# HPLC and CE: Principles and Practice

# HPLC and CE

## Principles and Practice

**Andrea Weston**
Dionex
Sunnyvale, California

**Phyllis R. Brown**
Department of Chemistry
University of Rhode Island
Kingston, Rhode Island

**Academic Press**

San Diego London Boston New York Sydney Tokyo Toronto

Front cover illustration by James Treubig, Jr.

This book is printed on acid-free paper. ♾



Academic Press
*a division of Harcourt Brace & Company*
525 B Street, Suite 1900, San Diego, California 92101-4495, USA
http://www.apnet.com

Academic Press Limited
24-28 Oval Road, London NW1 7DX, UK
http://www.hbuk.co.uk/ap/

Library of Congress Cataloging-in-Publication Data

HPLC and CE : fundamentals and applications / [edited] by Andrea Weston, Phyllis R. Brown.
p. cm.
Includes index.
ISBN 0-12-136640-5 (alk. paper)
1. High performance liquid chromatography. 2. Capillary electrophoresis. I. Weston, Andrea, date. II. Brown, Phyllis R.
QD79.C454H63 1997
543'.0871--dc21 96-49198
CIP

PRINTED IN THE UNITED STATES OF AMERICA
97 98 99 00 01 02 EB 9 8 7 6 5 4 3 2 1

# HPLC and CE

## Principles and Practice

*This book is dedicated to the memory of Dr. J. Calvin Giddings for his outstanding work in analytical chemistry and his unique contributions to the field of separations science. By devising the theoretical framework for chromatography, he made possible the development of high-performance liquid chromatography, which in turn opened doors to a new era of science, especially the birth of biotechnology.*

# Preface

In the early 1970s, I wrote the primer *High Pressure Liquid Chromatography*, as the technique was then called. At the time, there were few commercially available instruments and a paucity of articles and books on this technique. Since then, the literature on high-performance liquid chromatography, or HPLC, has exploded and almost every laboratory that deals with analytical problems has one or more liquid chromatography instruments. Approximately 1 million liquid chromatographic analyses or separations are performed daily. The growth of HPLC has been phenomenal, and HPLC is routinely used not only in the analysis of thermally labile, nonvolatile ionic compounds but for all types of molecules from the smallest ions to macromolecules. As predicted, HPLC is indispensable in biochemistry and all the biologically related sciences. It has also been found to be a powerful analytical tool in other areas such as inorganic chemistry and organic synthesis as well as in disciplines such as environmental monitoring, clinical chemistry, oceanography, and agricultural chemistry. It is especially important as an analytical, preparative, and process technique in industries where purity of products is required by law. Most important, HPLC has opened new horizons in separations and has helped make possible the development of biotechnology where there is a great need for ultrapure products.

In the 1980s, a new separations technique, capillary electrophoresis (CE), was developed. CE created great excitement and was initially expected to replace HPLC as the method of choice for ultratrace analyses. However, it became evident that CE was complementary to HPLC and filled a different niche in separations. Since each technique has advantages and disadvantages, it is important to understand the basic theory that underlies the separations in order to choose the right technique for a problem.

Therefore, when I was asked by Academic Press to update my book, I realized that a new section on CE was mandatory. I asked a former graduate student with extensive experience in CE, Andrea Weston, to collaborate with me. By the time the book was finished, it was considerably longer than previously planned.

The book is aimed at both novices and users of HPLC and CE to give them a solid understanding of basic principles, instrumentation, methods,

optimization of operation, and applications. In addition, the two techniques are compared so that users can rationally choose the appropriate technique or techniques for their analytical problems. As in my first book, we have included many illustrations to make the material easily understandable. Although this book is aimed at HPLC and CE practitioners, it can also be used for upper-level undergraduate or graduate courses. Moreover, it will be valuable for individual students, especially those from other scientific disciplines, who must use separations but have never studied the fundamentals of chromatography or electrophoresis.

Andrea and I thank all our colleagues who have helped us along the way, especially our husbands for their support, encouragement, and patience during the preparation of this book.

*Phyllis R. Brown*
*Kingston, Rhode Island*

# Contents

## CHAPTER 3 Instrumentation for High-Performance Liquid Chromatography

## CHAPTER 4 Capillary Electrophoresis

## CHAPTER 5 Separation in Capillary Electrophoresis



## CHAPTER 6 Instrumentation of Capillary Electrophoresis

## CHAPTER 7 Data Manipulation



## CHAPTER 8 Miniaturization

# CHAPTER 1

# High-Performance Liquid Chromatography

## 1.1 Introduction

"Chromatography is a method in which the components of a mixture are separated on an adsorbent column in a flowing system."[1] The adsorbent material, or stationary phase, first described by Tswett[1] in 1906, has taken many forms over the years, including paper,[2] thin layers of solids attached to glass plates,[3,4] immobilized liquids,[5] gels,[6] and solid particles packed in columns.[7] The flowing component of the system, or mobile phase, is either a liquid or a gas. Concurrent with development of the different adsorbent materials has been the development of methods more specific to particular classes of analytes.[6,8–10] In general, however, the trend in development of chromatography has been toward faster, more efficient systems.[11–20]

Liquid chromatography (LC), which is one of the forms of chromatography, is an analytical technique that is used to separate a mixture *in solution* into its individual components. As indicated by Tswett, the separation relies on the use of two different "phases" or "immiscible layers," one of which is held stationary while the other moves over it. Liquid chromatography is the generic name used to describe any chromatographic procedure in which the mobile phase is a liquid. The separation occurs because, under an optimum set of conditions, each component in a mixture will interact with the two phases differently relative to the other components in the mixture. High-performance liquid chromatography (HPLC) is the term used to describe liquid chromatography in which the liquid mobile phase is mechanically pumped through a column that contains the stationary phase. An HPLC instrument, therefore, consists of an injector, a pump, a column, and a detector.

This chapter introduces the basic theory and terminology governing chromatographic separations and the equations used to calculate the effectiveness of the analytical system. With this information, the best separation mechanism and column characteristics for a given problem can be chosen,

on the basis of the nature of the components in the mixture as well as the physical and chemical characteristics of the column.

## 1.2 Classification of Liquid Chromatographic Methods

There are two ways to classify liquid chromatographic methods. The first and more common classification is based on the mechanism of retention, and from this the chromatographic modes discussed in Chapter 2 are derived. For example, the normal-phase mode can be performed by taking advantage of either the adsorption mechanism or the partition mechanism. The gel-filtration mode is performed using the mechanism of size exclusion. The second classification discussed below is based on the separation principle and is found mostly in the literature published before the 1990s.

### 1.2.1 Classification According to Mechanism of Retention

The most popular classification scheme stems from the manner in which the analyte interacts with the stationary phase. With this approach, chromatography may be divided into five separation mechanisms: adsorption, partition, size exclusion, affinity, and ion exchange, as illustrated in Figure 1.1.

Adsorption chromatography is based on competition for neutral analytes between the liquid mobile phase and a neutral, solid stationary phase. The analytes interact with the stationary phase according to the premise "like likes like": polar solutes will be retained longest by polar stationary phases, and nonpolar solutes will be retained best by nonpolar stationary phases. In adsorption chromatography the solute molecules are in contact with both the stationary phase and the mobile phase, simultaneously. Under these conditions, the solutes are said to be in an anisotropic environment.

Partition chromatography is also based on competition for neutral analytes, but in this case the stationary phase is considered to be a neutral liquid. Owing to the instability of liquid stationary phases, true partition

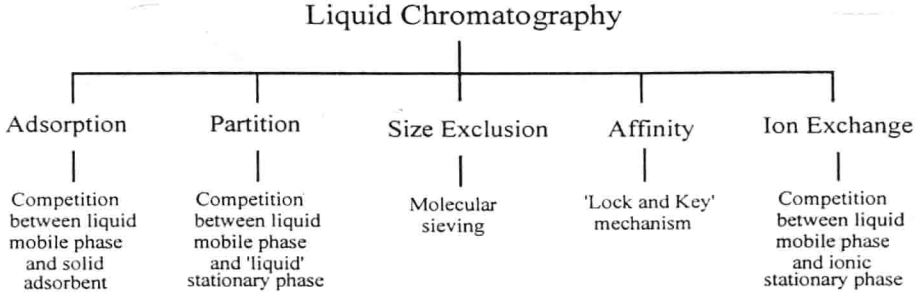


**Figure 1.1** Classification of chromatographic modes according to the retention mechanism.

chromatography is not commonly used in modern HPLC. Instead, long-chain ($C_{18}$) "bonded-phase" columns have been developed in which the long alkyl chains are considered to behave like a liquid. Thus, the process is termed partition when the solute is transferred from the bulk of one phase into the bulk of the other, so that the solute molecules are completely surrounded by molecules of one phase. Under these conditions, the solutes are said to be in an isotropic environment.

Ion-exchange chromatography (IEC) is based on the principle that opposites attract. Ion-exchange chromatography is used to separate charged analytes and therefore occurs as a result of interaction between a charged solute and an oppositely charged, solid stationary phase. Ion-exchange chromatography can be applied to any solute that can acquire a charge in solution. Thus, even carbohydrates, which are largely uncharged below pH 12, can be separated by ion-exchange chromatography at sufficiently high pH.

Size-exclusion chromatography (SEC) is based on the sieving principle. In SEC, the stationary phase particles are manufactured with a wide range of pore sizes, causing the stationary phase to behave like a molecular sieve. As a result of the sieving action, the solutes are separated on the basis of size, with the larger ones eluting first (BOCOF, big ones come out first).

Affinity chromatography is based on the lock-and-key mechanism prevalent in biological systems. The retention mechanism is very specific, but the technique is more time-consuming and more expensive than those employing other retention mechanisms.

The mechanisms described above form the basis for the chromatographic modes described in Chapter 2, namely, normal-phase, reversed-phase, size-exclusion, ion-exchange, and affinity chromatographies. However, other modes that are variations of those mentioned above, such as hydrophobic-interaction chromatography (HIC), chiral, ion-exclusion, and ion-pair chromatographies are also used and will be mentioned.

### 1.2.2 Classification According to Operating Method

The second classification scheme is less common than the first but is found in the literature. It is based on the operating method, or the mechanism by which the sample is removed from the column, and is therefore dependent on the nature of the mobile phase. This classification, which was introduced by Tiselius[21] in 1940, includes elution development, displacement development, and frontal analysis, as shown in Figure 1.2.[22] In practice, only elution and to a lesser extent displacement development are commonly used.

In elution development a small volume of sample is introduced onto the head of the column, and the components are adsorbed onto the stationary phase to various degrees. The solutes are eluted from the column using

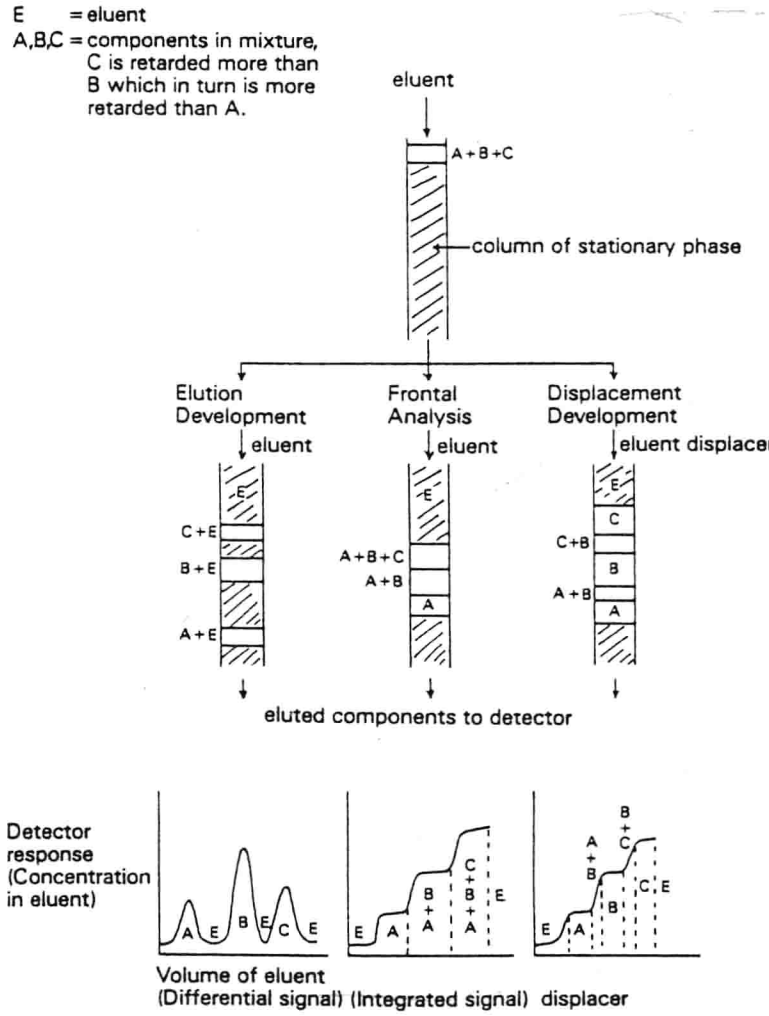


**Figure 1.2** Classification of chromatographic methods according to the operating method. (Reprinted from Ref. 22 with permission.)

a mobile phase which has a greater affinity for the sample components than for the stationary phase. Since the components can be completely separated with a zone of mobile phase between them, elution chromatography is commonly used for analytical separations where quantitation and characterization may be important.

Elution chromatography may be subclassified according to the "continuity" of the mobile phase. Isocratic elution is the term used when the sample is introduced onto the column and eluted from it under the same set of mobile phase conditions. Isocratic elution is the most common way