

PRACTICAL HPLC METHOD DEVELOPMENT

Second Edition

LLOYD R. SNYDER

LC Resources, Inc.
Walnut Creek, California

JOSEPH J. KIRKLAND

Rockland Technologies, Inc.
Newport, Delaware

JOSEPH L. GLAJCH

DuPont Merck Pharmaceutical Company
North Billerica, Massachusetts

A Wiley-Interscience Publication

JOHN WILEY & SONS, INC.

CONTENTS

| | |
|--|--------------|
| PREFACE | xix |
| GLOSSARY OF SYMBOLS AND TERMS | xxiii |
| 1 Getting Started | 1 |
| 1.1 Introduction, 1 | |
| 1.2 What Is Known Before Starting, 3 | |
| 1.2.1 Nature of the Sample, 3 | |
| 1.2.2 Separation Goals, 5 | |
| 1.3 Sample Pretreatment and Detection, 6 | |
| 1.4 Developing the Separation, 7 | |
| 1.4.1 Selecting an HPLC Method and Initial Conditions, 7 | |
| 1.4.2 Getting Started on Method Development, 10 | |
| 1.4.3 Improving the Separation, 13 | |
| 1.4.4 Repeatable Separation, 16 | |
| 1.5 Completing the HPLC Method, 17 | |
| 1.5.1 Quantitation and Method Validation, 17 | |
| 1.5.2 Checking for Problems, 18 | |
| 1.5.3 Method Ruggedness, 19 | |
| References, 20 | |
| 2 Basics of Separation | 21 |
| 2.1 Introduction, 21 | |

| | |
|---------|--|
| 2.2 | Resolution: General Considerations, 22 |
| 2.2.1 | Measurement of Resolution, 22 |
| 2.2.2 | Minimum Resolution, 25 |
| 2.3 | Resolution as a Function of Conditions, 27 |
| 2.3.1 | Effect of Solvent Strength, 31 |
| 2.3.2 | Effect of Selectivity, 34 |
| 2.3.2.1 | Changes in the Mobile Phase, 34 |
| 2.3.2.2 | Changes in the Column, 40 |
| 2.3.2.3 | Changes in Temperature, 40 |
| 2.3.3 | Effect of Column Plate Number, 41 |
| 2.3.3.1 | Column Conditions and Separation, 43 |
| 2.3.3.2 | Plate Number as a Function of Conditions, 46 |
| 2.3.3.3 | Extra-column Effects, 47 |
| 2.4 | Sample-Size Effects, 50 |
| 2.4.1 | Volume Overload: Effect of Sample Volume on Separation, 51 |
| 2.4.2 | Mass Overload: Effect of Sample Weight on Separation, 53 |
| 2.4.3 | Avoiding Problems Due to Too Large a Sample Size, 56 |
| 2.4.3.1 | Higher-Than-Expected Sample Concentrations, 56 |
| 2.4.3.2 | Trace Analysis, 56 |
| | References, 57 |

Detection Sensitivity and Selectivity

59

| | |
|---------|--|
| 3.1 | Introduction, 59 |
| 3.2 | UV Detection, 60 |
| 3.2.1 | General Considerations, 60 |
| 3.2.2 | Choice of Wavelength, 63 |
| 3.2.2.1 | Sample Absorbance as a Function of Molecular Structure, 63 |
| 3.2.2.2 | Mobile-Phase Absorbance as a Function of Composition, 66 |
| 3.2.3 | Signal, Noise, and Assay Precision, 71 |
| 3.2.4 | Maximizing Signal/Noise Ratio for Better Assay Precision, 73 |
| 3.2.5 | Detector Linearity, 76 |
| 3.2.6 | Diode-Array UV Detectors, 77 |
| 3.3 | Other HPLC Detectors, 80 |
| 3.3.1 | Universal Detection, 80 |
| 3.3.2 | Fluorescence Detection, 81 |
| 3.3.3 | Electrochemical Detection, 84 |
| 3.3.4 | Mass Spectrometer Detection (LC-MS), 89 |
| 3.3.4.1 | Mass Analyzers, 91 |
| 3.3.4.2 | Ionization Methods, 92 |

| | | |
|-------|--|----|
| 3.3.5 | Selecting the Mass Spectrometric Detector, | 95 |
| 3.3.6 | Less Common Detectors, | 96 |
| | References, | 97 |

| | | |
|----------|---|------------|
| 4 | Sample Preparation | 100 |
| 4.1 | Introduction, | 101 |
| 4.2 | Types of Samples, | 103 |
| 4.3 | Preliminary Processing of Solid and Semi-solid Samples, | 103 |
| 4.3.1 | Reducing Sample Particle Size, | 103 |
| 4.3.2 | Drying the Sample, | 108 |
| 4.3.3 | Filtration, | 108 |
| 4.4 | Sample Pretreatment for Liquid Samples, | 110 |
| 4.4.1 | Liquid-Liquid Extraction, | 110 |
| 4.4.1.1 | Theory, | 112 |
| 4.4.1.2 | Practice, | 114 |
| 4.4.1.3 | Problems, | 117 |
| 4.4.2 | Solid-Phase Extraction, | 119 |
| 4.4.2.1 | SPE vs. LLE, | 119 |
| 4.4.2.2 | SPE vs. HPLC, | 120 |
| 4.4.2.3 | Uses of SPE, | 120 |
| 4.4.2.4 | SPE Devices, | 121 |
| 4.4.2.5 | SPE Apparatus, | 125 |
| 4.4.2.6 | SPE Method Development, | 127 |
| 4.4.2.7 | Column Chromatography for Sample Pretreatment, | 139 |
| 4.4.3 | Membrane Separations, | 139 |
| 4.5 | Sample Pretreatment for Solid Samples, | 144 |
| 4.5.1 | Traditional Extraction Methods, | 145 |
| 4.5.2 | Newer Extraction Methods, | 147 |
| 4.5.2.1 | Supercritical Fluid Extraction, | 147 |
| 4.5.2.2 | Microwave-Assisted Solvent Extraction, | 151 |
| 4.5.2.3 | Accelerated Solvent Extraction, | 153 |
| 4.5.3 | Comparison of Methods for Extraction of Solids, | 154 |
| 4.6 | Column Switching, | 154 |
| 4.6.1 | Principle of Operation, | 158 |
| 4.6.2 | Developing a Column-Switching Method: General Considerations, | 160 |
| 4.6.3 | Examples of Column Switching for Sample Cleanup, | 160 |
| 4.7 | Derivatization, | 161 |
| 4.7.1 | Detectability, | 163 |
| 4.7.1.1 | UV Detection, | 165 |
| 4.7.1.2 | Fluorescence Detection, | 165 |
| 4.7.2 | Pre- and Post-column Derivatization, | 167 |
| 4.7.2.1 | Pre-column Derivatization, | 167 |
| 4.7.2.2 | Post-column Derivatization, | 168 |

4.7.3 Chiral Analysis by Derivatization, 169
References, 170

5 The Column 174

- 5.1 Introduction, 175
 - 5.2 Characteristics of Columns and Column Packings, 175
 - 5.2.1 Column-Packing Particles, 175
 - 5.2.1.1 Silica Packing Particles, 178
 - 5.2.1.2 Porous Polymers, 182
 - 5.2.1.3 Other Inorganic Supports, 184
 - 5.2.2 Column Configuration, 186
 - 5.2.3 Stationary Phases, 189
 - 5.2.3.1 Bonded Silanes, 189
 - 5.2.3.2 Other Stationary Phases, 192
 - 5.2.3.3 Retention of the Bonded Phase in RPC, 192
 - 5.2.3.4 Stability of Bonded-Phase Columns, 193
 - 5.2.4 Sources of Retention and Selectivity Variability, 203
 - 5.3 Column Specifications, 205
 - 5.3.1 Plate Number, 205
 - 5.3.2 Peak Asymmetry and Peak Tailing, 208
 - 5.3.3 Column Failure: How Long Should a Column Last?, 210
 - 5.3.4 Retention Reproducibility, 212
 - 5.3.5 Pressure Drop, 212
 - 5.3.6 Bonded-Phase Concentration (Coverage), 213
 - 5.4 Column Problems and Remedies, 214
 - 5.4.1 Retention and Resolution Irreproducibility, 214
 - 5.4.2 Band Tailing, 219
 - 5.4.3 Why Do Columns Die?, 223
 - 5.4.3.1 Column Frit Problems, 224
 - 5.4.3.2 Strongly Held Sample Components, 225
 - 5.4.3.3 Poorly Packed Columns, 226
 - 5.4.3.4 Pressure Effects, 226
 - 5.4.3.5 Chemical Attack, 227
 - 5.4.3.6 Other Factors, 227
 - 5.4.4 Suggested Column for Method Development, 229
- References, 230

6 Non-ionic Samples: Reversed- and Normal-Phase HPLC 233

- 6.1 Introduction, 234
- Part I: Reversed-Phase Chromatography 234**
- 6.2 Retention in Reversed-Phase Chromatography, 235
 - 6.2.1 Mobile-Phase Effects, 236
 - 6.2.1.1 Choice of % B, 237
 - 6.2.1.2 Mobile-Phase Strength, 239
 - 6.2.2 Column and Temperature Effects, 240

| | | |
|----------|--|------------|
| 6.3 | Selectivity in Reversed-Phase Chromatography, 242 | |
| 6.3.1 | Solvent-Strength Selectivity, 242 | |
| 6.3.2 | Solvent-Type Selectivity, 244 | |
| 6.3.3 | Column-Type Selectivity, 248 | |
| 6.3.4 | Temperature Selectivity, 251 | |
| 6.4 | Optimizing the Separation of Non-ionic Samples in Reversed-Phase Chromatography, 252 | |
| 6.4.1 | Getting Started, 253 | |
| 6.4.2 | Optimizing Selectivity, 254 | |
| 6.4.2.1 | Solvent Strength (% B) Effects, 255 | |
| 6.4.2.2 | Solvent-Type Effects Plus % B Effects, 255 | |
| 6.4.2.3 | Use of Organic Solvent Mixtures, 257 | |
| 6.4.2.4 | Column-Type Effects Plus % B Effects, 260 | |
| 6.4.2.5 | Combined Use of Different Solvents Plus Column Types, 260 | |
| 6.5 | Non-aqueous Reversed-Phase HPLC, 264 | |
| | Part II: Normal-Phase Chromatography | 266 |
| 6.6 | Retention and Selectivity in Normal-Phase Chromatography, 268 | |
| 6.6.1 | General Aspects, 268 | |
| 6.6.1.1 | Sample and Solvent Localization, 269 | |
| 6.6.2 | Mobile-Phase Effects, 271 | |
| 6.6.2.1 | Solvent Strength, 271 | |
| 6.6.2.2 | Mobile-Phase Selectivity, 273 | |
| 6.6.3 | Column-Type Effects, 276 | |
| 6.6.4 | Temperature Effects, 278 | |
| 6.6.5 | Use of Aqueous Mobile Phases for Hydrophilic Samples, 278 | |
| 6.7 | Optimizing the Separation of Non-ionic Samples in Normal-Phase Chromatography, 282 | |
| 6.7.1 | Initial Conditions, 282 | |
| 6.7.1.1 | Choice of Column, 282 | |
| 6.7.1.2 | Mobile Phase Solvents, 284 | |
| 6.7.2 | Adjusting Retention, 284 | |
| 6.7.3 | Optimizing Selectivity, 285 | |
| 6.7.4 | Other Considerations, 287 | |
| 6.7.4.1 | Slow Column Equilibration and Solvent Demixing, 287 | |
| 6.7.4.2 | Changes in Stationary-Phase Water Content, 288 | |
| | References, 289 | |
| 7 | Ionic Samples: Reversed-Phase, Ion-Pair, and Ion-Exchange HPLC | 292 |
| 7.1 | Introduction, 293 | |

- 7.2 Acidic and Basic Samples, 294
 - 7.2.1 Acid-Base Equilibria and Reversed-Phase Retention, 294
 - 7.2.2 Choice of Buffers, 296
 - 7.2.2.1 Buffer Capacity, 297
 - 7.2.2.2 Buffer UV Absorbance, 300
 - 7.2.2.3 Other Buffer Properties, 300
 - 7.2.2.4 Preferred Buffers, 301
 - 7.2.3 pK_a as a Function of Compound Structure, 301
 - 7.2.3.1 Preferred Mobile-Phase pH, 302
 - 7.2.4 Which HPLC Method is Best for Ionic Samples?, 303
- 7.3 Optimizing the Reversed-Phase Separation of Ionic Samples, 303
 - 7.3.1 Initial Experiments, 303
 - 7.3.2 Controlling Selectivity, 304
 - 7.3.2.1 pH, 305
 - 7.3.2.2 Solvent Strength (% B), 307
 - 7.3.2.3 Solvent Type, 307
 - 7.3.2.4 Temperature, 308
 - 7.3.2.5 Buffer Concentration, 309
 - 7.3.2.6 Amine Modifiers, 309
 - 7.3.2.7 Column Type, 311
 - 7.3.3 Special Problems, 311
 - 7.3.3.1 pH Sensitivity, 311
 - 7.3.3.2 Silanol Effects, 311
 - 7.3.3.3 Temperature Sensitivity, 313
 - 7.3.4 Summary, 313
- 7.4 Ion-Pair Chromatography, 317
 - 7.4.1 Basis of Retention, 318
 - 7.4.1.1 pH and Ion Pairing, 318
 - 7.4.1.2 Ion-Pair Reagent Concentration, 320
 - 7.4.1.3 Ion-Pair Reagent Type, 322
 - 7.4.2 Initial Experiments, 324
 - 7.4.3 Controlling Retention Range and Selectivity: Changes in % B, pH, and Ion-Pair Reagent Concentration, 327
 - 7.4.3.1 Retention Range, 327
 - 7.4.3.2 Selectivity, 328
 - 7.4.4 Other Changes in Selectivity, 332
 - 7.4.4.1 Solvent Strength (%B), 332
 - 7.4.4.2 Temperature, 333
 - 7.4.4.3 Buffer Concentration, 333
 - 7.4.4.4 Solvent Type, 333
 - 7.4.4.5 Buffer Type or Added Salt, 337
 - 7.4.4.6 Amine Modifiers, 337
 - 7.4.5 Special Problems, 337
 - 7.4.5.1 Artfactual Peaks, 337

| | |
|-----|--|
| | 7.4.5.2 Slow Column Equilibration, 338 |
| | 7.4.5.3 Poor Peak Shape, 339 |
| | 7.4.6 Summary, 339 |
| 7.5 | Ion-Exchange Chromatography, 341 |
| | 7.5.1 Basis of Retention, 342 |
| | 7.5.1.1 pH Effects, 343 |
| | 7.5.1.2 Salt or Buffer Type, 343 |
| | 7.5.1.3 Organic Solvents, 343 |
| | 7.5.1.4 Column Type, 343 |
| | 7.5.2 Method Development, 344 |
| | 7.5.3 Mixed-Mode Separations, 344 |
| | 7.5.4 Silica Columns, 346 |
| | References, 346 |

8 Gradient Elution

350

| | |
|-----|---|
| 8.1 | Introduction, 351 |
| 8.2 | Applications of Gradient Elution, 352 |
| | 8.2.1 Gradient Elution for Routine Analysis, 353 |
| | 8.2.1.1 Sample Retention Range, 353 |
| | 8.2.1.2 High-Molecular-Weight Sample Components, 353 |
| | 8.2.1.3 Late Eluters, 356 |
| | 8.2.1.4 Maximizing Detection Sensitivity, 356 |
| | 8.2.1.5 Dilute Sample Solutions, 356 |
| | 8.2.1.6 Alternatives to Gradient Elution, 358 |
| | 8.2.2 Gradient Elution for Method Development, 359 |
| | 8.2.2.1 Isocratic or Gradient Separation?, 359 |
| | 8.2.2.2 Estimating the Best Isocratic Conditions, 362 |
| | 8.2.2.3 Estimating the Best Gradient Conditions, 362 |
| 8.3 | Principles of Gradient Elution, 363 |
| | 8.3.1 Gradient vs. Isocratic Elution, 365 |
| | 8.3.2 Effect of Gradient Steepness, 367 |
| | 8.3.3 Effect of Gradient Range, 367 |
| | 8.3.4 Effect of Gradient Shape, 372 |
| | 8.3.4.1 Homologous or Oligomeric Samples, 372 |
| | 8.3.4.2 Chromatograms with Peak Bunching, 374 |
| 8.4 | Developing a Gradient Separation, 374 |
| | 8.4.1 Selecting Gradient Conditions, 376 |
| | 8.4.1.1 Gradient Steepness, 376 |
| | 8.4.1.2 Gradient Range, 376 |
| | 8.4.1.3 Gradient Shape, 377 |
| | 8.4.2 Varying Band Spacing, 377 |
| | 8.4.2.1 Gradient Steepness, 377 |

- 8.4.2.2 Solvent Type, 380
 - 8.4.2.3 Other Variables, 380
 - 8.4.3 Adjusting Column Conditions, 382
 - 8.5 Experimental Considerations, 385
 - 8.5.1 Effect of Equipment on Separation: System Dwell Volume, 386
 - 8.5.1.1 Equipment Differences, 386
 - 8.5.1.2 Changes in Separation for Different HPLC Systems, 387
 - 8.5.1.3 Minimizing the Effect of Equipment Dwell Volume, 390
 - 8.5.1.4 Determining the Dwell Volume, 392
 - 8.5.2 Reproducible Separation, 394
 - 8.5.2.1 Column Regeneration, 394
 - 8.5.2.2 Column Equilibration, 394
 - 8.5.2.3 Inaccurate Gradients, 395
 - 8.5.3 Baseline Problems, 396
 - 8.5.3.1 Drift, 396
 - 8.5.3.2 Artifactual Bands, 397
 - 8.6 Summary of Gradient Elution Method Development, 397
 - 8.6.1 Systematic Approach, 397
 - 8.6.2 Computer Simulation, 399
- References, 400

Systematic Approach to the Reversed-Phase Separation of Regular Samples

402

- 9.1 Introduction, 403
 - 9.1.1 Some Guiding Principles, 405
 - 9.1.1.1 Classifying the Sample, 406
 - 9.1.1.2 Initial Separation Conditions: The Column and Flow Rate, 406
 - 9.1.1.3 Initial Separation Conditions: The Mobile Phase, 407
 - 9.1.1.4 Other Initial Separation Conditions, 408
 - 9.1.1.5 Ensuring Accurate Retention Data, 408
 - 9.1.1.6 Confirming Good Column Performance, 409
 - 9.1.1.7 Peak Tracking, 410
- 9.2 Getting Started, 410
 - 9.2.1 Initial Conditions, 410
 - 9.2.2 Adjusting the Retention Range, 411
 - 9.2.2.1 Isocratic Separation, 411
 - 9.2.2.2 Gradient Separation, 414
 - 9.2.2.3 Early or Late Eluters, 416
 - 9.2.2.4 Very Hydrophobic Cations, 416

| | | |
|---------|---|-----|
| 9.2.2.5 | Complex Samples, | 417 |
| 9.2.2.6 | No Real Peaks, | 418 |
| 9.2.3 | Evaluating Peak Shape and Plate Number, | 418 |
| 9.3 | Completing Isocratic Method Development, | 420 |
| 9.3.1 | Optimizing Retention and Selectivity, | 420 |
| 9.3.1.1 | Sample A: An Easy Separation, | 422 |
| 9.3.1.2 | Sample B: A Typical Separation, | 422 |
| 9.3.1.3 | Sample C: A Difficult Separation, | 424 |
| 9.3.1.4 | Further Improvements in Separation, | 426 |
| 9.3.1.5 | Changing the Method for Later Samples or Applications, | 429 |
| 9.3.2 | Optimizing Column Conditions, | 430 |
| 9.4 | Alternative To Completing Isocratic Method Development, | 431 |
| 9.5 | Completing Gradient Method Development, | 433 |
| | References, | 437 |

10 Computer-Assisted Method Development 439

| | | |
|----------|---|-----|
| 10.1 | Introduction, | 439 |
| 10.1.1 | Summary of Commercial Method-Development Software, | 440 |
| 10.2 | Computer Simulation Software (DryLab), | 441 |
| 10.2.1 | Isocratic Separation Varying % B and Column Conditions, | 443 |
| 10.2.1.1 | Use of Other Variables for Changing Selectivity, | 445 |
| 10.2.2 | Gradient Separations, | 448 |
| 10.2.2.1 | Segmented Gradients, | 452 |
| 10.2.2.2 | Other Applications, | 452 |
| 10.3 | Software for Solvent-Type Optimization (ICOS, DIAMOND), | 455 |
| 10.4 | Grid-Search Software (PESOS), | 458 |
| 10.5 | Structure-Based Predictive Software, | 463 |
| 10.5.1 | ELUEX, | 463 |
| 10.5.2 | CHROMDREAM, | 465 |
| 10.5.3 | Special-Purpose Programs, | 465 |
| 10.6 | Method Ruggedness, | 467 |
| 10.7 | Peak Tracking, | 470 |
| 10.7.1 | Injection of Standards, | 470 |
| 10.7.2 | Retention and Area Comparisons, | 472 |
| 10.7.3 | Trends in Retention, | 473 |
| 10.7.4 | Spectral Identification, | 473 |
| 10.8 | Pitfalls, | 475 |
| | References, | 476 |

| | |
|--|------------|
| 11 Biochemical Samples: Proteins, Nucleic Acids, Carbohydrates, and Related Compounds | 479 |
| 11.1 Introduction, 480 | |
| 11.1.1 Primary Structure, 482 | |
| 11.1.1.1 Peptides and Proteins, 482 | |
| 11.1.1.2 Oligonucleotides and Nucleic Acids, 485 | |
| 11.1.1.3 Modified Oligonucleotides, 488 | |
| 11.1.2 Special Requirements of Biochemical HPLC, 488 | |
| 11.1.2.1 Columns, 488 | |
| 11.1.2.2 Sample Molecular Conformation, 492 | |
| 11.1.2.3 Sample Recovery: Mass and Bioactivity, 494 | |
| 11.1.2.4 Sample Handling and Pretreatment, 495 | |
| 11.1.2.5 Sample Detection, 497 | |
| 11.2 Separation of Peptide and Protein Samples, 497 | |
| 11.2.1 Reversed-Phase HPLC, 497 | |
| 11.2.1.1 Preferred Conditions for an Initial Separation, 498 | |
| 11.2.1.2 Variables for Changing Selectivity, 502 | |
| 11.2.1.3 Common Problems and Remedies, 507 | |
| 11.2.2 Ion-Exchange HPLC, 509 | |
| 11.2.2.1 Preferred Conditions for an Initial Separation, 512 | |
| 11.2.2.2 Variables for Changing Selectivity, 515 | |
| 11.2.2.3 Common Problems and Remedies, 515 | |
| 11.2.3 Hydrophobic Interaction Chromatography, 516 | |
| 11.2.3.1 Preferred Conditions for HIC Separation, 517 | |
| 11.3 Separation of Oligonucleotides, 519 | |
| 11.3.1 Ion-Pair HPLC, 520 | |
| 11.3.2 Ion-Exchange HPLC, 521 | |
| 11.4 Size-Exclusion Chromatography, 523 | |
| 11.4.1 The Basis of SEC Retention, 523 | |
| 11.4.2 Applications, 528 | |
| 11.4.3 Preferred Conditions for an SEC Separation, 530 | |
| 11.4.4 Common Problems and Remedies, 531 | |
| 11.4.5 Protein Folding, 533 | |
| References, 533 | |
| | |
| 12 Chiral Separations | 537 |
| 12.1 Introduction, 538 | |
| 12.1.1 Chiral Derivatization, 540 | |
| 12.1.2 Chiral Mobile-Phase Additives, 540 | |
| 12.1.3 Chiral Stationary Phases, 541 | |
| 12.1.4 Principles of Chiral Recognition, 542 | |

- 12.1.5 General Considerations for Chiral HPLC Method Development, 546
 - 12.1.5.1 Sample Information, 546
 - 12.1.5.2 Preparative Separations, 547
- 12.1.6 Selecting a Chiral Column, 547
- 12.2 Protein-Derived Chiral Stationary Phases for HPLC, 548
 - 12.2.1 Introduction, 548
 - 12.2.2 Background, 548
 - 12.2.3 Mechanism of Chiral Interactions, 550
 - 12.2.4 Characteristics of Protein-Based Chiral Columns, 550
 - 12.2.5 Adjusting Retention and Selectivity with the Mobile Phase, 552
 - 12.2.5.1 Organic Mobile-Phase Modifiers, 554
 - 12.2.5.2 pH, Ionic Strength, and Ion-Pairing Effects, 555
 - 12.2.6 Experimental Parameters, 559
 - 12.2.6.1 Mobile-Phase Effects, 559
 - 12.2.6.2 Sample Loading and Injection, 561
 - 12.2.6.3 Column Temperature, 561
 - 12.2.6.4 Column Configuration, 561
 - 12.2.6.5 Column Care and Stability, 563
 - 12.2.7 Application and Special Techniques, 563
 - 12.2.8 Systematic Method Development, 567
- 12.3 Polysaccharide (Carbohydrate) Columns, 568
 - 12.3.1 Introduction, 568
 - 12.3.2 Properties of Commercial Polysaccharide Phases, 568
 - 12.3.2.1 General Characteristics, 568
 - 12.3.2.2 Availability, 570
 - 12.3.3 Mechanisms of Chiral Interactions, 572
 - 12.3.4 Experimental Parameters, 576
 - 12.3.4.1 Mobile-Phase Selection, 576
 - 12.3.4.2 Temperature and Pressure Effects, 579
 - 12.3.4.3 Column Configuration and Operation, 579
 - 12.3.4.4 Sample Size, 581
 - 12.3.5 Applications, 581
 - 12.3.6 Strategy for Method Development, 581
- 12.4 Donor-Acceptor (Pirkle) Columns, 585
 - 12.4.1 Introduction, 585
 - 12.4.2 Properties of Commercial Donor-Acceptor CSPs, 586
 - 12.4.3 Mobile-Phase Conditions, 588
 - 12.4.3.1 Solvents, 591
 - 12.4.4 Method Development with Pirkle CSPs, 591
 - 12.4.4.1 Column, 591
 - 12.4.4.2 Mobile Phase, 591
 - 12.4.4.3 Derivatization, 592
 - 12.4.4.4 Effects of Temperature and Flow Rate, 594

| | | |
|-----------|--|------------|
| 12.5 | Cavity-Type Columns, 600 | |
| 12.5.1 | Introduction, 600 | |
| 12.5.2 | Method Development for Separations Using Underivatized CD Columns, 604 | |
| 12.5.2.1 | Separation Modes, 604 | |
| 12.5.2.2 | Reversed-Phase Mode, 604 | |
| 12.5.2.3 | Polar-Organic and Normal-Phase Modes, 608 | |
| 12.5.3 | Method Development with Derivatized Cyclodextrins, 610 | |
| | References, 613 | |
| 13 | Preparative HPLC Separation | 616 |
| 13.1 | Introduction, 616 | |
| 13.2 | Developing a Preparative HPLC Separation, 618 | |
| 13.2.1 | General Considerations, 618 | |
| 13.2.2 | Effect of Sample Size: Touching-Band Separation, 621 | |
| 13.2.3 | Optimizing Conditions for Preparative HPLC, 622 | |
| 13.2.4 | Gradient Separations, 625 | |
| 13.2.5 | Trace Recovery, 627 | |
| 13.3 | Practical Aspects of Preparative HPLC, 627 | |
| 13.3.1 | Sample Solubility, 627 | |
| 13.3.2 | Equipment Requirements, 628 | |
| 13.4 | Quantitative Prediction of Preparative HPLC Separation, 628 | |
| 13.4.1 | General Relationships, 629 | |
| 13.4.2 | Column Saturation Capacity, 631 | |
| 13.4.3 | Gradient Elution Separations, 631 | |
| 13.4.4 | Heavily Overloaded Separations, 632 | |
| 13.4.5 | Unusual Isotherm Behavior, 634 | |
| 13.5 | Summary and Example of Method Development for Preparative HPLC, 636 | |
| 13.5.1 | Process-Scale HPLC Separations, 640 | |
| | References, 641 | |
| 14 | Quantitation (Including Trace Analysis) | 643 |
| 14.1 | Introduction, 643 | |
| 14.1.1 | Accuracy, Precision, and Linearity, 644 | |
| 14.1.2 | Limits of Detection and Quantitation, 645 | |
| 14.2 | Measurement of Signals, 647 | |
| 14.2.1 | Noise, 647 | |
| 14.2.2 | Peak Height, 649 | |
| 14.2.3 | Peak Area, 650 | |
| 14.2.4 | Peak Height vs. Peak Area for Quantitation, 652 | |
| 14.3 | Quantitation Methods, 653 | |
| 14.3.1 | Normalized Peak Area, 654 | |

- 14.3.2 External Standard Calibration, 655
- 14.3.3 Internal Standard Calibration, 657
- 14.3.4 Method of Standard Addition, 660
- 14.4 Sources of Error in Quantitation, 660
 - 14.4.1 Sampling and Sample Preparation, 662
 - 14.4.2 Chromatographic Effects, 663
 - 14.4.3 Data System Effects, 665
- 14.5 Trace Analysis, 666
 - 14.5.1 Sample Preparation, 666
 - 14.5.2 Column Resolution, 667
 - 14.5.3 Sample Injection, 673
 - 14.5.4 Detection, 676
 - 14.5.5 Calibration, 678
 - 14.5.6 General Strategy, 680
- References, 683

15 Completing the Method: Validation and Transfer

- 15.1 Introduction, 686
 - 15.1.1 General Approach to Method Validation, 686
- 15.2 Accuracy, 687
 - 15.2.1 Comparison to a Standard, 688
 - 15.2.2 Analyte Recovery, 688
 - 15.2.3 Method of Standard Addition, 689
- 15.3 Precision, 690
- 15.4 Linearity, 691
- 15.5 Range, 694
- 15.6 Limit of Detection and Limit of Quantitation, 695
- 15.7 Specificity, 695
 - 15.7.1 Spiking of Potential Interferents, 697
 - 15.7.2 Sample Degradation, 697
 - 15.7.3 Peak Collection and Analysis, 698
 - 15.7.4 Additional On-Line Detection, 698
 - 15.7.5 Chromatographic Cross-Check, 700
 - 15.7.6 Changing HPLC Conditions, 700
- 15.8 Ruggedness, 701
- 15.9 Robustness, 702
- 15.10 Stability, 704
- 15.11 System Suitability, 705
- 15.12 Documentation of Validation Results and the Final Method, 706
- 15.13 Interlaboratory Crossover Studies (Transferability), 707
 - 15.13.1 Determining Equivalence, 708
- 15.14 Method Validation Protocol, 712
- References, 712

| | | |
|---------------------|---|------------|
| Appendix I | Plate Number and Resolution | 714 |
| Appendix II | Properties of Solvents Used in HPLC | 721 |
| Appendix III | Retention in Reversed-Phase and Normal-Phase HPLC as a Function of Sample Molecular Structure | 729 |
| Appendix IV | Preparing Buffered Mobile Phases | 735 |
| Appendix V | Characterizing the Differences Among C₁₈ or C₈ Reversed-Phase Columns from Different Suppliers | 740 |
| Appendix VI | Adjusting Mobile-Phase Water Content for Normal-Phase HPLC | 744 |
| Index | | 747 |