PRACTICAL HPLC METHOD DEVELOPMENT

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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 devel-

oped 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)
C_8, C_{18}	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. 8.2*a*); $k^* = 20/G_s$
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 chromatrogram (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. I.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)
Δ% 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. 2.10*a;* 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
TOFMS	time-of-flight mass spectrometry
TSP	thermospray (MS)
u	velocity of mobile phase through the column (cm/s); equal to L/t_0
V	baseline bandwidth (mL)
V_c	contribution to V from band broadening within the column; also, the baseline width (mL) of a peak for a small sample (Eq. 2.16)
V_R	retention volume (mL) (Eq. 2.14); also equal to $t_R F$
W	bandwidth (min) (Eq. 2.12)
W_c, W_s, W_{lc}, W_{fc}	contributions to W from the column, sampling, connecting tubing and flow cell, respectively (min) (Eq. 2.12)
X, X1, X2, X3	solutes of unspecified structure (Figs. 7.8, 7.17, and 7.19)
X_B	mole fraction of B-solvent in the mobile phase
ν	reduced velocity, equal to ud_p/D_m (Eq. 2.11)
σ	one standard deviation of a Gaussian curve; equal to 1/4 of baseline bandwidth
τ	detector time constant (s)
ϕ	volume fraction of B-solvent in the mobile phase; equal to 0.01% B

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