column Packing: Novel Bonded Phases /	266
11.3	Pumps /	266
11.4	Autosamplers /	267
11.5	Detectors /	267
11.6	HPLC Systems /	268
	11.6.1 Low-Dispersion Instruments /	268
	11.6.2 Ultra-High-Pressure LC /	268
	11.6.3 Multi-Dimensional LC /	268
	11.6.4 Parallel Analysis /	269
11.7	Lab-on-a-Chip /	269
11.8	Data Handling /	269
11.9	Regulatory Compliance /	269
11.10	Greener HPLC Methods /	270
11.11	Summary and Conclusions /	270
11.12	References /	271





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# PREFACE

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The idea for writing this basic HPLC book was probably born in the New York City subway system while I was a graduate student in the 1970s. Amidst the rumbling noise of the subway, I was reading “the green book”—*Basic Gas Chromatography* by McNair and Bonelli—and was immediately impressed with its simplicity and clarity. In the summer of 2004, I had just completed the editing of *Handbook of Pharmaceutical Analysis by HPLC* with Elsevier/Academic Press, and was toying with the idea of starting a book project on Fast LC and high-throughput screening. Several phone conversations with Heather Bergman, my editor at Wiley, convinced me that an updated book on modern HPLC, modeled after “the green book,” would have more of an impact.

This book was written with a sense of urgency during weekends and weekday evenings . . . through snow storms, plane trips, allergy seasons, company restructuring, and job changes. The first draft was ready in only 10 months because I was able to draw many examples from my previous publications and from my short course materials for advanced HPLC in pharmaceutical analysis given at national meetings. I am not a fast writer, but rather a methodical one who revised each chapter many times before seeking review advice from my friends and colleagues. My goal was to provide the reader with an updated view of the concepts and practices of modern HPLC, illustrated with many figures and case studies. My intended audience was the practicing scientist—to provide them with a review of the basics as well as best practices, applications, and trends of this fast-evolving technique. Note that this basic book for practitioners was written at both an introductory and intermediate level. I am also targeting the pharmaceutical analysts who constitute a significant fraction of all HPLC users. My focus was biased towards reversed-phase LC and pharmaceutical analysis. The scope of this book does not allow anything more than a cursory mention of the other applications.

Writing a book as a sole author was a labor of love, punctuated with flashes of inspiration and moments of despair. It would have been a lonely journey without the encouragement and support of my colleagues and friends. First and foremost, I would like to acknowledge the professionalism of my editor at John Wiley, Heather Bergman, whose enthusiasm and support made this a

happy project. I also owe much to my reviewers, including the 10 reviewers of the book proposal, and particularly to those whose patience I tested by asking them to preview multiple chapters. They have given me many insights and valuable advice. The list of reviewers is long:

Prof. David Locke of City University of New York (my graduate advisor); Prof. Harold McNair of Virginia Tech, whose “green book” provided me with a model; Prof. Jim Stuart of University of Connecticut; Drs. Lloyd Snyder and John Dolan of LC Resources; Drs. Raphael Ornaf, Cathy Davidson, and Danlin Wu, and Joe Grills, Leon Zhou, Sung Ha, and Larry Wilson of Purdue Pharma; Dr. Ron Kong of Synaptic; Drs. Uwe Neue, Diane Diehl, and Michael Swartz of Waters Corporation, Wilhad Reuter of PerkinElmer; Drs. Bill Barbers and Thomas Waeghe of Agilent Technologies; Drs. Krishna Kallary and Michael McGinley of Phenomenex; Dr. Tim Wehr of BioRad; John Martin and Bill Campbell of Supelco; Dr. Andy Alpert of PolyLC; Margie Dix of Springborn-Smithers Laboratories; Dr. Linda Ng of FDA, CDER; and Ursula Caterbone of MacMod.

Finally, I acknowledge the support and the unfailing patience of my wife, Cynthia, and my daughter, Melissa, for putting up with my long periods of distraction when I struggled for better ways for putting ideas on paper. To them, I pledge more quality time to come after 2006.

*Norwalk, Connecticut*

MICHAEL W. DONG

“[...] a concise and ‘to-the-point’ text, covering the broad topic of HPLC. While the book is not intended to be a comprehensive treatise, it addresses all major topics in HPLC and provides updated, practical information not found in other introductory texts. I found the author’s use of bullet points, tabulation and figures extremely effective in conveying practical ‘take-home’ messages and providing sound and definitive guidance.”

—Henrik T. Rasmussen, PhD

“This is a great introductory book on liquid chromatography. I especially like the intuitive explanations and clear figures.”

—John W. Dolan, PhD

“Dong’s book is especially written for the pharmaceutical industry. I am sure that it will be highly welcomed by the practitioners in this field, not only because of its numerous relevant examples of drug separations but also because of its focus on regulatory aspects.”

—Veronika Meyer, PhD



## INTRODUCTION

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1.1	Introduction	1
1.1.1	Scope	1
1.1.2	What Is HPLC?	2
1.1.3	A Brief History	3
1.1.4	Advantages and Limitations	4
1.2	Modes of HPLC	5
1.2.1	Normal-Phase Chromatography (NPC)	5
1.2.2	Reversed-Phase Chromatography (RPC)	7
1.2.3	Ion-Exchange Chromatography (IEC)	7
1.2.4	Size-Exclusion Chromatography (SEC)	9
1.2.5	Other Separation Modes	10
1.3	Some Common-Sense Corollaries	11
1.4	How To Get More Information	12
1.5	Summary	13
1.6	References	13

### 1.1 INTRODUCTION

#### 1.1.1 Scope

High-performance liquid chromatography (HPLC) is a versatile analytical technology widely used for the analysis of pharmaceuticals, biomolecules,

polymers, and many organic and ionic compounds. There is no shortage of excellent books on chromatography<sup>1,2</sup> and on HPLC,<sup>3-9</sup> though many are outdated and others cover academic theories or specialized topics. This book strives to be a concise text that capsulizes the essence of HPLC fundamentals, applications, and developments. It describes basic theories and terminologies for the novice and reviews relevant concepts, best practices, and modern trends for the experienced practitioner. While broad in scope, this book focuses on reversed-phase HPLC (the most common separation mode) and pharmaceutical applications (the largest user segment). Information is presented in a straightforward manner and illustrated with an abundance of diagrams, chromatograms, tables, and case studies and supported with selected key references or web resources.

Most importantly, this book was written as an updated reference guide for busy laboratory analysts and researchers. Topics covered include HPLC operation, method development, maintenance/troubleshooting, and regulatory aspects. This book can serve as a supplementary text for students pursuing a career in analytical chemistry. A reader with a science degree and a basic understanding of chemistry is assumed.

This book offers the following benefits:

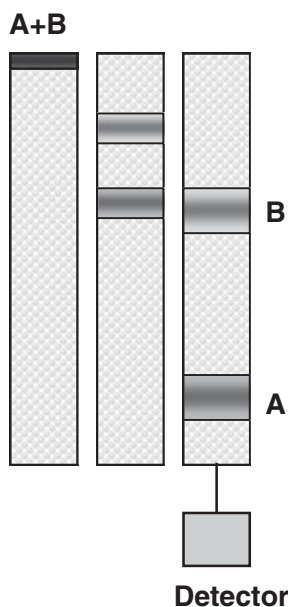
- A broad-scope overview of basic principles, instrumentation, and applications.
- A concise review of concepts and trends relevant to modern practice.
- A summary update of best practices in HPLC operation, method development, maintenance, troubleshooting, and regulatory compliance.
- A summary review of modern trends in HPLC, including quick-turnaround and “greener” methods.

### 1.1.2 What Is HPLC?

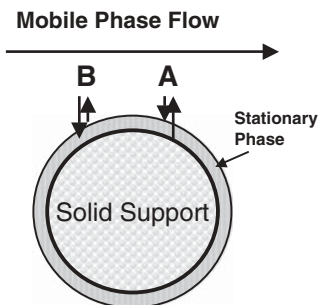
Liquid chromatography (LC) is a physical separation technique conducted in the liquid phase. A sample is separated into its constituent components (or analytes) by distributing between the mobile phase (a flowing liquid) and a stationary phase (sorbents packed inside a column). For example, the flowing liquid can be an organic solvent such as hexane and the stationary phase can be porous silica particles packed in a column. HPLC is a modern form of LC that uses small-particle columns through which the mobile phase is pumped at high pressure.

Figure 1.1a is a schematic of the chromatographic process, where a mixture of analytes A and B are separated into two distinct bands as they migrate down the column filled with packing (stationary phase). Figure 1.1b is a representation of the dynamic partitioning process of the analytes between the flowing liquid and a spherical packing particle. Note that the movement of component B is retarded in the column because each B molecule has stronger affinity for

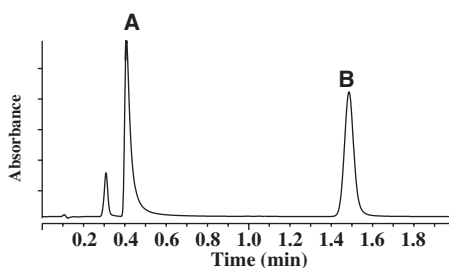
### 1a. Chromatography Process



### 1b. Partitioning



### 1c. The Chromatogram

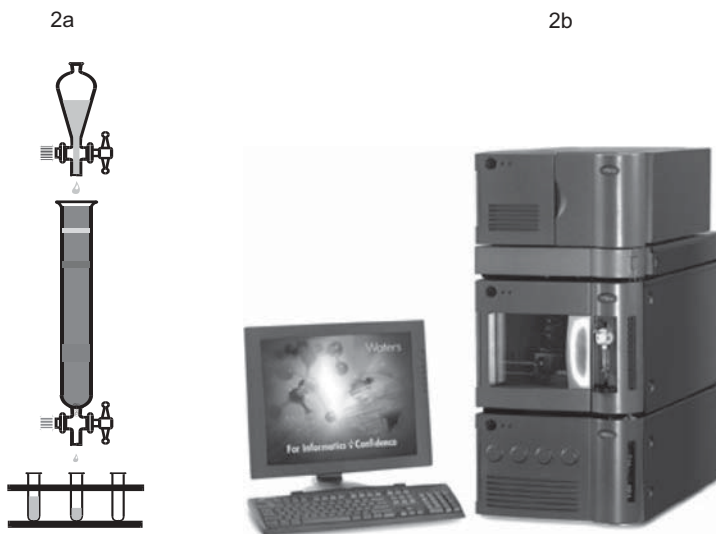


**Figure 1.1.** (a) Schematic of the chromatographic process showing the migration of two bands of components down a column. (b) Microscopic representation of the partitioning process of analyte molecules A and B into the stationary phase bonded to a spherical solid support. (c) A chromatogram plotting the signal from a UV detector displays the elution of components A and B.

the stationary phase than the A molecule. An in-line detector monitors the concentration of each separated component band in the effluent and generates a trace called the “chromatogram,” shown in Figure 1.1c.

#### 1.1.3 A Brief History

Classical LC, the term *chromatography* meaning “color writing,” was first discovered by Mikhail Tswett, a Russian botanist who separated plant pigments on chalk ( $\text{CaCO}_3$ ) packed in glass columns in 1903.<sup>10</sup> Since the 1930s, chemists used gravity-fed silica columns to purify organic materials and ion-exchange resin columns to separate ionic compounds and radionuclides. The invention of gas chromatography (GC) by British chemists A.J.P. Martin and co-workers in 1952, and its successful applications, provided both the theoretical foundation and the incentive for the development of LC. In the late 1960s, LC turned “high performance” with the use of small-particle columns that required high-pressure pumps. The first generation of high-performance liquid chromatographs was developed by researchers in the 1960s, including Horvath,



**Figure 1.2.** (a) The traditional technique of low-pressure liquid chromatography using a glass column and gravity-fed solvent with manual fraction collection. (b) A modern automated HPLC instrument (Waters Acquity UPLC system) capable of very high efficiency and pressure up to 15,000 psi.

Kirkland, and Huber. Commercial development of in-line detectors and reliable injectors allowed HPLC to become a sensitive and quantitative technique leading to an explosive growth of applications.<sup>10</sup> In the 1980s, the versatility and precision of HPLC rendered it virtually indispensable in pharmaceuticals as well as other diverse industries. The annual worldwide sales of HPLC systems and accessories approached three billion US\$ in 2002.<sup>11</sup> Today, HPLC continues to evolve rapidly toward higher speed, efficiency, and sensitivity, driven by the emerging needs of life sciences and pharmaceutical applications. Figure 1.2a depicts the classical technique of LC with a glass column that is packed with coarse adsorbents and gravity fed with solvents. Fractions of the eluent containing separated components are collected manually. This is contrasted with the latest computer-controlled HPLC, depicted in Figure 1.2b, operated at high pressure and capable of very high efficiency.

#### 1.1.4 Advantages and Limitations

Table 1.1 highlights the advantages and limitations of HPLC. HPLC is a premier separation technique capable of multicomponent analysis of real-life samples and complex mixtures. Few techniques can match its versatility and precision of <0.5% relative standard deviation (RSD). HPLC is highly automated, using sophisticated autosamplers and data systems for unattended analysis and report generation. A host of highly sensitive and specific detec-



**Table 1.1. Advantages and Limitations of HPLC**

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**Advantages**

- Rapid and precise quantitative analysis
- Automated operation
- High-sensitivity detection
- Quantitative sample recovery
- Amenable to diverse samples

**Limitations**

- No universal detector
  - Less separation efficiency than capillary GC
  - More difficult for novices
- 

tors extend detection limits to nanogram, picogram, and even femtogram levels. As a preparative technique, it provides quantitative recovery of many labile components in milligram to kilogram quantities. Most importantly, HPLC is amenable to 60% to 80% of all existing compounds, as compared with about 15% for GC.<sup>3,4</sup>

HPLC suffers from several well-known disadvantages or perceived limitations. First, there is no universal detector, such as the equivalence of flame ionization detector in GC, so detection is more problematic if the analyte does not absorb UV rays or cannot be easily ionized for mass spectrometric detection. Second, separation efficiency is substantially less than that of capillary GC, thus, the analysis of complex mixtures is more difficult. Finally, HPLC has many operating parameters and is more difficult for a novice. As shown in later chapters, these limitations have been largely minimized through instrumental and column developments.

## 1.2 MODES OF HPLC

In this section, the four major separation modes of HPLC are introduced and illustrated with application examples, each labeled with the pertinent parameters: column (stationary phase), mobile phase, flow rate, detector, and sample information. These terminologies will be elaborated later.

### 1.2.1 Normal-Phase Chromatography (NPC)

Also known as liquid-solid chromatography or adsorption chromatography, NPC is the traditional separation mode based on adsorption/desorption of the analyte onto a polar stationary phase (typically silica or alumina).<sup>3-5</sup> Figure 1.3a shows a schematic diagram of part of a porous silica particle with silanol groups (Si-OH) residing at the surface and inside its pores. Polar analytes migrate slowly through the column due to strong interactions with the silanol groups. Figure 1.4 shows a chromatogram of four vitamin E isomers in a palm



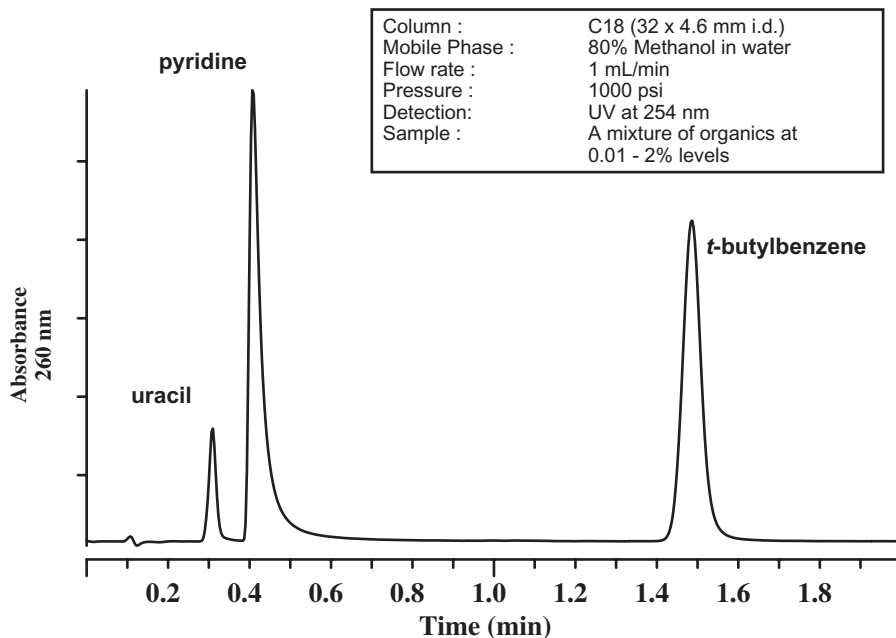
olein sample using a nonpolar mobile phase of hexane modified with a small amount of ethanol. It is believed that a surface layer of water reduces the activity of the silanol groups and yields more symmetrical peaks.<sup>3</sup> NPC is particularly useful for the separation of nonpolar compounds and isomers, as well as for the fractionation of complex samples by functional groups or for sample clean-up. One major disadvantage of this mode is the easy contamination of the polar surfaces by sample components. This problem is partly reduced by bonding polar functional groups such as amino- or cyano-moiety to the silanol groups.

### 1.2.2 Reversed-Phase Chromatography (RPC)

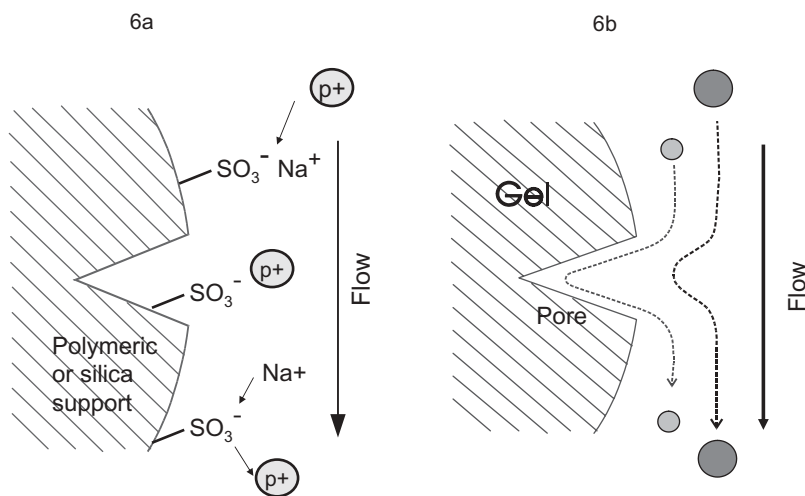
The separation is based on analytes' partition coefficients between a polar mobile phase and a hydrophobic (nonpolar) stationary phase. The earliest stationary phases were solid particles coated with nonpolar liquids. These were quickly replaced by more permanently bonding hydrophobic groups, such as octadecyl (C18) bonded groups, on silica support. A simplified view of RPC is shown in Figure 1.3b, where polar analytes elute first while nonpolar analytes interact more strongly with the hydrophobic C18 groups that form a "liquid-like" layer around the solid silica support. This elution order of "polar first and nonpolar last" is the reverse of that observed in NPC, and thus the term "reversed-phase chromatography." RPC typically uses a polar mobile phase such as a mixture of methanol or acetonitrile with water. The mechanism of separation is primarily attributed to solvophobic or hydrophobic interaction.<sup>12,13</sup> Figure 1.5 shows the separation of three organic components. Note that uracil, the most polar component and the most soluble compound in the mobile phase, elutes first. *t*-Butylbenzene elutes much later due to increased hydrophobic interaction with the stationary phase. RPC is the most popular HPLC mode and is used in more than 70% of all HPLC analyses.<sup>3,4</sup> It is suitable for the analysis of polar (water-soluble), medium-polarity, and some nonpolar analytes. Ionic analytes can be separated using ion-suppression or ion-pairing techniques, which will be discussed in Sections 2.3.4–2.3.6 in Chapter 2.

### 1.2.3 Ion-Exchange Chromatography (IEC)

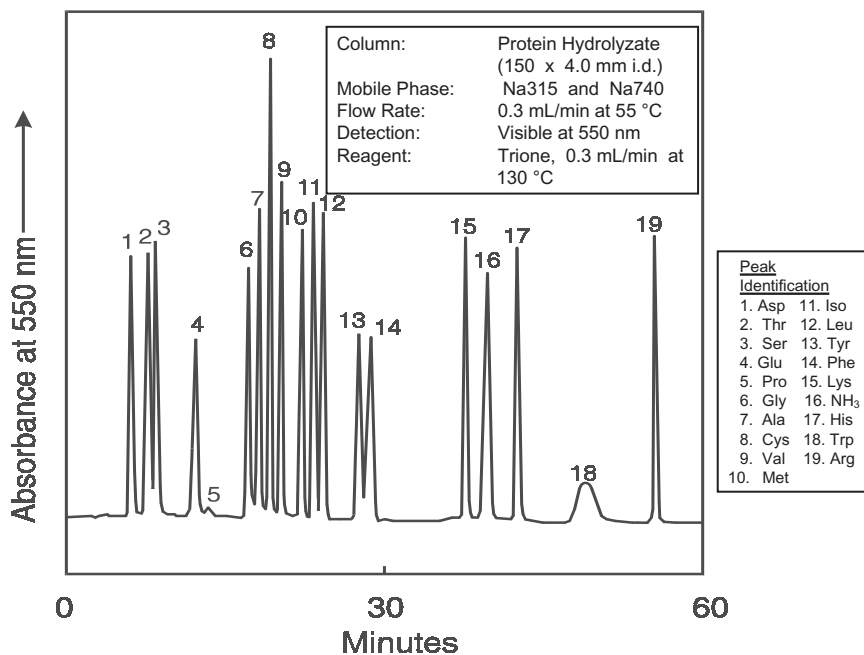
In ion-exchange chromatography,<sup>3-5</sup> the separation mode is based on the exchange of ionic analytes with the counter-ions of the ionic groups attached to the solid support (Figure 1.6a). Typical stationary phases are cationic exchange (sulfonate) or anionic exchange (quaternary ammonium) groups bonded to polymeric or silica materials. Mobile phases consist of buffers, often with increasing ionic strength, to force the migration of the analytes. Common applications are the analysis of ions and biological components such as amino acids, proteins/peptides, and polynucleotides. Figure 1.7 shows the separation of amino acids on a sulfonated polymer column and a mobile phase of



**Figure 1.5.** A reversed-phase HPLC chromatogram of three organic components eluting in the order of “polar first and nonpolar last.” The basic pyridine peak is tailing due to a secondary interaction of the nitrogen lone-pair with residual silanol groups of the silica based bonded phase. Figure reprinted with permission from reference 8, Chapter 2.



**Figure 1.6.** a. Schematic diagrams depicting separation modes of (a) ion-exchange chromatography (IEC), showing the exchange of analyte ion  $p^+$  with the sodium counter ions of the bonded sulfonate groups; (b) size-exclusion chromatography (SEC), showing the faster migration of large molecules.

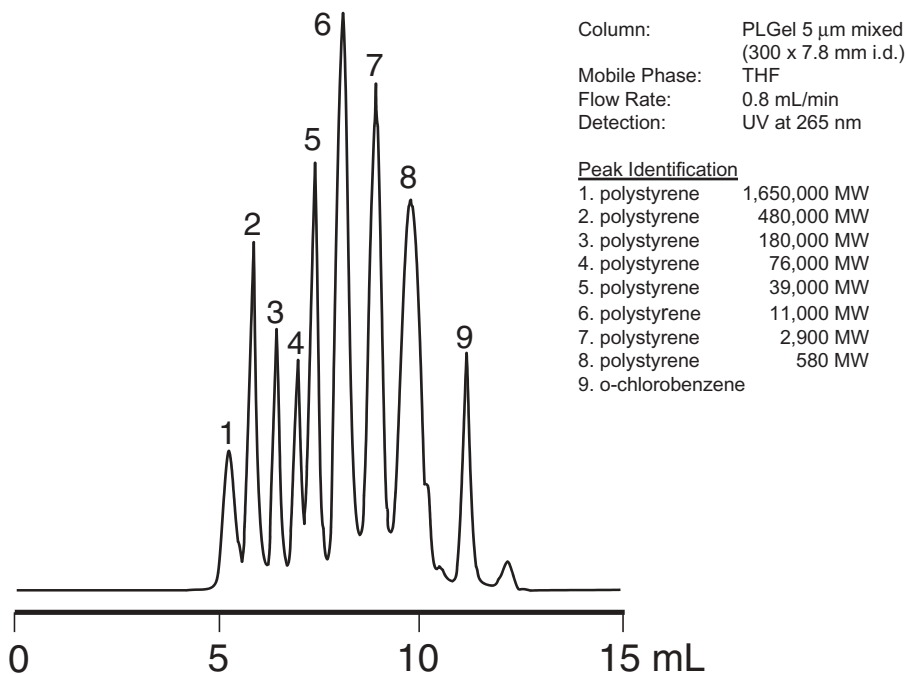


**Figure 1.7.** An ion-exchange HPLC chromatogram of essential amino acids using a cationic sulfonate column and detection with post-column reaction. Note that Na315 and Na740 are prepackaged eluents containing sodium ion and buffered at pH of 3.15 and 7.40, respectively. Trione is a derivatization reagent similar to ninhydrin. Chromatogram courtesy of Pickering Laboratories.

increasing sodium ion concentration and increasing pH. Since amino acids do not absorb strongly in the UV or visible region, a post-column reaction technique is used to form a color derivative to enhance detection at 550 nm. Ion chromatography<sup>14</sup> is a segment of IEC pertaining to the analysis of low concentrations of cations or anions using a high-performance ion-exchange column, often with a specialized conductivity detector.

### 1.2.4 Size-Exclusion Chromatography (SEC)

Size-exclusion chromatography<sup>15</sup> is a separation mode based solely on the analyte's molecular size. Figure 1.6b shows that a large molecule is excluded from the pores and migrates quickly, whereas a small molecule can penetrate the pores and migrates more slowly down the column. It is often called gel-permeation chromatography (GPC) when used for the determination of molecular weights of organic polymers and gel-filtration chromatography (GFC) when used in the separation of water-soluble biological materials. In GPC, the column is packed with cross-linked polystyrene beads of controlled pore sizes and eluted with common mobile phases such as toluene and tetrahy-



**Figure 1.8.** A GPC chromatogram of polystyrene standards on a mixed-bed polystyrene column. Chromatogram courtesy of Polymer Laboratories.

dofuran. Figure 1.8 shows the separation of polystyrene standards showing an elution order of decreasing molecular size. Detection with a refractive index detector is typical.

### 1.2.5 Other Separation Modes

Besides the four major HPLC separation modes, several others often encountered in HPLC or related techniques are noted below.

- *Affinity chromatography*<sup>9</sup>: Based on a receptor/ligand interaction in which immobilized ligands (enzymes, antigens, or hormones) on solid supports are used to isolate selected components from a mixture. The retained components can later be released in a purified state.
- *Chiral chromatography*<sup>16</sup>: For the separation of enantiomers using a chiral-specific stationary phase. Both NPC and RPC chiral columns are available.
- *Hydrophilic interaction chromatography (HILIC)*<sup>9</sup>: This is somewhat similar to normal phase chromatography using a polar stationary phase such as silica or ion-exchange materials but eluted with polar mobile