

Contents

Preface	<i>xv</i>
About the Book	<i>xvii</i>

Part I Optimization Strategies for Different Modes and Uses of HPLC 1

1.1	2D-HPLC – Method Development for Successful Separations	3
	<i>Dwight R. Stoll, Ph.D.</i>	
1.1.1	Motivations for Two-Dimensional Separation	3
1.1.1.1	Difficult-to-Separate Samples	3
1.1.1.2	Complex Samples	4
1.1.1.3	Separation Goals	4
1.1.2	Choosing a Two-Dimensional Separation Mode	4
1.1.2.1	Analytical Goals Dictate Choice of Mode	5
1.1.2.2	Survey of Four 2D Separation Modes	5
1.1.2.3	Hybrid Modes Provide Flexibility	7
1.1.3	Choosing Separation Types/Mechanisms	8
1.1.3.1	Complementarity as a Guiding Principle	8
1.1.3.2	Pirok Compatibility Table	9
1.1.3.3	Measuring the Complementarity of Separation Types	9
1.1.4	Choosing Separation Conditions	11
1.1.4.1	Starting with Fixed First-Dimension Conditions	11
1.1.4.2	Starting from Scratch – Flexible First-Dimension Conditions	13
1.1.4.3	Special Considerations for Comprehensive 2D-LC Methods	13
1.1.4.4	Rules of Thumb	13
1.1.5	Method Development Examples	14
1.1.5.1	Example 1 – Use of LC–LC to Identify an Impurity in a Synthetic Oligonucleotide	14
1.1.5.2	Example 2 – Comprehensive 2D-LC Separation of Surfactants	14
1.1.6	Outlook for the Future	17
	Acknowledgment	18
	References	18

1.2	Do you HILIC? With Mass Spectrometry? Then do it Systematically	23
	<i>Thomas Letzel</i>	
1.2.1	Initial Situation and Optimal Use of Stationary HILIC Phases	25
1.2.2	Initial Situation and Optimal Use of the “Mobile” HILIC Phase	28
1.2.2.1	Organic Solvent	28
1.2.2.2	Salts	31
1.2.2.3	pH Value	33
1.2.3	Further Settings and Conditions Specific to Mass Spectrometric Detection	35
1.2.4	Short Summary on Method Optimization in HILIC	36
	References	36
1.3	Optimization Strategies in LC–MS Method Development	39
	<i>Markus M. Martin</i>	
1.3.1	Introduction	39
1.3.2	Developing New Methods for HPLC–MS Separations	39
1.3.2.1	Optimizing the LC Separation	40
1.3.2.1.1	Optimizing for Sensitivity and Limit of Detection – Which Column to Take?	40
1.3.2.1.2	Optimizing Resolution vs. Sample Throughput	41
1.3.2.1.3	MS-Compatible Eluent Compositions and Additives	43
1.3.2.2	Optimizing Ion Source Conditions	44
1.3.2.3	Optimizing MS Detection	47
1.3.2.4	Verifying the Hyphenated Method	48
1.3.2.5	Method Development Supported by Software-based Parameter Variation	49
1.3.3	Transferring Established HPLC Methods to Mass spectrometry	50
1.3.3.1	Transfer of an Entire HPLC Method to a Mass Spectrometer	51
1.3.3.2	Selected Analysis of an Unknown Impurity – Solvent Change by Single-/Multi-Heartcut Techniques	52
	Abbreviations	54
	References	55
1.4	Chromatographic Strategies for the Successful Characterization of Protein Biopharmaceuticals	57
	<i>Szabolcs Fekete, Valentina D’Atri, and Davy Guillarme</i>	
1.4.1	Introduction to Protein Biopharmaceuticals	57
1.4.2	From Standard to High-Performance Chromatography of Protein Biopharmaceuticals	58
1.4.3	Online Coupling of Nondenaturing LC Modes with MS	62
1.4.4	Multidimensional LC Approaches for Protein Biopharmaceuticals	64
1.4.5	Conclusion and Future Trends in Protein Biopharmaceuticals Analysis	66
	References	67

- 1.5 Optimization Strategies in HPLC for the Separation of Biomolecules** 73
Lisa Strasser, Florian Füssl, and Jonathan Bones
- 1.5.1 Optimizing a Chromatographic Separation 73
 - 1.5.2 Optimizing the Speed of an HPLC Method 77
 - 1.5.3 Optimizing the Sensitivity of an HPLC Method 79
 - 1.5.4 Multidimensional Separations (See also Chapter 1.1) 80
 - 1.5.5 Considerations for MS Detection (See also Chapter 1.3) 81
 - 1.5.6 Conclusions and Future Prospects 83
References 84
- 1.6 Optimization Strategies in Packed-Column Supercritical Fluid Chromatography (SFC)** 87
Caroline West
- 1.6.1 Selecting a Stationary Phase Allowing for Adequate Retention and Desired Selectivity 88
 - 1.6.1.1 Selecting a Stationary Phase for Chiral Separations 88
 - 1.6.1.2 Selecting a Stationary Phase for Achiral Separations 90
 - 1.6.2 Optimizing Mobile Phase to Elute all Analytes 93
 - 1.6.2.1 Nature of the Cosolvent 93
 - 1.6.2.2 Proportion of Cosolvent 94
 - 1.6.2.3 Use of Additives 96
 - 1.6.2.4 Sample Diluent 97
 - 1.6.3 Optimizing Temperature, Pressure, and Flow Rate 97
 - 1.6.3.1 Understanding the Effects of Temperature, Pressure, and Flow Rate on your Chromatograms 97
 - 1.6.3.2 Optimizing Temperature, Pressure, and Flow Rate Concomitantly 99
 - 1.6.4 Considerations on SFC–MS Coupling 100
 - 1.6.5 Summary of Method Optimization 101
 - 1.6.6 SFC as a Second Dimension in Two-Dimensional Chromatography 102
 - 1.6.7 Further Reading 102
References 103
- 1.7 Strategies for Enantioselective (Chiral) Separations** 107
Markus Juza
- 1.7.1 How to Start? 108
 - 1.7.2 Particle Size 109
 - 1.7.3 Chiral Polysaccharide Stationary Phases as First Choice 110
 - 1.7.4 Screening Coated and Immobilized Polysaccharide CSPs in Normal-Phase and Polar Organic Mode 113
 - 1.7.5 Screening Coated and Immobilized Polysaccharide CSPs in Reversed-Phase Mode 116
 - 1.7.6 Screening Immobilized Polysaccharide CSPs in Medium-Polarity Mode 119

- 1.7.7 Screening Coated and Immobilized Polysaccharide CSPs under Polar Organic Supercritical Fluid Chromatography Conditions 120
- 1.7.8 Screening Immobilized Polysaccharide CSPs in Medium-Polarity Supercritical Fluid Chromatography Conditions 125
- 1.7.9 SFC First? 127
- 1.7.10 Are There Rules for Predicting Which CSP Is Suited for My Separation Problem? 127
- 1.7.11 Which Are the Most Promising Polysaccharide CSPs? 127
- 1.7.12 Are some CSPs Comparable? 129
- 1.7.13 “No-Go’s,” Pitfalls, and Peculiarities in Chiral HPLC and SFC 132
- 1.7.14 Gradients in Chiral Chromatography 133
- 1.7.15 Alternative Strategies to Chiral HPLC and SFC on Polysaccharide CSPs 133
- 1.7.16 How Can I Solve Enantiomer Separation Problems Without Going to the Laboratory? 135
- 1.7.17 The Future of Chiral Separations – Fast Chiral Separations (cUHPLC and cSFC)? 136
 - References 138

- 1.8 Optimization Strategies Based on the Structure of the Analytes 141**
Christoph A. Fleckenstein
 - 1.8.1 Introduction 141
 - 1.8.2 The Impact of Functional Moieties 142
 - 1.8.3 Hydrogen Bonds 143
 - 1.8.4 Influence of Water Solubility by Hydrate Formation of Aldehydes and Ketones 146
 - 1.8.5 Does “Polar” Equal “Hydrophilic”? 148
 - 1.8.6 Peroxide Formation of Ethers 150
 - 1.8.7 The pH Value in HPLC 151
 - 1.8.7.1 Acidic Functional Groups 152
 - 1.8.7.2 Basic Functional Groups 153
 - 1.8.8 General Assessment and Estimation of Solubility of Complex Molecules 155
 - 1.8.9 Octanol–Water Coefficient 157
 - 1.8.10 Hansen Solubility Parameters 160
 - 1.8.11 Conclusion and Outlook 162
 - Acknowledgments 163
 - References 163

- 1.9 Optimization Opportunities in a Regulated Environment 165**
Stavros Kromidas
 - 1.9.1 Introduction 165
 - 1.9.2 Preliminary Remark 165
 - 1.9.3 Resolution 167

- 1.9.3.1 Hardware Changes 167
- 1.9.3.1.1 Preliminary Remark 167
- 1.9.3.1.2 UHPLC Systems 168
- 1.9.3.1.3 Column Oven 168
- 1.9.3.2 Improving the Peak Shape 169
- 1.9.4 Peak-to-Noise Ratio 171
- 1.9.4.1 Noise Reduction 171
- 1.9.5 Coefficient of Variation, VC (Relative Standard Deviation, RSD) 171
- References 176

Part II Computer-aided Optimization 177

2.1 Strategy for Automated Development of Reversed-Phase HPLC Methods for Domain-Specific Characterization of Monoclonal Antibodies 179

Jennifer La, Mark Condina, Leexin Chong, Craig Kyngdon, Matthias Zimmermann, and Sergey Galushko

- 2.1.1 Introduction 179
- 2.1.2 Interaction with Instruments 181
- 2.1.3 Columns 182
- 2.1.4 Sample Preparation and HPLC Analysis 183
- 2.1.5 Automated Method Development 184
- 2.1.5.1 Columns Screening 185
- 2.1.5.2 Rapid Optimization 186
- 2.1.5.3 Fine Optimization and Sample Profiling 188
- 2.1.6 Robustness Tests 188
- 2.1.6.1 Selection of the Variables 189
- 2.1.6.2 Selection of the experimental design 190
- 2.1.6.3 Definition of the Different Levels for the Factors 191
- 2.1.6.4 Creation of the Experimental Set-up 191
- 2.1.6.5 Execution of Experiments 192
- 2.1.6.6 Calculation of Effects and Response and Numerical and Graphical Analysis of the Effects 192
- 2.1.6.7 Improving the Performance of the Method 194
- 2.1.7 Conclusions 196
- References 196

2.2 Fusion QbD[®] Software Implementation of APLM Best Practices for Analytical Method Development, Validation, and Transfer 199

Richard Verseput

- 2.2.1 Introduction 199
- 2.2.1.1 Application to Chromatographic Separation Modes 200
- 2.2.1.2 Small- and Large-Molecule Applications 200

2.2.1.3	Use for Non-LC Method Development Procedures	200
2.2.2	Overview – Experimental Design and Data Modeling in Fusion QbD	201
2.2.3	Analytical Target Profile	201
2.2.4	APLM Stage 1 – Procedure Design and Development	202
2.2.4.1	Initial Sample Workup	202
2.2.5	Chemistry System Screening	204
2.2.5.1	Starting Points Based on Molecular Structure and Chemistry Considerations	205
2.2.5.2	Trend Responses and Data Modeling	205
2.2.6	Method Optimization	207
2.2.6.1	Optimizing Mean Performance	207
2.2.6.2	Optimizing Robustness In Silico – Monte Carlo Simulation	210
2.2.6.3	A Few Words About Segmented (Multistep) Gradients and Robustness	213
2.2.7	APLM Stage 2 – Procedure Performance Verification	214
2.2.7.1	Replication Strategy	214
2.2.8	The USP <1210> Tolerance Interval in Support of Method Transfer	214
2.2.9	What is Coming – Expectations for 2021 and Beyond	216
	References	217

Part III Current Challenges for HPLC Users in Industry 219

3.1	Modern HPLC Method Development	221
	<i>Stefan Lamotte</i>	
3.1.1	Robust Approaches to Practice	222
3.1.1.1	Generic Systems for all Tasks	222
3.1.2	The Classic Reverse-phase System	225
3.1.3	A System that Primarily Separates According to π - π Interactions	227
3.1.4	A system that Primarily Separates According to Cation Exchange and Hydrogen Bridge Bonding Selectivity	227
3.1.5	System for Nonpolar Analytes	228
3.1.6	System for Polar Analytes	228
3.1.7	Conclusion	230
3.1.8	The Maximum Peak Capacity	230
3.1.9	Outlook	231
	References	231
3.2	Optimization Strategies in HPLC from the Perspective of an Industrial Service Provider	233
	<i>Juri Leonhardt and Michael Hausteil</i>	
3.2.1	Introduction	233
3.2.2	Research and Development	233
3.2.3	Quality Control	234

- 3.2.4 Process Control Analytics 235
- 3.2.5 Decision Tree for the Optimization Strategy Depending on the Final Application Field 237

- 3.3 Optimization Strategies in HPLC from the Perspective of a Service Provider – The UNTIE® Process of the CUP Laboratories 239**
Dirk Freitag-Stechl and Melanie Janich
 - 3.3.1 Common Challenges for a Service Provider 239
 - 3.3.2 A Typical, Lengthy Project – How it Usually Goes and How it Should not be Done! 239
 - 3.3.3 How Do We Make It Better? - The UNTIE® Process of the CUP Laboratories 241
 - 3.3.4 Understanding Customer Needs 241
 - 3.3.5 The Test of an Existing Method 242
 - 3.3.6 Method Development and Optimization 243
 - 3.3.7 Execution of the Validation 245
 - 3.3.8 Summary 248
 - Acknowledgments 249
 - References 249

- 3.4 Optimization Strategies in HPLC 251**
Bernard Burn
 - 3.4.1 Definition of the Task 252
 - 3.4.2 Relevant Data for the HPLC Analysis of a Substance (see also Chapter 1.8) 252
 - 3.4.2.1 Solubility 252
 - 3.4.2.2 Acidity Constants (pK_a) 257
 - 3.4.2.2.1 Polarity of Acidic or Alkaline Substances (see also Chapter 1.8) 257
 - 3.4.2.2.2 UV Spectra 259
 - 3.4.2.2.3 Influence on the Peak Shape 259
 - 3.4.2.2.4 Acid Constant Estimation 263
 - 3.4.2.3 Octanol–Water Partition Coefficient 263
 - 3.4.2.4 UV Absorption 270
 - 3.4.2.5 Stability of the Dissolved Analyte 272
 - 3.4.3 Generic Methods 278
 - 3.4.3.1 General Method for the Analysis of Active Pharmaceutical Ingredients 278
 - 3.4.3.2 Extensions of the Range of Application 279
 - 3.4.3.3 Limits of this General Method 279
 - 3.4.3.4 Example, Determination of Butamirate Dihydrogen Citrate in a Cough Syrup 279
 - 3.4.3.4.1 Basic Data 279
 - 3.4.3.4.2 Expected Difficulties 279
 - 3.4.3.4.3 HPLC Method 279

- 3.4.3.4.4 Example Chromatogram 279
- 3.4.4 General Tips for Optimizing HPLC Methods 279
 - 3.4.4.1 Production of Mobile Phases 284
 - 3.4.4.1.1 Reagents 284
 - 3.4.4.1.2 Vessels and Bottles 285
 - 3.4.4.1.3 Measurement of Reagents and Solvent 285
 - 3.4.4.1.4 Preparation of Buffer Solutions 286
 - 3.4.4.1.5 Filtration of Solvents and Buffer 286
 - 3.4.4.1.6 Degassing of Mobile Phases 287
 - 3.4.4.2 Blank Samples 287
 - 3.4.4.3 Defining Measurement Wavelengths for UV Detection 288
 - 3.4.4.4 UV Detection at Low Wavelengths 288
 - 3.4.4.4.1 Solvents 291
 - 3.4.4.4.2 Acids and Buffer Additives 292
 - 3.4.4.4.3 Drift at Solvent Gradients 294
 - 3.4.4.5 Avoidance of Peak Tailing 295
 - 3.4.4.6 Measurement Uncertainty and Method Design 302
 - 3.4.4.6.1 Weighing in or Measuring 302
 - 3.4.4.6.2 Dilutions 303
 - 3.4.4.6.3 HPLC Analysis 304
 - 3.4.4.6.4 Internal Standards 305
 - 3.4.4.7 Column Dimension and Particle Sizes 305
 - Reference 309

Part IV Current Challenges for HPLC Equipment Suppliers 311

- 4.1 Optimization Strategies with your HPLC – Agilent Technologies 313**
Jens Trafkowski
 - 4.1.1 Increase the Absolute Separation Performance: Zero Dead-Volume Fittings 314
 - 4.1.2 Separation Performance: Minimizing the Dispersion 314
 - 4.1.3 Increasing the Throughput – Different Ways to Lower the Turnaround Time 316
 - 4.1.4 Minimum Carryover for Trace Analysis: Multiwash 317
 - 4.1.5 Increase the Performance of What you have got – Modular or Stepwise Upgrade of Existing Systems 318
 - 4.1.6 Increase Automation, Ease of Use, and Reproducibility with the Features of a High-End Quaternary UHPLC Pump 319
 - 4.1.7 Increase Automation: Let your Autosampler do the Job 321
 - 4.1.8 Use Your System for Multiple Purposes: Multimethod and Method Development Systems 321
 - 4.1.9 Combine Sample Preparation with LC Analysis: Online SPE 322

- 4.1.10 Boost Performance with a Second Chromatographic Dimension: 2D-LC (see also Chapter 1.1) 323
- 4.1.11 Think Different, Work with Supercritical CO₂ as Eluent: SFC – Supercritical Fluid Chromatography (see also Chapter 1.6) 324
- 4.1.12 Determine Different Concentration Ranges in One System: High-Definition Range (HDR) HPLC 325
- 4.1.13 Automize Even Your Method Transfer from other LC Systems: Intelligent System Emulation Technology (ISET) 326
- 4.1.14 Conclusion 327
References 328

4.2 To Empower the Customer – Optimization Through Individualization 329

Kristin Folmert and Kathryn Monks

- 4.2.1 Introduction 329
- 4.2.2 Define Your Own Requirements 329
 - 4.2.2.1 Specification Sheet, Timetable, or Catalogue of Measures 329
 - 4.2.2.2 Personnel Optimization Helps to make Better Use of HPLC 331
 - 4.2.2.3 Mastering Time-Consuming Method Optimizations in a Planned Manner 332
 - 4.2.2.4 Optimizations at Device Level do not Always have to Mean an Investment 332
- 4.2.3 An Assistant Opens Up Many New Possibilities 333
 - 4.2.3.1 If the HPLC System must Simply be able to do more in the Future 333
 - 4.2.3.2 Individual Optimizations with an Assistant 333
 - 4.2.3.3 Automatic Method Optimization and Column Screening 334
 - 4.2.3.4 A New Perspective at Fractionation, Sample Preparation, and Peak Recycling 335
 - 4.2.3.5 Continuous Chromatography, a New Level of Purification 336
- 4.2.4 The Used Materials in the Focus of the Optimization 337
 - 4.2.4.1 Wetted vs. Dry Components of the HPLC 337
 - 4.2.4.2 Chemical Resistance of Wetted Components 338
 - 4.2.4.3 Bioinert Components 340
 - 4.2.4.3.1 Material Certification 340
- 4.2.5 Software Optimization Requires Open-Mindedness 340
- 4.2.6 Outlook 341

4.3 (U)HPLC Basics and Beyond 343

Gesa Schad, Brigitte Bollig, and Kyoko Watanabe

- 4.3.1 An Evaluation of (U)HPLC-operating Parameters and their Effect on Chromatographic Performance 343
 - 4.3.1.1 Compressibility Settings 343
 - 4.3.1.2 Solvent Composition and Injection Volume 346
 - 4.3.1.3 Photodiode Array Detector: Slit Width 348

4.3.2	“Analytical Intelligence” – AI, M2M, IoT – How Modern Technology can Simplify the Lab Routine	349
4.3.2.1	Auto-Diagnostics and Auto-Recovery to Maximize Reliability and Uptime	349
4.3.2.2	Advanced Peak Processing to Improve Resolution	350
4.3.2.3	Predictive Maintenance to Minimize System Downtime	353
	References	354
4.4	Addressing Analytical Challenges in a Modern HPLC Laboratory	355
	<i>Frank Steiner and Soo Hyun Park</i>	
4.4.1	Vanquish Core, Flex, and Horizon – Three Different Tiers, all Dedicated to Specific Requirements	356
4.4.2	Intelligent and Self-Contained HPLC Devices	362
4.4.3	2D-LC for Analyzing Complex Samples and Further Automation Capabilities (see also Chapter 1.1)	363
4.4.3.1	Loop-based Single-Heart-Cut 2D-LC	364
4.4.3.2	Loop-based Multi-Heart-Cut 2D-LC	364
4.4.3.3	Trap-based Single-Heart-Cut 2D-LC for Eluent Strength Reduction	366
4.4.3.4	Trap-based Single-Heart-Cut 2D LC–MS Using Vanquish Dual Split Sampler	367
4.4.4	Software-Assisted Automated Method Development	368
	Abbreviations	374
	References	374
4.5	Systematic Method Development with an Analytical Quality-by-Design Approach Supported by Fusion QbD and UPLC–MS	375
	<i>Falk-Thilo Ferse, Detlev Kurth, Tran N. Pham, Fadi L. Alkhateeb, and Paul Rainville</i>	
	References	384
	Index	385