HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF FATTY ACIDS AND MATHEMATICAL MODELING OF LIQUID CHROMATOGRAPHY

A Dissertation Presented to
The Faculty of the
Fritz J. and Dolores H. Russ
College of Engineering and Technology
Ohio University

In Partial Fulfillment
of the Requirement for the Degree
Doctor of Philosophy

By
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March 2001
ACKNOWLEDGEMENT

I am deeply grateful to my advisor, Dr. Tingyue Gu, for giving me the opportunity to do research for my Ph.D. degree. His guidance, encouragement, and assistance have made possible the completion of this work.

I want to thank Dr. John J. Kopchick for financially supporting the fatty acid project, providing very helpful suggestions on my research and serving on my advisory committee. Thanks are also due to Dr. Bruce Kelder who provided the recombinant animal cell samples used in my research. I also want to thank Dr. Darin Ridgway and Dr. Tiao Chang for serving on my advisory committee and providing helpful comments and suggestions.
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<tr>
<td>$a_i$</td>
<td>constant in Langmuir isotherm for component $i$, $b_i C_i^*$</td>
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<tr>
<td>$b_i$</td>
<td>constant in Langmuir isotherm for component $I$</td>
</tr>
<tr>
<td>$B_i$</td>
<td>Biot number of mass-transfer of a solute, $kR_p/(\varepsilon_p^2 D_p)$</td>
</tr>
<tr>
<td>$C_0$</td>
<td>Concentration of a solute used for nondimensionalization, $\max {C(t)}$ (mol \cdot L$^{-1}$)</td>
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<tr>
<td>$C_b$</td>
<td>Concentration of a solute in the bulk-fluid phase (mol \cdot L$^{-1}$)</td>
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<tr>
<td>$c_b$</td>
<td>$= C_b/C_0$</td>
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<td>$C_{m0}$</td>
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<td>$C_p$</td>
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<tr>
<td>$c_p$</td>
<td>$= C_p/C_0$</td>
</tr>
<tr>
<td>$c^\infty$</td>
<td>$= C^\infty/C_0$</td>
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<tr>
<td>$D_b$</td>
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<td>$D_m$</td>
<td>Intraparticle molecular diffusivity (m$^2$ \cdot s$^{-1}$)</td>
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<td>$D_p$</td>
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<tr>
<td>$Da^a$</td>
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<td>$Da^d$</td>
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\( d_m \) Diameter of a molecule
\( d_{pore} \) Macropore diameter of a particle
\( F^{ex} \) Size exclusion factor of a solute (\( F^{ex}=0 \) means complete exclusion)
\( k \) Film mass transfer coefficient of a solute (\( m \cdot s^{-1} \))
\( k_d \) Desorption rate constant
\( K_{sec} \) Distribution coefficient of a solute
\( L \) Column length (m)
\( MW \) Molecular weight of a solute
\( N \) Avogadro's number, \( 6.023 \times 10^{23} \) molecules per mole
\( N_c \) Number of interior collocation points
\( N_e \) Number of quadratic elements
\( N_s \) Number of components
\( Pec_L \) Peclet number of axial dispersion for a solute, \( vL/D_b \)
\( Q \) Mobile phase volumetric flow rate (\( m^3 \cdot s^{-1} \))
\( R \) Radial coordinate for a particle in cylindrical coordinate system
\( Re \) Reynolds number, \( (v e_b) \rho (2R_p)/\mu \)
\( r \) \( = \) \( R/R_p \)
\( R_m \) Radius of a molecule (m)
\( R_p \) Particle radius (m)
\( Sc \) Schmidt number, \( \mu /D_m \rho \)
\( Sh \) Sherwood number, \( k(2R_p)/D_m \)
\( T \) Absolute temperature (K)
t  Dimensional time (t = 0 is the moment a sample enters a column) (s)

t₀  Retention time of a very small molecule that can penetrate all macropores (s) for an SEC column, or dead volume time of unretained small molecules, such as salts and solvents for an RP-HPLC column.

t₉  Retention time of totally excluded large molecules, such as blue dextran (s)

t₉  Retention time of a solute (s)

v  Interstitial velocity, 4Q/(πd²εₜₜ) (m • s⁻¹)

V₀  Column void volume (m³)

Vₑ  Solute elution volume (m³)

Vₑ,₀  Elution volume at retention time t₀ (m³)

Vₑ,₉  Elution volume at retention time t₉ (m³)

Vₑ,R  Elution volume at retention time t₉ (m³)

Vₛₐₘₚ  Sample volume (L)

vₛ  Partial specific volume of a molecule (m³ • kg⁻¹)

V₁  Total volume of liquid phase in the column (m³)

Z  Column axial coordinate in cylindrical coordinate system

z  Z/L

_Greek Letters_

α (α') Parameter for the eluite-modulator correlation

β (β') Parameter for the eluite-modulator correlation

γ (γ') Parameter for the eluite-modulator correlation
\( \eta \) Dimensionless group, \( \varepsilon_p^a D_p L / (R_p^2 v) \)

\( \kappa \) Boltzmann's constant, \( 1.38 \times 10^{-23} \) (J \( \cdot \) K\(^{-1}\))

\( \lambda \) Ratio of the solute molecular diameter to the pore diameter, \( d_m / d_{pore} \)

\( \lambda_0 \) Ratio of the solute molecular diameter to the pore diameter when the solute is completely excluded

\( \mu \) Mobile phase viscosity (Pa \( \cdot \) s)

\( \xi \) Dimensionless constant, \( 3B\eta(1-\varepsilon_b) / \varepsilon_b \)

\( \rho \) Density of solvent (kg \( \cdot \) m\(^{-3}\))

\( \tau \) Dimensionless time, \( vt / L \)

\( \tau_{imp} \) Dimensionless time duration for a rectangular pulse of the sample, \( 4V_{samp} / (\pi d^2 L \varepsilon_b) \)

\( \tau_{delay} \) Dimensionless time it takes for the gradient front to reach the column inlet

\( \tau_{tor} \) Pore tortuosity

\( \varepsilon_b \) Bed void volume fraction

\( \varepsilon_p \) Particle porosity

\( \varepsilon_p^a \) Accessible particle porosity

\( \phi \) Phase ratio (stationary phase to mobile phase), \( (1-\varepsilon_b)(1-\varepsilon_p) / [\varepsilon_b + (1-\varepsilon_b)\varepsilon_p] \)
CHAPTER 1
INTRODUCTION

The biochemical and economic importance of fatty acids, especially essential fatty acids (EFA), account for widespread interest in their separation and analysis. Fatty acids are usually considered lipids. Lipids comprise a heterogeneous group of compounds. They have been divided on the basis of susceptibility to hydrolysis into two main groups, simple lipids such as fatty acids and prostaglandins which contain no hydrolysable bonds, and complex lipids which usually contain fatty acids esterified to polyfunctional alcohols such as glycerol (triglycerides), phospholipids and sphingolipids. The latter two groups are further esterified to highly polar groups that confer surface activity on the molecule.

The diversity of structure and function of lipids demands different analytical procedures to suit the chemical properties of each group and the environment in which it occurs. Before the advent of modern HPLC, thin layer chromatography (TLC) was used to separate involatile or thermolabile lipids, and gas chromatography (GC) was used extensively for quantitative separation and identification of volatile lipids or volatile lipid derivatives. HPLC is now used increasingly for the analysis of all classes of lipids. Good resolution basis of complex and thermolabile compounds can be obtained and intact material for further analysis can be easily recovered. HPLC on a reversed phase (RP) column using either a variable wavelength UV detector or a refractive index (RI) detector will yield useful results in most instances although other detectors such as the infrared
detector have been used. Liquid chromatography-mass spectrometry (LC-MS) can be used to identify complex lipids in a single analysis where several steps were required before.

Although HPLC is a very promising analytical method for lipids, very few studies in literature have been devoted to fatty acids, especially free fatty acids. This study will explore how to separate and analyze fatty acids in their free form to support the study on the metabolic pathway of oleic acid in recombinant animal cells. The results of this study can also be used or provide some useful information for the separation and analysis of fatty acids from other sources.

With the development of computer technology, mathematical modeling and the use of computer programs to facilitate HPLC method development have received much attention since the late 1970s. Many theories and mathematical models have been developed to simulate various chromatography processes. Among these models, the general rate model is considered the most comprehensive. In this work, a general rate model will be adopted to analyze, simulate and predict chromatography processes. The application of the general rate model to size-exclusion chromatography (SEC) and reverse-phase high-performance liquid chromatography (RP-HPLC) will be studied.
2.1 Definition of Fatty Acids

Fatty acids are a type of simple lipid. The term lipid describes a group of naturally occurring compounds, which have, in common, a good solubility in organic solvents such as chloroform, benzene, ethers and alcohol. Such diverse compounds as fatty acids and their derivatives, steroids, carotenoids, terpenes, and bile acids are included. In fact, a definition of this kind is positively misleading, since many of lipids are more soluble in water than in organic solvents. These compounds are found in all biological systems, in great variety and with enormous diversity of incidence and function. Lipids, which are solid at ambient temperatures, tend to be referred to as fats while those that are liquid are called oil (Kates, 1986). A more specific definition than one based simply on solubility is necessary. Most workers in this field would restrict the use of lipid to fatty acids and their naturally-occurring derivatives and to compounds related closely through biosynthetic pathways (e.g. prostaglandins) or by their functions (e.g. cholesterol) to fatty acid derivatives.

2.1.1 Synthesis of Fatty Acid

Fatty acids are compounds synthesized in nature via condensation of malonyl coenzyme A units by a fatty acid synthase complex. These form a homologous series of straight chain carbon compounds terminating with a methyl group at one end and a
carboxylic acid at the other. The carbon chain length of natural fatty acids varies typically from C4 to C30, with the majority lying between C10 and C22. Fatty acid chains are generally composed of an even number of carbon atoms by virtue of their biochemical synthesis. This involves the cellular enzyme complex (fatty acid synthetase) that uses the 2-carbon fragment acetic acid as a building block (Gurr and James, 1980). The product of this synthetase complex is palmitic acid (C16:0), and further chain elongation (including elongation of unsaturated fatty acids) and desaturation require a different complex of enzymes.

Desaturation of normal straight chain fatty acids via desaturase enzymes, yields an important family of fatty acids that contain one or more double bonds, of the cis-isomeric form. Where there is more than one double bond each pair is normally separated by single methylene unit (-CH\(_2\)-). The series of unsaturated fatty acids synthesized in plants is different from that synthesized in animals. This difference creates the requirement by animals for essential dietary fatty acids (EFA) produced in plants.

EFAs are fatty acids that are necessary for normal physiological function in animals and man. The essentiality of the EFAs lies not only in their physiological importance, but also in the fact that, like many vitamins, they cannot be synthesized de novo. They are derived either directly or in partially elaborated precursor form from the diet.

The so-called derived EFAs that can be oxidatively converted into eicosanoids are generally of a longer chain (usually C20) and are more unsaturated than their
common dietary precursors (Hammarström and Falardeau, 1977). The EFAs have the following general formula: \( \text{CH}_3[\text{CH}_2]_k[\text{CH}==\text{CHCH}_2]_m[\text{CH}_2]_n \text{COOH} \).

### 2.1.2 Nomenclature of Fatty Acids

The systematic nomenclature of fatty acids defines both the position and geometry of each double bond, counting from the terminal carboxylic acid function (alpha end of the molecule). For example, arachidonic acid is cis-5, cis-8, cis-11, cis-14-eicosatetraenoic acid and is sometimes abbreviated to all cis-\( \Delta^5,8,11,14 \) eicosatetraenoic acid. The \( \Delta \) is an abbreviation which denotes the position of a double bond numbered from the terminal carboxyl group.

A shorthand nomenclature recommended by Holman (1966) has been used. This system designates the chain length, number of double bonds, and the position of the double bond nearest to the terminal methyl group. This terminal carbon atom is referred to as the omega (\( \omega \)) carbon atom, as it occurs at the opposite end of the molecule to the alpha (\( \alpha \)) carbon atom that bears the terminal carboxyl function. A methylene-interrupted sequence in which the double bonds all have cis geometry is assumed. For linoleic acid, i.e. cis-9, cis-12-octadecadienoic acid, the shorthand is \( \text{C}18:2\omega6 \) (Figure 2.1). This shorthand system is most useful when considering the biochemical interrelationships between EFAs. Chain elongation and insertion of double bonds (desaturation) occur only at the carboxyl end of the molecule. Thus, all \( \omega6 \) EFAs are biochemically interrelated, and all members of the \( \omega3 \) series are also related to each other. Generally, no inter-
conversion can take place between the \( \omega 6 \) and \( \omega 3 \) series of fatty acids in animals, although such inter-conversions can take place in plants.

\[
\begin{array}{cccc}
18 & 12 & 9 & 1 \\
\text{CH}_3(\text{CH}_2)_4(\text{CH}=\text{CH})(\text{CH}_2)(\text{CH}=\text{CH})(\text{CH}_2)_7\text{COOH} \\
\omega & \omega 6 & \alpha
\end{array}
\]

Figure 2.1. Molecular formula and numbering system for linoleic acid (C18:2\( \omega 6 \)).

### 2.1.3 Prostaglandins

Prostaglandin belongs to a group of biologically active fatty acids, referred to as the eicosanoids, that are found naturally occurring in most tissues. Arachidonic acid (5, 8, 12, 14-cis-eicosatetraenoic acid) is the precursor for the synthesis of prostaglandin and other eicosanoids such as leukotrienes, thromboxanes and prostacyclins. Synthetic analogues of the naturally occurring substances with pharmacological activity are used in medicine. The field of prostaglandins and related eicosanoids is one of the most complicated areas of biological research that has ever existed. There is a bewildering array of chemical structures and an equally bewildering (and often widely differing) spectrum of biological activity.

Prostaglandin was the name given by Von Euler (1936) to the agent responsible for depression of blood pressure, contraction of intestinal smooth muscle, and relaxation of uterine smooth muscle. Its name (actually a misnomer) arose from the belief that it originated in the prostate. Von Euler recognized the unsaturated acidic lipid nature of prostaglandin, but it was not until the 1950s that chemical isolation and thus subdivisions
of nomenclature occurred. Prostaglandins E and F were isolated from extracts of sheep vesicular gland (Bergström and Sjövall, 1960a, 1960b) and were identified by the first functioning gas chromatography/mass spectrometry (GC/MS) instrument developed by Rhyhage (Bergström et al., 1963).

The principal series or types of prostaglandin are denoted by the letters E, F, etc. Initially by design, and sometimes by coincidence, this suffix has reflected the means by which the prostaglandins were first discovered. The E- and F-type prostaglandins were initially named because on partition between ether and phosphate buffer, the E types tended to be extracted into the ether and F types into the phosphate buffer (Hamberg, 1973). Chemically, E-type prostaglandin (PGE) is characterized by the presence of a keto group at position 9 on the cyclopentane ring, while F-type prostaglandin (PGF) has this keto function replaced by a hydroxyl group.

The usual naturally occurring prostaglandins have a Δ13, 14 (trans) double bond. In prostaglandins of the 1 series, (PGE₁, A₁ etc), this is the only double bond in the side chains. Prostaglandins of the 2 series have an additional Δ5-6 (cis) double bond, and member of the 3 series have a further Δ17-18 (cis) double bond.

2.2 The Inter-Conversion of Fatty Acids

The link between the EFAs and prostaglandins was forged in the mid 1960s. The groups of Van Dorp (Van Dorp et al., 1964) and Bergström (Bergström et al., 1964) simultaneously reported that prostaglandins (E2 and F2α) could be formed biosynthetically from arachidonic acid (20:4ω6). Eicosanoids derived from arachidonic
Dihomo-γ-linolenic acid (DGLA, C20:3ω6)

\[ \text{Prostaglandin synthase (PGS)} \]
\[ \text{PGG}_1 \]
\[ \text{PGS} \]
\[ \text{PGH}_1 \text{ (PG endoperoxide)} \]

Figure 2.2. Synthesis of one-series eicosanoids.

It is certainly true that the ultimate source of the precursors of the essential fatty acids (EFA) is a group of polyunsaturated fatty acids produced only in plants (largely linoleic, 18:2ω6, and α-linolenic, 18:3ω3). However, the same cannot be said with accuracy for the eicosanoids. With only a few minor exceptions, the eicosapolyenoic acids necessary as eicosanoid precursors are synthesized only in animals.

Natural essential fatty acids of the animal kingdom are traditionally regarded as the unsaturated fatty acids linoleic and α-linolenic that cannot be synthesized de novo by animals and the fatty acids derived from them. That is, plants synthesize de novo linoleic and α-linolenic acids and animals convert them to fatty acids that are more unsaturated and of longer chain length. The fatty acids derived from each one of these precursors constitute a family. Each family is independent and there is no direct crossover (Figure 2.4) (Mead and Willis, 1987).
Figure 2.3. Synthesis of two-series eicosanoids.

2.3 Analytical Methods of Lipids and Fatty Acids

The study of lipids and fatty acids has assumed considerable importance in recent years with the recognition that they are involved in many vital biological processes in animals, plants and microorganisms. Disturbances in lipid metabolism are known to accompany a variety of disease states, and the role of lipids in heart disease especially remains an unresolved controversy (Hadley, 1985).

Methods for the analysis of lipids and fatty acids are therefore of great importance for many research, clinical and quality control applications. Gas-liquid chromatography (GLC) and thin-layer chromatography (TLC) provided the springboard for the explosive growth of knowledge in the fields of lipid chemistry and biochemistry that has occurred over the last few decades (Kawashima et al., 1997, Medina et al., 1995, May and Hume, 1993, Duchateau et al., 1996, Lepage, and Roy, 1984). TLC has served lipid analysts well over the years, especially for lipid class separations, and the required equipment is inexpensive and versatile in that a wide range of adsorbents can be employed and complex agents, such as silver nitrate, can easily be incorporated into the
<table>
<thead>
<tr>
<th>γ-Linolenic acid</th>
<th>Linoleic acid</th>
<th>Palmitoleic acid</th>
<th>Oleic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-\text{2H})</td>
<td>(-\text{2H})</td>
<td>(-\text{2H})</td>
<td>(-\text{2H})</td>
</tr>
<tr>
<td>6, 9, 12, 15-18:4</td>
<td>6, 9, 12-18:3</td>
<td>6, 9-16:2</td>
<td>6, 9-18:2</td>
</tr>
<tr>
<td>+2C</td>
<td>+2C</td>
<td>+2C</td>
<td>+2C</td>
</tr>
<tr>
<td>8, 11, 14, 17-20:4</td>
<td>8, 11, 14-20:3</td>
<td>8, 11-18:2</td>
<td>8, 11-20:2</td>
</tr>
<tr>
<td>Dihomo-γ-Linolenic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-\text{2H})</td>
<td>(-\text{2H})</td>
<td>(-\text{2H})</td>
<td>(-\text{2H})</td>
</tr>
<tr>
<td>5, 8, 11, 14, 17-20:5</td>
<td>5, 8, 11, 14-20:4</td>
<td>5, 8, 11-18:3</td>
<td>5, 8, 11-20:3</td>
</tr>
<tr>
<td>Arachidonic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+2C</td>
<td>+2C</td>
<td>+2C</td>
<td>+2C</td>
</tr>
<tr>
<td>7,10,13,16,19-22:5</td>
<td>7,10,13,16-22:4</td>
<td>7,10,13-20:3</td>
<td>7,10-22:3</td>
</tr>
<tr>
<td>(-\text{2H})</td>
<td>(-\text{2H})</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2.4. Pathways of inter-conversion of polyunsaturated fatty acids.
layers. It is particularly useful for a rapid qualitative examination of a sample, for developing methodology, and for routine screening of large numbers of samples for the occurrence of certain compounds.

HPLC was at first rather neglected for such purposes, and is only recently coming rapidly into use. Certainly, it has the potential to become one of the major tools in the hands of those analysts who wish to determine lipid compositions and structure. In the past few years many separations of lipids by means of HPLC have been described that cannot be rivaled by other methods (Carelli et al., 1997, VanRollins, Hofer et al., 1993, Wynalda et al., 1979, Bailey and Southon, 1998). The resolving power of HPLC has never been in doubt. The main barrier to progress and wider use of the technique has always been the fact that lipids lack chromophores that facilitate spectrophotometric detection.

With the development of HPLC, it could now be claimed that there is no separation, which has been accomplished by TLC, that could not now be repeated with greater convenience and speed by HPLC, if detector limitations could be overcome (Christie, 1987). GLC is likely to remain the preferred method for the analysis of stable non-polar lipids or their derivatives with a molecular weight of up to about 700, as high resolution is attainable at lower capital and running costs than by means of HPLC. However, preparative-scale GLC, a technique that has always been considered for use only as a last resort (because of poor recoveries and doubtful reproducibility among other reasons), will fall into disuse as reversed-phase HPLC takes its place. Reviews of HPLC and other techniques in the analysis of lipids can be found in the literature (Runser, 1981,
2.4 High-Performance Liquid Chromatography

The term "high-performance liquid chromatography" or "HPLC" denotes a form of chromatography. In HPLC, a mobile liquid phase is forced under controlled high pressure through a relatively narrow bore column containing a stationary phase, which can be a solid surface or a liquid phase, the latter bonded chemically to an inert support. The technique has also been termed high-pressure liquid chromatography or high-speed liquid chromatography.

Modern technology has been applied to each stage of the separation process, from the design of the pumping and detection systems to the manufacture of the column packing materials and fittings, in order to improve the quality and reproducibility of the separations attainable and the accuracy of the quantification. The first essential equipment for HPLC is a solvent delivery system. This system comprises solvent reservoirs and filters with some means of degassing the solvents. Most important of all, a pulse-free pump (or pumps), capable of delivering solvents at precise specified flow rates under pressures of up to 6000psi; some means of generating a gradient in the composition of the mobile phase is desirable for many applications. Next, an injection system is needed to ensure that a sample can be introduced on to the column without releasing the pressure. The column itself is a polished stainless steel tube with the appropriate end couplings, and is packed with a micro-particulate adsorbent or other
phase. Finally, some form of detection system is required so that the progress of an analysis can be monitored, and each of the components of the sample can be quantified.

2.4.1 The Theory of HPLC

The process of liquid chromatography involves a partition of the components of a mixture between two phases, mobile liquid phase and a stationary liquid or solid phase. A dynamic equilibrium state is set up between the phases and this can be characterized by an equilibrium coefficient or capacity factor (k'), defined as the ratio of the amount or concentration of a given component in the stationary phase (C_s) to that in the mobile phase (C_m), i.e.

\[ k' = \frac{C_s}{C_m} \]  

(2-1)

This parameter can perhaps be seen to have more immediate relevance when it is defined in terms of the retention times (or volumes) of a solute, i.e.,

\[ k' = \frac{t-t_0}{t_0} \]  

(2-2)

where \( t = \) the retention time of a solute, and \( t_0 = \) the time required for the solvent (or an unretained solute) to move from one end of the column to the other.

As migration of the component only takes place when it is in the mobile phase, the rate of movement is inversely proportional to the capacity factor. Different
compounds exhibit different relative distributions and must migrate at different rates, so separation if possible. When the resolution is insufficient, it is necessary to alter the capacity factors by varying the selectivities towards either the stationary or mobile phases by changes in the elution conditions.

If there were no diffusion, each compound would migrate as a sharp band. In practice, components do diffuse and emerge from a column in the form of peaks, ideally with a Gaussian shape. The efficiency of a column can be calculated from the dimensions of the peaks by using the concept of plate heights, derived initially from distillation theory. If it is assumed that a peak indeed has an ideal Gaussian shape, the number of theoretical plates (N) in a column is determined by the retention time of the component (t_R), i.e. the time from sample injection until the peak for a component reaches its maximum height, and the width of the peak at half that height (w_h) by using the following relationship (Christie, 1987).

\[ N = 5.54 \times \left( \frac{t_R}{w_h} \right)^2. \]  \hspace{1cm} (2-3)

It follows that the wider a peak is at a given retention time, the lower the column efficiency. An understanding of the factors that cause peak broadening can therefore help to optimize the separation conditions for a particular analysis.

A number of factors contribute to broadening of chromatographic bands. Eddy diffusion occurs because of irregularities in the size and shape of the particles of packing material, and, in turn, irregularities are found in the size of the channels between
particles. Solvents will move more rapidly through a wide channel than through a narrow one, and any sample molecules in a narrow channel could be delayed. In general, the smaller the diameter of the particles, the higher the efficiency of the column that should be attainable.

Further effects on the broadening of peaks are known as mass transfer phenomena and can be considered in terms of both the stationary and mobile phases. With a liquid stationary phase, the stationary phase mass transfer effect is a consequence largely of the depth of the liquid phase. Molecules which enter the liquid phase can diffuse further down into the layer and in consequence spend a longer time there than molecules which remain closer to the surface. When these molecules eventually enter the mobile phase, they will be overtaken by the bulk of the material and peak broadening again is the result. For adsorption chromatography, molecules are also retarded by some of the sites on the adsorptive surface that are more active than others.

Mobile-phase mass transfer causes broadening of peaks for two main reasons. Firstly, not all molecules in a particular flow stream will move at the same rate, since those near the channel walls will travel more slowly than those in the center of the stream. Secondly, because of irregularities in the shapes of the particles, there will always be some regions where the mobile phase is static. It may be necessary for molecules to spend time diffusing through such dead volumes before they can enter the moving stream again.

Simple diffusion can have an effect on the broadening of bands. Longitudinal diffusion, i.e. in the direction of flow, brings about a symmetrical band broadening,
although this is only troublesome when molecules have a long residence time on the column when very slow flow-rates are used. Radial diffusion, which occurs in all directions, causes band spreading indirectly at the column walls as molecules in this region will travel more slowly than those in mid-stream. With an ideal Gaussian distribution, sample size should have no effect on peak width, as there is then a linear relationship between the concentrations of compounds in the stationary and mobile phases. More often, however, there is a non-linear distribution isotherm and sample size can have a noticeable effect on the width, shape and retention time of a peak. This may be manifested by the appearance of skewed peaks, which exhibit tailing or fronting. Similarly, badly shaped peaks may be observed if the solvent flow-rate is either too high or too low.

Many factors, which are external to the column, also have an effect on peak broadening. The influence of temperature on a separation is proportional to the enthalpy of the transfer from the mobile to the stationary phase, and tends to be small (much less in fact than in GLC) but significant. Most HPLC separations are run at ambient temperatures, but thermostatically controlled ovens are available for some purposes. For example, higher temperatures may increase the solubility of compounds that are otherwise only sparingly soluble in the liquid phase, and so will facilitate analysis. Increased temperatures will generally reduce solvent viscosity and this can also improve resolution. In practice, it is usually easier and more effective to use gradient elution techniques than temperature-programming in HPLC to achieve the same results.
Other external effects on column efficiency are observed as a consequence of the fact that the tubing and fittings between the injection valve and the detector have a finite volume. Resolution can be improved by good design of the equipment, and by keeping the length and the diameter (ideally 0.25 mm or 0.01 in) of the connecting tubing to the minimum (Christie, 1987).

2.4.2 Modes of Separation in HPLC

There are six main modes of separation in HPLC that appear to be of interest to lipid analysts, i.e. adsorption, reversed-phase liquid partition, normal phase liquid partition, ion-exchange, gel permeation and chiral-phase chromatography. Separations in all of these modes arise from molecular interactions between the solute and the mobile and stationary phases, in which various types of force are involved. Ionic forces arise when positively or negatively charged species are present; for example, a cation in the mobile phase will be strongly attracted to an anionic sample molecule. Polar forces are the result of uneven charge distributions (dipoles) over molecules, when electron-withdrawing or electron-donating substituent groups are present. In liquid-solid chromatography, molecules with such dipoles will initially be held strongly by the adsorbent, but will be released as the polarity of the mobile phase is increased, as this will compete for the adsorptive sites. Lastly, there are dispersive forces, which are weak impermanent electrical charges, and they may come into play to a limited extent, usually during reversed-phase chromatography. With a complex sample, some or all of these forces may have some importance.
2.4.2.1 Adsorption Chromatography

Silica gel has been widely used by lipid analysts as an adsorbent in low-pressure column chromatography and in TLC, and now has innumerable uses in HPLC. It is a porous solid and the surface area is inversely related to the size of the pores in the particles, 6 nm being the standard pore size of most purposes.

One specialized type of adsorption chromatography of particular importance in lipid analysis is a form of complexation chromatography, i.e. silver ion or argentation chromatography. Silica gel impregnated with silver nitrate has been used in many forms of adsorption chromatography, including HPLC, to separate lipids according to the number and configurations of the double bonds in acyl or alkyl moieties (Guha, and Janak, 1972, Morris, 1966). The principle of the method is that silver ions interact reversibly with double bonds (cis more strongly than trans) to form polar complexes; the greater the number of double bonds in a molecule, the stronger the complex formation and the longer it is retained.

2.4.2.2 Reversed-Phase Liquid Partition Chromatography

The term reversed-phase implies that the stationary phase is a non-polar liquid and the mobile phase is a more polar solvent. HPLC in the reversed-phase mode has been much used for the separation of lipids within a single lipid class (Whorton et al., 1979, Batta et al., 1984, Nagayo and Mizuno, 1979, Aveldano et al., 1983, Cohen and Cohen, 1991, Smith et al., 1980). These lipids are separated principally according to the sum of the chain-lengths of the fatty acyl or alkyl moieties, together with an appreciable
dependence on the number and configuration of any double bonds. Separation depends on differences in the equilibrium distribution coefficients of the various components between the two phases. By far the most widely used stationary phase consists of octadecylsilyl (C18 or ODS) groups, linked to a silanol surface by covalent bonds.

2.4.2.3 Normal-Phase Liquid Partition Chromatography

A number of liquid stationary phases of higher polarity are available, consisting of a substituent group, such as diol, nitrile, nitro, methylcyano or phenylcyano, bonded chemically via a short alkyl bridge or spacer to a silanol support. Only a few of these appear to have been used to a significant extent in the analysis of lipids by means of HPLC. The nature of the bond between the support and the organic group is important in that it determines the stability of the phase under various elution conditions.

The nature of the separations can be similar to that obtained with silica gel, especially when eluted with non-polar solvents. Indeed, the term normal-phase chromatography is sometimes used to encompass both adsorption and normal-phase partition chromatography, with some justification as bound water is probably a major factor influencing the separations obtained with both. Bonded phases tend to give much more reproducible separations with less tailing of peaks, and they also equilibrate much more rapidly with the mobile phase in gradient applications.
2.4.2.4 Ion-Exchange Chromatography

The cellulose-based ion-exchange media, diethylaminoethyl (DEAE)-and triethylaminoethyl (TEAE)-cellulose (anion exchangers) and carboxymethyl (CM)-cellulose (cation exchanger), have been much used by lipid analysts in low-pressure column applications for the separation of polar complex lipids (Christie, 1982). Although silica gel based bonded phases containing the appropriate functional groups are available for HPLC; they do not appear to have been applied to lipid separations. On the other hand, bonded phases (silica gel-based) with a variety of different amine groups (anion exchangers) or with sulphonic acid. In essence, the process of ion exchange can be considered as a competition between the solute ions and counterions present in the mobile phase for fixed sites of opposite charge on a support. The quality and extent of a given separation can be manipulated by varying the nature and concentration of the counterion or by varying the pH of the mobile phase.

2.4.2.5 Size-Exclusion Chromatography

This technique, which is also known as gel-permeation chromatography or gel-filtration chromatography, differs from the others above in that the separations achieved are based mainly on the size (and to a limited extent, on the shape) of the solute molecules. The stationary phase is generally an inert porous silica or polymer matrix, such as a dextran or polystyrene, in which the sizes of the pores are carefully controlled by the manufacturer, within limits that are predetermined for particular applications. During chromatography, small molecules may diffuse out of the mobile phase into the
pores so their progress is retarded relative to that of larger molecules. The smaller the molecule, the further it will be able to penetrate into the pores and the more it will be retained. The largest molecules obviously elute first. The mobile phases in gel-permeation chromatography are chosen mainly on the basis of their capacity to solubilize the compounds of interest, although again not all resin-based stationary phases can be sued with organic solvents.

With a gel of a given type, the retention time of a compound of known molecular weight can be predicted with reasonable accuracy; conversely, the molecular weights of unknown compounds can be predicted from retention times determined experimentally. Components tend to elute as sharp bands, so are often detected relatively easily. Elution times are usually short, and solvent gradients are rarely required. The resolution attainable with the technique is limited to compounds that differ in molecular weight by at least 10%.

2.4.2.6 Chiral-Phase Chromatography

A number of stationary phases consisting of various chiral molecules, which are bonded chemically to a matrix of silica gel, have been described, and they have been used in HPLC columns for the separation of enantionmeric compounds, avoiding any need for the separation of diastereoisomeric derivatives. The forces involved are highly complex, and depend on the natures of the bound molecules and of the solutes. To date, only a few applications to lipids have been described.
2.4.3 Mobile Phases

The choice of mobile phase is dependent on the nature of the separation mode adopted. For lipid analyses of most kinds, organic rather than aqueous eluents are almost invariably required. In formulating mixed solvents, it is essential that they are fully miscible with each other. Otherwise droplets of a second phase may be trapped in the system to appear at an inopportune moment and ruin a separation. For reproducible results when mixing solvents to prepare an eluent, it is important to measure out each of the solvents separately before mixing (and not to use a single measuring cylinder and make up each solvent to the appropriate mark). It is essential that none of the constituents of the mobile phase interact with the stationary phase and affect it adversely.

All solvents can contain impurities, some of which (e.g. antioxidants) are indeed introduced deliberately by manufacturers to improve stability. For HPLC use, the solvents should be the highest quality available. Special HPLC grade solvents should be used when possible and this implies that they contain low levels of UV-absorbing materials, which could otherwise give high background values with UV detectors. Solvents should be stored in the dark in a flameproof cabinet, and in a cool dry place. The inlet lines from solvent reservoirs should incorporate filters to remove any particulate matter, including dust and bacteria, in solvents.

2.4.3.1 Solvent Selection

The physical properties of solvents are the first consideration in solvent selection. The physical properties of some solvents, which are used frequently by lipid analysts, are
listed in Table 2.1 (Snyder and Kirkland, 1979). The polarity of a solvent is probably one of the first factors to consider. Where two solvents appear to be equally suited to a purpose, it is usually recommended that the one of lower viscosity be selected. Also, the solvent should not be so volatile that it tends to evaporate at an appreciable rate in the reservoir. Certain solvents have spectroscopic properties, which are particularly suitable for some purposes can be used with UV detection below 205nm, where most other solvents are opaque.

Solvents such as hexane, chloroform, ethers, methanol, isopropanol and toluene have been used for decades in the isolation and analysis of lipids. Since the advent of HPLC, acetonitrile has found many uses in the analysis of lipids, especially in the reversed-phase mode, for separations according to the chain-lengths of the fatty acyl groups.
Table 2.1. The physical properties of some organic solvents of special interest to lipid analysts (Christie, 1987).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Refractive index (25°C)</th>
<th>Boiling point (°C)</th>
<th>Polarity factor</th>
<th>Viscosity (cP, 25°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2,4-Trimethylpentane</td>
<td>1.389</td>
<td>99</td>
<td>0.1</td>
<td>0.47</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>1.372</td>
<td>69</td>
<td>0.1</td>
<td>0.30</td>
</tr>
<tr>
<td>n-Heptane</td>
<td>1.385</td>
<td>98</td>
<td>0.2</td>
<td>0.40</td>
</tr>
<tr>
<td>Toluene</td>
<td>1.494</td>
<td>110</td>
<td>2.4</td>
<td>0.55</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>1.350</td>
<td>35</td>
<td>2.8</td>
<td>0.24</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>1.405</td>
<td>66</td>
<td>4.0</td>
<td>0.46</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.326</td>
<td>65</td>
<td>5.1</td>
<td>0.54</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.359</td>
<td>78</td>
<td>4.3</td>
<td>1.08</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>1.384</td>
<td>82</td>
<td>3.9</td>
<td>1.90</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>1.397</td>
<td>118</td>
<td>3.9</td>
<td>2.60</td>
</tr>
<tr>
<td>Water</td>
<td>1.333</td>
<td>100</td>
<td>10.2</td>
<td>0.89</td>
</tr>
<tr>
<td>Acetone</td>
<td>1.356</td>
<td>56</td>
<td>5.1</td>
<td>0.30</td>
</tr>
<tr>
<td>Butan-2-one</td>
<td>1.376</td>
<td>80</td>
<td>4.7</td>
<td>0.38</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>1.370</td>
<td>77</td>
<td>4.4</td>
<td>0.43</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>1.341</td>
<td>82</td>
<td>5.8</td>
<td>0.34</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>1.370</td>
<td>118</td>
<td>6.0</td>
<td>1.10</td>
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<td>Chloroform</td>
<td>1.443</td>
<td>61</td>
<td>4.1</td>
<td>0.53</td>
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<td>dichloromethane</td>
<td>1.421</td>
<td>40</td>
<td>3.1</td>
<td>0.41</td>
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<td>1,2-Dichloroethane</td>
<td>1.442</td>
<td>83</td>
<td>3.5</td>
<td>0.78</td>
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</table>
2.4.3.2 Removal of Dissolved Air from Solvents

All solvents contain dissolved air, the solubility of which is increased at high pressures. With the sudden release of pressure at the end of the chromatographic column, bubbles can form that cause pressure fluctuations and interfere with detection. Dissolved air can also have deleterious effects on solutes, for example by causing autoxidation of double bonds in lipids. It is, therefore, desirable to remove all dissolved gases from solvents prior to an analysis.

Perhaps the most common method of achieving this is refluxing solvents for a short time. However, this method can be hazardous, additional cooling time is needed before the solvent is used, and the solvent must be purged continuously with an inert gas, otherwise air will rapidly be taken up again. Air can also be removed by subjecting solvents to a vacuum for a time, and this method is often preferred for aqueous eluents, although again they must be purged with an inert gas to ensure that air does not redissolve. A less effective method is to use ultrasonic vibrations. The simplest and least hazardous method of removing air from solvents is to purge them with helium gas, which displaces any dissolved gases but which itself is virtually insoluble in solvents. All that is necessary is to purge the solvents with helium via a porous stainless steel frit or filter at a flow-rate of about 100 ml/min for about 10 min, then to reduce the flow-rate to a trickle to prevent any redissolution of air.
2.5 Mathematical Models for Liquid Chromatography

There are many mathematical models of different complexities for liquid chromatography. These models can be classified into three general categories: equilibrium theory, plate models and rate models (Ruthven, 1984).

Equilibrium theory assumes a direct local equilibrium between the mobile phase and the stationary phase, neglecting the mass transfer resistance. It provides the retention times of the solutes, but it fails to predict the peak shapes accurately if the mass transfer effects are significant (Gu, 1995). Plate theory was first introduced to chromatography by Martin and Synge (1941). In many aspects, plate models are not adequate to describe multicomponent chromatography because the equilibrium stages may not be assumed equal for different solutes. Rate models contain a rate expression that describes the interfacial mass transfer between the mobile phase and the stationary phase. A rate model usually consists of two sets of differential mass balance equations, one for the bulk-fluid phases, the other for the particle phase. Different rate model have varying complexities (Ruthven, 1984). In this work, a general rate model proposed by Gu (1995) will be used to study the SEC and RP-HPLC processes.
CHAPTER 3
PURPOSE OF THIS STUDY

This work consists of two parts. The first part is the analysis of the fatty acids from recombinant animal cells. The second part is the simulation of liquid chromatography processes.

For the first part, the objectives are developing an effective process to prepare fatty acid samples from recombinant animal cells for RP-HPLC analysis and establishing an RP-HPLC method to analyze these fatty acid samples. Free fatty acids only account for a very small part of the total fatty acids in animal cells. Before RP-HPLC analysis, a process needs to be developed to release these fatty acids and isolate them from other impurities. Such a fatty acid isolation process together with the RP-HPLC method could also be adapted to serve as a fatty acid production process on a large scale.

For the second part, the purpose of this work is to simulate two kinds of liquid chromatography processes: size exclusion chromatography and reverse-phase liquid chromatography. These two kinds of chromatography are two important techniques used in the purification of biological products. However, equipment investment and operational expense for chromatographic processes constitutes a major part of the production cost of biological products. A good mathematical model will help to greatly reduce the time and cost for the method development of chromatography processes. The general rate model was developed based on the general consideration of mass transfer process in various liquid chromatographic processes. Its application in a specific...
chromatographic process needs to be based on the characteristics of the process studied. In this study, procedures will be developed to simulate these two chromatographic processes using the general rate model. The effects of various model parameters will be analyzed. Methods to estimate these model parameters will be established. The validity of the general rate model for these two chromatographic processes will be studied by the comparison of the model predicted results and the experimental results.
CHAPTER 4
HPLC ANALYSIS OF FATTY ACID STANDARDS

4.1 Introduction

Fatty acids are the distinctive structural components of lipids, and as their compositions and biological functions can vary widely in tissues or cells, methods for their isolation and analysis are of great significance.

As part of the strategy for the elucidation of cell metabolic pathway or the identification of species of phospholipids, triacylglycerols and other complex lipids, it is usually necessary either to analyze the fatty acids composition or hydrolyze the lipids and chromatograph the resulting fatty acids for quantitation and identification. ODS phases, ideally as 5 µm particles nowadays, have been preferred for the HPLC separations with acetonitrile - water or methanol - water mixtures as the mobile phase. Acetonitrile tends to have a greater effect than methanol in reducing the retention times of unsaturated components.

It should be recognized that individual fatty acids can only be identified tentatively by one method alone, and that it is often advisable to confirm the identity of particular components using a second method (e.g. spectroscopic procedures) (Wübert et al., 1988, Ahern et al., 1983). In order to analyze fatty acids, it is first necessary to isolate fatty acids from other impurities in the sample.

Nowadays, HPLC methods should almost certainly be preferred for the isolation of specific fatty acids. This is because HPLC does offer a number of advantages over
other techniques. A wide range of column packing materials is available for specific applications, and the columns can be used many times. Most separations of interest can be achieved at ambient temperature under anaerobic conditions, so HPLC is particularly well suited to compounds with reactive functional groups. In addition, resolution tends to fall off only slowly with increasing sample size; analysis times can be short; retention times of compounds under set conditions are reproducible; there is a sound theoretical base to the technique; and the nature of the equipment implies that it is capable of a high degree of automation, especially with respect to the quantification of separated components.

Because the separations are usually carried out at ambient temperature, HPLC methods do have a number of valuable applications in analysis of fatty acids with labile functional moieties, such as hydro-peroxide groups or cyclopropene rings whereas unwanted rearrangements occur at the high temperatures required for GLC analysis. High specificity and sensitivity are possible if fluorescent derivatives of fatty acids are prepared for HPLC analysis or if a low range wavelength detector is used, and this can sometimes be the best method available for very small samples.

Although ethyl esters and other fatty acid derivatives have been used in the analysis of fatty acids, if pure free fatty acids are needed for further analysis or other purposes, it is not appropriate to convert fatty acids to their derivatives. In this case, it is desirable to establish a process to separate and analyze free fatty acids.
4.2 Fatty Acid Standards Used for HPLC Analysis

In order to analyze fatty acids from real cell samples using HPLC, it is necessary to have an idea about what kinds of fatty acids are possibly present in the real sample. This study was to support the study on the fatty acids metabolic pathway of recombinant animal cells that were prepared by Dr. Bruce Kelder at Edison Biological Institute of Ohio University. Dr. Kelder provided the following fatty acids metabolic pathway of the recombinant mouse cells (Figure 4.1).

According to this metabolic pathway, some fatty acids (including some prostagladins) were chosen to act as fatty acids standards for the HPLC analysis. These standards are listed in Table 4.1. The molecular structures of these fatty acids are shown in Figure 4.2.

4.3 HPLC System

The fatty acids analysis was performed on a Waters (Milipore Corp., Bedford, MA) dual-pump gradient HPLC system. A Vydac brand C18 RP-HPLC column (5µm, 300Å pore size, 0.46cm x 25cm) was used to analyze the fatty acids concentrations. The mobile phase was the water solution of acetonitrile (ACN). Two kinds of solution were used in a gradient mode: solution A: 25% acetonitrile + 75% water, solution B: pure acetonitrile. Acetic acid (0.12%v/v) was added to each mobile phase solutions A and B to adjust the pH and prevent the ion exchange effect. The mobile phase changes from 100% A to 100% B linearly over 80 minutes. The flow rate was 1 ml/min from 0 to 80 minutes and then changes to 2ml/min. The run time for each analysis was 95 minutes. A Waters
Figure 4.1. Long chain fatty acid synthesis.
Table 4.1. Fatty acid standards used for HPLC analysis.

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<td>20:2ω6</td>
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<td>Di-homo-γ-Linolenic acid (DGLA)</td>
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</tr>
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<td>20:4ω6</td>
<td>4</td>
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<td>Eicosapentaenoic acid (EPA)</td>
<td>20:5ω3</td>
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</tr>
<tr>
<td>Adrenic acid</td>
<td>22:4ω6</td>
<td>4</td>
</tr>
<tr>
<td>Docosapentaenoic acid (DPA)</td>
<td>22:5ω3</td>
<td>5</td>
</tr>
<tr>
<td>Docosahexaenoic acid (DHA)</td>
<td>22:6ω3</td>
<td>6</td>
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<tr>
<td>Prostaglandin F$<em>{1α}$ (PGF$</em>{1α}$)</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Prostaglandin E$_1$ (PGE$_1$)</td>
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<td>1</td>
</tr>
<tr>
<td>Prostaglandin E$_2$ (PGE$_2$)</td>
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</tr>
<tr>
<td>Prostaglandin E$_3$ (PGE$_3$)</td>
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Figure 4.2. The molecular structure of the fatty acids used as standards.
Photodiode Array detector was used to detect the concentration of the fatty acids. The wavelength range was set to 191.5 ~ 300nm. The absorbance at 195nm was used to calibrate the fatty acids concentrations.

4.4 Results and Discussions

In order to identify fatty acids in the real cell sample profiles, the retention times of various standard fatty acids need to be obtained under the same operation conditions as that used for real cell sample analysis. Because the retention times sometimes change slightly from run to run, especially when newly made mobile phase solutions were added to the mobile phase reservoirs, standard fatty acids mixture was re-injected to confirm the retention times when necessary.

In addition, retention time alone cannot identify a fatty acid because, very often, different chemicals may have the same retention time. So other criteria is needed to confirm the identification of the peaks in the real sample HPLC profiles. In this study, the absorbance spectra over the wavelength range of 191.5nm-300nm were adopted to help identify the fatty acids for real samples.

4.4.1 Retention Times

The retention times of the standard fatty acids were first obtained one by one under the same operational conditions. After that, a mixture of the standards was injected to obtain the RP-HPLC profile of the fatty acid standards.
The mobile phase may introduce some impurity peaks to the HPLC profiles. Besides, the analysis is under a gradient mode. This will cause the fluctuation of the base. These factors will influence the analysis results, especially when the concentrations of some fatty acids in the real samples are very low. So it is necessary to subtract these background artifacts from the HPLC profiles. The software used in the HPLC system supports the ability to subtract the effects of the mobile phase on a standard or sample. This “blank subtract” capability can be used when the HPLC profile is affected by solvent gradient, system peaks, and contamination in the mobile phase. Blank subtraction, in effect, removes the chromatographic artifact from the data set, resulting in a baseline-corrected chromatogram. In order to do blank subtraction, a 3D blank HPLC profile should be obtained under the same operation conditions as that under which the profiles of fatty acids standards and real samples are obtained. The blank subtraction operation will subtract the named 3D gradient blank chromatogram from the selected chromatogram. Blank baseline subtraction can be used when the chromatogram of the standard or sample cannot be properly integrated due to small noise peaks or a drifting noisy baseline, or the gradient blank includes characteristics that are worth subtracting (for example, small noise peaks).

This action subtracts all identical chromatographic data (primarily the solvent-related artifacts) and generates a new 3D chromatogram that is the difference between the blank and the standard or sample. Blank baseline subtraction does not improve the signal to noise ratio of the signal. It only removes the background signal. The benefits
from performing blank baseline subtraction include the generation of a chromatogram that:

- has a baseline closer to 0 AU (a flatter baseline than the original 3D chromatogram).
- has fewer artifacts (no extra peaks, less drift).
- is easier to integrate.

4.4.1.1 The HPLC Profiles of the Fatty Acid Standards

The blank gradient chromatogram is shown in Figure 4.3. Figures 4.4 and 4.5 are the chromatograms of the fatty acid standards and one of the real cell samples. After the blank subtraction, the new chromatograms for the standards and sample are shown in Figures 4.6 and 4.7.

4.4.1.2 The Retention Times of the Fatty Acid Standards

According to Figure 4.4, the retention times of the various fatty acid standards are listed in Table 4.2. The values in Table 4.2 are the typical retention times of the fatty acids standards. These values can change slightly from run to run. Generally the fluctuation is in the range of 0.1 minutes.
Table 4.2. The retention times of various fatty acids stands.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Retention Time (min)</th>
<th>Fatty Acid</th>
<th>Retention Time (min)</th>
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<td>60.10</td>
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<td>DGLA</td>
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<td>OA</td>
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<td>DHA</td>
<td>56.38</td>
<td>EDA</td>
<td>69.92</td>
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</table>

4.4.2 Absorbance Spectra

Absorbance spectra were used in combination with retention time to identify a fatty acid peak in the RP-HPLC profile. The absorbance of fatty acids is mainly due to the double bond and ring structure in the molecule. The contribution of the carbon chain length to the absorbance spectra is negligible. So different fatty acids with the same number of double bonds have almost the same UV spectra. The difference between the UV spectra of fatty acids with different numbers of double bonds is obvious. Figures 4.8-4.10 are the UV spectra of fatty acids with 2, 4, 5 double bonds, respectively. The comparison of the UV spectra of fatty acids with different number of double bonds is shown in Figures 4.11 (after normalization) and 4.12 (before normalization).
Figure 4.3. The HPLC chromatogram of a blank gradient run.
Figure 4.4. The chromatogram of the fatty acid standards before baseline subtraction.
Figure 4.5. The chromatogram of a real sample before baseline subtraction.
Figure 4.6. The chromatogram of the fatty acid standards after baseline subtraction.
Figure 4.7. The chromatogram of a real sample after baseline subtraction.
Figure 4.8. Comparison of the UV spectra of LA and EDA.

Figure 4.9. Comparison of the UV spectra of AA and adrenic acid.
Figure 4.10. Comparison of the UV spectra of EPA and DPA.

Figure 4.11. Comparison of the UV spectra (after normalization) of fatty acids with different numbers of double bonds and similar chain length.
Figure 4.12. UV sensitivity of fatty acids with different numbers of double bonds.

4.4.3 Calibration Curves

HPLC can analyze fatty acids qualitatively and quantitatively if the quantitative correlation is established between the peak area and the quantity of the fatty acid. The calibration curves including the correlation equations of these fatty acids are shown in Figures 4.13 to 4.18. The data used for these calibration graphs are listed in Tables 4.3 to 4.5. From these results, it can be concluded that the linearity of these calibration curves is good. The UV responses of the fatty acids with different numbers of double bond are very different. The more double bonds in the molecular structure, the larger the response.
Figure 4.13. UV absorbance calibration curves of PGF$_{1a}$ and PGE$_3$ at 195 nm.
Figure 4.14. UV absorbance calibration curves of PGE$_1$ and PGE$_2$ at 195 nm.
Figure 4.15. UV absorbance calibration curves of EPA, GLA, DHA, and AA at 195 nm.
Figure 4.16. UV absorbance calibration curves of DPA and LA at 195 nm.
Figure 4.17. UV absorbance calibration curves of DGLA and adrenic acid at 195 nm.
Figure 4.18. UV absorbance calibration curves of OA and EDA at 195 nm.
Table 4.3. Data used in Figures 4.13 and 4.14.

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CHAPTER 5

HPLC ANALYSIS OF FATTY ACIDS IN RECOMBINANT ANIMAL CELL SAMPLES

5.1 Sample Preparation

Before the fatty acids from the cell samples can be separated and analyzed using RP-HPLC, they must first be extracted and transferred into a solvent that is suitable for HPLC analysis. However, free fatty acids only constitute a very small part of the total fatty acids present in the recombinant animal cells. Most fatty acids exist in the cells in the form of complex lipids. Some fatty acids are attached to proteins by hydrogen bond.

No literature was found on the separation and analysis of fatty acids from recombinant animal cells. Most studies on the separation and analysis of fatty acids used plant oil, animal tissues, fish and microbial cells as the fatty acid resources. In these studies, the first step to separate fatty acids is always the extraction of total lipids from these materials with organic solvents. Then the lipid extracts are hydrolyzed to free fatty acids for further separation and analysis. In literature, fatty acids are usually converted directly or indirectly to their derivatives for separation and analysis (Vaghela and Kilara, 1995, Bligh, and Dyer, 1959, Grima et al., 1994, Gimenez et al., 1998). The methyl ester derivatives are the simplest in structural terms, and they have proved of great value with GLC where their properties are well documented and understood.

It is obviously that those methods found in literature are complex and time consuming and not suitable for the purpose of this study. The size of the cell samples
used in this study is very small (around 1 million cells per sample). Too many processing steps will be not suitable. On the other hand, the purpose of this study is to support the study on the fatty acids metabolic pathways in the recombinant animal cells. Sometimes, the cell samples are prepared under different conditions, and the concentration difference of different fatty acids present in the cell samples is very large. So the sample preparation method should be more general, flexible, rapid and able to recover those fatty acids that are present in the cell samples at very low concentration level.

In this study, when developing the sample preparation method for RP-HPLC analysis, the following three factors were considered.

- In order to obtain the complete fatty acids profile of the cell samples, the fatty acids need to be released from the complex lipids or the complex compounds. This purpose was achieved by the saponification step.

- In order to protect the HPLC column and other parts of the HPLC system, salts need to be removed from the sample before HPLC analysis. This purpose was achieved by the solvent extraction step. This step also got rid of most highly hydrophilic substances that could introduce some unwanted artifact peaks in the HPLC profiles.

- In order to reduce the artifact peaks in the HPLC profiles caused by the sample solvent, a proper sample solvent is needed to dissolve the fatty acid extract resulted from the solvent extraction step. This purpose was achieved by the solvent change step.
5.1.1 Saponification

The fatty acids are often part of large aggregates from which they are relatively easily extractable. On the other hand, the fatty acids forming complex lipids are usually constituents of membranes, where they occur in a close association with such compounds as proteins and polysaccharides, with which they interact, and they are not extracted so readily. A saponification step is needed to release the fatty acids.

5.1.1.1 The Selection of Solvent System

In order to release fatty acids from cells, it is necessary to find solvents that will not only dissolve the fatty acids readily but will overcome the interactions between the fatty acids and the cell matrix. According to literature, fatty acids can be released from the complex lipids with relative ease by hydrolysis (or saponification) of the ester bonds under basic conditions. In the case of recombinant animal cells, the same result can be expected.

In order to release fatty acids, a solvent is needed to support the saponification reaction and dissolve the sodium salts of the fatty acids. In this study, 0.5N sodium hydroxide in the mixture of 96% ethanol-4% water is used to saponify the fatty acids. The 96% ethanol-4% water system is used for the following reasons:

- Studies in this area indicate that 96% ethanol - 4% water system is best for the saponification reaction.

- Ethanol can help to break up the complexes formed by fatty acids and proteins.
• Ethanol can also deactivate the enzymes of the cell to prevent the change of the fatty acid profile of the sample.

• Biological cells have a wide range of fatty acid profiles. Among these fatty acids, some are hydrophobic while others are hydrophilic. Ethanol is suitable for both kinds of fatty acids.

• Ethanol and water are miscible. Water assists the extraction by causing swelling of the biopolymers and it is an essential component of any extractant.

• Ethanol has a lower toxicity compared to most other organic solvents.

5.1.1.2 The Procedure of Saponification

The saponification procedure includes the following three steps:

• Add 1ml 0.5N NaOH 96%ethanol-4%water (v/v) solution to the cell sample (containing around 1 million recombinant mouse cells).

• Use an ultrasonic homogenizer to disrupt the cells.

• Replace the air in the test tube with nitrogen.

• Leave the sample under room temperature for 8 hours. Vortex the samples 10 seconds every one hour.

• Centrifuge the sample for 5 minute at 7000rpm.

• Transfer the supernatant to a 10ml glass tube and replace the air in the glass tube with nitrogen.
5.1.2 Extraction

The purpose of the solvent extraction step is to separate fatty acids from salts and other unwanted water-soluble impurities. Care should be taken to make sure that the phase separation is complete and there are no bubbles on the wall of the test tube before taking the top phase.

5.1.2.1 Selection of Solvents

In order to extract fatty acids from the 96%ethanol-4%water system resulted from the saponification step. A third organic solvent is needed to achieve phase separation. In this study, ethyl acetate was used as the third solvent for the following reasons:

• Ethyl acetate, ethanol and water can form a two-phase system to achieve the separation of fatty acids from unwanted salts.
• Ethyl acetate has lower viscosity that will help to achieve phase separation.
• Ethyl acetate is volatile. It is easy to be removed in the following solvent change step.

In this work, chloroform was also tested for the extraction step. Since chloroform is heavier than water, it is more difficult to take out the chloroform phase without disturbing the phase boundaries.
5.1.2.2 Procedure of Solvent Extraction

The procedure of solvent extraction includes the following steps.

- Add 1ml 0.6N HCl-water to the test tube that contains the supernatant from the saponification step. This operation will change all the fatty acids from their sodium salts to their free acid forms.
- Add 3ml ethyl acetate to the test tube.
- Vortex the test tube for 1 minute.
- Leave the sample under room temperature for 30 minutes.
- Transfer the top phase to another glass tube for the use of the following solvent change step.

5.1.3 Solvent Change

The purpose of this step was to transfer the fatty acids from the ethyl acetate-ethanol-water solution to pure methanol for RP-HPLC analysis. This step is necessary because of the following reasons:

- In the solvent extraction step, in order to increase the recovery rate of the fatty acids, 3ml ethyl acetate was used. So the concentrations of some fatty acids in the extractant, especially those highly unsaturated fatty acids were too low to be analyzed by HPLC directly. One purpose of the solvent change step was to reduce the sample volume.
- When using ethyl acetate or ethanol as the solvent for HPLC analysis, the baseline of the chromatogram was very noisy and there were always some
artifact peaks in the HPLC profiles. Methanol greatly improved the HPLC baseline.

The procedure of solvent change was as following:

- Gently blow the top phase from the extraction step using nitrogen under 30°C.
- When most ethyl acetate was blown out from the solution, transfer the sample to lyophilizer to get rid of the remaining water.
- Dissolve the residues into 350μl HPLC grade methanol and centrifuge the sample.
- Take 300μl supernatant and transfer it to another test tube.
- Displace the air in the tube with nitrogen, seal the tube and keep it in a -20°C freezer for HPLC analysis.

5.2 RP- HPLC Analysis

RP-HPLC analysis of real sample was conducted under the same operation as that under which the fatty acid standards were analyzed. For each sample, the final extract from the extraction step in 5.1.2 was transferred to methanol. For each run, the injection volume was 100μl. The remaining 200μl could be used to repeat the result or collect the peaks from the profile for further use.

The cell samples used in this work were recombinant mouse cells prepared by Dr. Bruce Kelder of Edison Biotechnology Institute at Ohio University. Two kinds of cell samples were analyzed: Δ12 cells and negative control cells. Δ12 cells were cultured
with the addition of Δ12 desaturatase while negative control cells were cultured without the addition of Δ12 desaturatase. Figures 5.1 and 5.2 are the RP-HPLC profiles for these two types of cell samples, respectively.

By comparing the RP-HPLC profiles of these two types of cell samples, it can be seen that the ratio of linoleic acid concentration to oleic acid concentration is obviously increased in the Δ12 cells.

5.3 Some Considerations

During extraction, as in many other aspects of analysis, it is possible to inadvertently introduce contaminants or to bring about some unwanted change in the composition of the samples. Autoxidation of double bonds in fatty acids, for example, is particularly troublesome and care must be taken at all steps in the analysis of cell samples, not just during storage and extraction, to eliminate the problem.

5.3.1 Storage and Extraction of Cell Samples

Before any analysis of cell samples can be commenced, it is necessary to extract the cell samples. Ideally, this should be done immediately after the cell samples are collected. If this is not possible, the cell samples should be stored in a freezer at -20°C so that they do not deteriorate significantly. Frozen cell samples should not be allowed to thaw before being homogenized with the extracting solvent. Sample extracts should not be left in the dry state, but should be dissolved in a small volume of solvent. Air should be excluded by flushing with a stream of nitrogen.
Figure 5.1. The RP-HPLC profile of the A12 cell sample.
5.3.2 Minimizing Auto-oxidation

If they are not protected, polyunsaturated fatty acids will auto-oxidize very rapidly in air, and it may not be possible to obtain an accurate analysis by chromatographic means. The mechanism of auto-oxidation involves attack by free radicals and is exacerbated by strong light and metal ions. Once initiated, the reaction proceeds auto-catalytically. Linoleic acid is auto-oxidized twenty times as rapidly as oleic acid, and each additional double bond in a fatty acid can increase the rate of destruction by two- to three-fold. As the reaction causes double bonds to migrate, forming conjugated systems that absorb strongly at wavelengths in the UV region of the spectrum, this can interfere when cell extracts are analyzed by means of HPLC with UV detection.

Wherever possible, samples should be handled in an atmosphere of nitrogen. Usually it is sufficient to ensure that nitrogen lines are freely available so that the air can be flushed out of glass containers. Small volumes of solvent can be evaporated by carefully directing a stream of nitrogen onto the surface of the solvent. This should not be done too vigorously or at too high a temperature. Under optimum conditions, fatty acids should not change in composition and structure during extraction or storage.

5.3.3 Contaminants and Artifacts in Extraction Procedures

All solvents, including from time to time those grades that are nominally of high purity, can contain contaminants, and any such impurities can be troublesome. It should not be forgotten that water is an important solvent with many uses in the
chromatographic analysis of lipids, and that it too should meet a high standard. Microbial growth in water of insufficient purity can introduce contaminants, which may block filters and frits in HPLC equipment.

Other extraneous lipid-like materials can be introduced accidentally into cell samples from a variety of sources. Plastic-ware of all kinds (other than that made from Teflon or PTFE) can be especially troublesome and should be avoided, since plasticizers (diesters of pathalic acid usually) are very easily leached out. They tend to co-chromatograph with lipids, so they may spread confusion and obscure compounds of interest in chromatograms, since they absorb very strongly in the UV range.
CHAPTER 6
IDENTIFICATION OF FATTY ACIDS IN REAL CELL SAMPLE PROFILES

6.1 Introduction

Retention time and absorbance spectra are two criteria adopted in this study to identify the fatty acid peaks in the HPLC profiles of real cell samples. Peaks with the same retention times are not necessarily the same substances. On the other hand, different fatty acids with the same number of double bonds have very similar absorbance spectra. Either retention time or absorbance match alone is not enough to identify the fatty acids in the HPLC profiles.

In this study, before each series of samples was analyzed by RP-HPLC, a mixture of fatty acid standards with known concentrations was injected to obtain the HPLC profile for the fatty acid standards. From this profile, a fatty acids library was established according to the information of concentration, retention time, and absorbance spectra of the standard fatty acids. After the injection of the real sample, a comparison of the profile against the fatty acids library was made to identify the fatty acids peaks and the concentration of different fatty acids in the real sample.

6.2 Comparison of Retention Times

The retention times of a fatty acid may vary slightly from run to run. However the difference is very small, especially when the peak is not so large. Generally speaking, the retention time fluctuation of a fatty acid in different runs is less than 0.1 minute. When
matching the real sample profiles against the standard library, setting a peak window
time of 0.3 minutes was enough for retention time identification.

6.3 Comparison of Absorbance Spectra

Photodiode array (PDA) detectors produce spectra that, in combination with good
chromatography, can be used for compound identification (qualitative analysis). In
qualitative procedures, the spectrum from a peak in the HPLC profile was matched
against an existing spectra library of fatty acid standards. PDA software used the spectral
contrast technique to quantitate the degree of differences between spectra. The results are
reported as the Match Angle.

6.3.1 Spectral Contrast Principles

Spectral contrast is the technique that PDA software uses to compare spectral
shapes. The PDA software uses the spectral contrast technique for spectrum matching
and purity testing. The technique quantifies differences in spectral shapes by converting
spectra to vectors and comparing the vectors. The technique allows for analysis of co-
elution, noise, photometric error, high sample concentration, and solvent effects during
spectral comparisons.

Spectrum matching and purity testing results are used in compound confirmation
to identify sample unknowns. In addition, multi-component peak purity is used to
determine the number of spectrally distinct compounds that co-elute in a peak.
With the spectral contrast technique, PDA software uses UV absorbance spectra from a PDA detector to:

- Match spectra from an eluting analyte with known spectra (spectrum matching) in the spectra library.
- Detect co-elution by matching all spectra within a peak (peak purity)
- Determine the number of spectrally distinct compounds within a peak (multi-component peak purity)

6.3.2 Spectral Contrast Procedure

The process the PDA software used for the spectra contrast includes the following steps:

- Performing baseline correction by interpolating lift-off and touchdown spectra to obtain a baseline spectrum for each sampling time within a peak and subtracting baseline spectra from the corresponding absorbance spectra within a peak.
- Converting baseline-corrected spectra to vectors.
- Comparing two spectra by measuring the differences in vector direction.

For spectrum matching, the peak apex spectrum is compared against spectra in a library. For peak purity, the peak apex spectrum is compared against all spectra within the peak. For multi-component peak purity, combinations of two, three, or four peak spectra are compared against all other spectra within the peak.
• Determining how much of the spectral differences can be attributed to nonideal conditions inherent in the detection process.

6.3.3 Spectral Contrast Angle

The spectral contrast angle is the difference in direction between two spectral vectors. Spectra that have the same shape have vectors that point in the same direction. Spectra that have different shapes have vectors that point in different directions. The angle between the two vectors, the spectral contrast angle, quantifies the magnitude of the shape difference between the spectra. A low spectral contrast angle, near 0 degree, indicates little shape difference. A spectral contrast angle of 1 degree corresponds to a difference that is barely discernible on a video display terminal. The maximum spectral contrast angle, 90 degrees, indicates that the two spectra do not overlap at any wavelength.

6.3.4 Threshold Angle - Threshold Criteria Parameter

Spectral contrast measures the mismatch between two spectra (for example, known spectra versus sample spectra) using the Match Angle. Spectral contrast also measures the spectral inhomogeneity of a peak using the Purity Angle. Detector noise, co-elution, photometric error, high sample concentration, or spectral variations induced by variations in solvent composition are all nonideal effects that change the shape of absorbance spectra. These various effects cause chemically pure, baseline-resolved peaks to include a small level of spectral inhomogeneity. The magnitude of this inhomogeneity
varies with peak height. Such inhomogeneity causes pure peaks to have a Purity Angle greater than zero, and results in the comparison of spectra for the same compound to have a Match Angle greater than zero.

Threshold criteria parameter is used to give an estimation of these nonideal contributions during peak purity testing and spectrum matching processes. When selecting Noise + Solvent as the threshold criteria in the PDA processing method, the threshold criteria parameter equals the sum of Noise Angle and solvent angle.

6.3.4.1. Noise Angle

The Noise Angle accounts for instrument statistical and thermal variations. PDA software calculates a Noise Angle by extracting spectra from a user-defined section (the noise interval) of the baseline and combining spectra to yield the root-mean-squared (RMS) noise absorbance for each spectral channel.

The Noise Angle value can vary between 0.01 and 90 degrees. Noise Angle of 0.01 degree indicates that the maximum absorbance of both matched spectra are near 1 AU; Noise Angle of 90 degrees indicates that the maximum absorbance of one or both matched spectra are at or below detector baseline noise. In general, the Noise Angle varies in inverse proportion to the highest absorbance in the matched spectra. In other words, Noise Angle is inversely proportional to compound concentration. The higher the compound concentration, the lower the Noise Angle. Conversely, the lower the Noise Angle, the more selective the spectral comparison.
6.3.4.2. Solvent Angle

The solvent angle accounts for spectral shape changes due to photometric error and solvent composition. The solvent angle parameter allows to compensate for solvent-induced spectral changes and the effects of residual small photometric errors. If replicate injections of a pure compound result in a spectral contrast angle greater than the Noise Angle, then the spectral contrast angle is being affected by photometric error, changes in solvent, or a combination of both. At high absorbance (generally greater than 1 AU), a combination of effects can produce slight departures from Beer’s law due to photometric error. Photometric errors at this level have a negligible effect on quantitation; however, they can be a dominant, although small, source of spectral inhomogeneity. The presence of photometric error increases Match Angle or Purity Angle values. To minimize the effects of photometric error for all spectral contrast operations, the maximum spectral absorbance of a compound should be less than 1 AU. A change in solvent, pH, or composition can affect spectra. Solvent changes of this type can be the results of inefficient solvent mixing from multiple reservoirs or mobile phase changes during gradient chromatography.

For peak purity determinations, the solvent angle must be set so that the Purity Threshold is greater than the Purity Angle for a chemically pure standard. When the Solvent Angle is properly set, the Purity Angle is less than the corresponding Purity Threshold for the standards. The Purity Angle and Purity Threshold values cannot be used to prove chemical purity. A Purity Angle greater than the Purity Threshold only warns of a possible co-elution.
For library match determinations, the solvent angle must be set so that the match threshold is greater than the Match Angle for a chemically pure standard. When the solvent angle is properly set, the Match Angle is less than the corresponding match threshold for standards.

When interpreting results, compare the Purity Angle or Match Angle to the Purity Threshold or match threshold to determine if the spectra are similar or different, or to determine if a peak is spectrally homogeneous. The threshold criteria parameter is the limit of detection of shape differences between two spectra. When the noise interval and solvent angle are set correctly:

- The Match Angle between spectra from different injections of the same compound does not exceed the match threshold angle.
- The Purity Angle of a peak containing a single compound does not exceed the Purity Threshold angle.

If a spectra contrast angle is less than the corresponding match or Purity Threshold, then a spectral shape difference can be attributed to nonideal effects and cannot be attributed to a true spectral shape difference. Otherwise, if a spectral contrast angle is greater than the corresponding match or Purity Threshold, then a spectral shape difference should be attributed to a true spectral shape difference.
6.3.5 Spectral Shape Difference

When there is a spectral shape difference between spectra of the same compound, the difference can be the result of the following nonideal effects:

- **Noise** - Statistical and thermal variations add noise to the absorbance measurements made by the PDA detector. The magnitude of the absorbance difference caused by statistical and thermal variations can be predicted from instrument noise (as measured in the baseline region of the chromatogram).

- **Photometric Error** - Departure from Beer's law (called photometric error) occurs at high compound concentrations due to a combination of nonideal effects. For all spectral contrast operations, the maximum spectral absorbance of a compound should be less than 1 AU to minimize the effects of photometric error.

- **Solvent Changes** - The spectra of most compounds can be sensitive to solvent pH and composition. The solvent contribution can be added to the absorbance measurement of the PDA detector.

To assess the significance of spectral shape difference, the shape variation due to the above-mentioned nonideal effects must be determined using the spectral contrast technique.

The match results can be interpreted by comparing the Match Angle with the match threshold. When the Match Angle is less than the match threshold, no difference in
spectral shape has been detected. When the Match Angle is greater than the match threshold, either the spectra are not from the same compound, or the spectra are affected by the presence of a co-eluting component.

6.3.6 Peak Purity Calculation - Detecting Co-elution with PDA Detector

The PDA software computes peak purity from an integrated peak. The integration parameters define the start and end spectra for baseline correction. The results of the peak purity operation are Purity Angle and threshold angle. Their values can range from 0 to 90 degrees. The threshold angle is the sum of the purity Noise Angle and solvent angle. This value is the largest Purity Angle that can be due to co-elution, noise, photometric error, high sample concentration, or solvent contribution alone, and not due to an actual difference in spectral shape. To calculate peak Purity Angles, the spectral contrast technique

- Performs baseline correction.
- Compares each spectrum within the peak against the peak apex spectrum by measuring the differences in vector direction.
- Calculates the Noise Angle for each match.
- Calculates the Purity Angle as the weighted root mean square of the Purity Angles obtained from the individual spectra matches. The Purity Angle is weighted to give more significance to spectra with more absorbance.
• Combines individual Noise Angle across the peak to produce a purity Noise Angle.

• Adds the Noise Angle to the solvent angle to obtain the threshold angle (when the threshold angle is set to noise + solvent).

To interpret peak purity results, compare the Purity Angle with the Purity Threshold. A Purity Angle less than the Purity Threshold means that no significant differences between spectra could be detected and there is no spectroscopic evidence for co-elution. A Purity Angle greater than the Purity Threshold means that the peak is not spectrally homogeneous and there is spectroscopic evidence for co-elution.

6.4 Multi-Component Peak Purity

By comparing the apex spectrum to all other spectra within a peak, spectral contrast and peak purity distinguish between a peak containing a single compound and a peak containing more than one compound. Multi-component peak purity is an extension of peak purity, beginning with the spectral contrast and peak purity operations. Multi-component peak purity extends these two operations by comparing combinations of spectra from within the peak to the other spectra within the same peak. As a result of these comparisons, multi-component peak purity helps to determine the number of spectrally distinct components within a single chromatographic peak. Multi-component peak purity is enabled using the purity passes parameter in the PDA processing method. On the first pass, the apex spectrum is compared to all other spectra within the peak. This comparison results in the generation of the computation of the Purity Angle and Purity
Threshold value for the peak. For the second purity pass, the PDA software selects the two model spectra, the apex spectra and maximum impurity spectra, derived from the first purity pass. For each spectrum within the peak, the PDA software performs a detailed comparison. PDA software compares the shape of each peak spectra to range of shapes that can occur from the additive combination of apex spectrum and maximum impurity spectrum. Each comparison results in a spectrum contrast angle and Noise Angle for that peak spectrum. This set of comparisons yields a new Purity Angle and Purity Threshold. The maximum impurity spectrum from this second purity pass is the peak spectrum that is most dissimilar to the additive combination of apex spectrum and maximum impurity spectrum from the first purity pass. Purity pass #1 tests the following hypothesis: Does this peak contain only one spectrally compound? Similarly, Purity #2 tests the following hypothesis: Does this peak contain only two spectrally distinct compounds?
CHAPTER 7

GENERAL RATE MODEL OF LIQUID CHROMATOGRAPHY

7.1 Introduction

Liquid chromatography is a very important tool for the separation and analysis of biochemicals. Although there are many kinds of liquid chromatography modes, according to the interaction between the solutes and the stationary surface, liquid chromatography can be classified into two categories. One kind of liquid chromatography, which is usually referred to as size-exclusion chromatography (SEC), separates solutes according to the molecular sizes of their solute. The interaction between the stationary surfaces and the solute molecules can usually be neglected in an SEC column. In another category, the separations are achieved due to the different interaction forces between the solutes and the stationary surfaces. Most often, the two chromatographic mechanisms exist in a chromatography process. So a mathematical model that includes the two factors is usually necessary to describe accurately the performance of a chromatography column.

The purpose of a mathematical model is to help understand chromatography mechanism, simulate the chromatography process, optimize the operational parameters and predict the performance of a chromatography system. Thus, a successful chromatography model should meet the following requirements:
- The model reflects the real chromatography mechanisms such as size-exclusion effect, mass transfer resistance and the interaction forces between the solutes and the stationary surfaces.

- There exists a method to solve the model equations

- The model parameters should be easy to obtain mathematically or experimentally.

Although there are many mathematical models for liquid chromatography, very few existing models consider interfacial film mass transfer and intraparticle diffusion, although some consider axial dispersion (Antia and Horvath, 1989, Pitts Jr., 1976, Kang and McCoy, 1989). The general rate model is considered the most comprehensive one because it takes into account various mechanisms that may exist in chromatography column including size-exclusion, interaction rate, and all kinds of mass transfer resistance. In this work, a general rate model (Gu et al., 1992, Gu, 1995) is used to study two kinds of chromatography process: size-exclusion chromatography and RP-HPLC. Neglecting the interaction between the solutes and macropore surfaces, SEC is considered the simplest chromatography mode. For this reason, the application of the general rate model on SEC will be first studied.

7.2 Model Assumptions

The general rate model proposed by Gu, et al. (1992) considers the following mechanisms in a chromatography column.
interactions between solutes and stationary surface,
size-exclusion effects,
axial dispersion in the bulk-fluid phase,
interfacial film mass-transfer between the stationary and mobile phases,
and
diffusion of solutes within the macropores of the packing particles.

In this work, the following assumptions are made to formulate the model:

- The column is isothermal.
- There is no interaction between different solutes.
- Diffusion and mass-transfer coefficients remain constant.
- Packing particles can be treated as spherical and uniform in size.
- The packing density is even along the column.
- Diffusion in the radial direction is negligible.
- An instantaneous local equilibrium exists between the macropore surfaces and the stagnant fluid inside macropores of the particles in RP-HPLC.

7.3 Model Formulation

When establishing the model formulations, it is assumed that there are Ns components in the mobile phase. Based on the model assumptions, the following governing equations can be formulated from the differential mass balances for each of the Ns components in the bulk-fluid and the particle phases (Gu, et al., 1992).
The following equation describes the second order kinetics,

\[ \frac{\partial C_{pi}^*}{\partial t} = k_{a_1} C_{pi} (C^\infty - \sum_{j=1}^{N_x} C_{pj}^*) - k_{dl} C_{pi}^* , \]  

(7-3)

With the assumption that there exists an instantaneous local equilibrium between the micropore surfaces and the stagnant fluid inside the macropores of the packing particles, both sides of Eq. (7-3) can be set to zero. The initial conditions for the partial differential equation systems are:

\[ t = 0, \ C_{bi} = C_{bi} (0, Z), \ C_{pi} = C_{pi} (0, R Z). \]  

(7-4,5)

\[ Z = 0, \ \frac{\partial C_{bi}}{\partial Z} = \frac{V}{D_{bi}} [C_{bi} - C_{fl} (t)], \ Z = L, \ \frac{\partial C_{bi}}{\partial Z} = 0. \]  

(7-6,7)

The boundary conditions are:

\[ R = 0, \ \frac{\partial C_{pi}}{\partial R} = 0; \ R = R_p, \ \frac{\partial C_{pi}}{\partial R} = k_{i} \frac{1}{\varepsilon_p D_{pi}} (C_{bi} - C_{pi,R=R_p}). \]  

(7-8,9)
$C_{pi}^*$ is the concentration of component i in the solid phase of the adsorbent based on the unit volume of the solid, excluding pores. Concentrations $C_{bi}$ and $C_{pi}$ are based on the unit volume of mobile phase fluid. In order to nondimensionalize the governing equations, the following dimensionless parameters are introduced:

\[
c_{bi} = \frac{C_{bi}}{C_{oi}}, \quad c_{bi} = \frac{C_{bi}}{C_{oi}} \quad c_{pi} = \frac{C_{pi}}{C_{oi}}, \quad r = \frac{R}{R_p}, \quad z = \frac{Z}{L}, \quad \tau = \frac{v t}{L}, \quad Pe_Li = \frac{v L}{D_{bi}}
\]

\[
Bi_i = k_i R_p / \epsilon_{pi}^* D_{pi}, \quad \eta_i = \epsilon_{pi}^* D_{pi} L / R_p^2 v, \quad \xi_i = 3 B_i \eta_i (1 - \epsilon_b) \epsilon_b
\]

The Eqs. (7-1) to (7-3) can now be transformed into the following dimensionless forms:

\[
- \frac{1}{Pe_Li} \frac{\partial^2 c_{bi}}{\partial z^2} + \frac{\partial c_{bi}}{\partial z} + \frac{\partial c_{bi}}{\partial \tau} + \xi_i (c_{bi} - c_{pi, r=1}) = 0 \quad (7-10)
\]

\[
\frac{\partial}{\partial \tau} \left[ (1 - \epsilon_p) c_{pi}^* + \epsilon_{pi}^* c_{pi} \right] - \eta_i \left[ \frac{1}{r^2} \frac{\partial}{\partial r} \left( r^2 \frac{\partial c_{pi}^*}{\partial r} \right) \right] = 0 \quad (7-11)
\]

\[
\frac{\partial c_{pi}^*}{\partial \tau} = Da_i^* c_{pi} \left( c_i^\infty - \sum_{j=1}^{N_k} \frac{C_{oj}}{C_{pi}} \right) - Da_i^d c_{pi}^* \quad (7-12)
\]

where $Da_i^* = L(k_{ai} C_{oi}) / v$, $Da_i^d = L k_{ai} / v$, $c_i^\infty = C^\infty / C_{oi}$.

The initial and boundary conditions are:

\[
\tau = 0, \quad c_{bi} = c_{bi} (0, z), \quad c_{pi} = c_{pi} (0, r, z), \quad (7-13,14)
\]

\[
z = 0, \quad \partial c_{bi} / \partial z = Pe_Li \left[ c_w \frac{C_{ai}(t)}{C_w} \right], \quad (7-15)
\]

\[
z = 1, \quad \partial c_{bi} / \partial z = 0, \quad (7-16)
\]
If the adsorption and desorption rates are sufficiently high, Eq. (7-3) reduces to the following common multicomponent Langmuir isotherm assuming the adsorption saturation capacities \( C_i^\infty \) are the same for all the components.

\[
C_{pi}^\ast = \frac{a_i C_{pi}}{1 + \sum_{j=1}^{N_s} b_j C_{pj}}, \quad \text{i.e. } c_{pi}^\ast = \frac{a_i c_{pi}}{1 + \sum_{j=1}^{N_s} (b_j C_{oj}) c_{pj}}
\]  

(7-19)

where \( a_i \) and \( b_i \) are Langmuir constants.

A popular eluite-modulator relationship has been given for both electrostatic and hydrophobic interactions as follows (Melander et al., 1989),

\[
\log_{10} k' = \alpha' - \beta' \log_{10} C_m + \gamma' C_m
\]  

(7-20)

where \( \alpha' \), \( \beta' \) and \( \gamma' \) are experimental correlation parameters, \( k' \) is the adsorption capacity factor, and \( C_m \) is the concentration of the modulator in the mobile phase. Since separation by reversed phase chromatography is based on attraction between hydrophobic groups on the solute and a hydrophobic matrix (Wheelwright, 1991), Eq. 7-20 can be used for RP-HPLC. Note that \( b \), the adsorption equilibrium constant in the Langmuir isotherm, is used in the general rate model instead of the capacity factor \( k' \).
According to the definition of capacity factor, it is easy to show that for an isocratic elution with a dilute sample, i.e., it observes the linear range of the Langmuir isotherm (Gu, et al, 1992),

\[ k' = \phi C^\infty b \]  \hspace{1cm} (7-21)

where \( \phi \) is the phase ratio (the stationary phase particle skeleton volume to mobile phase volume including the particle macropores). Thus, the following equation that is used in the general rate model can be obtained.

\[ \log_{10} b = \alpha - \beta \log_{10} C_m + \gamma C_m \]  \hspace{1cm} (7-22)

where \( \alpha = \alpha' - \log_{10}(\phi C^\infty) \), \( \beta = \beta' \) and \( \gamma = \gamma' \). It is assumed that eluites do not interfere with each other's correlation parameters (\( \alpha, \beta, \gamma \)). The adsorption saturation capacities for all the eluites are considered to be the same and they are not affected by the modulator concentration.

This general rate model was solved using the finite element method with quadratic elements for the discretization of the bulk-fluid equations, and the orthogonal collocation method for the particle-phase equations. The resulting ordinary differential equation (ODE) system was solved using a public domain ODE solver called VODE (Brown et al., 1989).
CHAPTER 8
APPLICATION OF THE GENERAL RATE MODEL IN SIZE EXCLUSION CHROMATOGRAPHY

8.1 Introduction

Size exclusion chromatography (SEC) is also referred to as gel permeation or gel filtration chromatography (Hagel, 1992, Hagnauer, 1987). It separates macromolecules on the basis of their relative sizes or hydrodynamic volumes. Since its introduction in 1964 (Moore, 1964), SEC has proven to be an important tool for the analysis and separation of macromolecules such as proteins and polymers. SEC is now widely used for the preparative and large-scale separation and purification of macromolecules (Yamamoto, 1991, Burnouf, 1991).

Many commercial bioseparation processes consist one or more steps of SEC (Wheelwright, 1995). Because SEC does not rely on any binding between solutes and the stationary phase, its feed volume is very limited compared to other forms of chromatography such as reversed-phase and ion-exchange chromatography. This is the reason why commercial scale SEC columns tend to be very large, with bed volumes reaching hundreds of liters. Because soft gels are typically used as the packing media, column height expansion is limited due to pressure limitations. Thus, large SEC columns tend to have large diameters in order to accommodate large feed volumes. In such columns, diffusion and mass transfer effects can be significant.
A number of monographs have been published (Barth et al., 1994, Barth et al., 1996; Barth and Boyes, 1992; Chicz and Regnier, 1990; Yau et al., 1989) on the theories and applications of SEC. Several mathematical models that consider mass transfer effects exist in the literature (Yau et al., 1979, Kim and Johnson, 1984, Koo and Wankat, 1988, Gu, 1995). The Kim and Johnson model introduced a pore volume fraction to account for the size exclusion effect of the particles. Similar to this, Gu (1995) proposed the use of an accessible particle porosity (i.e., accessible macropore volume fraction for a macromolecule) to describe the effect of size exclusion in the general rate model which considers axial dispersion, interfacial film mass-transfer and intraparticle diffusion. Similar general rate models were used by Liapis and Arve for affinity chromatography (Liapis, 1990, Arve and Liapis, 1987) and by Yu and Wang for ion-exchange chromatography (Yu and Wang, 1989).

In this work, a personal computer (PC) based Fortran 77 software program (Gu, 1995) using the general rate model was used for the simulation and scale-up of SEC. The effects of various model parameters on the performance of SEC will be analyzed. Methods used to obtain the values of these parameters will be established. The validity of the general rate model will be tested by the comparison of the model predicted results and the experimental results.

8.2 Mathematical Model

The general rate model for SEC considers the following three mass transfer processes in the SEC column.
- Axial dispersion in the bulk-fluid phase,
- Interfacial film mass-transfer between the stationary and mobile phases,
- Diffusion of solutes within the macropores of the packing particles.

Most model assumptions made in Chapter 7 are used here to model the SEC process except that the interactions between the solutes and the stationary surface are neglected (e.g. there is no binding between the solutes and the stationary phase). For all the components, the concentration on the stationary surface is zero.

With these assumptions, we conclude that, when the general rate model described in Chapter 7 is used to simulate the SEC processes without binding reactions, the mobile phase governing equation remains unchanged, while the dimensionless particle phase governing equation will be transformed into the following Eq. (8-1)

\[
\frac{\partial c_{pi}}{\partial \tau} = \frac{\eta_i}{\varepsilon_{pi}^*} \left[ \frac{\partial^2 c_{pi}}{\partial r^2} + \frac{2}{r} \frac{\partial c_{pi}}{\partial r} \right]
\]

(8-1)

The expressions for initial and boundary conditions remain unchanged. The dimensionless feed concentration profiles at the column inlet is as follows,

\[
\frac{C_f(\tau)}{C_0} = \begin{cases} 1, & 0 \leq \tau \leq \tau_{\text{imp}} \\ 0, & \tau > \tau_{\text{imp}} \end{cases}
\]

(8-2)

where \(\tau_{\text{imp}}\) is the dimensionless time duration for a rectangular sample pulse.
8.3 Model Input Parameters

The input parameters for the Fortran 77 code include the number of components, the number of elements (Ne), the number of interior collocation points (Nc), \( \tau_{\text{imp}} \) (injection volume in terms of dimensionless feed time), particle porosity (\( \epsilon_p \)), the bed void volume fraction (\( \epsilon_b \)), the Peclet number (\( \text{Pe}_L \)), the \( \eta \) number, the Biot number (Bi), the maximum concentration (\( C_0 \), usually the feed concentration for simple column operations) and the size exclusion factor (\( F^{CS} \)).

8.3.1 Numerical Parameters (Ne and Nc)

The selection of the number of elements, Ne, greatly affects the computer simulation. Small Ne usually causes oscillation of the simulated elution peaks. However, if Ne is too large, excessive computation time will be needed. In this work, a trial and error method was used to determine the value of Ne. First, a smaller Ne was used, if the simulated peaks oscillated, a larger Ne would be used to run the program again.

The value of Nc does not affect the stability of the numerical solution much. Usually, two interior collocation points (Nc=2) are needed, especially when \( D_p \) values are small. Sometimes one interior collocation point is sufficient for practical applications.

8.3.2 Bed Void Volume Fraction (\( \epsilon_b \))

\( \epsilon_b \) depends on the size of the packing particles, as well as the packing procedure. In this work, \( \epsilon_b \) was treated as a constant for different columns with the same packing material. \( \epsilon_b \) can be obtained experimentally according to the following relationship,
where \( t_d \) is the retention time of very large molecules such as blue dextran which is completely excluded from the macropores. \( t_d \) is also known as the dead-volume time.

**8.3.3 Particle Porosity (\( \epsilon_p \))**

The value of \( \epsilon_p \) can be calculated from the retention time or the elution volume of a small molecule whose size is smaller than the lower exclusion limit of the porous particles. The relationship between the retention time of a small solute (\( t_0 \)) and \( \epsilon_p \) is shown in Eq. (8-4)

\[
\begin{align*}
\tau_0 &= \tau_d \left[ 1 + \frac{(1 - \epsilon_b)\epsilon_p}{\epsilon_b} \right] \\
\text{Eq. (8-4)}
\end{align*}
\]

**8.3.4 Accessible Particle Porosity for a Solute (\( \epsilon_{p^*} \))**

The accessible particle porosity represents the accessible macropore volume fraction (vs. the total particle volume) for a particular solute. \( \epsilon_{p^*} \) value for a typical macromolecule such as a protein is less than \( \epsilon_p \). This means that the protein molecule can penetrate some larger pores while it is excluded from smaller pores. If a macromolecule has \( \epsilon_{p^*} = 0 \), it means that this molecule is completely excluded from the macropores. Blue
dextran is an example. The $\varepsilon_p$ value of a solute differing from that of another solute is a key factor responsible for the separation of molecules in an SEC column. The value of $\varepsilon_p$ for a solute can be obtained from its retention time ($t_R$) using Eq. (8-5).

$$t_R = t_d \left[ 1 + \frac{(1 - \varepsilon_b)\varepsilon_p}{\varepsilon_b} \right]$$  \hspace{1cm} (8-5)

### 8.3.5 Peclet Number (PeL)

According to the definition of Peclet number ($Pe_L = vL/D_b$), its value can be calculated from the axial dispersion coefficient ($D_b$). However, the value of $D_b$ is not easy to measure experimentally. In this work, $Pe_L$ was calculated according to the Chung and Wen (1968) correlation for a fixed bed.

$$Pe_L = \frac{L}{2R_p \varepsilon_b} \left( 0.2 + 0.011Re^{0.48} \right), \quad 10^3 \leq Re \leq 10^3$$  \hspace{1cm} (8-6)

where $Re = (2R_p)v_p/\mu$. When $Re$ is small, the contribution to $Pe_L$ from the second term in the brackets is negligible. For instance, the elimination of the second term produces a 0.8% error when $Re$ equals 0.02. In all the experiments of this work, $Re$ is less than 0.02. Therefore, Eq. (8-6) can be written as
8.3.6 \( \eta \) Number

According to its definition, in order to calculate the value of \( \eta \) number \([\eta = \varepsilon_p^* \frac{D_p L}{(R_p^2 \nu)}]\), the value of effective diffusivity \( (D_p) \) is needed. \( D_p \) affects peak widths in chromatograms. \( D_p \) can be obtained from the molecular diffusivity \( (D_m) \) (Satterfield et al., 1973, Boyer and Hsu, 1992). In this work, the following correlation (Satterfield et al., 1973) is used.

\[
D_p = \frac{D_m}{\tau_{tor}} \left( 1 - 2.104 \lambda + 2.09 \lambda^2 - 0.95 \lambda^3 \right)
\]

In Eq. (8-8), in order to calculate the value of \( D_p \), the value of the pore tortuosity \( (\tau_{tor}) \), the molecular diffusivity \( (D_m) \) and the ratio of the solute molecular diameter to the pore diameter \( (\lambda) \) are needed. The value of \( \tau_{tor} \) for gas diffusion into porous materials is easy to obtain (Satterfield and Sherwood, 1963). However, no rigorous expression of \( \tau_{tor} \) is available for liquids. The particle tortuosity factor varies from about 1.5 to over 10 (Geankoplis, 1993). A reasonable range for many commercial porous solid is about 2-6 (Geankoplis, 1993, Satterfield, 1970). In this work, the value of \( \tau_{tor} \) will be obtained experimentally.
The molecular diffusivity ($D_m$) of large spherical molecules is given by the Stokes-Einstein equation (Bird et al., 1960) as

$$D_m = \frac{\kappa T}{6\pi \mu R_m}$$  \hspace{1cm} (8-9)

where $\kappa$ is the Boltzmann constant and $T$ is the absolute temperature.

The radius of a solute molecule can be obtained from its specific volume ($v_s$) and its molecular weight based on the assumption that the protein is spherical. It can be written as

$$R_m = \left[\frac{3(MW)v_s}{4\pi N}\right]^{1/3}.$$  \hspace{1cm} (8-10)

According to Marshall (1978), the $v_s$ values of proteins are in a narrow range (0.728–0.751). If $v_s$ is assigned an average value of 0.7384, then,

$$R_m(\text{Å}) = 0.66 (\text{MW})^{1/3}$$  \hspace{1cm} (8-11)

Usually proteins in solutions are hydrated and this results in an increase in their size. Marshall recommended 0.2 g water per g protein as a typical hydration rate. Tanford (1980) proposed the following relationship between the molecular weight of globular proteins and the hydrodynamic radius,
where the hydrated specific volume is calculated from \( V_{s.h} = 0.7384 \text{ cm}^3\text{g}^{-1} + (0.2 \text{ g water per g protein}) \times (1 \text{ cm}^3\text{g}^{-1 \text{ water}}) \) (Cantor and Schimmel, 1980).

Based on Eqs. (8-8), (8-11) and experimental data for some organic substances including proteins such as bovine serum albumin (BSA), Polson (1950) proposed the following semi-empirical relationship for organic substances with MW greater than 1,000.

\[
D_m (m^2/s) = 2.74 \times 10^{-9} (MW)^{-1/3} \tag{8-13}
\]

Eq. (8-13) will be used to calculate \( D_m \) in this work because of its simplicity and satisfactory accuracy for proteins.

The pore diameter of the gel (\( d_{\text{pore}} \)) may be provided by manufacturers, but for most soft porous materials, it is usually unavailable. In this case, an approximation for the pore diameter of the gel can be obtained from the upper exclusion limit of the gel. For polymers, the value of \( \lambda \) is a function of the molecular weight of a solute (Yau et al., 1989). A simple method was used to calculate the value of \( \lambda \) for a solute assuming spherical molecules, cylindrical pores, and equal partial specific volume,
where $\lambda_0 = 0.35$ according to Stegeman et al. (1991). This equation was derived using Eq. (8-12) by assuming that when the solute diameter reaches 35% of the pore diameter, it is unable to penetrate the pore (Stegeman et al., 1991). In this work, the MW of upper exclusion limit for the Bio-Rad P60 gel (Bio-Rad Laboratories, Hercules, CA, USA) will be set to 67,000, which is the MW of BSA. This is because experiments showed that BSA has very limited access to the pores of P60 gel used in this work. The $\epsilon_\phi$ value for BSA is only 0.03 (Li et al., 1998), which means only a small fraction of the pores are large enough for BSA to penetrate. The P60 gel has a nominal size exclusion MW range of 3,000 to 60,000 according to its vendor. If 60,000 is chosen instead of 67,000, there will be no significant error in the simulated elution profiles.

### 8.3.7 Biot Number (Bi) for Mass Transfer

The value of Bi $[Bi = kR_p / (\epsilon_\phi D_p)]$ can be obtained from the effective diffusivity ($D_p$) and the film mass-transfer coefficient ($k$). The value of $D_p$ can be calculated from Eq. (8-8) because, under normal experimental conditions of an SEC column, the Reynolds number is usually very small. Several correlations can be employed to estimate the value of the film mass-transfer coefficient ($k$) in terms of the Sherwood number (Sh) for small Re. The following equation (Wilson and Geankoplis, 1966) seems to be most convenient since viscosity cancels out in $Re \cdot Sc$,
\[ Sh = \frac{1.09}{\varepsilon_b} (Re \cdot Sc)^{0.33} = 1.37(\varepsilon_b v R_p / D_m)^{0.33} / \varepsilon_b, \quad 0.0015 \leq Re \leq 55 \quad (8-15) \]

where \( Sh = (2R_p)k/D_m \), \( Sc = \mu/D_m \rho \) and \( Re = (2R_p)\rho(\varepsilon_b v) / \mu \). After \( Sh \) value is obtained, \( k \) value can be calculated from \( k = Sh \cdot D_m / (2R_p) \).

### 8.3.8 Size-Exclusion Factor \((F_{ex})\)

The size-exclusion factor \((F_{ex} = \varepsilon_{p}^{*}/\varepsilon_{p})\) introduced by Gu (1995) actually has the same value of the distribution coefficient \((K_{SEC})\). The separation capacity of an SEC column can be characterized by \( K_{SEC} \), which is defined (Hussain et al., 1991) using solute elution volume \((V_e)\),

\[ K_{SEC} = \frac{V_e - V_0}{V_t - V_0} \quad (8-16) \]

\( K_{SEC} \) can also be written as,

\[ K_{SEC} = \frac{t_R - t_d}{t_0 - t_d} \quad (8-17) \]

Inserting Eqs. (8-4) and (8-5) into Eq. (8-17) yields,
Thus, $F_{ex}$ has the same value as $K_{SEC}$. $F_{ex}$ can be readily calculated using $\varepsilon_p^a$ and $\varepsilon_p$ values obtained from a small column and remains unchanged during the scale-up procedure.

8.4 Effects of Mass Transfer Parameters on SEC Performance

It is beneficial to find out the sensitivities of parameters in the model system. The results can indicate which parameters are relatively important and should be more accurately estimated for the model system, and which parameters do not require rigid estimation.

8.4.1 Effect of the Peclet Number ($Pe_L$)

The value of $Pe_L$ represents the extent of the axial dispersion. As $Pe_L$ approaches infinity, the axial dispersion becomes negligible, indicating a plug flow. The influence of $Pe_L$ on the simulated chromatogram is shown in Figure 8.1. Parameters used in computer simulation to obtain Figure 8.1 are listed in Table 8.1. In addition, $\varepsilon_p = 0.6$, $\varepsilon_b = 0.26$, $F_{ex} = 0.8$ and $\tau_{imp} = 0.03$ were used. From Figure 8.1, it can be seen that, when $Pe_L$ becomes larger, the simulated peak becomes sharper. When $Pe_L$ exceeds 1,000, its influence on peak width becomes relatively insignificant. This case is always true in this work. Because the value of $Re$ is quite small, the $Pe_L$ value calculated from Eq. (8-5) is above 1,000.
Figure 8.1. The effect of $\text{Pe}_L$ number
8.4.2 Effect of the Biot Number (Bi)

The value of Bi reflects the characteristic ratio of the external film mass-transfer rate to the intraparticle diffusion rate. The effect of Bi on the elution peak is shown in Figure 8.2. It appears that Bi plays almost no part in the overall peak broadening effect when its value is greater than 50. This large Bi value indicates that the mass transfer process is limited by intraparticle diffusion. Interfacial mass transfer resistance is negligible in such cases.

8.4.3 Effect of the η Number

The η number \[ \eta = \varepsilon^D_p \frac{L}{(R_p^2 \nu)} \] plays an important role in the peak skewness and peak width. Figure 8.3 shows that the peak shape is sensitively affected by the value of η. The larger the η value is, the sharper the peak. When η decreases, the simulated peak first broadens then appears skewed. Small η value means slow elution rate. In order to obtain sharp elution peaks, the elution rate should be controlled within a certain limit.

8.4.4 Effect of Particle Radius (R_p)

The gel particle radius is a very important factor that affects peak broadening. From Figure 8.4, it is seen that a smaller particle radius makes the simulated peak stiffer and hence provides a better resolution. In Figure 8.4, the \( R_p/2 \) peak means that it is calculated using dimensionless parameters \( Pe_L, Bi \), and η values that reflect a reduction of 50% in particle radius (see Table 8.2). The drawback of a small particle size is that column pressure goes up. This may result in excessive bed compression.
Figure 8.2. The effect of Biot number
Figure 8.3. The effect of η number.
Table 8.1. Parameter values used for the study of the effects of $\text{Pe}_L$, $\text{Bi}$ and $\eta$.

<table>
<thead>
<tr>
<th>Figures</th>
<th>Simulation Parameters</th>
<th>Numerical Parameters</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$\text{Pe}_L$</td>
<td>$\text{Bi}$</td>
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<td>Figure 8.1</td>
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</tr>
<tr>
<td></td>
<td>500</td>
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</tr>
<tr>
<td></td>
<td>1000</td>
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<tr>
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<td></td>
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<td></td>
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<td>100</td>
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<tr>
<td>Figure 8.3</td>
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</table>
Figure 8.4. The effect of the particle radius ($R_p$).
8.4.5 Effect of the Effective Diffusion Coefficient \((D_p)\)

Figure 8.5 shows the influence of \(D_p\) on peak broadening. It is seen that a larger \(D_p\) value gives a sharper peak. Eq. (8-7) shows that \(D_p\) increases with the decrease of \(\lambda\) when \(0<\lambda<1\). According to Eq. (8-13), a lower \(\lambda\) value implies a larger pore diameter of the packing particle. So a larger pore size results in sharper peaks. However, selection of pore size also relies on how effective the pore size can discriminate against different solute molecules to be separated.

![Diagram showing the effect of effective diffusion coefficient (\(D_p\))](image)

Figure 8.5. The effect of effective diffusion coefficient \((D_p)\)
8.4.6 Effect of the Pore Tortuosity ($\tau_{\text{tor}}$)

According to Eq. (8-8), the pore tortuosity influences the performance of an SEC column through $D_p$. $D_p$ increases with the decrease of $\tau_{\text{tor}}$. Therefore, a larger pore tortuosity gives a broader peak. The effect of $\tau_{\text{tor}}$ is shown in Figure 8.6.

![Figure 8.6. The effect of particle tortuosity.](image-url)
The $\tau_{tor}$ value range is quite broad. It is not easily estimated (Li et al., 1998). Thus in this work, it will be correlated by matching experimental elution profile from a small column with the profile calculated using computer simulation with the general rate model. The same $\tau_{tor}$ value is used for larger beds with the same packing material. By adjusting $\tau_{tor}$, some errors resulting from the estimation of diffusional mass transfer parameters may be alleviated to a certain degree.

Table 8.2. Parameter values used for the study of the effects of $R_p$, $D_p$ and $\tau_{tor}$.

<table>
<thead>
<tr>
<th>Figures</th>
<th>Physical Parameters</th>
<th>Simulation Parameters</th>
<th>Numerical Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\text{Pe}_L$</td>
<td>$\text{Bi}$</td>
</tr>
<tr>
<td>Figure 8.4</td>
<td>$R_p/2$</td>
<td>1000</td>
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<td></td>
<td>$R_p$</td>
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<td>10</td>
</tr>
<tr>
<td></td>
<td>$2R_p$</td>
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<td>Figure 8.5</td>
<td>$D_p/2$</td>
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<tr>
<td></td>
<td>$D_p$</td>
<td>500</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>$2D_p$</td>
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<td>5</td>
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<td>Figure 8.6</td>
<td>$6\tau_{tor}$</td>
<td>500</td>
<td>60</td>
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<td>$3\tau_{tor}$</td>
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<td>30</td>
</tr>
<tr>
<td></td>
<td>$2\tau_{tor}$</td>
<td>500</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>$\tau_{tor}$</td>
<td>500</td>
<td>10</td>
</tr>
</tbody>
</table>
8.5 Experimental Method

The validity of a model can be judged by its ability to predict actual experimental results. In this work, the Bio-Rad P60 gel was used as the packing material. Three glass columns were employed: a 1.5 cm x 80 cm Bio-Rad column (Bio-Rad Laboratories, Hercules, CA, USA), a 5 cm x 70 cm Bio-Rad column and a 4.4 cm x 50 cm Amicon column (Amicon, Inc., Beverly, MA, USA). BSA, myoglobin and ovalbumin (SIGMA Chemical Company, St. Louis, MO, USA) were used. All experiments were carried out at ambient temperature using a Cole Parmer Marsterflex pump (Cole Parmer Instrument Co., Chicago, IL, USA). Fractions of the column effluent were collected using a Bio-Rad 2110 fraction collector. Protein concentration analysis was done using a Beckman DU640 spectrophotometer (Beckman Instrument, Inc., Fullerton, CA, USA).

The elution volume is more reliably measured than the retention time, because the elution volume is more stable for a solute in different runs. Therefore, in order to calculate $\varepsilon_b$, $\varepsilon_p$ and $\varepsilon_p^a$ Eqs. (8-3) to (8-5) are rewritten as,

\[
V_{e,d} = \pi d^2 L \varepsilon_b / 4 \tag{8-19}
\]

\[
V_{e,0} = V_{e,d} \left[ 1 + \frac{(1 - \varepsilon_b) \varepsilon_p}{\varepsilon_b} \right] \tag{8-20}
\]

\[
V_{e,R} = V_{e,d} \left[ 1 + \frac{(1 - \varepsilon_b) \varepsilon_p^a}{\varepsilon_b} \right] \tag{8-21}
\]
In this work, $\varepsilon_b$, $\varepsilon_p$, $\varepsilon^*_p$ and $\tau_{tor}$ were obtained using the small column. Since these parameters are properties of the gel particles rather than a bed, the same values obtained from a small column can be used for a large bed as long as the same gel is used and the bed compression is not very different between the two beds.

### 8.6 Results and Discussions

#### 8.6.1 Calibration Curve

Figure 8.7 shows the experimental calibration curve for protein samples on a 1.5 cm x 30 cm (bed dimensions) glass column packed with the Bio-Rad P60 gel. The sample molecules were BSA, ovalbumin, myoglobin and L-tryptophan. Tryptophan (MW=204) was small enough for P60 gel such that it was not excluded by any pores. This curve was used to calculate the values of $\varepsilon_p$ and $\varepsilon^*_p$. Blue Dextran was used to measure the void volume fraction of the column ($\varepsilon_b$). The values of $\varepsilon_b$, $\varepsilon_p$, and $\varepsilon^*_p$ for a protein (such as ovalbumin and myoglobin) were calculated according to Eqs. (8-19) to (8-21), respectively using experimental elution volumes of blue dextran, tryptophan, and the solute protein. The elution volumes were easily obtained by measuring the retention volumes on experimental chromatograms. The results are listed in Table 8.3.
Figure 8.7. The calibration curve for a small SEC column (1.5 cm x 30 cm bed dimensions).
Table 8.3. Values of physical parameters used in scale-up.

<table>
<thead>
<tr>
<th>Protein</th>
<th>MW</th>
<th>$\varepsilon_p$</th>
<th>$F_{ex}$</th>
<th>$D_m \times 10^{11}$ (m$^2$·s$^{-1}$)</th>
<th>$D_p \times 10^{11}$ (m$^2$·s$^{-1}$)</th>
<th>$\varepsilon_b$</th>
<th>$\varepsilon_p$</th>
<th>$\tau_{tor}$</th>
<th>$R_p \times 10^6$ (m)</th>
</tr>
</thead>
<tbody>
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<td>Myoglobin</td>
<td>16890</td>
<td>0.23</td>
<td>0.35</td>
<td>10.7</td>
<td>2.98</td>
<td>0.27</td>
<td>0.66</td>
<td>2.0</td>
<td>67.5</td>
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<tr>
<td>Ovalbumin</td>
<td>43500</td>
<td>0.08</td>
<td>0.12</td>
<td>7.8</td>
<td>1.65</td>
<td></td>
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</table>

8.6.2 Determination of the Pore Tortuosity ($\tau_{tor}$)

$\tau_{tor}$ value for the P60 gel was obtained by matching a model calculated peak profile with its corresponding experimental peak profile on the small column (1.5 cm x 27.3 cm bed dimensions). An assumed $\tau_{tor}$ value was first used in the computer program to calculate the peak profile. If the simulated peak had a wider band width compared to that of the experimental peak, a smaller $\tau_{tor}$ was used to run the computer program again until the two peaks match. For Bio-Rad P60 gel used in this work, the value of $\tau_{tor}$ was found to be 2.0 in Figure 8.8(a).

8.6.3 Scale-Up Procedure

If a large column is to be built or purchased, the column’s performance can be evaluated using the computer software. Three elution experiments can be carried out using a small column with the same packing. In this work, elution experiments were carried out using blue dextran, a small molecule (such as an amino acid) and some of the proteins to be separated. With the experimental elution volumes for these molecules, $\varepsilon_b$, $\varepsilon_p$, and $\varepsilon_p^*$ (for individual proteins) values can be easily calculated using Eqs. (8-19) to
In practice, a calibration curve similar to Figure 8.7 can be produced. Not all solutes need to be experimentally tested for their \( \varepsilon_p^a \) values, since their values may be interpolated using the calibration curve.

In the general rate model, with an assumed \( \tau_{\text{tor}} \) value, \( D_p \) can be calculated using Eq. (8-8) and because it is a particle-specific parameter, it can be used for both small and large columns for the same protein. Thus, \( \eta \) is readily calculated using its definition, \( \eta = \varepsilon_p^a D_p L / (R_p^2 v) \). \( R_p \) value is usually from the vendor of the packing material. \( P_eL \) is conveniently calculated using Eq. (8-7). In order to calculate the Biot number for mass transfer, \( Bi = kR_p / (\varepsilon_p^a D_p) \), the film mass transfer coefficient \( k \) for a protein is needed. It is calculated from the \( Sh \) value obtained from Eq. (8-15).

The \( \varepsilon_o, \varepsilon_p, \varepsilon_p^a \) and \( \tau_{\text{tor}} \) values obtained for the small column can be then used to calculate the elution profiles for a larger column. Different bed size and operational conditions can be simulated.
Figure 8.8 (a). Comparison between experimental results and model predictions for a single component elution on a small column (1.5 cm x 27.3 cm bed dimensions).
8.6.4 Experimental Scale-up Example

To validate the scale-up procedure, a small column (1.5cm x 27.3cm bed dimensions) packed with P60 gel was used. Comparisons between the model calculated and the experimental results on the small column are shown in Figures 8.8(a) and (b).

Figure 8.8(a) shows the results for a single-component elution. $\tau_{\text{tor}}$ value was adjusted to allow a good fit between model calculated and experimental data. This $\tau_{\text{tor}}$ value was then used for all subsequent model calculations. Figure 8.8(b) shows the results for a binary elution on this column. Figure 8.8(b) shows that the model predictions and the experimental results match very well in terms of retention time, peak width and peak height.

Three larger columns (4.4 cm x 29.5 cm, 5.0 cm x 29.5 cm, 5.0 cm x 42 cm in bed dimensions) packed with the same P60 gel were used to compare scale-up predictions using single-component and binary elutions. The results are shown in Figures 8.9-8.11. The parameters used for these figures are listed in Table 8.4.

From these figures it can be concluded that the agreement between the model predictions and the experimental results is very good. The volumetric scale-up factor is about 15.6:1 between the small 1.5cm x 27.3cm column and the larger 5.0 cm x 42 cm column.
Figure 8.8 (b). Comparison between experimental results and model predictions for a binary elution on a small column (1.5 cm x 27.3 cm).

This scale-up method hinges heavily on the assumption that the small column and the larger column have the same $\varepsilon_b$, $\varepsilon_p$, $\varepsilon_p^*$ and $\tau_{ir}$ values. This requires that the two columns have the same packing density which means the two beds should be operated at similar pressures. To maintain the validity of this assumption, the small bench column should be chosen in such a way that it has a similar bed height and operating pressure as the large column. If the bed compression is not a problem, such precautions are relaxed.
Figure 8.9 (a). Comparison between experimental results and model predictions for single component elution on a large column (4.4 cm x 29.5 cm).
Figure 8.9 (b). Comparison between experimental results and model predictions for a binary elution on a large column (4.4 cm x 29.5 cm).
Figure 8.10 (a). Comparison between experimental results and model predictions for single component elution on a large column (5.0 cm x 29.5 cm).
Figure 8.10 (b). Comparison between experimental results and model predictions for a binary elution on a large column (5.0 cm x 29.5 cm).
Figure 8.11. Comparison between experimental results and model predictions for a binary elution on a large column (5.0 cm x 42 cm).
Table 8.4. Parameter values used in Figures 8-11.

<table>
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<tr>
<th>Figures</th>
<th>Proteins</th>
<th>Operation Parameters</th>
<th>Simulation Parameters</th>
<th>Numerical Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>d (cm)</td>
<td>L (cm)</td>
<td>( v \times 10^4 ) (m/s)</td>
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<td>27.3</td>
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</table>

Of course for very large columns, it is hard to maintain perfect flow patterns. To compensate for this, a relatively larger bench column should be used such that similar irregular flow patterns occur. Or, a larger \( \tau_{\text{tor}} \) value (than that obtained from a small column with rather good flow patterns) is used for the scale-up predictions of a very large column since a larger \( \tau_{\text{tor}} \) value will result in more diffused peaks. This will compensate poorer performance of a large column due to irregular flows. By doing so, the rate model became a semi-empirical model.
8.7 Conclusions

A procedure was developed using a Fortran 77 software program based on a general rate model for the scale-up of SEC columns. Parameter sensitivities were studied using computer simulation. Methods used to estimate the values of these parameters were established. The tortuosity was treated as an adjustable parameters. Using the elution data from a few simple runs on a small column some physical parameters were obtained. Together with other mass transfer parameters evaluated using existing mass transfer correlations, the value of the tortuosity was obtained by matching the model-calculated results with the experimental results for the small column using a trial and error method. The elution performance of a much larger column can be predicated a priori. The validity of the general rate model in the simulation and scale-up of SEC process was demonstrated by scaling up an SEC column from 15 cm x 30 cm to 5.0 cm x 42 cm (bed dimensions) with a volumetric scale-up factor of 15.5 to 1.
CHAPTER 9

APPLICATION OF THE GENERAL RATE MODEL IN RP-HPLC

9.1 Introduction

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a very popular chromatography mode. RP-HPLC is usually the first choice for most regular samples. RP-HPLC columns are efficient, stable, and reproducible, so RP-HPLC is typically more convenient and rugged than other forms of chromatography and is more likely to result in a satisfactory final separation. In RP-HPLC, a gradient mobile phase is often used in order to obtain a satisfactory separation especially for the following kinds of samples:

- Samples with a wide $k'$ range
- Samples composed of large molecules [e.g., with molecular weights above 1000 and especially, samples of biological origin]
- Samples containing late-eluting interferences that can either foul the column or overlap subsequent chromatograms.
- Dilute solutions of the sample dissolved in a weak solvent (e.g., aqueous sample solutions for injection onto a reversed-phase column).

However, gradient chromatography is not a simple technique. The difficulties of reproducing the results, optimizing the conditions and scaling-up gradient elution separations are well known. Especially at the early stage of method development, in order to obtain a suitable gradient mode, many trial-and-error runs are needed. This is
very time and money consuming. A good mathematical model can facilitate the process development and optimization of chromatography. In this chapter, study will be made on the application of the general rate model in the simulation of RP-HPLC process.

9.2 Mathematical Model

In this work, when applying the general rate model to gradient RP-HPLC analysis of fatty acids, according to the properties of the system, it was assumed that there was no size exclusion effect in the column. Based on these additional model assumptions, when using the general rate model for RP-HPLC, all the $e^*_p$ in the particle phase governing equation will be replaced by $e_p$. Thus, Eq. (7-10) remain unchanged while Eq. (7-11) will be converted into the following form:

$$
\frac{\partial}{\partial \tau} \left[ (1 - e_p) c_{pi}^* + e_p c_{pi} \right] - \eta_i \left[ \frac{1}{r^2} \frac{\partial}{\partial \tau} \left( r^2 \frac{\partial c_{pi}}{\partial \tau} \right) \right] = 0
$$

(9-1)

In this model, the modulator is designated as the last component in a multicomponent (Ns components) system. It does not bind with the stationary phase. Its concentration affects the $b$ values of the eluities.
9.3 Initial and Boundary Conditions for RP-HPLC

For the eluites \((i = 1, 2, \ldots, N_s-1)\), this model has the following initial conditions:

\[
\tau = 0, \quad c_{bi} = c_{pi} = c_{pi}^* = 0, \quad (9-2)
\]

For the modulator \((i = N_s)\), this model has the following initial conditions:

\[
\tau = 0, \quad c_{bi} = c_{pi} = \frac{C_m}{C_{0i}} = c_{m0}, \quad c_{pi}^* = 0 \quad (9-3)
\]

The dimensionless feed concentration profiles for the boundary conditions at the column inlet are as follows.

For the eluites \((i = 1, 2, \ldots, N_s-1)\),

\[
C_f(\tau)/C_{0i} \begin{cases} 
1 & 0 \leq \tau \leq \tau_{\text{imp}} \\
0 & \tau > \tau_{\text{imp}}
\end{cases} \quad (9-4)
\]

For the modulator \((i = N_s)\),

\[
C_f(\tau)/C_{0i} \begin{cases} 
= \frac{C_{m0}}{C_{0i}} & -\infty < \tau \leq \tau_{\text{imp}} \\
\geq (\text{or } \leq) \frac{C_{m0}}{C_{0i}} & \tau > \tau_{\text{imp}}
\end{cases} \quad (9-5)
\]
For elutes, $C_{Oi}$ is the sample concentration for the rectangular sample pulse. For the modulator, its reference concentration value $C_{m0}$ can take any convenient value, such as the modulator concentration in the column before a gradient takeoff. In this work, $C_{m0} = 1$.

9.4 Estimation of Model Parameters

From Chapter 8, a conclusion can be drawn that, when using the general rate model to simulate liquid chromatography, most important is to establish the methods used to conveniently and accurately estimate the values of the input parameters used in this model. After the values of these parameters are calculated or obtained experimentally, the simulation should be quite straightforward. All one needs to do is just to input these parameters and run the model program on a personal computer.

From the analysis above, it can be concluded that one of the critical factors for a successful simulation of RP-HPLC using the general rate model is parameter estimation. Three types of parameter are needed to carry out model calculations: column property parameters, mass transfer parameters and the eluite-modulator relationship parameters. In this section, the methods used to estimate these parameters will be discussed.

9.4.1 Bed Void Fraction and Particle Porosity

Bed void fraction ($\varepsilon_b$) and porosity ($\varepsilon_p$) are the two most important column property parameters. These two parameters are needed as model input parameters and are also used to calculate mass transfer parameters. The methods used to estimate these
parameters for an SEC column can also be used for an RP-HPLC column. According to Unger (1979), the bed void fraction of a column packed with 5 µm silica-based particles is 0.4. Then the particle porosity can be easily calculated from the following equation (Gu and Zheng, 1999).

\[ \varepsilon_p = \frac{Q t_0}{V_b - \varepsilon_b} (1 - \varepsilon_b) \]  

(9-6)

In this work, \( Q = 1 \text{ ml/min}, t_0 = 2.80 \) (obtained from the blank gradient chromatogram in Figure 4.3), thus \( \varepsilon_p = 0.45 \).

9.4.2 Mass Transfer Parameters

According to the analysis in Chapter 8, mass transfer parameters only influence the height and width of the peaks in the SEC chromatogram. Although RP-HPLC is different from SEC in retention mechanism, a similar conclusion can be drawn for RP-HPLC from the analysis of the model simulation results. In this work, the effects of mass transfer parameters for RP-HPLC were studied using the general rate model by changing one parameter one time. Figures 9.1 to 9.3 depict the influences of \( \text{Pe}_L, \text{Bi} \) and \( \eta \), respectively. Data used to generate these figures are listed in Table 9.1. From these figures, it can be seen that the effects of the mass transfer parameters are very small. In fact, the values of the mass transfer parameters in RP-HPLC usually fall into these ranges in which their influences on the RP-HPLC can be neglected. Methods used to estimate these parameters are the same as those used in the SEC simulation in Chapter 8.
Figure 9.1. The effect of Pe_L on RP-HPLC.
Figure 9.2. The effect of Biot number on RP-HPLC.
Figure 9.3. The effect of \( \eta \) number on RP-HPLC.
Table 9.1. Parameters used in Figures 9.1–9.4.

<table>
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</tr>
<tr>
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<td>1000</td>
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<tr>
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</tbody>
</table>
9.4.3 Eluite-Modulator Relationship Parameters

In the general rate model, the correlation parameters $\alpha$, $\beta$ and $\gamma$ in the eluite-modulator relationship equation, Eq. (7-22) are needed to calculate the elution profiles of RP-HPLC. From Eqs. (7-20) and (7-22), the following equation can be derived.

$$\log_{10} k' = (\alpha - \log_{10} \phi C^e) - \beta \log_{10} C_m + \gamma C_m$$  \hspace{1cm} (9-7)

The capacity factor ($k'$) of an eluite at a fixed mobile-phase modulator concentration ($C_m$) can be calculated with a single isocratic run using the following equation (Snyder and Kirkland, 1974):

$$k' = \frac{(t_R - t_0)}{t_0}. \hspace{1cm} (9-8)$$

From Eqs. (9-3) and (9-4), the values of $\beta$ and $\gamma$ can be correlated using a plot of $k'$ versus $C_m$ when several isocratic runs are performed with different mobile-phase modulator concentrations. The value of $\alpha$ can also obtained this way if the values of adsorption saturation capacity ($C^e$) and the phase ration ($\phi$) are known. However, the value of $\alpha$ cannot be obtained directly. Determination of $\alpha$ is critical in the simulation of RP-HPLC. The effect of $\alpha$ on the RP-HPLC profile is analyzed using the general rate model. The results are depicted in Figure 9.4. The parameters used in Figure 9.4 are listed in Table 9.1. From Figure 9.4, it can be concluded that RP-HPLC profile is very
sensitive to the change of $\alpha$ value. The phase ratio can be easily calculated using the following equation:

$$\phi = (1 - \varepsilon_b)(1 - \varepsilon_p)/[\varepsilon_b + (1 - \varepsilon_b)\varepsilon_p]$$  \hspace{1cm} (9.5)

![Graph showing the effect of $\alpha$ on RP-HPLC](image-url)

Figure 9.4. The effect of $\alpha$ on RP-HPLC.
The adsorption capacity is the maximum molar amount of the eluite adsorbed onto the stationary phase per unit volume of the particle skeleton (Eq. 9-6):

$$C^\infty = \frac{w_s}{(MW)V_b(1 - \varepsilon_b)(1 - \varepsilon_p)}$$

(9-6)

where $w_s$ is defined as the column saturation capacity (mg of eluite) corresponding to a very concentrated equilibrium concentration. Snyder and Stadalius (1986) obtained the value of $w_s$ using a method which was based on the small retention time difference between two gradient runs, one with a small sample and the other with a large sample.

From the above analysis, all other parameters used in the general rate model, except $\alpha$ can be easily measured, calculated or estimated. Although the value of $\alpha$ can be obtained using the above-mentioned method, in this work, it is obtained using a trial and error method. This is because the RP-HPLC profile is very sensitive to $\alpha$ (Figure 9.4). It is not easy to obtain the accurate $\alpha$ values for each fatty acid using above-mentioned methods. Similar to the tortuosity factor in SEC, $\alpha$ is the only adjustable parameters used in this work to simulate RP-HPLC using the general rate model. Thus the $\alpha$ values for each fatty acid can be conveniently obtained by matching the model calculated peak profile with its corresponding experimental peak profile for an isocratic elution or a gradient elution. For each fatty acid, an assumed $\alpha$ was first used in the computer program to calculate its peak profile. If the calculated retention time was larger than that of the experimental peak, a smaller $\alpha$ was used to run the computer program again until the retention times of the two peaks match.
9.5 Experimental Results

Experiments were carried out using the same HPLC system as that used for fatty acids analysis in Chapter 4. The simulation procedure includes the following three steps:

- Perform several isocratic runs to obtain the values of $k'$, $\beta$, and $\gamma$ for each fatty acid.
- Obtain the values of $\alpha$ for each fatty acid by peak matching.
- Using the values of $\alpha$, $\beta$, and $\gamma$ obtained in the above two steps to predict the retention times of each fatty acid for a gradient elution, and compare the model prediction results with the experimental results.

9.5.1 Determination of the Eluite-Modulator Relationship Parameters

In order to obtain the values of the eluite-modulator relationship parameters, firstly, four isocratic runs were performed to obtain the $k' - C_m$ curves for each fatty acids. The results are plotted in Figures 9.5 and 9.6. The data used in these two figures are listed in Table 9.2 and Table 9.3. From these two figures, it can be seen that, for each fatty acid, the correlation between $\log_{10}k'$ and $C_m$ has very good linearity. From these results, the values of $\beta$ and $\gamma$ for each fatty acid were obtained.

These values are listed in Table 9.4. By matching the model calculated results and the experimental results using the procedure described in Section 9.4.3, the values of $\alpha$ for each fatty acids are obtained and listed in Table 9.4.
Figure 9.5. Capacity factor versus acetonitrile concentration for EPA, GLA, DHA, AA and DPA.
Figure 9.6. Capacity factor ($k'$) versus acetonitrile concentration for LA, DGLA, ADR, OA and EDA.
Table 9.2. Data used in Figure 9.5.

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<tr>
<th>C_m (v/v)</th>
<th>Logk'</th>
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</thead>
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<td>EPA</td>
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<tr>
<td>0.663</td>
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<td>0.700</td>
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Table 9.3. Data used in Figure 9.6.

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Table 9.4. The values of $\alpha'$, $\beta'(\beta)$, $\gamma'(\gamma)$ and $\alpha$ for fatty acids.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>$\alpha'$</th>
<th>$\beta'(\beta)$</th>
<th>$\gamma'(\gamma)$</th>
<th>$\alpha$</th>
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<tbody>
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9.5.2 Comparison of Model Predictions and Experimental Results

After all the model parameters for each fatty acid are measured, calculated, or estimated, the RP-HPLC profile was calculated using the general rate model for a gradient elution. An RP-HPLC run was performed using the operational conditions specified in the model calculation. Comparison between the calculated results and the experimental results was made. The model predicted results are depicted in Figures 9.7–9.16. Figure 9.17 is the experimental RP-HPLC profile for a mixture of the ten fatty acid standards.
The column parameters and operational parameters are listed in Table 9.5. The model input parameters for each fatty acid are listed in Tables 9.6 and 9.7. The retention times obtained from the model calculations and the RP-HPLC run are listed in Table 9.8. The calculated PeL values are around 25000 for all these fatty acids. This large PeL value causes unnecessary difficulties in numerical calculation. In this work, the Peclet numbers for all fatty acids are set to 1000. Although Peclet number has some influences on the peak width and peak height, its effect on the retention time is negligible when its value is 1000 and above.

From these results, it can be concluded that the model predicted retention times and the experimental retention times match very well. Although the model predicted peak width does not match the experimental results, the general rate model could still be an effective tool to predict the performance of RP-HPLC. In practice, the prediction of the retention time is much more important for HPLC separation and analysis. The following reasons can result in the difference between the model calculated peak width and the experimental peak width.

The first reason is that the mass transfer and retention mechanisms in an RP-HPLC column are very complex. It is very difficult to describe these mechanisms accurately. The second reason is that α is the only adjustable parameters used in the simulation process. Retention time is the first consideration when determining the value of α for each fatty acid. In this study, the column gave very sharp peaks because the column was a high efficiency analytical column with very small sample volume. Such peaks are difficult to simulate because of stiff numerical difficulties. The computer
software was intended for scale-up to preparative and large scale columns which do not have very stiff peaks.

Table 9.5. The column properties and operational parameters.

<table>
<thead>
<tr>
<th>Column Length</th>
<th>Column Diameter</th>
<th>Packing</th>
<th>Particle Diameter</th>
<th>ε_b</th>
<th>ε_p</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 cm</td>
<td>4.6 mm</td>
<td>C18</td>
<td>5 μm</td>
<td>0.4</td>
<td>0.45</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Mobile Phase</th>
<th>Gradient (ACN% v/v)</th>
<th>Flow Rate</th>
<th>Sample Volume</th>
<th>Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °</td>
<td>ACN-H₂O</td>
<td>25%-100% over 80 min</td>
<td>1 ml/min</td>
<td>100 μl</td>
<td>195 nm</td>
</tr>
</tbody>
</table>

Table 9.6. Parameters used for model calculations (1).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ACN</th>
<th>EPA</th>
<th>GLA</th>
<th>DHA</th>
<th>AA</th>
<th>DPA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>41.0</td>
<td>302.0</td>
<td>278.0</td>
<td>328.0</td>
<td>304.0</td>
<td>330.3</td>
</tr>
<tr>
<td>d_m (Å)</td>
<td>4.97</td>
<td>9.66</td>
<td>9.40</td>
<td>9.93</td>
<td>9.68</td>
<td>9.95</td>
</tr>
<tr>
<td>λ x 10²</td>
<td>1.66</td>
<td>3.22</td>
<td>3.13</td>
<td>3.31</td>
<td>3.23</td>
<td>3.32</td>
</tr>
<tr>
<td>D_m x 10¹⁰ (m² • s⁻¹)</td>
<td>7.95</td>
<td>4.08</td>
<td>4.2</td>
<td>3.97</td>
<td>4.07</td>
<td>3.97</td>
</tr>
<tr>
<td>D_p x 10¹⁰ (m² • s⁻¹)</td>
<td>1.92</td>
<td>0.95</td>
<td>0.98</td>
<td>0.92</td>
<td>0.95</td>
<td>0.92</td>
</tr>
<tr>
<td>k x 10⁴ (m • s⁻¹)</td>
<td>8.01</td>
<td>5.14</td>
<td>5.23</td>
<td>5.05</td>
<td>5.13</td>
<td>5.04</td>
</tr>
<tr>
<td>Pe_L</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>η</td>
<td>1375.0</td>
<td>682.6</td>
<td>703.1</td>
<td>662.7</td>
<td>681.0</td>
<td>661.3</td>
</tr>
<tr>
<td>Bi</td>
<td>23.2</td>
<td>30.0</td>
<td>29.7</td>
<td>30.3</td>
<td>30.0</td>
<td>30.4</td>
</tr>
<tr>
<td>α'</td>
<td>0</td>
<td>2.9556</td>
<td>2.9587</td>
<td>3.2731</td>
<td>3.258</td>
<td>3.4429</td>
</tr>
<tr>
<td>β'</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Table 9.7. Parameters used for model calculations (2).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>LA</th>
<th>DGLA</th>
<th>ADR</th>
<th>OA</th>
<th>EDA</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW</td>
<td>308</td>
<td>306</td>
<td>332</td>
<td>282</td>
<td>318</td>
</tr>
<tr>
<td>$d_m$ (Å)</td>
<td>9.72</td>
<td>9.70</td>
<td>9.97</td>
<td>9.44</td>
<td>9.83</td>
</tr>
<tr>
<td>$\lambda \times 10^2$</td>
<td>3.24</td>
<td>3.23</td>
<td>3.32</td>
<td>3.15</td>
<td>3.28</td>
</tr>
<tr>
<td>$D_m \times 10^{10}$ (m$^2$ s$^{-1}$)</td>
<td>4.06</td>
<td>4.07</td>
<td>3.96</td>
<td>4.18</td>
<td>4.01</td>
</tr>
<tr>
<td>$D_p \times 10^{10}$ (m$^2$ s$^{-1}$)</td>
<td>9.45</td>
<td>9.47</td>
<td>9.20</td>
<td>9.75</td>
<td>9.34</td>
</tr>
<tr>
<td>$k \times 10^4$ (m s$^{-1}$)</td>
<td>5.12</td>
<td>5.12</td>
<td>5.03</td>
<td>5.22</td>
<td>5.08</td>
</tr>
<tr>
<td>$P_{EL}$</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>$\eta$</td>
<td>677.8</td>
<td>679.4</td>
<td>659.9</td>
<td>699.5</td>
<td>670.1</td>
</tr>
<tr>
<td>Bi</td>
<td>30.1</td>
<td>30.0</td>
<td>30.4</td>
<td>29.7</td>
<td>30.2</td>
</tr>
<tr>
<td>$\alpha'$</td>
<td>3.2764</td>
<td>3.4982</td>
<td>3.7473</td>
<td>3.7359</td>
<td>3.9097</td>
</tr>
<tr>
<td>$\beta'$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$\gamma'$</td>
<td>-3.6319</td>
<td>-3.8469</td>
<td>-4.0699</td>
<td>-3.8074</td>
<td>-3.9881</td>
</tr>
</tbody>
</table>

Table 9.8. Comparison between the model predictions and experimental results for fatty acids.

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EPA</td>
</tr>
<tr>
<td>Model Prediction</td>
<td>52.79</td>
</tr>
<tr>
<td>Experimental Result</td>
<td>52.83</td>
</tr>
<tr>
<td>Fatty Acid</td>
<td>LA</td>
</tr>
<tr>
<td>Model Prediction</td>
<td>60.26</td>
</tr>
<tr>
<td>Experimental Result</td>
<td>60.09</td>
</tr>
</tbody>
</table>
Figure 9.7. The model predicted profile for EDA

Figure 9.8. The model predicted profile for GLA.
Figure 9.9. The model predicted profile for DHA.

Figure 9.10. The model predicted profile for AA.
Figure 9.11. The model predicted profile for DPA.

Figure 9.12. The model predicted profile for LA.
Figure 9.13. The model predicted profile for DGLA.

Figure 9.14. The model predicted profile for adrenic acid.
Figure 9.15. The model predicted profile for OA.

Figure 9.16. The model predicted profile for EDA.
Figure 9.17. Experimental RP-HPLC profile of fatty acid standards.
This work consists of two parts. The first part is the method development for the separation and analysis of fatty acids from recombinant animal cells using RP-HPLC. The second part is the application of the general rate model to the simulation of two liquid chromatography processes: SEC and RP-HPLC.

10.1 Fatty Acid Separation and Analysis

In this part, a process was developed for the separation and analysis of various underivatized fatty acids in recombinant animal cells. These fatty acids are the metabolites of oleic acid. This method consists of four steps: saponification, solvent extraction, solvent change and RP-HPLC analysis.

The saponification step was used to release fatty acids from complex lipids. In this method, 0.5N sodium hydroxide in the mixture of 96% ethanol-4% water was used to saponify the fatty acids. Here, ethanol was used to support the saponification reaction and dissolve the sodium salts of the fatty acids. First, 1ml 0.5N NaOH 96%ethanol-4%water (v/v) solution was added to the cell sample (containing around 1 million recombinant animal cells). After disrupting the cells with an ultrasonic homogenizer, the sample was left at room temperature for 8 hours. Finally, the sample was centrifuged at 7000rpm for 5 minute and the solid residue was discarded.
The purpose of the solvent extraction step is to separate fatty acids from salts and other unwanted water-soluble impurities. 1ml 0.6N HCl-water solution was added to the supernatant from the saponification step. This operation changed the fatty acids from their sodium salts to their free acids form. The free fatty acids were then extracted by adding 3ml ethyl acetate to the system.

The purpose of the solvent change step was to transfer free fatty acids from the ethyl acetate-ethanol solution to pure methanol for the HPLC injection. The top phase was gently blew with nitrogen under 30°C. When most ethyl acetate was blow out from the solution, the sample was lyophilized to get rid of the remained water. The sample was then re-dissolved into 350μl HPLC grade methanol for RP-HPLC analysis.

The RP-HPLC analysis was performed on a C18 RP-HPLC column using a gradient ACN-H₂O mobile phase. The fatty acids were monitored by measuring ultraviolet light absorption at 195 nm (absorption mainly by double bonds) and identified by the comparison of retention times and adsorption spectra between the sample profile and the fatty acid standards.

This method successfully resolved these fatty acids and the reproducibility was very good. It can be used to separate and analyze fatty acids on an analytical scale. However, its feasibility for the preparative separation and purification of fatty acids need to be further studied.
10.2 Mathematical Modeling of SEC and RP-HPLC

In this part, the general rate model was adopted to simulate two kinds of liquid chromatography processes: size exclusion chromatography and reverse-phase chromatography. The effects of various parameters were analyzed using the mathematical model. Methods to estimate the model parameters and the procedures using the general rate model to simulate and predict the results of these two types of chromatography process were developed. The validity of these two simulation procedures was verified by the comparison between the model predictions and the experimental results for larger SEC and RP-HPLC columns.

For SEC simulation, protein standards were used as sample solutes. After process analysis, the particle tortuosity was used as an adjustable parameter and was obtained by matching the model-predicted results with the experimental results for a small column. All other model parameters can be easily obtained experimentally or from empirical equations.

An assumed $\tau_{0r}$ value was first used in the computer program to calculate the peak profile. If the simulated peak had a wider band width compared to that of the experimental peak, a smaller $\tau_{0r}$ was used to run the computer program again until the two peaks match. Three larger columns (4.4 cm x 29.5 cm, 5.0 cm x 29.5 cm, 5.0 cm x 42 cm in bed dimensions) packed with the same P60 gel were used to compare scale-up predictions using single-component and binary elutions. The agreement between the model predictions and the experimental results for the three columns are very good.
For very large columns, it is hard to maintain perfect flow patterns. To compensate for this, a relatively larger bench column should be used to obtain the value of $\tau_{\text{tor}}$ such that similar irregular flow patterns occur. Or, a larger $\tau_{\text{tor}}$ value (than that obtained from a small column with rather good flow patterns) is used for the scale-up predictions of a very large column since a larger $\tau_{\text{tor}}$ value will result in more diffused peaks. This will compensate poorer performance of a large column due to irregular flows. By doing so, the rate model became a semi-empirical model.

For RP-HPLC simulation, the fatty acid standards were used as sample solutes to be separated. The eluite-modulator relationship parameter $\alpha$ was used as an adjustable parameter. Similar to SEC, all other parameters used in the general rate model can be easily measured, calculated or estimated. Although the value of $\alpha$ can also be obtained experimentally, in this work, it is obtained using a trial and error method. This is because the RP-HPLC profile is very sensitive to $\alpha$. It is not easy to obtain the accurate $\alpha$ values for each fatty acid using above-mentioned methods. The $\alpha$ values for each fatty acid can be conveniently obtained by matching the model calculated peak profile with its corresponding experimental peak profile in an isocratic elution or a gradient elution. For each fatty acid, an assumed $\alpha$ was first used in the computer program to calculate its peak profile. If the calculated retention time is larger than that of the experimental peak, a smaller $\alpha$ was used to run the computer program again until the retention times of the two peaks match.

The simulation procedure includes the following three steps: Perform several isocratic runs to obtain the values of $k'$, $\beta$, and $\gamma$ for each fatty acid; Obtain the values of
α for each fatty acid by peak matching; Using the values of α, β, and γ obtained in the above two steps to predict the retention times of each fatty acid for a gradient elution, and compare the model prediction results with the experimental results.

The model predicted retention times and the experimental retention times match very well. However the model predicted peak width is larger than the experimental results. In this study, the column gave very sharp peaks because the column was a high efficiency analytical column with very small sample volume. Such peaks are difficult to simulate because of stiff numerical difficulties. The computer software was intended for scale-up to preparative and large-scale columns which do not have very stiff peaks. For future study on the simulation of RP-HPLC using the general rate model, large molecule protein standards and preparative columns are recommended.
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ABSTRACT

LI, ZHIGUO. Ph.D. March 2001
Chemical Engineering

High-Performance Chromatography Analysis of Fatty Acids and Mathematical Modeling of Liquid Chromatography (176 pp.)

Director of Dissertation: Tingyue Gu

The purposes of this study are to develop a process to analyze fatty acids in recombinant animal cells using reversed-phase chromatography and simulate liquid chromatography processes using the general rate model.

A method was developed for the separation and analysis of various fatty acids in recombinant animal cells. The cell sample was saponified with 0.5M NaOH in 96% ethanol and then extracted with acidified ethyl acetate. After extraction, the sample was dried and dissolved in HPLC-grade methanol. After centrifugation to remove insoluble impurities, the sample was applied to a C18 RP-HPLC column to separate and identify fatty acids using a gradient ACN-H$_2$O mobile phase. The fatty acids were monitored by measuring ultraviolet light absorption at 195 nm and identified by retention time and adsorption spectrum comparison. This method successfully resolves these fatty acids and can be used either analytically or preparatively. The fatty acids separation and analysis process developed in this work can be adapted to quantitatively analyze fatty acids from others sources.

The general rate model was used to simulate liquid chromatography processes. Two kinds of liquid chromatography processes were studied: size exclusion chromatography and reverse-phase chromatography. The effects of various parameters