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Countercurrent Chromatography

THEORY AND PRACTICE

edited by

N. Bhushan Mandava

Yoichiro Ito

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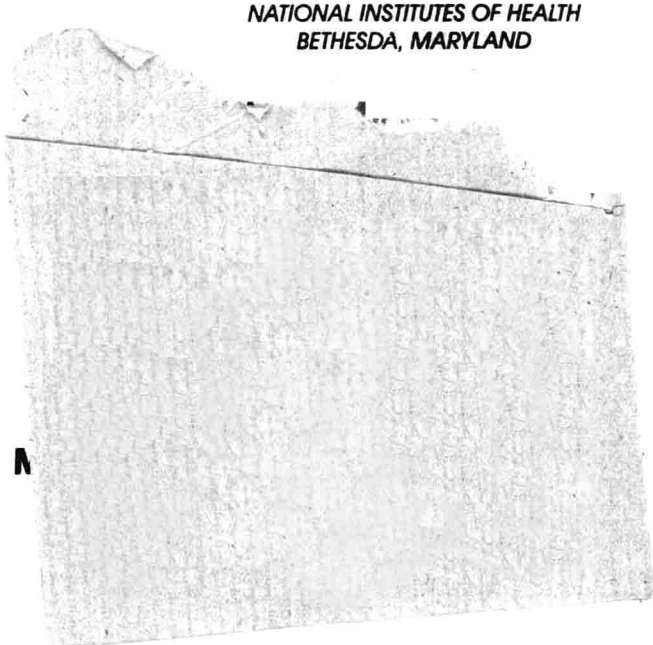

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Preface

Historically, the separation of similar molecules by their differential solubility between two immiscible phases has provided the basis for several highly selective separatory techniques, such as paper chromatography, countercurrent distribution (CCD), and liquid-liquid chromatography. The great selectivity of these techniques arises from the fact that the partition coefficient is the reflection of the intermolecular forces which can be strongly modified by conditions of temperature and phase composition. Thus, by a judicious choice of conditions, highly specific interactions can often by exploited that permit the resolution of selected components from very complex mixtures.

In separation science, several chromatographic methods, such as paper chromatography, high-resolution column chromatography, and high-performance liquid chromatography (HPLC), were developed on the basis of liquid-liquid partition principles, but these methods require a solid support that sometimes presents such problems as loss of samples by adsorption and chemical degradation of compounds. To date, more limited recognition has been attained by alternative separation systems, also based on solvent-solvent partitioning but not requiring the use of a solid support matrix. These include the classic separatory funnel, cascade extraction train, partition chromatography, Craig's CCD apparatus, cascade percolator train, and Ronor columns, to name a few older partition methods. Except for partition chromatography and CCD, they did not gain wide acceptance in laboratory scale separations despite the fact that some did

show very good resolving power. The fragility and inconvenience of the apparatus, and its unadaptability to separations of varying scale, may account for some of these failures.

The technology of countercurrent chromatography (CCC) evolved from keen observation that two immiscible liquids flowing countercurrently in a helical tube rotating in an acceleration field become uniformly segmented in the coils of the helix. Separation of both soluble and particulate samples was demonstrated. Although tedious and limited to microgram samples, the value of the technique was immediately acknowledged by the commercial development of an instrument called the coil planet centrifuge by Sanki Engineering, Ltd. (Japan).

In the late 1960s, a systematic program to design and evaluate an extensive series of separation devices was initiated. With the exception of droplet countercurrent chromatography and locular countercurrent chromatography, the instruments are based on a helical coil which is acted on by either a gravitational or centrifugally induced acceleration field. Aside from their mechanical features, the devices may be classified on the basis of the cyclic variation in the direction and intensity of this field relative to the coil. The more recently designed chromatographic devices incorporate an ingenious flow-through system which permits high-speed rotation of the coil without kinking of the flexible influent and effluent lines, thereby avoiding the need for an often troublesome rotating fluid seal. With the introduction of a new family of high-performance countercurrent chromatographic devices, this field of separation science is now comparable in many respects to other chromatographic systems, especially to HPLC, in speed, resolution, efficiency, and sample size as well as in separation capabilities.

The process of countercurrent chromatography is essentially liquid-liquid chromatography in which the stationary liquid bed is retained in the column by an acceleration field rather than by a solid supporting matrix. Adsorption effects are thereby eliminated. The technique is particularly advantageous in the preparative (mg to g) range for polar and labile organic compounds and bioparticulate materials such as cells and cell fragments. Virtually any two-phase system, either aqueous or nonaqueous, may be employed, and some CCC instruments are particularly useful for the two-phase aqueous polymer systems.

This book consists of essentially two parts. An introductory chapter covers the basic chromatographic principles, which are generally applicable to any chromatographic science. Part I, dealing with the theory and instrumentation, is divided into three chapters on historical developments that have led to what is now known as countercurrent chromatography, instrumentation that describes the state of the art in the design of various CCC devices, and theory of countercurrent chromatography. Part II deals with applications

of countercurrent chromatography. This section discusses the application of droplet countercurrent chromatography, rotation locular countercurrent chromatography, and centrifugal countercurrent chromatography to natural products isolation. Other applications of countercurrent chromatography include agrochemicals, drugs, peptides, and biopolymers, each of which is described in a separate chapter. Chapter 12, at the end of the book, was introduced to show how promising CCC is for the future with tandem methods such as CCC/MS as powerful techniques of separation science. A glossary of terms commonly used in chromatography and a list of CCC instruments now commercially available are included in Part III.

The contributors, who are specialists in their respective fields, deserve special credit for illustrating the power of CCC in their specialties. We express our sincere thanks to all contributors for their ready cooperation and for presenting their work very lucidly and candidly. Finally, we thank our spouses for their patience and understanding while we completed the tasks of writing and editing this book. The publisher's support and the series editor's enthusiasm for this book are greatly appreciated.

N. Bhushan Mandava
Yoichiro Ito

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chapter one

Introduction to Chromatography

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In preparation for a description of countercurrent chromatography (CCC), it is convenient to summarize briefly the general principles which underlie all forms of chromatography. The theoretical foundations of countercurrent chromatography resemble most closely those of liquid chromatography and countercurrent distribution, and for that reason the theory of elution chromatography will receive attention in preference to other techniques. Frontal and displacement chromatography, treated in terms of solute fronts, are less applicable to countercurrent chromatography and will be mentioned only briefly.

The function of the theoretical treatment is to describe the behavior of solutes, which in elution chromatography involves solute zone profiles and positions. As discussed by Morris and Morris, this requires two kinds of information: (a) the properties of the solutes and (b) the properties of the chromatographic system. Although the specific properties of solutes are of less interest in the chromatography of macromolecules, polar molecules, and ionic molecules than the properties of the chromatographic systems, the solute properties are important in explaining the behavior of small molecules in nonpolar solvent systems [28. Topics covered include:

1. General chromatographic principles, theory, and fundamental differences in chromatographic systems as they relate to chromatographic retention and ideal linear chromatography.

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2. Liquid-liquid partition chromatography. This part will be treated in more detail because the same phenomena are found in coutercurrent chromatography. Included are solvent systems, support materials, some description of the operation of chromatography, and a few examples of applications.
3. Countercurrent distribution. The basic principles and processes will be discussed, but the details of application will not be included.

The treatment of other chromatographic systems—liquid-solid chromatography based on adsorption principles, gel permeation (size exclusion) chromatography, ion-exchange chromatography, and affinity chromatography—will be avoided because they have little application in countercurrent chromatography.

I. HISTORICAL DEVELOPMENTS

The origins of the chromatography, discussed by Novak and Janak [29], go back to Runge's experiments [1] on capillary analysis. Just before the turn of the century, Davy [2] observed changes in the composition of crude petroleum when it came in contact with rocks displaying adsorptive activity. These reports can be considered as part of the development of chromatography. It was only about 80 years ago that Tswett [3] devised methods for the separation of petroleum extracts on columns of inorganic adsorbents and coined the word "chromatography." He demonstrated the separation of the petroleum ether extract of the coloring substances of leaves into a series of green and yellow bands. The apparatus was a column of calcium carbonate in a glass tube, through which the light petroleum solvent was made to percolate continuously. The importance of Tswett's work was not recognized until the beginning of the 1930s, when Kuhn et al. [4] applied chromatographic principles to the separation of natural substances.

Another milestone in the development of chromatography was reached in 1941 when Martin and Synge [5] reported their discovery of liquid-liquid partition chromatography. In their work one liquid was used as a sorbent and another liquid was allowed to percolate through the former, thus making the technique a chromatographic process. Their work established the foundations for chromatography and set a precedent for the development of other forms of chromatography and other separation techniques, including:

1. Paper chromatography
2. Countercurrent chromatography
3. Ion-exchange chromatography
4. Electrophoresis

5. Thin-layer chromatography
6. Gel permeation chromatography
7. Gas-liquid chromatography
8. High-performance liquid chromatography

It is said that when one of the great discoveries in chromatographic science was submitted for publication, the journal of a renowned chemical society rejected it. The importance of the work was eventually emphasized by a Nobel Prize. Such instances are not uncommon in the history of scientific society publications. The paper on brassinolide [25] was rejected by the journals of two learned societies. After it was published in *Nature* and its impact was realized, the journals of those societies carried news stories.

Stahl [6] notes the comment of a well-known critic: "Only amateurs consider that an idea must be brand new in order to be good; important in reality is not he who first had the idea but he who expressed it better." This comment applies to the work of Twsett, who not only coined the term "chromatography" but also defined his method as a general means of separating substances, including colorless substances.

It was in 1906 that Goppelsroeder [7] published his book entitled *Suggestions for the Study of Capillary Analysis, Based on Capillarity and Adsorption Phenomena.* Subsequently (38 years later) Consden et al. [8] developed a practical technique which is known as paper chromatography. The chronological developments in separation techniques after Tswett's discovery of chromatography, based first on adsorption principles and then on partition phenomena, are shown in Table 1.

II. CHROMATOGRAPHIC PRINCIPLES

Martin [24] defines chromatography as "the uniform percolation of a fluid through a column of more or less finely divided substance, which selectively retards, by whatever means, certain components of the fluid." As Morris and Morris noted, this definition includes mixtures of gases and liquids, and avoids any description of the mechanisms responsible for selective retardation. The term "column" refers to various experimental arrangements, from the most commonly used cylindrical tube to the thin sheets of porous material used in paper chromatography [28]. The words "finely divided substance" denote a packing material responsible for the separation process in adsorption chromatography or refer to a support for another active phase in partition chromatography. As Morris and Morris point out, the features listed are common to chromatographic separations: (a) they are cascade processes and (b) separation results from the differential migration of the components of the mixture through the two-phase system.

Table 1 Chronological Developments in Separation Techniques

Separation system	Authors	Year	Ref.
Partition chromatography	Martin and Synge	1941	[5]
Paper chromatography (PC)	Consden, Gordon, and Martin	1944	[8]
Countercurrent distribution (CCD)	Craig	1944	[9]
Ion-exchange chromatography (IEC)	Mayer and Thompkins	1947	[10]
	Samuelson	1963	[11]
Electrophoresis	Haugaard and Kroner	1948	[12]
Thin-layer chromatography (TLC)	Ismailov and Shraiber	1938	[13]
	Kirchner, Miller, and Keller	1951	[14]
Gel permeation chromatography (GPC)	Barrer	1945	[15]
	Porath and Flodin	1959	[16]
Gas chromatography (GC)	Claesson	1946	[17]
	James and Martin	1952	[18]
High-performance liquid chromatography (HPLC)			[19]

In words of Novák and Janák [29], "Chromatography can be characterized as a separation method based on the differential migration of solutes through a system of two phases, one of which is mobile." Chromatographic systems can therefore be classified [29] according to:

1. The state of aggregation of the phases
2. The physical arrangement of the phases, and
3. The mechanism underlying the distribution equilibrium

The four possible systems derived from solid, liquid, and gaseous phases are liquid-liquid, liquid-solid, gas-liquid, and gas-solid chromatography. Of the two systems, liquid-liquid and liquid-solid, that may be called "liquid chromatography," it is liquid-liquid chromatography whose principles are directly applicable to countercurrent chromatography. The physical arrangements of the phases in chromatography can be classified as columnar or planar systems. Depending

on the geometry of the chromatographic column support, the columnar systems can be further divided into such types as packed columns, capillary columns, and so forth (Table 2).

In liquid-liquid systems of all types, they [29] note that an important feature is the way in which the stationary liquid phase is formed. There are various ways of achieving a stationary liquid phase. As shown in the following sections, it is produced in countercurrent chromatography by surface tension or by centrifugal force, as in droplet countercurrent chromatography (DCCC) or in rotation locular countercurrent chromatography (RLCCC), respectively.

Chromatographic systems may be divided into two categories, two-dimensional and three-dimensional, in which the solute-solvent interactions take place on the surface or in the interior of the phase. These two geometries characterize "adsorption chromatography" and "partition chromatography," respectively [29].

A chromatographic system is characterized by a distribution isotherm, which is a curve showing the equilibrium relationship of concentration in the stationary phase to concentration in the mobile phase, at a constant temperature. When the distribution constant is independent of the solute concentration, the distribution isotherm (see Section IV.D) is linear. The term "linear chromatography" is applied to this case. When the distribution constant varies with solute concentration, a curved (nonlinear) isotherm is observed, giving "nonlinear chromatography."

III. CHROMATOGRAPHIC TECHNIQUES

The chromatographic system allows us to perform the following three types of operation:

1. Elution chromatography
2. Frontal chromatography, and
3. Displacement chromatography (with a solid adsorbent)

Table 2 Examples of the Geometrical Types of Chromatographic Systems

Columnar	Planar
Packed columns	Paper chromatography
Capillary columns	Thin-layer chromatography
Liquid-liquid columns	

In elution technique, the mixture of solutes is introduced at the column inlet onto the stationary phase (solid support in liquid chromatography or stationary liquid phase in CCC). The solute bands begin to migrate as the mobile phase starts passing through the stationary phase, and begin to separate, depending on the migration velocities of the individual components. The mobile phase, being essentially nonsorbed on the stationary phase, does not interfere with that mechanism. The migration velocities are inversely proportional to the corresponding distribution coefficients. In the chromatogram (Fig. 1a), the distances of the peak maxima from the

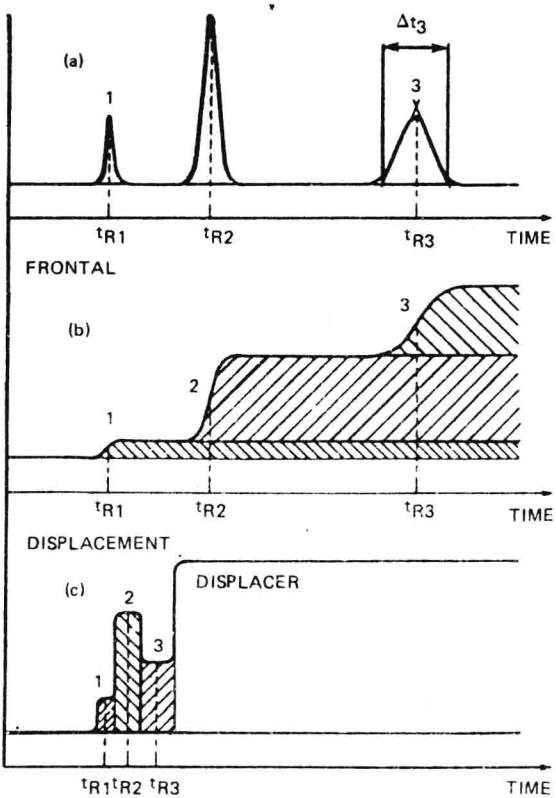

Fig. 1 Chromatographic records: (a) elution chromatography; (b) frontal chromatography; (c) displacement chromatography. R = response. (From J. Novák and J. Janák; in *Liquid Column Chromatography*, (Z. Deyl, K. Macek, and J. Janák, eds.), Elsevier, Amsterdam, 1975, pp. 3–10.)

starting point depend on the solute properties, and the peak areas are proportional to the total amounts of the components.

The status of frontal chromatography (Fig. 1b) appears to be well defined by Morris and Morris [28]: "Although this method of chromatographic analysis is not strictly a practical separation method, the simple theoretical treatment forms a useful introduction to the more complex methods of operation." The mobile phase is the sample mixture itself, flowing continuously. The column is eventually saturated with each solute. The first solute to reach saturation in the column appears pure in the effluent. Other solutes, successively achieving saturation of the column, then mix in the output, which finally returns to the input mixture composition.

In displacement technique (Fig. 1c), the mobile phase (in contrast to that in elution chromatography) is adsorbed more strongly than any of the solutes in the sample mixture, so that each solute follows those less strongly adsorbed. Each emerging zone is internally pure but touches others at its boundaries. In elution chromatography each zone not only contains, in the ideal case, a single component but also is separated by distance along the column axis and by time of elution from its neighboring zones.

In elution and displacement methods, components may be recovered either by elution from the column or by stopping the run before zones reach the exit and extracting portions of the column packing. Displacement and frontal methods have a place in the historical development, but modern practice in liquid chromatography is essentially restricted to the elution techniques of open columns, high performance liquid chromatography (HPLC), and CCC.

IV. DEFINITIONS AND EQUATIONS

A. Capacity Ratio D_C and Distribution Constant K_C

During the progress of the chromatographic elution process, the effluent composition leaving the column outlet is described mathematically as either a differential (Fig. 2a) or an integral (Fig. 2b) form. In this chapter and the following one, the subscript C will be used to mark quantities defined for liquid-liquid partition chromatography. The subscript D will indicate those defined for countercurrent distribution. The reason for that is that the distribution coefficient is defined differently in liquid-liquid partition chromatography and in countercurrent distribution. In chromatography, it is usually expressed as the concentration in the stationary phase divided by the concentration in the mobile phase. In countercurrent distribution, it is usually defined as the concentration in the upper phase (usually, but not always, mobile) divided by the concentration in the lower phase, so that the two are most often, but not always, reciprocals of each other.