



PRACTICAL
HPLC METHOD
DEVELOPMENT

SECOND EDITION

Lloyd R. Snyder • Joseph J. Kirkland • Joseph L. Glajch

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Second Edition

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PREFACE

The first edition of this book, published in 1988, described systematic procedures for developing an HPLC method, based on the best information available at that time. HPLC method development involves several essential steps: sample pretreatment, detection of sample bands, choosing separation conditions, quantitation, and method validation. Our earlier edition emphasized choosing separation conditions with little attention to other important areas. The goals of the present book are to present up-to-date insights on choosing separation conditions and to provide a more complete treatment of all aspects of HPLC method development. Chapter 3 addresses detection, Chapter 4 sample pretreatment, Chapter 14 quantitation, and Chapter 15 method validation.

To quote from the first edition, our recommendation was “to minimize the use of information on sample structure and carry out method development for most samples in the same general way.” Favored conditions for an initial separation were suggested, and the use of this initial run as a basis for the next experiment was described. These two runs were then used to select conditions for the next experiment, and the process was continued until an acceptable separation was achieved. This systematic iterative procedure can be described as “enlightened trial and error.” At that time we felt that most samples encountered in typical laboratories could be handled in this fashion. Nevertheless, a number of important exceptions to this approach were indicated, especially samples of biological origin, mixtures of enantiomers, and preparative separations. Since 1988 the relative importance of these exceptions has increased greatly. In this second edition we

give these specialized applications of HPLC much more attention (Chapters 11 to 13).

Finally, the science of HPLC separation and means for the development of HPLC methods have continued to receive study by numerous groups around the world. Since 1988 a large body of new and valuable information has become available. This has led to important insights and improvements relating to HPLC columns and method development that are not covered in the first edition. The present book addresses each of these limitations of the first edition. All aspects of the subject are now covered, and the information provided has been supplemented with the literature that has appeared since 1987. Chapters 5 to 10 provide a reasonably complete account of how HPLC separation depends on the choice of column and other separation conditions.

Unfortunately, one result of these various enhancements to the first edition is a book that is now more than twice as long. Because the various parts of the present book are strongly interrelated, it is useful at this point to provide an overview of our subject matter and to point out how different chromatographers can use this book most effectively. Chapter 1 provides an introduction to how method development is carried out for any sample, and this should be read before proceeding further. Chapter 2 offers a review of the basics of HPLC separation, plus important background information that the chromatographer should know before beginning method development. Less experienced chromatographers will benefit by first studying Chapter 2, plus a selective reading of Sections 3.1 and 3.2 on UV detection, before reading further.

Following a review by the reader of Chapters 1 to 3, the next step depends on the nature of the sample: (a) biological samples (peptides, proteins, oligonucleotides, etc.), (b) enantiomeric samples, (c) samples requiring

pretreatment, or (d) other samples. For samples that cannot be injected directly into the HPLC system, Chapter 4 provides a comprehensive review of the aims and means of various pretreatment procedures. Readers with an interest in biological samples will find Chapters 8 (gradient elution) and 11 (biological samples) most useful at this point. Workers attempting to separate enantiomeric mixtures (chiral compounds) will want to read Chapter 12.

The majority of chromatographers, whose samples are neither biological samples nor enantiomeric mixtures, will benefit by first reading Chapter 9, which deals with a systematic approach for the separation of most samples by reversed-phase HPLC. *The recommendations of Chapter 9 have been changed in important ways since the appearance of the first edition.* We believe that these latest method-development recommendations will prove even more efficient and practical than those described earlier.

Chapter 5 gives an overview of HPLC columns, including their design, specifications, column care, and troubleshooting. If any problems are encountered during method development as described in Chapter 9, or if that approach does not result in an acceptable separation, Chapters 6 to 8 (non-ionic and ionic samples, gradient elution) provide a thorough discussion of how the separation of typical samples can be achieved by varying additional separation conditions beyond those discussed in Chapter 9. These chapters are useful when the separation of the sample proves more challenging, either because of sample complexity or the chemical similarity of sample components. When the recovery of purified compounds by HPLC is required, separations developed on an analytical scale can be scaled up using information contained in Chapter 13 (preparative HPLC). However, Chapter 13 is best read after examining Chapters 6 to 9.

Chapter 10 deals with the use of computer programs that can facilitate HPLC method development. For the most part, these commercially available programs use a small number of starting experiments as a basis for computer predictions of separation as a function of separation conditions. In this way, the user can carry out the majority of method development experiments with the computer instead of much more slowly and with greater effort in the laboratory. Although the use of computers in this way began in the late 1970s, users have been slow to adopt this approach to method development. However, the popularity of these programs has been on a sharp increase since 1990, and today they are widely accepted as an important tool for HPLC method development.

We have made further use of computer simulation for some examples presented in this book. In several cases, data from the literature or from our individual laboratories could be used to show experiments where one or more variables were changed. Because of the documented reliability of computer simulation, these examples are equivalent to real experiments.

After conditions have been adjusted for an acceptable separation, additional steps are required for use of the method in quantitative analysis (Chapter 14). Finally, the resulting method must be evaluated to show that it consistently provides accurate and precise results and can be transferred to other laboratories and personnel. Chapter 15 on method validation, concludes the second edition, except for some useful appendices referred to in the text.

The size of the present book presents a challenge to the reader in terms of locating material that is relevant to a particular sample or problem. Often, it will be necessary to look in different places for the total answer to a particular question. As an aid in this information search, we have provided extensive cross-referencing to other parts of the

book. This can have the undesirable effect of breaking up the continuity of the text when the book is being read for general background and information. For this reason we suggest that the reader ignore cross-references unless the topic being discussed is of special interest. We have also taken special care to provide a comprehensive index, which should be an effective tool in the use of this book. Again, cross-referencing is used as much as possible for convenience to the reader.

It is our belief that research dealing with HPLC method development is now beginning to level off. The technique of HPLC is over 30 years old and is in a mature stage of development and application. Many of the pioneers in this field have moved on to other areas, such as capillary electrophoresis, supercritical fluid chromatography, and field-flow fractionation. For this reason we believe that the present book should remain useful and reasonably up-to-date for the next decade or two. As in the first edition, all the contributors to this edition are active practitioners of HPLC method development. Much of the advice or recommendations offered in this book is the result of our personal observations in the laboratory.

Finally, we would like to acknowledge the support of our families and thank numerous friends and associates for their invaluable contribution to this book. First, we must acknowledge the contributions of those contributing to certain chapters: Ira Krull and Mike Szulc (Chapter 3), Ron Majors and Greg Slack (Chapter 4), Barry Boyes and Andy Alpert (Chapter 11), and John Kern and Karin Kirkland (Chapter 12). Second, the painstaking review of individual chapters was carried out by a large group of chromatographers, some of whom have read most of the book: John Dolan, Sal Fusari, Tom Jupille, and Derek Southern. Others have provided important inputs to one or more individual chapters: Andy Alpert, Brian Bidlingmeyer,

Pete Carr, Tom Catalano, Bill Cooper, Geoff Cox, Andrew Deputy, Joe DeStefano, John Dorsey, Roger W. Giese, J. Mark Green, Dick Henry, Jeff Hurst, Pavel Jandera, John P. Larmann, Jr., Gordon Marr, Donald Parriott, Dennis Saunders, Peter Schoenmakers, Marilyn Stadalius, Gyula Vigh, Tom Waeghe, Chuck Whitney and Reed C. Williams. This book owes much to our reviewers, and for this dedicated (but inadequately reimbursed) effort the authors are deeply appreciative.

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GLOSSARY OF SYMBOLS AND TERMS

The units assumed in this book for all symbols are given below, unless the quantity is dimensionless. Where a defining equation appears in the book, the equation number is given in parentheses after the definition below.

Common Terms

A

absorbance ([Eqs. 3.1, 3.2](#)); also area; also weak solvent or gradient from A → B

ACN

acetonitrile

B (%B)

refers to the strong solvent in a binary-solvent mobile phase (% v/v)

B, BH⁺

a basic solute (Chapter 7)

C₈, C₁₈

chain length (octyl or octadecyl) of alkyl bonded phase

CD

cyclodextrin

CV

coefficient of variation (usually in %); [Eq. 15.3](#)

d_c

column internal diameter (cm)

d_p

particle diameter (μm)

DAD

diode-array detector

EC

electrochemical (detector)

F

flow rate (mL/min)

FL

fluorescence (detector)

G_S

gradient steepness parameter ([Eq. 8.2a](#)); $k^* = 20/G_S$

h'

peak height

HP

Hewlett-Packard

HPLC

high-performance liquid chromatography

ID

internal diameter, d_C

IEC

ion-exchange chromatography

IPC

ion-pair chromatography

k

retention factor ([Eq. 2.4](#))

k^*

effective or average value of k in gradient elution ([Eq. 8.1](#))

k_a, k_z

values of k for first (a) and last (z) bands in the chromatogram

L

column length (cm)

LC-MS

liquid chromatography-mass spectrometry

M

molecular weight

MC

methylene chloride

MeOH

methanol

MS

mass spectrometric

MTBE

methyl-*t*-butyl ether

N

column plate number ([Eqs. 2.8-2.8b](#))

N'

noise ([Eq. 3.3](#), [Fig. 3.3](#))

NARP

non-aqueous reversed-phase HPLC

NPC

normal-phase chromatography

P

column pressure drop (usually psi) ([Eq. 2.9](#))

pK_a

acidity constant for an acid or protonated base

PAH

polyaromatic hydrocarbon

R_s

resolution ([Eq. 2.1](#))

RI

refractive index

RPC

reversed-phase chromatography

S

signal; [Eq. 3.3](#); [Fig. 3.3](#); also, parameter defined by [Eq. 6.1](#)

t_D

delay or dwell time (min in gradient elution); equal to V_D/F

t_G

gradient time (min)

t_R

retention time (min) ([Fig. 2.2](#)); equal to $t_0(1 + k)$

t_{Ra}, t_{Rz}

retention times t_R for first (a) and last (z) bands in a chromatogram (min) ([Fig. 8.6a](#))

t_0

column dead time (min) ([Eq. 2.5](#))

t_1, t_2

retention times for adjacent bands 1 and 2 (min)

TEA

triethylamine

TFA

trifluoroacetic acid

THF

tetrahydrofuran

UV

ultraviolet

V_D

delay or dwell volume (mL); volume between gradient mixer and column inlet (including mixer volume)

V_m

column dead volume (mL) ([Eq. 2.6](#)); V_m is the volume of mobile phase inside the column, not including any solvent attached to the stationary phase

V_{max}

maximum sample volume (mL) ([Eq. 13.1](#))

V_s

sample volume (mL)

w

sample weight (mg); also, bandwidth at half-height (min)

W_{max}

maximum weight of injected sample (mg) that does not overload the column ([Eq. 2.17](#))

w_s

saturation capacity of the column (mg) ([Eq. 13.4](#))

W

baseline bandwidth (min) ([Fig. 2.2](#))

W_{th}

contribution to baseline bandwidth as a result of a large sample weight (min) ([Eq. 13.2](#))

W_0

baseline bandwidth for a small sample (min) ([Eq. 13.2](#))

W_1, W_2

baseline bandwidths for adjacent bands (min)

$W_{1/2}$

bandwidth at half-height (min) ([Fig. 1.1](#))

α

separation factor, equal to k_2/k_1 , where k_1 and k_2 refer to k values for adjacent bands 1 and 2

Δt_R

$t_{Rz} - t_{Ra}$ (min)

$\Delta\% B$

change in % B during gradient elution

ϵ

molar absorptivity

ϵ^0

strength of a solvent or solvent mixture for normal-phase HPLC

η

viscosity (cP)

Less Common Items

A, B, C

constants in [Eq. 2.11](#); the values of $A, B,$ and C vary with the value of k but should be approximately constant for other changes in conditions or for other solutes

A', B', C'

constants in [Eq. 2.10](#); values of $A', B',$ and C' vary with conditions and with the sample

A'', B'', C''

constants in [Eq. 2.10a](#); values of $A'', B'',$ and C'' vary with conditions and with the sample

C

band concentration at maximum (mol/L) ([Eq. 3.2](#))

C_0

analyte concentration in injected sample (mol/L)

CI

chemical ionization (MS)

DCA

direct current amperometry

DMA

N,N-dimethyl-1-naphthylamide; also, dimethylaniline

EI

electron ionization (MS)

ELS

evaporative light scattering

ESI

electrospray ionization (MS)

EtOAc

ethyl acetate

FAB

fast-atom bombardment (MS)

FD

field desorption (MS)

h

reduced plate height, equal to H/d_p ([Eq. 2.11](#))

HB

hydroxybenzoic acid ([Figs. 7.8](#), [7.17](#), and [7.19](#))

HFBA

heptafluorobutyric acid

IPA

isopropanol

k_w

value of k for water as mobile phase ([Eq. 6.1](#))

LCEC

liquid chromatographic electrochemical (detection)

LD

laser desorption (MS)

LSIMS

liquid secondary ion mass spectrometry

MALDI

matrix-assisted laser desorption ionization (MS)

MP

methyl paraben ([Figs. 7.8](#), [7.17](#), and [7.19](#))

$[P^-]_m$

concentration of ion-pair reagent P^- in mobile phase (mmol/L)

PAD

pulsed amperometric detection

PBP

polar bonded phase

PD

plasma desorption (MS)

PP

propyl paraben ([Figs. 7.8](#), [7.17](#), and [7.19](#))

PTH

phenylthiohydantoin

R^+ , R^-

charged functional groups in anion and cation ion-exchange columns, respectively ([Eqs. 7.4](#) and [7.5](#)) [e.g., $-N(CH_3)_3^+$ and $-SO_3^-$]

RF

response factor

TBA⁺

tetrabutylammonium ion

tBME

see MTBE

TMS

trimethylsilyl; also C_1

TNB

1,3,5-trinitrobenzene

Statistical test

analysis of variance (ANOVA)

Dixon criteria

Q-test

t-test

Step-gradient

Sterically-protected phases

Steroid sample

Strength, *see Column strength, Solvent strength*

Strong ion exchange column

Strongly retained components

Substituent polarity

Sugars, *see Carbohydrates*

Supercritical fluid extraction (SFE)

CO₂

collection methods

density programming

equipment

extractor diagram

fluids

method development

phase diagram

polar organic modifiers

restrictor

variables

Supported-liquid membrane enrichment

Supports, *see also Silica*

alumina

graphitized carbon

inorganic

porous polymers

silica

zirconia

Surfactants for biomolecules

Suspensions

Synthetic polymers

Syringe injection

System suitability

t-Test

Tailing, *see Band shape*

Tailing factor

Tangent skimming method

Temperature

- band shape in ion-pair chromatography

- band width and shape for proteins

- cavity-type chiral column effects

- chiral separations

- control of

- effect on column stability

- ion-pair chromatography

- ionic samples

- loss of bonded phase

- NPC

- peptides and proteins

- RPC

- selectivity

- sensitivity

- separation of tryptic digest

- variability

Tetrabutylammonium ion

Tetrahydrofuran (THF)

Thermostatting

Theoretical plate number, *see Plate number*

Thin-layer cell

Thin-layer chromatography

Tissue plasminogen activator (TPA)
Total exclusion
Total permeation
Touching band separation
Trace analysis
 accuracy
 calibration
 column switching
 concentration
 detection
 goals
 gradient elution
 precision
 preferred conditions
 recovery
 sample injection
 sample preparation
 sample size effects
Transferability, method
Triazine herbicides
Tricine buffers
Tricyclic antidepressants
Triethylamine (TEA)
 buffers
 ion-pair gradient elution
Trifluoroacetic acid (TFA)
 basic peptides separation
 ion-pair reagent
 selectivity effects
 1,1,2-Trifluorotrchloroethane (FC113)
Tris buffers
Troubleshooting, *see also Problems*
 column

- peptides
- size-exclusion chromatography
- Tryptic digest
- Ultrafiltration
- Ultrasonication
- Universal detectors
- Unretained compound
- Unstable sample
- Uracil, unretained
- UV, *see Detector*
- UV cut-off
- Vacuum filtration
- Validation
 - problems
- Valley-to-height method, resolution
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