

# Selectivity and Detectability Optimizations in HPLC

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## PREFACE

Selectivity and detectability optimizations are two primary goals sought by chromatographers. Unfortunately, no text to date deals fully with this subject. Chromatographic theory does not provide adequate support either, since the resolution equation does not directly account for selectivity of multiple compounds. Separation on an HPLC column is a very complex process—especially when one is dealing with compounds which are not from a homologous series. Most of these compounds can have a variety of functional groups and exhibit acidic, basic, or neutral properties. Isomeric structure or high molecular weight can further influence separations.

Selectivity can be described as the ability of a column–mobile phase combination to separate a variety of components in a reasonable period of time. For most practical purposes, a reasonable period of time is considered to be less than 30 minutes. It must be recognized that certain complex separations will require more time, whereas other separations can be achieved in less than one minute. In any event, the key is resolution of all components of interest. The first step in optimizing selectivity is selection of a stationary phase(s). Once this is done, further improvements must result from optimization of the mobile phase composition by choosing the right components, composition, and additives. This process can be based on current scientific knowledge or acquired knowledge, that is, experimentation. As we obtain more knowledge of this process we can better rationalize the optimization steps. This book attempts to pool the current knowledge on the subject in order to provide some insights into the optimization process.

Detectability is a function of the ability of the selected detector and its electronics to reduce noise and peak width. The selection of a given detector not only can help to optimize detectability but can help with selectivity by eliminating signals from the unwanted peaks. Further optimization necessitates reducing peak width. In general, decreasing the peak width helps to improve the signal. Several approaches to minimizing peak width and enhancing the signal are discussed in this book.

The scope of selectivity and detectability optimizations is described in Chapter 1. The next two chapters deal with the physicochemical basis of retention and the studies performed to improve our understanding of

separation mechanisms. Chapter 4 provides background information by discussing the conventional approaches to mobile-phase selection and optimization. The next four chapters describe approaches used to optimize separation by adsorption or normal-phase, reversed-phase, ion-exchange, and ion-pairing chromatography. Applications in select areas such as those pertaining to macromolecules, especially in the field of biotechnology, and isomers (including chiral separations) are covered in Chapters 9 and 10. Computerized approaches to selectivity (Chapter 11) describe unattended optimizations that could further improve our understanding of this area. Chapters 12 and 13 deal with selective detectors and approaches to optimize detectability.

Prior to tackling complex problems pertaining to selectivity and detectability optimizations, scientific texts by Snyder and Kirkland; Horvath; Poole and Schuette; and Hearn, duly referenced in this book, should be used by the reader to acquire a sound foundation in HPLC.

I wish to express my thanks to Lloyd Snyder for his contribution to this book, and to a host of people, both here at CIBA-GEIGY and in the international chromatographic community, for their support in completion of this book. Special thanks go to my family, especially my wife, Fay, for her help in many different ways.

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## CHAPTER

### 1

## THE SCOPE OF SELECTIVITY AND DETECTABILITY OPTIMIZATION IN HPLC

High-pressure liquid chromatography (HPLC) has revolutionized the field of separations. The compounds that once were considered, because of volatility, polarity, or thermal instability, too difficult to separate by techniques such as gas-liquid chromatography (GLC) can be easily separated by HPLC within a short period of time. Furthermore, proteins and other macromolecules produced in biotechnology can also be resolved. Major improvement in this field has resulted from the development of bonded columns with small particle size; a large number of theoretical plates are possible with a 5- to 10-cm column packed with particles of 3  $\mu\text{m}$  or less (1). As a matter of fact, the improvement of resolution obtained when particle size of columns decreased from 30 to 10  $\mu\text{m}$  or less led several chromatographers to describe this technique as high-performance liquid chromatography, which has the same abbreviation as high-pressure liquid chromatography. For all practical purposes, most of the high-pressure liquid chromatography performed today can be described as high-performance liquid chromatography. Therefore, in this book no distinction is made between these terms because the primary objective of the book is to describe ways to improve performance in liquid chromatography by considering various means of optimizing selectivity and detectability.

### 1.1. SELECTIVITY AND DETECTABILITY

Selectivity of an HPLC column can be described as its ability to separate components with slight variations in structure or molecular weight. For compounds with the same molecular weight, the structural differences may involve no more than compounds that are mirror images, that is, optical isomers resulting from the presence of one or more asymmetric carbon atoms. Other examples of high selectivity in HPLC relate to separation of compounds that differ only in the position of a double bond or in isotopic composition, for example, deuterated polyaromatic hydrocarbons can be separated from their protiated analogs.

Detectability relates to the minimum quantity of material than can be detected, which in turn relates to the separation efficiency of the column, that is, minimum band broadening and the sensitivity of the detector. Therefore it is possible to improve detectability by improving separations even when the same detector is being used; this requires narrowing the band width (Chapter 13).

To optimize selectivity and detectability it is necessary to understand the physicochemical basis of retention (Chapter 2) and the retention mechanism involved in HPLC separations (Chapter 3). The classic approaches to mobile phase selection and optimization (Chapter 4) greatly improve our understanding of the complex separations. The approaches taken by various scientists to optimize separations include adsorption/normal-phase (Chapter 5), reversed-phase (Chapter 6), and ion-exchange (Chapter 7) separations. Difficult separations are exemplified by ion-pair (Chapter 8), macromolecular (Chapter 9), and isomeric compound (Chapter 10) separations. These separations constitute some of the interesting applications in HPLC. A chemometric approach to optimizing selectivity with the help of computers is discussed next (Chapter 11) and the last two chapters concern the new and sensitive detectors and optimization detectability with the detectors most commonly used in HPLC.

## 1.2. MODES OF HPLC

HPLC separations for compounds with molecular weight lower than 2000 can be simply classified into three categories for the explicit purpose of improving selectivity: (a) normal-phase or adsorption separations, (b) reversed-phase separations, and (c) ion-exchange separations.

Classical adsorption chromatography as it is generally practiced with gravity-fed columns or on thin-layer chromatography (TLC) plates is not commonly used in HPLC. Because HPLC columns have to be preequilibrated with the mobile phase, they cease to behave as a true adsorption phase since some of the components from the mobile phase are retained by the column to reach an equilibrium state. This frequently leads to a mixed mechanism separation based on adsorption and partition against the retained solvent components.

Normal-phase separations require a stationary phase that is more polar than the mobile phase. However, this technique is less frequently used in HPLC. Chromatographers take unusual steps to make their separations reversed phase; over 65% of HPLC separations are conducted in the reversed phase (RP), that is, the stationary phase is less polar. Hence this technique has received the broadest coverage in this book.

Although it is generally agreed that separation in RP-HPLC or RPLC occurs primarily because of hydrophobic or solvophobic effects, the secondary effects of stationary phase and the weak dispersive forces (dependent upon solute molar volume) should not be ignored (2). Underivatized surface silanols and hydro-organic solvents further affect the separation process; some specific solvation effects in that layer may control selectivity to a large extent.

Ion-exchange separations entail ionic compounds (cations or anions) or compounds that can be made ionic by pH adjustment or other means. Separations of inorganic salts with conductivity-type detectors have been labeled ion chromatography. This is unfortunate since it could suggest that this is a different form of chromatography. It should be noted that inorganic salts can also be chromatographed with other modes of chromatography, for example, RPLC after appropriate complexation.

Size-exclusion chromatography is generally used for separations of compounds of molecular weight over 2000 (macromolecules). However, macromolecules of biological interest are generally separated by RPLC or a technique called affinity chromatography.

### 1.3. THEORETICAL CONSIDERATIONS

Since the main objective in HPLC is to resolve the maximum number of components, there is a constant thrust to improve resolution, that is, separation between two or more components. Unfortunately, the resolution equation is limited to separation of two components:

$$\text{resolution} = R_s = \frac{1}{4} \sqrt{N} \left[ \frac{\alpha - 1}{\alpha} \right] \left[ \frac{k'}{1 + k'} \right]$$

where  $N$  = number of theoretical plates

$\alpha$  = separation factor

$k'$  = peak capacity factor

It is patently clear that, individually, none of the terms ( $N$ ,  $\alpha$ , or  $k'$ ) in the resolution equation is sufficient to describe resolution (3). Unfortunately, many chromatographers equate resolution with  $N$  or even  $k'$ . It is erroneous to describe a separation involving a large number of components or theoretical plates as high resolution; these situations can be best described as separation requiring high peak capacity or large number of theoretical plates. The separation factor,  $\alpha$ , is the most important component of the resolution equation; however, it is limited in this equation to separation of one pair of peaks, generally the closest pair. Hence its value is mostly limited to separation of compounds in homologous series as it does not provide a good

indication of resolution achieved for components with different structures and/or functional groups.

It is important to recognize there is a significant overlap in chromatograms for which relative component spacing is governed by random factors. Giddings et al. (4) calculate only 36% recovery for 100 components from a system with a peak capacity of 200. A 90% recovery would require a peak capacity of 1900 or twenty million theoretical plates. In those circumstances in which a single component can be isolated as a single-component peak (80% probability), the probability that two components can be simultaneously isolated is only  $(0.80)^2$  or 0.64. Thus, the probability of isolating 10 components simultaneously is quite small  $[(0.80)^{10} = 0.107]$ . Consequently, with systems of enormous resolving power, it is difficult to resolve a small number of components simultaneously. This has significant implications with respect to the way we handle data and design optimization procedures.

Much improvement is being made in general equipment to minimize band broadening. It is important to eliminate or minimize dead volumes. Special injectors with minimum dead volume are being tested to optimize separations. Similarly, detectors with small cell volumes are being developed to minimize postcolumn band broadening. As shown by the following equation, band broadening can be minimized by giving consideration to each component:

$$W_T^2 = \underbrace{W_i^2}_{\text{injector}} + \underbrace{W_f^2}_{\text{fittings}} + \underbrace{W_d^2}_{\text{detector}} + \underbrace{W_c^2}_{\text{column}}$$

Major improvements are needed in conventional columns (4.6 mm i.d. or equivalent) used for HPLC. Even though columns are available that can provide more than 10,000 plates/column or more than 100,000 plates/m, large column-to-column variations can be found between manufacturers and from one batch to the next (5). Further improvements require better understanding of column variabilities and the separation processes occurring in the column. This will help select desirable characteristics of a given column from a given manufacturer for optimum separation.

#### 1.4. VARIOUS ROUTES TO HIGH RESOLUTION

There are many examples of impressive separations in HPLC, for example, separation of 150 polynuclear aromatic hydrocarbons (6) or a large number of components of coal fractions (7) or 300 peptides from a single tryptic digest (8). For difficult chromatographic separations, it is common to look for high  $N$  or  $k'$ ; however, what is really needed is high selectivity. An excellent example as

to why it is not always necessary to produce a high number of plates is provided in the separation of toluene, nitrobenzene, and *m*-dinitrobenzene (9). A 6-m  $\times$  1-mm-i.d. column packed with Zorbax BP Sil 7-8  $\mu$ m provided 270,000 theoretical plates with a baseline separation of these three components in 300 minutes with methylene chloride as mobile phase; however, the same components can be separated in 15 seconds on a 10-cm  $\times$  1-mm-i.d. column packed with the same material (less than 1500 theoretical plates) with hexane-methanol (99:1) mobile phase. Not only can a reasonable separation be obtained in 15 seconds, a run for 60 seconds can separate five additional components with various substituents. The important difference in the above separation is the selectivity provided by the mobile phase.

High selectivity is achieved by optimal selection of the mobile phase, which is unique to HPLC as compared to GLC; the end result is good separations in a more reasonable time. This is accomplished through appropriate selections of particle size (for packed columns), column diameter/length, pressure drop, flow velocity, stationary phase, that is, sorption or other suitable separation property, solvent viscosity, peak capacity, and total number of theoretical plates.

#### 1.4.1. Optimum Column Selection (Conventional versus Small-Particle versus Microbore Columns)

High resolution in reversed-phase HPLC can be achieved by use of conventional columns ( $< 30 \mu$ m), small particle columns (3  $\mu$ m), or microbore columns. However, column selection is dependent upon whether one wants to optimize  $N$ ,  $k'$ , or analysis time. To maximize the effective peak number achieved in a given analysis time at constant  $N$ , the largest calculated value of  $k'$  is approximately equal to 6 (10). At  $k' \cong 6$ , the highest peak capacity of 74 was offered by the conventional column and lowest analysis time by the small-particle column. The latter had a peak capacity of 54 and an analysis time one-fifth that of a conventional column (11).

The largest number of plates per meter was obtained on the 3- $\mu$ m column (119,000 plates/m). This column also generated the largest number of plates per unit time (99 plates/second); however, it was poor in terms of plates generated per unit pressure drop. At "optimum practical" flow ( $2 \times$  optimum flow), conventional columns provided the largest number of plates per unit time and the highest column performance as measured by impedance. Several lengths of individually packed microbore columns can be connected to produce columns with a high plate count—a distinct advantage at a fixed pressure of 5000 psi. Under these conditions, the microbore column produces 318,000 theoretical plates with analysis time of 33 hours ( $k' = 64$ ) and a peak capacity of 282. The conventional column provides a peak capacity of 125 and



analysis time of less than 1 hour. For a fixed number of theoretical plates (10,000), the column length and analysis time are shortest for 3- $\mu\text{m}$  particle columns (84 mm, 224 seconds).

### 1.4.2. Optimum Analysis Time

Comparison between the resolving power of a packed and open tubular column indicates that open tubular columns have better resolving power than packed columns in a given analysis time, if the column diameter for the open tubular column is the same as the particle diameter in the packed column (12). The speed of analysis is strongly dependent on the particle size of the packing materials for the packed column and column diameter for open tubular columns. It can be calculated that open tubular columns with i.d.  $\geq 31\ \mu\text{m}$  would never meet the separation speed of a 5- $\mu\text{m}$  particle column having  $h$  (reduced plate height) = 2 and  $v$  (reduced velocity) = 5. For  $k'$  between 0 and 10,  $a \leq 7.5\text{-}\mu\text{m}$ -i.d. open tubular column with the same peak capacity as a 5- $\mu\text{m}$  particle size column should be used if the faster analysis time is desired.

It is possible to generate a large number of plates, for example,  $1 \times 10^6$  plates with the smaller i.d. columns. However, in practice, it is much easier to achieve the gain in column resolving power by using microparticles (3–5  $\mu\text{m}$ ) packed columns than by using microbore open tubular columns. The columns packed with 3- $\mu\text{m}$  particles provide shorter analysis time than those packed with 5- and 10- $\mu\text{m}$  particles for solute pairs with  $\alpha$  of about 1.03.

The following equation can assist in optimization of analysis time (13):

$$t_{R(n)} = \frac{1}{\Delta P} \frac{\Phi}{d_p^2} (1 + k'_n) (L_{\text{req}})^2 \eta$$

where  $t_{R(n)}$  = retention time of the strongest retained component

$\Delta P$  = pressure at which the system is operated

$\Phi$  = column resistance parameter

$d_p$  = particle size of the column packing

$k'_n$  = capacity factor of the last eluting component

$L_{\text{req}}$  = minimum column length required for the separation

$\eta$  = viscosity of the mobile phase

To obtain optimum analysis time, the operating pressure should be the maximum allowable pressure with the given equipment. The particle size ( $d_p$ ) should be the smallest size available except for certain separations.  $L_{\text{req}}$  is dependent mainly on the selectivity of the HPLC system; if  $L_{\text{req}}$  is the same for the two, then the system with the smallest  $(1 + k'_n) \eta$  factor, should be favored.