High Performance Liquid Chromatography in Biochemistry

Edited by
Agnes Henschen, Klaus-Peter Hupe,
Friedrich Lottspeich, Wolfgang Voelter

High Performance Liquid Chromatography in Biochemistry

Edited by
Agnes Henschen, Klaus-Peter Hupe,
Friedrich Lottspeich, Wolfgang Voelter

Editors:

Dr. Agnes Henschen
Max-Planck-Institut für Biochemie
D-8033 Martinsried

Dr. Friedrich Lottspeich
Max-Planck-Institut für Biochemie
D-8033 Martinsried

Dr.-Ing. Klaus-Peter Hupe
Hewlett-Packard GmbH
D-7517 Waldbronn 2

Prof. Dr. Wolfgang Voelter
Abt. für Physikalische Biochemie
Physiologisch-chemisches Institut der Universität
D-7400 Tübingen

Editorial Director: Dr. Hans F. Ebel
Production Manager: Dipl.-Ing. (FH) Hans Jörg Maier

Library of Congress Card No. 85-5053

Deutsche Bibliothek Cataloguing-in-Publication Data

High performance liquid chromatography in biochemistry / ed. by Agnes Henschen ... –
Weinheim; Deerfield Beach, Florida; Basel: VCH, 1985.
ISBN 3-527-26057-9 (Weinheim, Basel)
ISBN 0-89573-066-9 (Deerfield Beach)
NE: Henschen, Agnes [Hrsg.]

Composition: Filmsatz Unger, D-6940 Weinheim
Printing: betz-druck GmbH, D-6100 Darmstadt-Arheiligen
Bookbinding: Wilhelm Osswald + Co., D-6730 Neustadt
Printed in the Federal Republic of Germany

Preface

It is hard to imagine what it would mean to research in biochemistry if chromatography were not available as an analytical tool.

Liquid chromatography, in all it's various modes, has become particularly indispensable. Above all, the high performance version of liquid chromatography (HPLC) has brought completely new dimensions to the analytical work of this and all other areas of chemistry. The features most important for the analysis (separation efficiency, speed of analysis and lower level of detection) have all gained improvement of at least one order of magnitude over the last 10 years. The method can now be carried out on a microscale, with small amounts of sample.

The roots of what is today called high performance liquid chromatography go back to the early 1960s. It was then that two crucial elements of the technique came together: 1. A better understanding of the kinetics and thermodynamics of the separation process, leading to column packings and columns of much higher selectivity and efficiency. 2. The development of instrumentation which turned the sequential steps of the analysis process (previously carried out separately and manually) into a continuous, automated procedure. The first instruments to appear on the market as a result of this trend were an Amino Acid Analyser, which was developed by Moore et al. *), and a Nucleic Acid Analyser, based on work by Horvath et al. *). Biochemistry can thus be regarded, from an instrumental and application point of view, as the cradle of HPLC. From there it grew and spread out over almost all areas of chemistry.

Today the technique has matured to such an extent that it can be applied routinely with a high degree of reliability and reproducibility even to the very complex analytical problems typical for biochemistry. This has initiated the use of HPLC by an ever increasing number of biochemists, a trend which is likely to continue. The time therefore seems right for a self-contained appraisal of the technique with special regard to its application in biochemistry.

In this book it has been our aim to give an introduction to the theoretical and instrumental principles of HPLC, followed by a detailed treatment of its application to the various groups of compounds of biochemical interest.

München, Waldbronn and Tübingen,
October 1984

A. Henschen
K.-P. Hupe
F. Lottspeich
W. Voelter

*) Bibliography at end of Chapter 1.

List of Contributors

Bauer, Hermann, Dr.
Abt. für Physikalische Biochemie
Physiologisch-chemisches
Institut der Universität
Hoppe-Seyler-Straße 1
D-7400 Tübingen
Chapter 8

Engelhardt, Heinz, Prof. Dr.
Angewandte Physikalische Chemie
der Universität des Saarlandes
Bau 9
D-6600 Saarbrücken
Chapter 2

Henschen, Agnes, Dr.
Max-Planck-Institut für Biochemie
D-8033 Martinsried
Chapter 4

Hostettmann, Kurt, Prof. Dr.
Ecole de Pharmacie
Université de Lausanne
Place du Château
CH-1005 Lausanne
Chapter 14

Hostettmann, Maryse, Dr.
Ecole de Pharmacie
Université de Lausanne
Place du Château
CH-1005 Lausanne
Chapter 14

Hupe, Klaus-Peter, Dr.-Ing.
Hewlett-Packard GmbH
Postfach 1280
D-7517 Waldbronn 2
Chapters 1, 3

Kronbach, Thomas, Dr.
Biozentrum der Universität
Klingelbergstraße 70
CH-4056 Basel
Chapter 12

Liebich, Hartmut, Dr.
Medizinische Klinik
der Universität
Otfried-Müller-Straße
D-7400 Tübingen
Chapter 13

Lottspeich, Friedrich, Dr.
Max-Planck-Institut für Biochemie
D-8033 Martinsried
Chapter 4

Meyer, Hans Detlef, Dr.
Institut für Klinische Chemie
der Ludwig-Maximilians-Universität
Klinikum Großhadern
Postfach 701 260
D-8000 München 70
Chapter 10

Müller, Hartmut, Dr.
Dynamit Nobel
Forschung und Entwicklung
Geb. 50
D-5210 Troisdorf
Chapter 2

Rozing, Gerard, Dr.
Hewlett-Packard GmbH
Postfach 1280
D-7517 Waldbronn 2
Chapter 3

Schön, Uwe, Dr.
Dynamit Nobel
D-5216 Niederkassel
Chapter 2

Schott, Herbert, Prof. Dr.
Institut für Organische Chemie
der Universität
Auf der Morgenstelle 18
D-7400 Tübingen 1
Chapter 9

Schrenker, Helge, Dipl.-Ing.
Hewlett-Packard GmbH
Postfach 1280
D-7517 Waldbronn 2
Chapter 3

Steinmüller, Dirk
Botanisches Institut der Universität
Lehrstuhl für Pflanzenphysiologie
Kaiserstraße 12
D-7500 Karlsruhe
Chapter 7

Tevini, Manfred, Prof. Dr.
Botanisches Institut der Universität
Lehrstuhl für Pflanzenphysiologie
Kaiserstraße 12
D-7500 Karlsruhe
Chapter 7

Voelter, Wolfgang, Prof. Dr.
Abt. für Physikalische Biochemie
Physiologisch-chemisches
Institut der Universität
Hoppe-Seyler-Straße 1
D-7400 Tübingen
Chapters 5, 8, 11, 12

Zech, Karl, Dr.
Byk Gulden Lomberg
Byk-Gulden-Straße 2
D-7750 Konstanz
Chapter 6

List of Chromatographic Symbols

A_s	asymmetry factor
A_c	free cross-sectional area of the column
B_0	column permeability
c	concentration
c_M	solute concentration in the mobile phase
c_S	solute concentration in the stationary phase
dp	particle size
D_M	diffusion coefficient in the mobile phase
E	extraction constant
F	flow-rate
h	height equivalent to a theoretical plate (HETP)
h_{min}	minimum plate height
H	height equivalent to one effective plate
k	capacity ratio (capacity factor, mass distribution ratio)
K	distribution coefficient, partition coeffient
K^*	adsorption coefficient
l	column length
n	number of theoretical plates
N	number of effective plates
Osp	specific surface area
r	column radius
R	gas constant
R_D	response factor
R_S	resolution
t_M	solvent hold-up time (dead time)
t_R	retention time
t_R'	adjusted retention time
T	absolute temperature
u	linear flow velocity
u_{min}	flow velocity at minimum plate height
u_x	linear migration velocity
Vol%	volumetric mixing ratio
V	peak volume
V_D	detector volume
V_e	elution volume (SEC)
V_g	gradient volume
V_M	void volume, volume of mobile phase
V_p	pore volume (volume within the particles)
V_R	retention volume
V_S	volume of stationary phase
V_z	interstitial volume (volume between particles)
W	peak area

w_b	width of a peak at its base
w_i	width of peak at height of inflection point
α	separation factor (selectivity)
β	phase ratio
γ	geometry factor
ΔP	pressure drop along the column
ε_0	eluting strength in eluotropic series
ε_T	total porosity
η	eluent viscosity
λ	Eddy diffusion coefficient, packing factor
σ	peak standard deviation
σ^2	peak variance
σ_l	peak standard deviation in units of length
σ_t	peak standard deviation in units of time
σ_v	peak standard deviation in units of volume
τ	detector time constant
ρ	packing density
ϕ	average pore diameter
Ψ	column resistance factor
ω	geometry factor

Contents

1 Fundamental Chromatographic Relationships

by *Klaus-Peter Hupe*

1 Fundamental Chromatographic Relationships

1.1 Introduction

This chapter gives a brief introduction to the formal relationships of the liquid chromatographic process and explains the terms and definitions used in this book. It shows how the performance of a system can be evaluated and enables the reader to define and to describe the conditions so that an analysis can be reproduced by other laboratories. Additionally, it will provide a basis for understanding the thermodynamic and kinetic mechanisms of the various chromatographic modes and phase systems dealt with in Chapter 2.

For in-depth study of the theory of liquid chromatography, the reader is referred to text books on this subject [1, 2, 3].

Nomenclature and symbols used throughout this book are in accordance with the recommendations of the American Society for Testing and Materials (ASTM) [4] as commented on in detail by L. S. Ettre [5].

1.2 Retention

Chromatography is a separation process. The analysis is accomplished by first separating a mixture into its individual components and then monitoring these with a detector for quantitative determination and/or qualitative identification. Optimizing the chromatographic process implies generating sufficient resolution between adjacent components as quickly as possible. In practice, the technical limitations of the pump and equipment available add a third variable: the column back-pressure.

The individual constituents of a mixture are separated as a result of their different physical and chemical interactions with the mobile phase (the solvent) and with the stationary phase (the column packing).

Times and volumes are connected via the mobile phase volumetric flow-rate (F):

$$F = \frac{V_R}{t_R} = \frac{V_M}{t_M} \quad \text{(mL/min)} \tag{1}$$

The velocity at which the mobile phase moves through the column is called the linear flow velocity (u) and is interrelated with the flow-rate (F) via the free cross-sectional area (A_c) of the column:

$$u = \frac{F}{A_c} = \frac{l}{t_M} \tag{2}$$

where l is the column length.

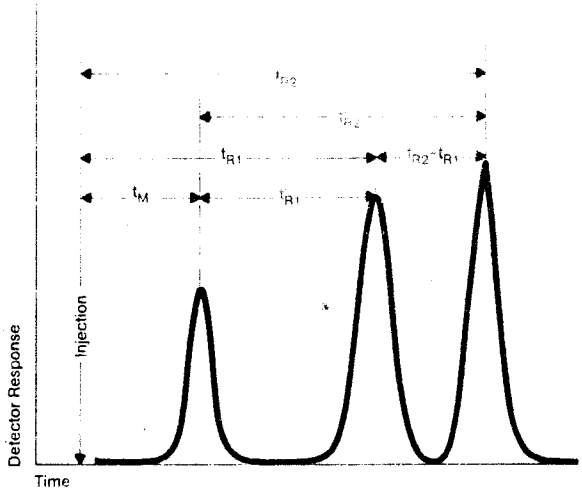

Figure 1-1. Definition of chromatographically relevant times.

According to Figure 1-1,

t_R the retention time (the time elapsed between injection and elution of a particular component);

V_R the retention volume (the volume of mobile phase passed through the column during t_R);

t_M the hold-up time (the time taken for the unretarded solvent front to elute from the column);

V_M the void volume of the column accessible to the solvent molecules.

On their way through the column, the solute molecules continually fluctuate between the mobile phase (solvent) and the stationary phase (column packing). While in the mobile phase they move at the linear velocity u in the stationary phase they do not move at all. Their apparent mean migration velocity is defined as:

$$u_x = \frac{l}{t_R} . \tag{3}$$

Consequently, the retention time (t_R) can be divided into the time that the molecules spend in the mobile phase (t_M) and in the stationary phase (t'_R):

$$t_R = t_M + t'_R . \tag{4}$$

The ratio between t'_R and t_M is an important characteristic describing the thermodynamic relationship between a solute and a given chromatographic system (where system is equal to mobile phase plus stationary phase). Under equilibrium conditions, this ratio also indicates the relative numbers of solute molecules to be found in the stationary and mobile phases. For this reason, it is called the mass distribution ratio; unfortunately, it is more commonly known as the capacity ratio or the capacity factor (k):

$$k = \frac{t'_R}{t_M} = \frac{t_R - t_M}{t_M} = \frac{V_R - V_M}{V_M} . \tag{5}$$

Rearranging equation (5) shows more clearly the relationship between retention time and capacity factor:

$$t_R = t_M(k + 1) . \tag{6}$$

t_R is a characteristic value for a given compound and can, for a given flow-rate, be used for its identification.

The capacity factor is interrelated with the distribution coefficient via the phase ratio. The distribution coefficient (K) is defined as the ratio of the concentration (c) of a solute in the stationary (S) and mobile (M) phases respectively:

$$K = \frac{c_S}{c_M} . \tag{7}$$

The phase ratio equals the interstitial volume of the mobile phase (V_M) divided by the stationary phase volume (V_S):

$$\beta = \frac{V_M}{V_S} . \tag{8}$$

For adsorption chromatography, V_S can be replaced by the surface area of the stationary phase.

It follows that:

$$K = k \cdot \beta . \tag{9}$$

The capacity factor also determines the ratio between the linear flow velocity (u) of the mobile phase and the mean migration velocity (u_x). Combination of equations (2), (3) and (6) gives:

$$\frac{u_x}{u} = \frac{1}{k + 1} . \tag{10}$$

k can vary between 0 and infinity. For $k = 0$ it follows that $u_x = u$, i. e. the solute passes the column unretained. For $k = \infty$, equation (10) gives $u_x = 0$, i. e. the solute is absorbed irreversibly and will not be eluted under the chosen conditions.

The ratio of the capacity factors of two solutes 1 and 2 is called the separation factor α, sometimes also referred to as selectivity:

$$\alpha = \frac{k_2}{k_1} \quad (k_2 > k_1) . \tag{11}$$

1.3 Separation

According to Figure 1-1, the separation of the maxima of two adjacent components increases with the time difference $(t_{R2} - t_{R1})$ between their maxima as they elute from the column. Applying equation (6) to two adjacent compounds, 1 and 2, and subtracting the first equation from the second gives:

$$t_{R2} - t_{R1} = t_M(k_2 - k_1) \,. \tag{12}$$

Substituting t_M from equation (2) leads to:

$$t_{R2} - t_{R1} = \frac{1}{u}(k_2 - k_1) \cdot l \,. \tag{13}$$

This relationship shows that the retention time difference necessary for separation increases with an increasing difference in the capacity factors of compounds 1 and 2; and, for a given velocity, with increasing column length (l).

1.4 Dispersion

1.4.1 The Characterization of Zone Spreading

The separation process described in the previous chapter has an antagonistic effect due to the dispersion of the individual solute zones during their migration through the column.
According to Figure 1-2, this effect is commonly characterized by the width (w_b) of a peak at its base (intersection of tangents through inflection points and base line) or at the height of the inflection points (w_i). For a Gaussian peak:

$$w_b = 2 \cdot w_i = 4\sigma \tag{14}$$

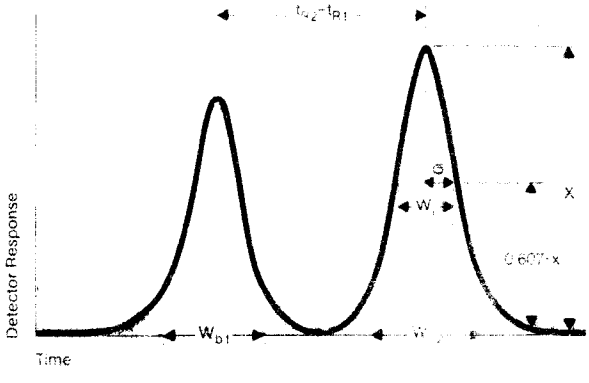

Figure 1-2. Terms describing separation and dispersion.

where σ is the standard deviation of the emerging peak measured in units of length, time or volume [6]. It should be noted that the graphical determination of the standard deviation for a peak in a given chromatographic trace gives accurate results only for truly Gaussian peaks. For the majority of peaks found in practice, which tend to be non-Gaussian, this value must be calculated via the second central moment.

Both theory and practice show that the peak width increases with the square root of the distance migrated within the column. For a completely eluted substance this distance is the column length (l), so that:

$$\sigma \approx \sqrt{l}; \quad \sigma^2 \approx l \tag{15}$$

where σ^2 is the peak variance.

In order to turn this relationship into an equation, a proportionality factor has to be introduced, leading to:

$$\sigma^2 = h \cdot l. \tag{16}$$

Equation (13) and (16), respectively, feature the two basic phenomena of the chromatographic process: separation and dispersion. Both effects increase with column length. The corresponding proportionality factors, $(k_2 - k_1)$ and h, are of paramount importance for the understanding, description and optimization of any chromatographic analysis.

Factor h in equation (16) comprises all those effects which cause the individual molecules of a uniform solute band to migrate through the column with differing velocities. As a consequence of this increasing dispersion and dilution, the solute will elute from the column in a volume of mobile phase larger than that in which it was initially dissolved and with a lower concentration.

It is unfortunate that, in the evolution of chromatographic nomenclature, this factor has been named "the height equivalent to a theoretical plate" (HETP). This has confused students and teachers of chromatography since its introduction, because it has only a little in common with the separation plates found in distillation columns. A more appropriate name would be "dispersion coefficient".

From a theoretical point of view, however, the plate concept is very useful. It assumes that a chromatographic column is mathematically equivalent to a plate column where an equilibrium is established for the solute between the stationary and the mobile phase on each plate. The column length (l) can then be thought of as being made up of a number of theoretical plates (n), the height of which is h.

It then follows that:

$$n = \frac{l}{h}. \tag{17}$$

This equation gives the standard measurement of column efficiency.

It is an important goal for any chromatographic analysis to operate columns under conditions such that they yield a high number of theoretical plates for a given length and, therefore, a small plate height. The progress HPLC has made over the last 15 years can be appreciated by the fact that in 1965 a good "HP"LC-column had a theoretical plate height not less than 1 mm. Today one expects a good column to have an h-value of 20 µm or less, which represents a 50-fold improvement.

Substituting equation (16) into equation (17) leads to:

$$n = \left(\frac{l}{\sigma_l}\right)^2 = \left(\frac{t_R}{\sigma_t}\right)^2 \tag{18}$$

σ_l and σ_t being the standard deviation of the peak in units of length and time respectively.

In the theory of the chromatographic process, the retention time is defined as the time the solute spends in the column. If the retention time is to be determined from a chromatogram, it must be taken into account that the time between injection of the sample and its appearance in the detector also includes the time that the solute has spent outside the column in the flow paths of the system. Consequently, the time determined from the chromatogram is longer than the retention time, as is the dispersion determined as the standard deviation of the peak. The plate number which is obtained when these values are substituted into equation (18) is called the "apparent plate number" and is usually lower than the theoretical plate number.

According to equation (18), and based on the formal definition of the retention time, an unretained peak ($t_R = t_M$) generates a certain number of theoretical plates (n). However, t_M has the same value for all peaks in the chromatogram and so does not contribute to differential retention and separation. Therefore a term equivalent to n, but based instead on the adjusted retention time (t_R'), has bee defined:

$$N = \left(\frac{t_R'}{\sigma_t}\right)^2 \tag{19}$$

N is called the number of effective plates and

$$H = \frac{l}{N} \tag{20}$$

is the height equivalent to one effective plate.

Combining equations (18) and (19), and substituting the capacity factor from equation (5), leads to the relation between the theoretical and the effective plate number:

$$N = n \cdot \left(\frac{k}{k+1}\right)^2 \tag{21}$$

For $k = 0$ it follows that $N = 0$.

1.4.2 The van Deemter Equation

There are three essential phenomena causing the dispersion of a chromatographic peak on its way through the column:

a) The multipath effect. Due to the tortuous nature of the flow through the packed bed, solute molecules taking different routes will become increasingly far apart. The best means of keeping this effect small is to pack columns carefully, with small particles of narrow size distribution, resulting in a packed bed of high uniformity.