

Contents

Foreword V

Preface IX

List of Contributors XXV

Structure of the Book XXXI

1 Fundamentals of Optimization 1

1.1 Principles of the Optimization of **HPLC** Illustrated by
RP-Chromatography 3
Stavros Kromidas

1.1.1 Before the First Steps of Optimization 3

1.1.2 What Exactly Do We Mean By "Optimization"? 5

1.1.3 Improvement of Resolution ("Separate Better") 6

1.1.3.1 Principal Possibilities for Improving Resolution 8

1.1.3.2 What has the Greatest Effect on Resolution? 10

1.1.3.3 Which Sequence of Steps is Most Logical When Attempting
an Optimization? 11

1.1.3.4 How to Change k , α , and N 17

1.1.3.4.1 Isocratic Mode 17

1.1.3.4.2 Gradient Mode 18

1.1.3.4.3 Acetonitrile or Methanol? 19

1.1.4 Testing of the Peak Homogeneity 22

1.1.5 Unknown Samples: "How Can I Start?"; Strategies and Concepts 35

1.1.5.1 The "Two Days Method" 36

1.1.5.2 "The 5-Step Model" 39

1.1.6 Shortening of the Run Time ("Faster Separation") 48

1.1.7 Improvement of the Sensitivity
("To See More", i.e. Lowering of the Detection Limit) 48

1.1.8	Economics in HPLC ("Cheaper Separation")	48
1.1.9	Final Remarks and Outlook	51
	<i>References</i>	57
1.2	Fast Gradient Separations	59
	<i>Uwe D. Neue, Yung-Fong Cheng, and Ziling Lu</i>	
1.2.1	Introduction	59
1.2.2	Main Part	59
1.2.2.1	Theory	59
1.2.2.2	Results	61
1.2.2.2.1	General Relationships	61
1.2.2.2.2	Short Columns, Small Particles	62
1.2.2.2.3	An Actual Example	64
1.2.2.3	Optimal Operating Conditions and Limits of Currently Available Technology	66
1.2.2.4	Problems and Solutions	67
1.2.2.4.1	Gradient Delay Volume	67
1.2.2.4.2	Detector Sampling Rate and Time Constant	68
1.2.2.4.3	Ion Suppression in Mass Spectrometry	69
	<i>References</i>	70
1.3	pH and Selectivity in RP-Chromatography	71
	<i>Uwe D. Neue, Alberto Méndez, KimVan Tran, and Diane M. Diehl</i>	
1.3.1	Introduction	71
1.3.2	Main Section	71
1.3.2.1	Ionization and pH	71
1.3.2.2	Mobile Phase and pH	73
1.3.2.2.1	Buffer Capacity	74
1.3.2.2.2	Changes of pK and pH Value in the Presence of an Organic Solvent	76
1.3.2.3	Buffers	78
1.3.2.3.1	Classical HPLC Buffers	78
1.3.2.3.2	MS-Compatible pH Control	79
1.3.2.4	Influence of the Samples	79
1.3.2.4.1	The Sample Type: Acids, Bases, Zwitterions	80
1.3.2.4.2	Influence of the Organic Solvent on the Ionization of the Analytes	81
1.3.3	Application Example	81
1.3.4	Troubleshooting	85
1.3.4.1	Reproducibility Problems	85
1.3.4.2	Buffer Strength and Solubility	86
1.3.4.3	Constant Buffer Concentration	86
1.3.5	Summary	87
	<i>References</i>	87

- 1.4** **Selecting the Correct pH Value for HPLC** 89
Michael McBrien
- 1.4.1 Introduction 89
- 1.4.2 Typical Approaches to pH Selection 90
- 1.4.3 Initial pH Selection 91
- 1.4.4 Basis of pK_a Prediction 92
- 1.4.5 Correction of pH Based on Organic Content 93
- 1.4.6 Optimization of Mobile Phase pH Without Chemical Structures 94
- 1.4.7 A Systematic Approach to pH Selection 96
- 1.4.8 An Example – Separation of 1,4-Bis[(2-pyridin-2-ylethyl)thio]butane-2,3-diol from its Impurities 97
- 1.4.9 Troubleshooting Mobile Phase pH 102
- 1.4.10 The Future 102
- 1.4.11 Conclusion 103
- References* 103
- 1.5** **Optimization of the Evaluation in Chromatography** 105
Hans-Joachim Kuss
- 1.5.1 Evaluation of Chromatographic Data – An Introduction 105
- 1.5.2 Working Range 105
- 1.5.3 Internal Standard 106
- 1.5.4 Calibration 107
- 1.5.5 Linear Regression 107
- 1.5.6 Weighting Exponent 110
- 1.5.7 In Real Practise 111
- 1.5.8 Drug Analysis 111
- 1.5.9 Measurement Uncertainty 112
- 1.5.10 Calibration Line Through the Origin 115
- References* 115
- Calibration Characteristics and Uncertainty – Indicating Starting Points to Optimize Methods 117
Stefan Schömer
- 1.6.1 Optimizing Calibration – What is the Objective? 117
- 1.6.2 The Essential Performance Characteristic of Calibration 118
- 1.6.3 Examples 118
- 1.6.3.1 Does Enhanced Sensitivity Improve Methods? 118
- 1.6.3.2 A Constant Variation Coefficient – Is it Good, Poor or Just an Inevitable Characteristic of Method Performance? 122
- 1.6.3.3 How to Prove Effects Due to Matrices – May the Recovery Function be Replaced? 133
- 1.6.3.4 Having Established Matrix Effects – Does Spiking Prove Necessary in Every Case? 136

1.6.3.5	Testing Linearity – Does a Calibration Really Need to Fit a Straight Line?	139
1.6.3.6	Enhancing Accuracy – Obtaining 'Robust' Calibration Functions with Weighting	143
	References	147
2	Characteristics of Optimization in Individual HPLC Modes	149
2.1	RP-HPLC	151
2.1.1	Comparison and Selection of Commercial RP-Columns	151
	Stavros Kromidas	
2.1.1.1	Introduction	151
2.1.1.2	Reasons for the Diversity of Commercially Available RP-Columns – First Consequences	151
2.1.1.2.1	On Polar Interactions	156
2.1.1.2.2	First Consequences	156
2.1.1.3	Criteria for Comparing RP-Phases	174
2.1.1.3.1	Similarity According to Physico-chemical Properties	174
2.1.1.3.2	Similarity Based on Chromatographic Behavior; Expressiveness of Retention and Selectivity Factors	175
2.1.1.3.3	Tests for the Comparison of Columns and Their Expressiveness	181
2.1.1.4	Similarity of RP-Phases	195
2.1.1.4.1	Selectivity Maps	196
2.1.1.4.2	Selectivity Plots	200
2.1.1.4.3	Selectivity Hexagons	205
2.1.1.4.4	Chemometric Analysis of Chromatographic Data	229
2.1.1.5	Suitability of RP-Phases for Special Types of Analytes and Proposals for the Choice of Columns	233
2.1.1.5.1	Polar and Hydrophobic RP-Phases	233
2.1.1.5.2	Suitability of RP-Phases for Different Classes of Substances	237
2.1.1.5.3	Procedure for the Choice of an RP-Column	248
	References	253
2.1.2	Column Selectivity in RP-Chromatography	254
	Uwe D. Neue, Bonnie A. Alden, and Pamela C. Iraneta	
2.1.2.1	Introduction	254
2.1.2.2	Main Section	255
2.1.2.2.1	Hydrophobicity and Silanol Activity (Ion Exchange)	255
2.1.2.2.2	Polar Interactions (Hydrogen Bonding)	259
2.1.2.2.3	Reproducibility of the Selectivity	261
	References	263

- 2.1.3 The Use of Principal Component Analysis for the Characterization of Reversed-Phase Liquid Chromatographic Stationary Phases 264
Melvin R. Euerby and Patrik Petersson
- 2.1.3.1 Introduction 264
- 2.1.3.2 Theory of Principal Component Analysis 265
- 2.1.3.3 PCA of the Database of RP Silica Materials 267
- 2.1.3.3.1 PCA of Polar Embedded, Enhanced Polar Selectivity, and AQ/Aqua Phases 269
- 2.1.3.3.2 PCA of Perfluorinated Phases 270
- 2.1.3.4 Use of PCA in the Identification of Column/Phase Equivalency 271
- 2.1.3.5 Use of PCA in the Rational Selection of Stationary Phases for Method Development 277
- 2.1.3.5.1 Proposed Solvent/Stationary Phase Optimization Strategy 278
References 279
- 2.1.4 Chemometrics – A Powerful Tool for Handling a Large Number of Data 280
Cinzia Stella and Jean-Luc Veuthey
- 2.1.4.1 Introduction 280
- 2.1.4.2 Chromatographic Tests and Their Importance in Column Selection 280
- 2.1.4.3 Use of Principal Component Analysis (PCA) in the Evaluation and Selection of Test Compounds 281
- 2.1.4.3.1 Physicochemical Properties of Test Compounds 281
- 2.1.4.3.2 Chromatographic Properties of Test Compounds 284
- 2.1.4.4 Use of PCA for the Evaluation of Chromatographic Supports 285
- 2.1.4.4.1 Evaluation of Chromatographic Supports in Mobile Phases Composed of pH 7.0 Phosphate Buffer 286
- 2.1.4.4.2 Evaluation of Chromatographic Supports in Mobile Phases Composed of pH 3.0 Phosphate Buffer 289
- 2.1.4.5 How a Chromatographic Test can be Optimized by Chemometrics 291
- 2.1.4.5.1 Test Compounds 291
- 2.1.4.5.2 Mobile Phases 292
- 2.1.4.5.3 Chromatographic Parameters and Batch (Column) Reproducibility 292
- 2.1.4.6 Conclusion and Perspectives 295
References 295

- 2.1.7.6 Hydrogen Bonding 345
- 2.1.7.7 Some Practical Considerations 345
- 2.1.7.8 Future Aspects 347
 - References* 347

- 2.2 Optimization in Normal-Phase HPLC 349
 - Veronika R. Meyer*

- 2.2.1 Introduction 349
- 2.2.2 Mobile Phases in NP-HPLC 350
- 2.2.3 Stationary Phases in NP-HPLC 354
- 2.2.4 Troubleshooting in Normal-Phase HPLC 356
 - References/Further Reading* 357

- 2.3 Optimization of GPC/SEC Separations by Appropriate Selection of the Stationary Phase and Detection Mode 359
 - Peter Kilz*

- 2.3.1 Introduction 359
- 2.3.2 Fundamentals of GPC Separations 360
 - 2.3.2.1 Chromatographic Modes of Column Separation 362
 - 2.3.2.2 GPC Column Selection Criteria and Optimization of GPC Separations 364
 - 2.3.2.2.1 Selection of Pore Size and Separation Range 364
 - 2.3.2.2.2 Advantages and Disadvantages of Linear or Mixed-Bed Columns 365
 - 2.3.2.3 HighSpeed GPC Separations 367
 - 2.3.3 The Role of Comprehensive Detection in the Investigation of Macromolecular Materials 369
 - 2.3.3.1 Coupling of Liquid Chromatography with Information-Rich Detectors 371
 - 2.3.3.2 Copolymer GPC Analysis by Multiple Detection 372
 - 2.3.3.3 Simultaneous Separation and Identification by GPC-FTIR 375
 - 2.3.3.4 Application of Molar Mass-Sensitive Detectors in GPC 377
 - 2.3.3.4.1 Light-Scattering Detection 377
 - 2.3.3.4.2 Viscometry Detection 379
- 2.3.4 Summary 380
 - References* 381

- 2.4 Gel Filtration/Size-Exclusion Chromatography (SEC) of Biopolymers – Optimization Strategies and Troubleshooting 383
 - Milena Quaglia, Egidijus Machtejevas, Tom Hennessy, and Klaus K. Unger*

- 2.4.1 Where Are We Now and Where Are We Going? 383
- 2.4.2 Theory in Brief 384
- 2.4.3 SEC vs. HPLC Variants 387

2.4.4	Optimization Aspects in SEC of Biopolymers	388
2.4.4.1	Column Selection and Optimal Flow Rate	388
2.4.4.2	Optimization of the Mobile Phase	392
2.4.4.3	Sample Preparation	394
2.4.4.4	Sample Viscosity and Sample Volume – Two Critical Parameters at Injection	395
2.4.4.5	Detection Methods	396
2.4.5	Applications	397
2.4.5.1	High-Performance SEC	397
2.4.5.2	Determination of Molecular Weight	398
2.4.5.3	Gel Filtration as a Tool to Study Conformational Changes of Proteins	398
2.4.5.4	Gel Filtration in Preparative and Process Separations (Downstream Processing)	399
2.4.5.5	SEC Columns Based on the Principle of Restricted Access and Their Use in Proteome Analysis	400
	References	403
2.5	Optimization in Affinity Chromatography	405
	<i>Egbert Müller</i>	
2.5.1	Introduction to Resin Design and Method Development in Affinity Chromatography	405
2.5.2	Base Matrix	408
2.5.3	Immobilization Methods	409
2.5.4	Activation Methods	409
2.5.5	Spacer	412
2.5.6	Site-Directed Immobilization	415
2.5.7	Non-Particulate Affinity Matrices	416
2.5.8	Affinity Purification	417
2.5.9	Factorial Design for the Preparation of Affinity Resins	419
2.5.10	Summary of Immobilization	423
	References	423
2.6	Optimization of Enantiomer Separations in HPLC	427
	<i>Markus Juza</i>	
2.6.1	Introduction	427
2.6.2	Basic Principles of Enantioselective HPLC	427
2.6.2.1	Thermodynamic Fundamentals of Enantioselective HPLC	429
2.6.2.2	Adsorption and Chiral Recognition	430
2.6.2.3	Differences to Reversed-Phase and Normal-Phase HPLC	433
2.6.2.4	Principles for Optimization of Enantioselective HPLC Separations	433
2.6.3	Selectors and Stationary Phases	433
2.6.4	Method Selection and Optimization	440

- 2.6.4.1 Cellulose and Amylose Derivatives 441
- 2.6.4.2 Immobilized Cellulose and Amylose Derivatives 443
- 2.6.4.3 Stationary Phases Derived from Tartaric Acid 444
- 2.6.4.4 n-Acidic and n-Basic Stationary Phases 444
- 2.6.4.5 Macrocyclic Selectors, Cyclodextrins, and Antibiotics 446
- 2.6.4.6 Proteins and Peptides 450
- 2.6.4.7 Ruthenium Complexes 450
- 2.6.4.8 Synthetic and Imprinted Polymers 450
- 2.6.4.9 Metal Complexation and Ligand-Exchange Phases 451
- 2.6.4.10 Chiral Ion Exchangers 451
- 2.6.5 Avoiding Errors and Troubleshooting 452
 - 2.6.5.1 Equipment and Columns – Practical Tips 452
 - 2.6.5.2 Detection 454
 - 2.6.5.3 Mistakes Originating from the Analyte 454
- 2.6.6 Preparative Enantioselective HPLC 454
 - 2.6.6.1 Determination of the Loading Capacity 455
 - 2.6.6.2 Determination of Elution Volumes and Flow Rates 456
 - 2.6.6.3 Enantiomer Separation using Simulated Moving Bed (SMB) Chromatography 458
 - 2.6.6.3.1 Principles of Simulated Moving Bed Chromatography 458
 - 2.6.6.3.2 Separation of Commercial Active Pharmaceutical Ingredients by SMB 459
- 2.6.7 Enantioselective Chromatography by the Addition of Chiral Additives to the Mobile Phase in HPLC and Capillary Electrophoresis 461
- 2.6.8 Determination of Enantiomeric Purity Through the Formation of Diastereomers 462
- 2.6.9 Indirect Enantiomer Separation on a Preparative Scale 462
- 2.6.10 Enantiomer Separations Under Supercritical Fluid Chromatographic (SFC) Conditions 462
- 2.6.11 New Chiral Stationary Phases and Information Management Software 463
- 2.6.12 Summary 463
 - References* 464
- 2.7 Miniaturization 467**
 - 2.7.1 mLC/NanoLC – Optimization and Troubleshooting 467
 - Jürgen Maier-Rosenkranz*
 - 2.7.1.1 Introduction 467
 - 2.7.1.2 Sensitivity 467
 - 2.7.1.2.1 Influence of Column Length 467
 - 2.7.1.2.2 Influence of Column Internal Diameter 467
 - 2.7.1.2.3 Influence of Stationary Phase 469
 - 2.7.1.3 Robustness 469

2.7.1.3.1	System Choice	469
2.7.1.3.2	Capillary Connections	472
2.7.1.3.3	Precautions Against Blocking	477
2.7.1.3.4	Testing for Leakages	478
2.7.1.3.5	Guard Column Switching and Sample Loading Strategies	478
2.7.1.4	Sensitivity/Resolution	483
2.7.1.4.1	Column Dimensions	483
2.7.1.4.2	Packing Materials/Surface Covering	484
2.7.1.4.3	Detectors	484
	<i>References</i>	486
2.7.2	Microchip-Based Liquid Chromatography – Techniques and Possibilities	487
	<i>Jorg P. Kutter</i>	
2.7.2.1	Introduction	487
2.7.2.2	Techniques	488
2.7.2.2.1	Pressure-Driven Liquid Chromatography (LC)	488
2.7.2.2.2	Open-Channel Electrochromatography (OCEC)	488
2.7.2.2.3	Packed-Bed Electrochromatography	488
2.7.2.2.4	Microfabricated Chromatographic Beds (Pillar Arrays)	489
2.7.2.2.5	In Situ Polymerized Monolithic Stationary Phases	489
2.7.2.3	Optimization and Possibilities	490
2.7.2.3.1	Separation Performance	490
2.7.2.3.2	Isocratic and Gradient Elution	491
2.7.2.3.3	Tailor-Made Stationary Phases	492
2.7.2.3.4	Sample Pretreatment and More-Dimensional Separations	492
2.7.2.3.5	Issues and Challenges	492
2.7.2.4	Application Examples	493
2.7.2.5	Conclusions and Outlook	496
	<i>References</i>	496
2.7.3	Ultra-Performance Liquid Chromatography	498
	<i>Uwe D. Neue, Eric S. Crumbach, Marianna Kele, Jeffrey R. Mazzeo, and Dirk Sievers</i>	
2.7.3.1	Introduction	498
2.7.3.2	Isocratic Separations	499
2.7.3.3	Gradient Separations	502
	<i>References</i>	505

- 3 Coupling Techniques 507**
- 3.1 Immunochromatographic Techniques 509**
Michael G. Weller
- 3.1.1 Introduction 509
- 3.1.2 Binding Molecules 509
- 3.1.3 Immunoassays 511
- 3.1.4 Immunochromatographic Techniques 511
- 3.1.4.1 Affinity Enrichment (AffinitySPE) 513
- 3.1.4.2 "Weak Affinity Chromatography"
(True Affinity Chromatography) 519
- 3.1.4.3 Biochemical Detectors 520
- 3.1.5 Examples 522
- 3.1.5.1 Example 1: Affinity Extraction (AffinitySPE) 522
- 3.1.5.2 Example 2: "Weak Affinity Chromatography" (WAC) 523
- 3.1.5.3 Example 3: Biochemical Detection 525
- References* 525
- 3.2 Enhanced Characterization and Comprehensive Analyses
by Two-Dimensional Chromatography 527**
Peter Kilz
- 3.2.1 Introduction 527
- 3.2.2 How Can I Take Advantage?— Experimental Aspects 529
- 3.2.3 2D Data Presentation and Analysis 533
- 3.2.4 The State-of-the-Art in 2D Chromatography 535
- 3.2.5 Summary 539
- References* 540
- 3.3 LC/MS – Hints and Recommendations on Optimization and
Troubleshooting 541**
Friedrich Mandel
- 3.3.1 Optimization of the Ionization Process 541
- 3.3.2 Lost LC/MS Peaks 542
- 3.3.2.1 Mobile Phase pH at the Edge of the Optimum Range 543
- 3.3.2.2 Ion-Pairing Agents in the HPLC System 543
- 3.3.2.3 Ion Suppression by the Sample Matrix or Sample
Contaminants 544
- 3.3.3 How Clean Should an LC/MS Ion Source Be? 544
- 3.3.4 Ion Suppression 545
- References* 549

- 3.4 LC-NMR Coupling 551
Klaus Albert, Manfred Krucker, Karsten Putzbach, and Marc D. Grynbaum
- 3.4.1 NMR Basics 551
- 3.4.2 Sensitivity of the NMR Experiment 552
- 3.4.3 NMR Spectroscopy in Flowing Systems 553
- 3.4.4 NMR Probes for LC-NMR 553
- 3.4.5 Practical Realization of Analytical HPLC-NMR and Capillary-HPLC-NMR 554
- 3.4.6 Continuous-Flow Measurements 555
- 3.4.7 Stopped-Flow Measurements 557
- 3.4.8 Capillary Separations 559
- 3.4.9 Outlook 560
References 563

- 4 **Computer-Aided Optimization** 565

- 4.1 Computer-Facilitated HPLC Method Development Using **DryLab**[®] Software 567
Lloyd R. Snyder and Loren Wisley
- 4.1.1 Introduction 567
- 4.1.1.1 History 569
- 4.1.1.2 Theory 570
- 4.1.2 DryLab Capabilities 570
- 4.1.2.1 DryLab Operation 570
- 4.1.2.2 Mode Choices 571
- 4.1.3 Practical Applications of DryLab[®] in the Laboratory 572
- 4.1.4 Conclusions 584
References 585

- 4.2 **ChromSword**[®] Software for Automated and Computer-Assisted Development of HPLC Methods 587
Sergey Calushko, Vsevolod Tanchuk, Irina Shishkina, Oleg Pylypchenko, and Wolf-Dieter Beinert
- 4.2.1 Introduction 587
- 4.2.1.1 Off-Line Mode 587
- 4.2.1.2 On-Line Mode 587
- 4.2.2 ChromSword[®] Versions 587
- 4.2.3 Experimental Set-Up for On-Line Mode 588
- 4.2.4 Method Development with ChromSword[®] 588
- 4.2.4.1 Off-Line Mode (Computer-Assisted Method Development) 588
- 4.2.4.2 On-Line Mode – Fully Automated Optimization of Isocratic and Gradient Separations 592
- 4.2.4.2.1 Software Functions for Automation 597

5.3	Separation of Complex Sample Mixtures	669
	<i>Knut Wagner</i>	
5.3.1	Introduction	669
5.3.2	Multidimensional HPLC	670
5.3.3	Techniques for Multidimensional Separations	672
5.3.3.1	Off-Line Technique	672
5.3.3.2	On-Line Technique	672
5.3.4	On-Line Sample Preparation as a Previous Stage of Multidimensional HPLC	674
5.3.5	Fields of Application of Multidimensional HPLC	675
5.3.5.1	What can be Realized?– A Practical Example	676
5.3.6	Critical Parameters of Multidimensional HPLC	682
	<i>References</i>	683
5.4	Evaluation of an Integrated Procedure for the Characterization of Chemical Libraries on the Basis of HPLC-UV/MS/CLND	685
	<i>Mario Arangio, Federico R Sirtori, Katia Marcucci, Giuseppe Razzano, Maristella Colombo, Roberto Biancardi, and Vincenzo Rizzo</i>	
5.4.1	Introduction	685
5.4.2	Materials and Methods	686
5.4.2.1	Instrumentation	686
5.4.2.2	Chemicals and Consumables	686
5.4.2.3	High-Throughput Platform (HTP1) Method Set-up	688
5.4.2.4	Chromatographic Conditions	688
5.4.2.5	Mass Spectrometer and CLND Conditions	689
5.4.2.6	Data Processing and Reporting	689
5.4.2.7	Multilinear Regression Analysis for the Derivation of CLND Response Factors	690
5.4.3	Results and Discussion	691
5.4.3.1	Liquid Chromatography and UV Detection	691
5.4.3.2	Mass Spectrometric Method Development	692
5.4.3.3	CLND Set-Up	693
5.4.3.4	Validation with Commercial Standards	693
5.4.3.5	Validation with Proprietary Compounds	695
5.4.4	Conclusions	699
	<i>References</i>	700
	Appendix	703
	Subject Index	729