A USER'S GUIDE

introduction to peptide and protein hplc (volume 1)

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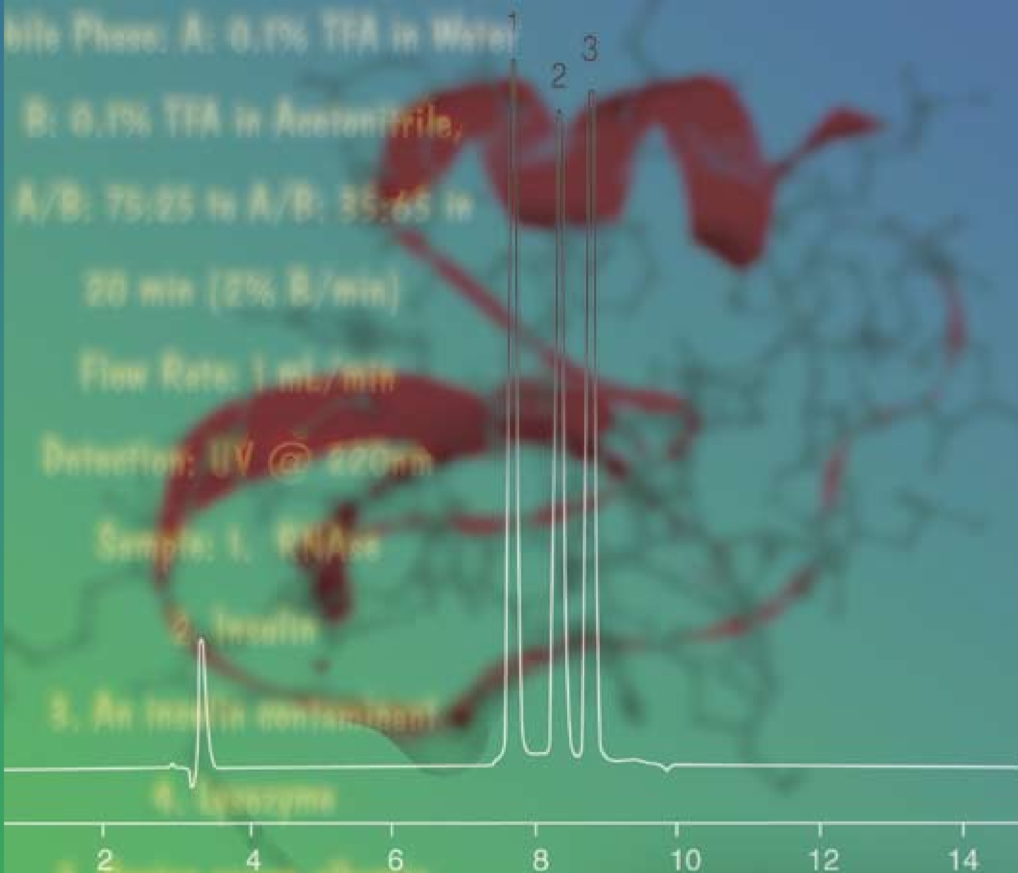
A USER'S GUIDE

Introduction to Peptide and Protein HPLC

volume 1



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A User's Guide:

INTRODUCTION TO PEPTIDE AND PROTEIN HPLC

Timothy P. Bradshaw, Ph.D.

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A User's Guide: Introduction to Peptide and Protein HPLC

PREFACE

Over the past decade, tremendous progress has been made in the analysis of peptides and proteins by high performance liquid chromatography (HPLC). This phenomenon has been driven, in part, by a greater appreciation of the crucial roles these biomolecules play both in normal cell physiology and disease states. Indeed, peptides and proteins are now considered an important class of therapeutics used in treating diseases ranging from diabetes to cancer. The use of HPLC for peptide and protein analysis has also exploded as a result of the myriad of novel column supports and chemistries now commercially available.

The first goal of this guidebook is to acquaint those new to peptide and protein HPLC with some basic principles of the technique, especially as they relate to these biomolecules. Some of the special features of peptides and proteins that distinguish them from other molecules and have relevance to their analysis are also discussed. Secondly, I briefly discuss the five most common modes of HPLC (size-exclusion, ion-exchange, reversed phase, hydrophobic interaction, and affinity chromatography) used in peptide and protein analysis. I describe typical separation conditions and, in some cases, provide buffer compositions and conditions for initial attempts at chromatography. Hopefully, this type of information will prove useful as one begins to design methods for individual application needs. After this discussion, the attention turns to Small-Bore chromatography. This topic is particularly relevant today as researchers attempt to separate and identify the minute quantities of peptides and proteins typically contained in biological samples. Finally, in the last couple of sections, Phenomenex presents some thoughts on column selection and beginning method development.

The guidebook is obviously not a comprehensive treatment of the very broad subject of peptide and protein HPLC. Instead, it is a limited overview that is meant to facilitate this analytical approach while dispelling its mystery as well as some preconceived and often erroneous notions (such as the inevitable loss of bioactivity

believed to be associated with certain modes of chromatography). I have relied heavily on the wealth of information available in the literature, and the user is encouraged to consult the materials listed in the Sources and Suggested Reading section. Of particular importance is the work of Mant and Hodges, which provides in-depth discussions from which I draw frequently. Their book, entitled *High-Performance Liquid Chromatography of Peptides and Proteins*, provides a great deal of practical advice and may be regarded as "a teaching manual for both the novice HPLC user as well as more experienced chromatographers".

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Section 1.

General HPLC Theory and Terminology

Section 1 contains:

- **Basic Principles**
- **Theoretical Plates (N) and HETP (H)**
- **Two Key Events in HPLC Separation**
- **Retention Time and Retention Factors**
- **Band (Peak) Broadening**
- **Resolution**

Basic Principles

For efficient use of HPLC to achieve the separation and purification of compounds, it is critical that one possess a basic understanding of the physical parameters and theoretical considerations upon which the resolving power of the method is based. Indeed, such knowledge is often essential in order for the researcher to develop the best separation methods possible while solving the various problems that can arise during the practical use of HPLC. Before some basic theory of HPLC is discussed below, it should be noted that undesirable chromatography can often be attributed to instrument limitations in addition to the stationary phase within the column itself and/or the method employed for separation. For example, the valves, tubing, and detector may hold relatively large volumes of mobile phase, thus contributing to band broadening through diffusion effects.

In Figure 1, a number of important chromatographic terms and principles are illustrated. This chromatogram is typical of the elution profiles for two pure compounds, denoted as Peak A and Peak B. Here the concentration of the two solutes eluting from the column, based on the response of the detector (e.g., fluorescent, UV or radiochemical), is plotted against time. After injection onto an HPLC column, any sample components that do not interact with the stationary phase would elute at time t_o in the void volume (v_o) which is characteristic for that column. This void volume represents both the interstitial volume between the particles of the bonded phase and the available volume within the particle pores themselves. The retention times, $t_{r(A)}$ and $t_{r(B)}$, for the two sample components shown in Figure 1 are the times from injection to the times of maximum concentration in the eluted peaks. Similarly, the retention volumes are the amounts of solvent required for their elution. We will discuss retention time in more detail later.

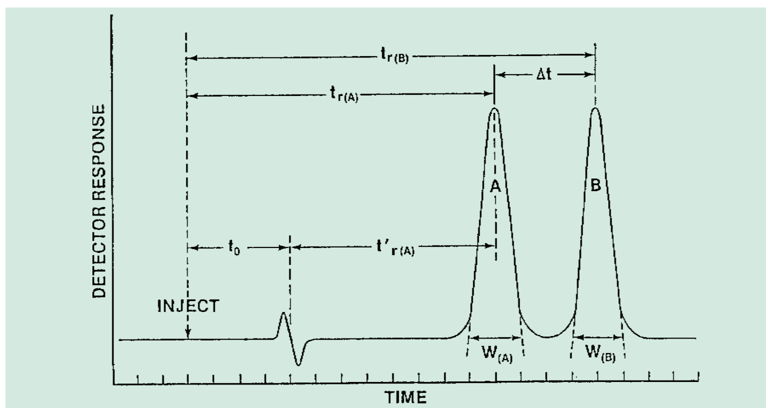


Figure 1. *Important chromatographic parameters, and how they are measured.* $\mathbf{t_{r(A)}}$ *represents the retention time of peak* ***A*** *(measured in minutes);* $\mathbf{t_{r(B)}}$ *is the retention time of peak B (measured in minutes); Δt is the difference in retention time of peaks* ***A*** *and* ***B***; $\mathbf{t_o}$ *is the elution time of a sample component which is not retained on the column (has no interaction with the stationary phase), commonly called the void time of the column;* $t'_{r(A)}$ *is the adjusted retention time of for peak* ***A*** ($\mathbf{t'_{r(A)} = t_{r(A)} - t_o}$); $\mathbf{W_{(A)}}$ *is the width at base of peak* ***A*** *(measured in minutes); and* $\mathbf{W_{(B)}}$ *is the width at base of peak* ***B***. *Reprinted with permission from Reference 1.*

Theoretical Plates (N) and HETP (H)

The number of theoretical plates (N) has traditionally been used as a measure of column efficiency. This value relates chromatographic separation to the theory of distillation. It may be visualized as a series of hypothetical layers within the column in which a solute is in an equilibrium process of adsorption-desorption between the two relevant phases.

$$N = 16(t_r/W)^2$$

where t_r is the retention time and W is the peak width at baseline.

Generally, the measurement of peak width at half height has been found to be most useful, since it can be carried out on peaks not completely resolved, peaks that exhibit tailing, or peaks that are otherwise asymmetrical in shape.

$$N = 5.54(t_r/W½)^2,$$ where W½ is peak width at half height

The value of N is a useful measure of the performance of a chromatographic column, and in general the more theoretical plates, the better the column. The number of theoretical plates can be calculated for any peak in a given separation, with each calculation resulting in a slightly different value. The value for N is to a

first approximation, independent of retention time; however, it is proportional to column length. Therefore, height equivalent to a theoretical plate (HETP) is the better measure of column efficiency since it allows for a comparison between columns of different lengths.

$$H = HETP = L/N$$ where L is the length of the column, usually in mm.

This value compares the extent that a given solute band has diffused during its migration down the column; thus efficient columns will have small H numbers. A small value for H is desirable such that as many plates as possible are present within the column. For a typical HPLC column, well-packed with 5 μm particles, HETP or H values usually range from 0.01-0.03 mm.

Two Key Events in HPLC Separation

The separation of a mixture of peptides and proteins during an HPLC run is the culmination of two events that occur during the chromatographic process. The first deals with solute retention and has its foundation in the concept of mass distribution along the stationary phase. Specifically, as solutes move down the length of the column, carried by a controlled solvent flow rate that is delivered by the HPLC pumps, the individual components of the mixture interact with the mobile phase and stationary phase to different extents. The second event involves band (or peak) broadening as the solutes travel down and through the chromatographic matrix. This dispersion is controlled by the diffusivity of the solutes and includes all the kinetic processes associated with the adsorption-desorption events combined with the molecular motion of the solute within the stationary phase. Together, these effects lead to band broadening which progressively increases during the transit through the column.

Retention Time and Retention Factor

The retention of a solute is that time required for the compound to pass, following injection, from one end of the column through the other end of the column and into the detector where it is monitored as an eluted peak. As stated above, in order for any two compounds to be resolved they must interact with the column matrix such that they are retained for distinct periods of time (t_r), eluting in different solvent volumes (V_r). The retention factor (k') is a measure of the degree of retention and can be calculated (assuming equal flow rates) by the following equation:

$$k' = (V_r - V_o)/V_o = (t_r - t_o)/t_o$$

where k' is the number of column volumes required to elute a particular solute (it is a unitless number); V_o and t_o represent the void volume and void time, respectively.

The retention factor is thus directly related to the partition coefficient of an analyte between the mobile and stationary phases. A related concept is that of selectivity (α), which can be defined as the relative separation between adjacent solute peaks. This number is the ratio of the retention factors for the two peaks:

$$\alpha = k'_1/k'_2$$

Band (Peak) Broadening

Band broadening (or peak spreading) is a generally unavoidable consequence of liquid chromatography. This undesirable phenomenon occurs as the solute experiences dilution during transit through the column. The extent of this effect is reflected in the column efficiency which, as discussed above, is illustrated by the height equivalent to a theoretical plate (HETP). There are four major factors (Figure 2) that contribute to band broadening: (1) eddy diffusion, (2) longitudinal diffusion from Brownian motion, (3) complications due to mass transfer, i.e., resistance to mass transfer in both the stationary phase and mobile phase, and (4) extra-column effects, particularly excessive dead volume within the system. These factors contribute to band broadening by increasing the H number. The control of these various forces that promote band broadening in HPLC represents one of the obvious challenges for the researcher. These processes can be partially offset by using columns that are well-packed with uniform particles possessing narrow, uniform pore sizes. Moreover, peptides and proteins have relatively small molecular

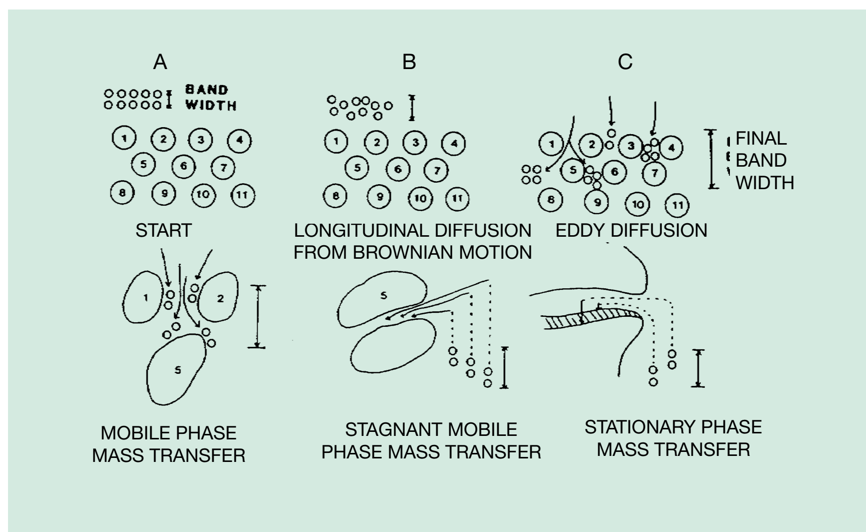


Figure 2. *Various contributions to the molecular dispersion of a peak zone for a peptide solute with porous HPLC sorbent. Reprinted with permission from Reference 2.*

diffusivities in HPLC systems, therefore better column efficiencies can often be attained by careful attention to mass transfer kinetics. A more thorough discussion of this topic can be found from the recommended reading list at the end of this introductory guide.

Resolution

Resolution is defined as the extent between separation of two chromatographic peaks. In practice one usually strives to have the best resolution possible within the constraints of complexity of the mixture of solutes as well as the maximum practical length of the column itself. Thus, resolution (R_s) can be described as a measure of how well a given HPLC column (and system) separates the two components.

$$R_s = \text{difference in retention time / average peak base width}$$

$$= 2(t_{r(B)} - t_{r(A)}) / (W_A + W_B)$$

For two symmetrical peaks, if the value for resolution (R_s) equals 1, then the peaks will be almost completely separated, having an overlap of only 2 %. Resolution values greater than 1.5 indicate complete separation.

Although its discussion is beyond the scope of this guide, resolution can also be described in terms of an equation which includes three factors: the selectivity factor α, the capacity factor k', and the plate number, N. Thus:

$$R_s = \text{(selectivity factor) x (retention factor) x (column efficiency factor)}$$

$$R_s = \alpha\ k^1\ N$$

Each of these three factors can be optimized independently, with selectivity playing the most important role with respect to resolution. Column efficiency is second in importance and, as described earlier, can be maximized by the use of longer columns, by decreasing the particle size of the solid support, or by adjusting the flow rate. The retention factor typically does not play a major role in affecting resolution.

Section 2.

Peptides and Proteins: General Aspects

Section 2 contains:

- **Introduction**
- **Basis for Separation**

Introduction

Proteins are the most abundant biomolecules present in all living cells, contributing up to fifty percent of the cell's dry weight. Proteins serve a variety of purposes, including metabolic (e.g., enzymes), structural (e.g., collagen), transport (e.g., hemoglobin), and defense (e.g., antibodies) functions. These important macromolecules are made up of long chain polymers of the basic building blocks called amino acids. Almost all proteins are constructed entirely from the same set of 20 common amino acids, linked in characteristic sequences which impart to them their specific functions.

Figure 3 (next page) shows the 20 common amino acid building blocks of proteins. Amino acids are covalently coupled via peptide (amide) bonds to form the linear peptide chain (Figure 4). Each amino acid has in common a carboxyl group and an amino group bonded "a primary carbon". They differ from each other by their side chains (or R groups) which vary dramatically in structure, size, electric charge and solubility in water. Thus, the properties of proteins and peptides are directly related to the primary sequence of amino acids from which they are derived, and which will be a major determinant in the type of chromatographic technique necessary to achieve their separation and/or purification. The amino acids shown in Figure 3 can be grouped into families based on the properties of the side chains. There are four main families: (1) nonpolar or hydrophobic R groups, (2) polar but uncharged R groups, (3) negatively charged R groups found in acidic amino acids, and (4) positively charged R groups found in basic amino acids.

Amino acids are ionizable in aqueous solution; those that have a single carboxyl group and a single amino group exist at physiological pH (7.4) as the fully ionized zwitterion. Furthermore, many peptides and proteins also contain ionizable acidic and basic side chains such that their charge and polarity are a function of the pH. Every protein, at a certain pH called the isoelectric pH, or isoelectric point (pI), will contain an equal number of positive and negative charges, and thus will be electrically neutral. At this pH, the protein is in its least soluble state in aqueous solution.

R GROUPS

NONPOLAR, HYDROPHOBIC

Amino acid	Abbr.	Code	MW
Alanine	Ala	A	89
Valine	Val	V	117
Leucine	Leu	L	131
Isoleucine	Ile	I	131
Phenylalanine	Phe	F	165
Tryptophan	Trp	W	204
Methionine	Met	M	149
Proline	Pro	P	115

POLAR, UNCHARGED

Amino acid	Abbr.	Code	MW
Glycine	Gly	G	75
Serine	Ser	S	105
Threonine	Thr	T	119
Cysteine	Cys	C	121
Tyrosine	Tyr	Y	181
Asparagine	Asn	N	132
Glutamine	Gln	Q	146

POLAR, ACIDIC

Amino acid	Abbr.	Code	MW
Aspartic acid	Asp	D	133
Glutamic acid	Glu	E	147

POLAR, BASIC

Amino acid	Abbr.	Code	MW
Lysine	Lys	K	146
Arginine	Arg	R	174
Histidine	His	H	155

Figure 3. *Structural formulas of the 20 most common amino acids.*

Peptide bonds absorb ultraviolet (UV) light strongly in the 200-220 nm range. This property explains why UV detection is routinely used for the identification and quantitation of peptides. Moreover, most proteins contain varying amounts of the amino acids tyrosine, tryptophan and phenylalanine, whose aromatic side chains absorb UV light in the 250-280 nm range. The presence of these residues allows for the detection and quantitation of proteins at higher wavelengths, thereby minimizing the background signal generated by mobile phases during HPLC.

In this introductory guide, we will consider the HPLC separation of both proteins and peptides. Although there is no explicit distinction between polypeptides and proteins, we will consider polypeptides to consist of 2-50 amino acids. As polypeptide chains grow toward 50 amino acids in size, they usually adopt particular three-dimensional structures in aqueous solution and are called proteins. For example, bovine insulin (MW~5700) is formed from the disulfide linkage of two polypeptides containing 21 and 30 amino acids each.

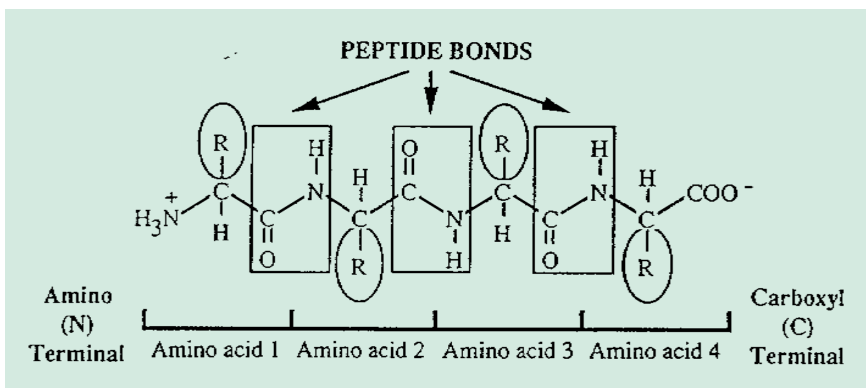


Figure 4. *Peptide structural formula.*

It should be noted that conformation can dramatically influence the chromatography of peptides as well as proteins, and should be a consideration when selecting separation modes and conditions. The potential for secondary structure increases with increasing chain length, and is particularly important in cyclic peptides. For example, cyclosporin A is a cyclic undecapeptide that is the primary immunosuppressive drug used in organ transplant patients. This unusual peptide adopts a conformation at 37 °C such that strong intramolecular hydrogen bonds are formed, thereby yielding an extremely hydrophobic molecule requiring high concentrations of organic solvent for elution in reversed phase HPLC. Similarly, some peptides may internalize hydrophobic residues to stabilize the folded structure in aqueous solution, giving rise to molecules that appear less hydrophobic in solution, and thus are more weakly retained in reversed phase HPLC than their unfolded counterparts.

Basis for Separation

The tremendous amount of structural diversity possible among peptides and proteins allows for the differences observed in their physical, chemical and functional properties. These differences in behavior also provide the molecular basis for their separation and/or purification by today's modern HPLC methods. The peptide and protein properties that have been shown to be useful to this end include size, shape, charge, hydrophobicity, and function.

The molecular weights of proteins vary widely from a couple of thousand daltons upwards to several million, and can sometimes yield the most straightforward, effective approach to their separation. Besides total molecular weight, proteins also adopt specific tertiary structures which must also be considered in the use of size-exclusion chromatography to design successful separation methodologies. For example, a compact globular protein will exhibit an apparent molecular size much smaller than its denatured, unfolded counterpart. Obviously, protein shape is dramatically influenced by the chemical environment.

The differing acid-base properties of both peptides and proteins discussed earlier frequently becomes the basis for their separation and identification by HPLC. The net charge experienced by a given protein is greatly influenced by the pH of the solution. A protein will be negatively charged at pH values above its pI, and positively charged at pH values below its pI. Ion-exchange chromatography exploits these differences in the acid-base behavior of peptides and proteins, and is often used as a separation step in their purification.

Besides the number of charged groups that peptides and proteins contain, the relative hydrophobicity of the molecules is also an important consideration in their chromatography. It is the interactions between peptides/proteins and hydrophobic stationary phases that provide the basis of reversed phase chromatography and hydrophobic interaction chromatography, two of the most widely used and powerful modes employed in HPLC applications today.

Additionally, we have already described how the unique sequence of amino acids present in a protein determines its specific function. The binding interaction between active proteins and their substrates or target molecules is often exploited in affinity chromatography, providing the researcher with another powerful, highly selective technique for separation and/or purification.

Section 3.

HPLC Techniques for the Analysis of Peptides and Proteins

Section 3 contains:

- **Size-Exclusion Chromatography**
- **Ion-Exchange Chromatography**
- **Reversed Phase Chromatography**
- **Hydrophobic Interaction Chromatography**
- **Affinity Chromatography**

The routine use of HPLC in the analysis of peptides and proteins is a relatively recent event; however, the basic separation mechanisms involved in the resolution of these molecules are very similar to those used in classical (gravity) chromatography.

Below we examine the use of a number of the most widely used HPLC methods for achieving both separation and purification of peptides and proteins. After spending some time describing the theory and practical aspects of utilizing these various HPLC modes in the laboratory, later sections will include important considerations in developing separation methods.

A. Size-Exclusion Chromatography (SEC)

Section 3A contains:

- **Theory**
- **Use of Denaturants**
- **Mobile Phase, Solid Support, and Sample Capacity**
- **Limitations of SEC**
- **Column Cleaning, Re-equilibration and Storage**

One of the earliest realizations in analytical science was the possibility of separating molecules by exploiting differences in their apparent molecular sizes. Since the introduction of Sephadex in 1959, size-exclusion chromatography (also referred to as gel-filtration or gel-permeation chromatography) has been a widely used laboratory tool in the separation of proteins. Because harsh elution conditions are not required, size-exclusion chromatography (SEC) does not inactivate enzymes, and thus is routinely employed as an important step in their purification. The widespread use of SEC can also be attributed to the speed, simplicity and versatility of the method. The HPLC mode of this type of liquid chromatography became possible in the 1980's only with the advent of semi-rigid (e.g., polystyrene) or rigid (e.g., silica) particles with uniform pores large enough

to be entered by proteins. Besides its use for separating and/or purifying proteins that differ significantly in size, there are two other important utilities of size-exclusion chromatography. SEC can also be used to change the buffer environment of a sample (as in the desalting of a protein before other studies or chromatographic procedures) and, more importantly, as a means of estimating the molecular sizes and shapes of proteins.

Theory

Size-exclusion column packing materials consist of particles containing pores of well-defined size. As the mobile phase flows over and through these particles, it carries along with it solutes which, depending on size, may flow into and out of the pores. Unlike other HPLC techniques, SEC does not depend on any selective interaction with the stationary phase. Thus, if a mixture of proteins or peptides is applied to a size-exclusion column that has been correctly packed with porous material of a specific pore size, these molecules will be separated by differences in their sizes, or more precisely, hydrodynamic volumes (defined by their Stokes radii) (Figure 5).

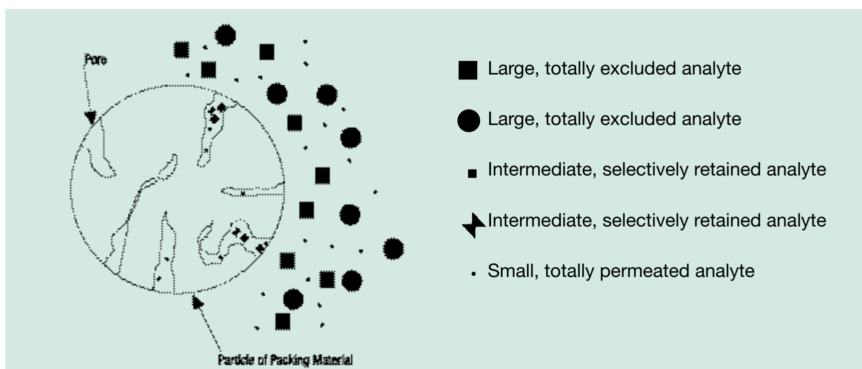


Figure 5a. *Separation of Analytes in Gel-Filtration Chromatography.*

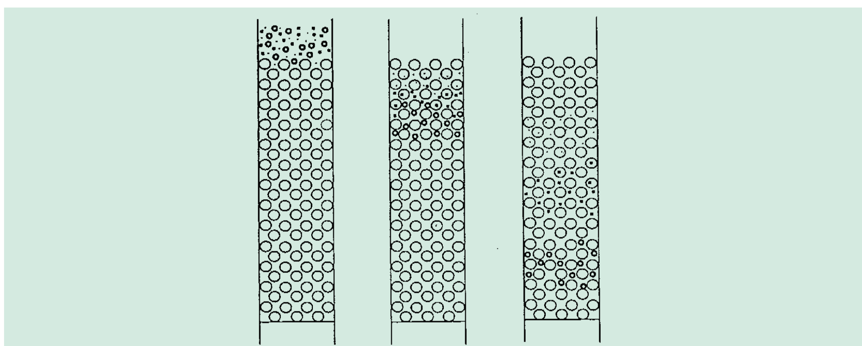


Figure 5b. *Schematic representation of gel-filtration chromatography. Molecules of different size in the frame are separated according to size during migration through the gel-filtration matrix as shown in the middle and right frames.*

Size-exclusion columns have published exclusion limits measured in daltons, and proteins whose molecular weights are larger than the exclusion limit are excluded (i.e., are too large to enter the pores) and elute in the void volume (V_o) of the column. The pores of a particle are differentially accessible to smaller proteins which then may elute after the void volume. The volume within the pores of the gel is called the internal volume (V_i), whereas the total permeation volume (V_t) of the column is defined as the sum of the void volume and the internal volume:

$$V_t = V_o + V_i \text{ (}V_o\text{ here is the excluded volume)}$$

In ideal SEC, there is a linear relationship between the elution volume (V_e) of a protein and the logarithm of its molecular size (actually its Stokes radius), such that it is possible to estimate (within ~10 %) the molecular weights for a series of proteins that elute between the void volume and the total permeation volume of a given HPLC column. The elution volume (V_e) can be described by:

$$V_e = V_o + K_D V_i, \quad K_D = \frac{(V_e - V_o)}{(V_t - V_o)}$$

The distribution coefficient (K_D) is a constant which describes the elution of solutes by SEC and which will be proportional to the logarithm of their molecular weights (MW). Plots of K_D against log MW are typically linear between K_D values of 0.15-0.80. Calibration curves, from which the molecular weight of an individual protein can be estimated, can also be constructed by plotting log MW vs. elution volume or retention time, K_D (Figure 6). The linearity of the plots offers a measure of the range of protein molecular weights that can be accommodated by the column (i.e., the column quality), and generally the lower the slope of the curve, the better the resolution between proteins of different molecular weights. One important reminder is that this approach to molecular weight determination is based on the assumption that the protein is globular and symmetrical in shape.

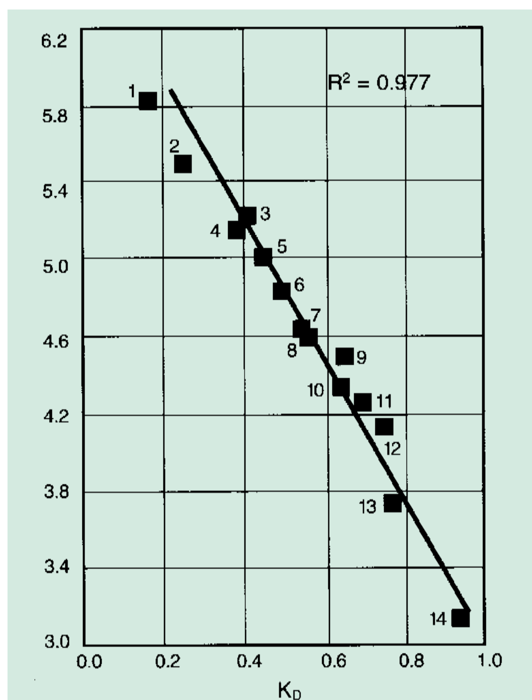


Figure 6. *Molecular weight calibration curve using BioSep™-SEC-3000.*

Moreover, the use of instruments capable of delivering accurate, reproducible flow rates is often essential for obtaining meaningful data from complex samples with this mode of chromatography. Otherwise, peak identification based on retention times alone becomes unreliable.

Use of Denaturants

For more accurate determinations of protein molecular weight, denaturants (e.g., 0.1 % SDS or 6 M guanidine hydrochloride) are sometimes included in the mobile phase. In addition to disrupting the aggregates formed by many proteins in solution, these agents also tend to promote uniform (rod-like) conformations, thereby promoting separations that approximate ideal SEC. Moreover, these denaturants may also aid in reducing interactions between the solutes and the column matrix.

Besides its use in molecular weight determinations, SEC experiments in which multiple sets of mobile phase conditions (in as many runs on the same column) are employed have been used in the demonstration of tertiary and quaternary structure in proteins. Denaturants similar to those described above disrupt these higher orders of structure, giving rise to several peaks when multiple subunit proteins are present.

Mobile Phase, Solid Support and Sample Capacity

With respect to optimum chromatographic results (at least in terms of accurate protein size estimation), the most important parameters of the stationary phase relate to pore volume and the uniformity of the pore size and particle shape. Furthermore, the separation of proteins or peptides by SEC by a mechanism dependent strictly on Stokes radius (ideal SEC) happens only when there is no interaction between the solutes and this solid support. Most silica-based size-exclusion columns, although end-capped to minimize nonspecific interactions, are weakly anionic and slightly hydrophobic in nature, which can result in compounds being retained longer (or shorter) than would be expected based on their known molecular weights. Thus, in many SEC applications for peptides and proteins, it is imperative that the researcher suppress these electrostatic and hydrophobic column effects by the addition of salts to the mobile phase. However, high concentrations of salts (>0.5 M) may increase hydrophobic interactions between proteins and the solid support. Working at pH values less than 7.0 will also help by minimizing the formation of silanolate anions. The use of an inert surface (i.e., polymer) is a viable alternative and will prevent most solute-stationary phase interactions, thereby yielding ideal SEC separations. BioSep™ silicas show no nonspecific interaction or hydrophobic interactions.

The SEC of proteins is typically conducted between 4 °C and 37 °C in mobile phases containing buffers of low ionic strength (less than or equal to 0.1 M) in near physiological pH ranges so as to preserve biological activity of the macromolecule. Silica resins are stable in the pH range of 2 to 7.5. A typical isocratic SEC elution buffer is 50 mM KH_2PO_4 (pH 6.5) containing 100 mM KCl. Inclusion of such counter-ions helps stabilize the protein, thus maintaining structural integrity. Flow rates between 0.5 - 1.0 mL/min are employed in SEC for the resolution of proteins on a number of stationary phases, although particular columns may require slightly slower rates. Generally, slower flow rates yield better resolution (up to a point).

Much effort has been concentrated in the area of optimization of particle size and shape. Today’s size-exclusion column beads/particles are small (5-10 µm), spherical, and have uniform size distributions (±10 %) to maximize resolution. An excess in pore size distribution results in increased band broadening. It is the pore diameter which dictates the size-exclusion limits, and usually a given molecule will travel more quickly through the column with smaller pores. Column efficiency is critical in SEC since, unlike modes based on specific solute-matrix interactions, the molecules are eluted isocratically without the aid of bind-and-release-type mechanisms. Well-packed HPLC SEC columns containing small particles minimize the void volume while maximizing the accessible volume and, as a result, allow for greatest resolution potential.

Due to the nature of the mechanisms responsible for separations in this mode of HPLC, SEC has the lowest loading capacity in terms of both sample mass and sample volume. Protein samples should be concentrated up to but not beyond

the point of precipitation for optimum performance (a general rule of thumb is that the sample should be less than twice as viscous as the elution buffer). The sample volume should be between 1 - 5 % of the packed column bed volume. As sample volumes exceed 5 % of the bed volume, resolution decreases dramatically in SEC. However, volumes less than 1 % generally do not improve resolution significantly.

Limitations of SEC

Besides the load volume restrictions noted above, other important disadvantages of SEC include the time required for elution of the peptide or protein of interest, the dilution of the sample, and the lack of resolution sometimes encountered. Each of these limitations is founded on the fact that there is no specific binding of molecules to the matrix in this mode of chromatography. The extended run times experienced in SEC are also due to the requirement of long narrow columns and slow flow rates (see above) to effect adequate resolution. Although longer columns improve resolution, it is increased only proportionally to the square root of the column length (i.e., doubling the column length will result in only a 40 % increase in resolution). Connecting columns of different pore size or exclusion limit is a common method for improving resolution over a wide range of molecular weights.

Column Cleaning, Re-equilibration and Storage

Size-exclusion supports should be cleaned and re-equilibrated after each chromatographic run. Cleaning to remove any remaining peptide or protein is usually carried out by washing with several column volumes of 0.1 M phosphate buffer (pH 3.0). Solutes bound through hydrophobic interactions are usually removed using an acetonitrile gradient. Finally, stubborn sample components can be eluted through use of washes containing 0.5 % SDS or 6 M guanidinium thiocyanate. Polymer-based (but not silica-based) columns can also be cleaned with 0.2 N NaOH. Cleaning the column in the reverse direction at half the normal flow rate is usually recommended.

If the column is to be stored for more than 48 hours before its next use, 3-5 column volumes of a solution containing an anti-microbial agent (e.g., 0.02 % sodium azide, 10 % MeOH) should be run through (after washing the column with water to remove salt-containing buffer). Before using the column again, the support must be thoroughly equilibrated in the buffer to be employed in the subsequent chromatography. Literature describing the cleaning and storage conditions specific for the column of interest is often available from the manufacturer and should always be consulted if possible.

B. Ion-Exchange Chromatography

Section 3B contains:

- **Mechanism and Principles**
- **Stationary Phase Characteristics**
- **Ion-Exchange Supports**
- **Choice of Mode and General IEC Conditions**
- **Sample Application and Column Elution**
- **Column Regeneration and Storage**

Ion-exchange chromatography (IEC) was the first type of chromatography to be developed into a high performance mode, and although the pressure limits of the early columns were quite low, major advances were made in this area in the 1970's when silica-based ion-exchange resins were introduced. The popularity of ion-exchange HPLC in peptide analysis has traditionally lagged behind its use in protein purification protocols, primarily due to the universal utility and acceptance of reversed-phase HPLC in peptide separations. However, many researchers now consider IEC to be the most promising alternative to the reversed-phase mode in peptide work.

Ion-exchange chromatography is often an integral step in protein purification procedures since virtually all of these macromolecules carry surface charges allowing for their adsorption onto the solid support. Another feature that has made IEC a favorite among researchers is the fact that biological activity is almost always preserved, along with the fact that this mode of chromatography represents a facile way for concentrating dilute protein samples into much more concentrated solutions.

Mechanism and Principles

We saw earlier how most peptides and proteins, by virtue of their functional groups, possess either net positive or negative charges that vary with pH. Proteins and peptides are positively charged at pH values below their pI and negatively charged at pH values above their pI. Ion-exchange chromatography effects the separation of peptides and proteins by taking advantage of these pH-dependent charges. Specifically, the mechanism of separation in IEC depends primarily on reversible, electrostatic (or ionic) interactions between these charged solutes (and salts) in the mobile phase and a charged ion-exchange group contained on the stationary phase.

There are two classes of IEC: anion-exchange and cation-exchange. In anion-exchange chromatography (Figure 7), negatively-charged peptide or protein molecules compete with negatively charged mobile-phase ions for the positive groups coupled on the column matrix. In contrast, cation-exchange chromatography involves the competition of positively charged peptide or protein molecules with positively charged mobile-phase ions for negatively charged sites on the stationary phase. For example, in anion-exchange chromatography the solid support of

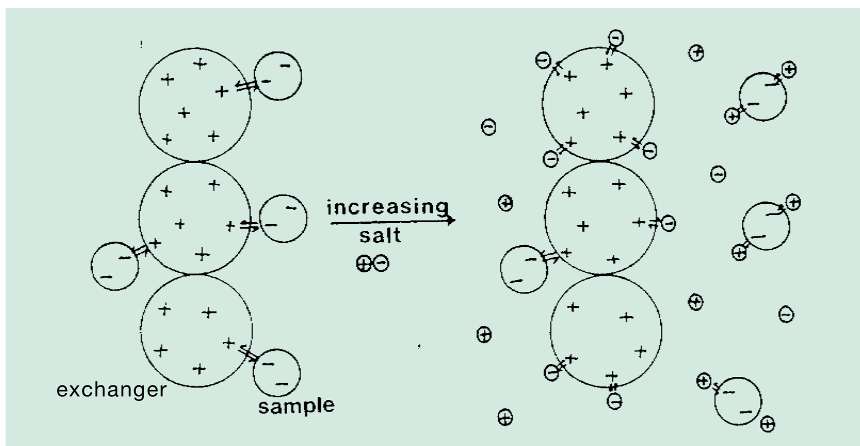


Figure 7. *Anion-exchange chromatography. A simplified representation of the dynamic equilibrium existing between an anion-exchange stationary phase and the ions in the mobile phase. Reprinted with permission from Reference 3.*

the column is initially positively charged and in equilibrium with the negatively-charged salts or counterions (e.g., 0.1 M Cl^-, where Cl^- is the counter ion from NaCl or KCl) present in the mobile phase. Under appropriate pH conditions (see below), a negatively charged peptide or protein may displace the chloride ion and bind to the column. To elute these molecules, equilibrium chemistry is applied as a higher concentration of the counterion (e.g., 1 M Cl^-) is included in a new mobile phase wash (gradient) step, thus effectively competing for the positively charged column sites and displacing the peptide or protein. The technique may also utilize, in addition to increases in ionic strength, changes in pH to achieve the best resolution in the shortest time possible.

Ion-exchange chromatography occurs as a multi-step process, including the movement of the peptide or protein solute from the mobile phase into the stationary phase environment, ionic binding to the solid support, and finally, the selective displacement and elution of the solute. Ultimately, separation occurs because those charged solutes displaying relatively weak interactions with the ion-exchange stationary phase will be retained less on the column, eluting earlier than those charged solutes that react more strongly with the column and elute later. It is important to note that the diffusional movement (mass transfer) of solutes through the stationary phase is relatively slow in IEC.

Stationary Phase Characteristics

Since the introduction of silica-based gels nearly two decades ago, many other types of solid supports have been developed (e.g., polystyrene cross-linked with divinylbenzene, alumina and recently, a polyester-polyamine co-polymer) which meet the requirements of high-performance IEC of peptides and proteins. Chief among these requirements is the ability of the resin to withstand the high pressures experienced in the packing, operation and regeneration of IEC columns (as much as several thousand psi). Most analytical HPLC columns contain small particles (5-10 μm) for adequate resolution; preparative columns may contain particles up to 25 μm in diameter.

The porous particles that make up most HPLC resins possess large surface areas thereby generating columns with enormous binding capacities. In fact, many ion-exchange resins can bind up to 40 % of their own wet weight of an average-sized (~40,000 dalton) protein. In practice, only 10-20 % of the maximum column capacity should be used where optimal resolution is essential. Since almost all peptide and protein IEC is performed using gradient or step elution, this mode of HPLC has few sample volume limitations.

IEC column exclusion limits (pore size) provided by the manufacturer should be noted, since proteins larger than these limits will not bind efficiently because they will be unable to penetrate the pores. Fortunately, most IEC columns have pore sizes greater than 300 Å, so exclusion is rarely a problem.

Ion-Exchange Supports

Ion-exchange columns are categorized as either anionic or cationic based on the type of functional group displayed on the solid support (Table 1).

Table 1. *Grouping of ion-exchangers based on functional group type. Reprinted with permission from Reference 4.*

Anion-exchanger	Typical functional group	Cation-exchangers	Typical functional group	Mix bed exchangers
Weak	Polyamino	Weak	Carboxymethyl	Amino + carboxy
Moderate	Diethylaminoethyl	Moderate	Phospho	Carboxy + sulpho
Strong	Quaternary ammonium	Strong	Sulpho	Quat + sulpho

As described above, anion-exchange columns are derivatized with positively charged groups, typically tertiary or quaternary amines. The diethylaminoethyl (DEAE) group is a widely used tertiary amine in anion-exchange matrices (e.g., BioSep™-DEAE-P, see Section 5, page 57) for peptide and protein separations. Recently, the quaternary amine groups that make up the ionic binding sites of

Shodex® QA resins have gained popularity in these applications. Cation-exchange columns usually contain a carboxymethyl (CM) group (e.g., Shodex® CM), although the sulfomethyl (Shodex® SP) is now commercially available. The pH of the mobile phase determines the ionization state of each of these functional groups, and thus the surface charge of the solid support. Figure 8 shows the effect of pH on the surface charge for the groups listed in Table 1. Those groups that are weakly basic (DEAE) or weakly acidic (CM) can bind peptides and proteins via relatively low affinity interactions; therefore, elution may be achieved through mild, non-denaturing conditions. It is important to point out that the terms “weak", “moderate" and “strong", when applied to ion-exchange resins, do not refer to the strength of the ionic bond

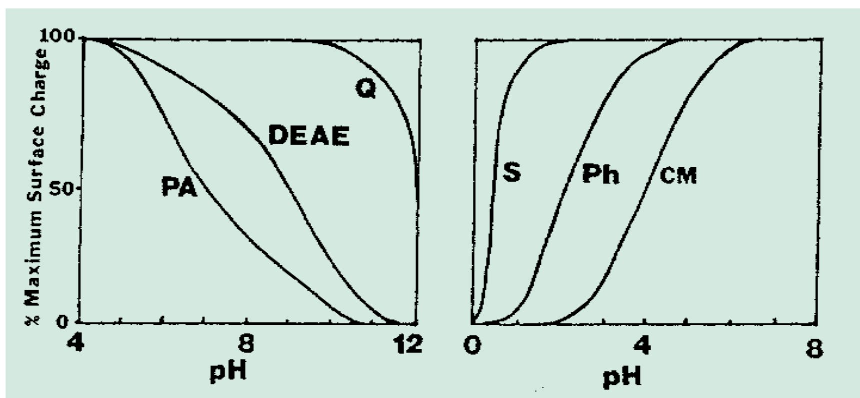


Figure 8. *Idealized representation of the variation of surface charge with pH for various acid and base groups on ion-exchangers. Reprinted with permission from Reference 4.*

formed during the chromatography. Instead these terms relate only to the pK_a's of the functional groups contained on the stationary phase.

Choice of Mode and General IEC Conditions

When the pI of the peptide or protein of interest is known, the type of ion-exchanger, as well as the useful range of mobile phase pH, may be readily identified. At pH values above the pI, these molecules are negatively charged, while at pH values below their pI, they are positively charged. Acidic proteins and peptides (pI < 6) are usually chromatographed on anion-exchange columns since they are negatively charged; when they are negatively charged (on anion-exchange columns), basic proteins and peptides (pI > 8) make similar change to basic proteins when they are positively charged (on cation-exchange columns). It is important to remember that at its pK_a, an ion-exchange resin is only 50 % ionized; therefore, the pH of the mobile phase should be at least one pH unit away from this value in order to maintain 90 % of the full charge on the column. With weak and moderate ion-exchange resins, the binding surface becomes more negative as the pH rises.

Thus, for anion-exchangers, the pH must be lower than the pKa; for cation-exchangers, the pH must be higher.

In selecting conditions for this mode of chromatography, it is critical to maximize the binding between an oppositely charged solute and the ion-exchanger. If the column or the protein is not fully charged, sample mass capacity will suffer. The extended usable pH range of strong exchangers makes them more versatile than the weak exchanger counterparts. However, few separations are performed at such pH extremes, and the different selectivity of the weak exchangers may be preferable in a given separation. For the successful use of IEC in the separation of peptides and proteins, it is imperative that the researcher possess a working knowledge of all of the above parameters such that the binding of the molecule of interest is different from those of the rest of the components of the sample.

From the above discussion, it should be obvious that the choice of mobile phase pH in IEC is critical for proper column performance and separation results. In fact, the pH of the buffer is probably more important than its ionic strength, since this characteristic will dictate the behavior of not only the ion-exchange resin, but the peptide or protein solutes as well. For example, small adjustments in mobile phase pH can be used to exert fine control over solute retention (Figure 9). The pH to be used determines, in part, the buffer which makes up the mobile phase. Some commonly used buffers in anion- and cation-exchange chromatography, along with their useful pH ranges, are shown in Table 2.

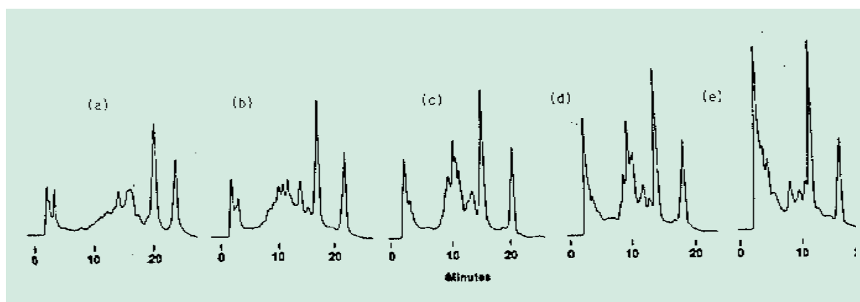


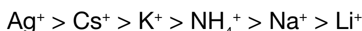
Figure 9. *Effect of pH change on the anion-exchange purification of lipoxidase. Identical typical anion-exchange conditions were used for all separations. (a) pH 10 with 1,3-diaminopropane-HCl, (b) pH 9 with 1,3-diaminopropane-HCl, (c) pH 8 with Tris-HCl, (d) pH 7 with triethanolamine-HCl, (e) pH 6 with piperazine-HCl. Reprinted with permission from Reference 5.*

Table 2. *Buffers used for HPIEC.*

Buffer		pK_a	pH range
Phosphate			
	pK_1	2.1	1.5 - 2.7
	pK_2	7.2	6.6 - 7.8
	pK_3	12.3	11.7 - 12.9
Citrate			
	pK_1	3.1	2.5 - 3.7
	pK_2	4.7	4.1 - 5.3
	pK_3	5.4	4.8 - 6.0
Formate		3.8	3.2 - 4.4
Acetate		4.8	4.2 - 5.4
Piperazine		5.7	5.0 - 6.0
MES	(2-[N-morpholino]ethanesulfonic acid)	6.1	5.5 - 6.7
bis-TRIS	(bis[2-hydroxyethyl]iminotris-[hydroxymethyl]methane	6.5	5.8 - 7.2
BIS-TRIS	propane	6.8	6.4 - 7.3
PIPES	(piperazine-N,N'-bis [2-ethanesulfonic acid)	6.8	6.1 -7.5
BES	(N,N'-bis[2-hydroxethyl]-2-aminoethanesulfonic acid)	7.1	6.4 - 7.8
HEPES	(N-[2-hydroxyethyl]piperazine-N'--ethanesulfonic acid])	7.5	6.8 - 8.2
Triethanolamine		7.8	7.3 - 7.7
TRIS (tris[hydroxymethyl] aminomethane)		8.3	7.7 - 8.9
Diethanolamine		8.9	8.4 - 8.8
Ammonia		9.2	8.6 - 9.8
Borate		9.2	8.6 - 9.8
Ethanolamine		9.5	9.0 - 9.5
Piperazine		9.7	9.5 - 9.8
Diethylamine		10.5	9.9 - 11.1
Piperidine		11.1	10.6 - 11.6
Phosphate		12.3	11.8 - 12.0

Of course, the other key determinant of solute retention is the ionic strength of the mobile phase. As we described in an earlier example, the salts contained in the buffer solution represent the source of counterions which compete with the peptide or protein for the charged binding sites on the resin. An "activity series" of ions describes the relative affinities of the counterions for the charged columns sites, thus giving an indication of their contribution to solvent strength:

For cation-exchangers:



$Ag^{+} > Cs^{+} > K^{+} > NH_4^{+} > Na^{+} > Li^{+}$

For anion exchangers:

$NO^{-3} > PO_4^{-3} > CN^{-} > HCO^{-3} > HCOO^{-} > CH_3COO^{-}$

Therefore, if a peptide or protein is strongly adsorbed to the ion-exchange column, elution may be improved by selecting a stronger counterion. It is important to mention that poor resolution can usually be improved by using longer columns, different gradient shapes and compositions (i.e., pH and counterion) as well as slower flow rates.

Sample Application and Column Elution

Before injection onto the pre-equilibrated ion-exchange column, the sample should be in low ionic strength buffer that is roughly the same as the starting mobile phase and lower than that at the start of the gradient. It is important to remember that any deviation in sample pH will affect not only the charge of the peptide or protein, but can have profound effects on both the loading capacity and resolving power of IEC. If the elution characteristics of the solute of interest are known, introduction in a buffer possessing an ionic strength slightly below that required for desorption from the support will aid in purification protocols, since those sample components displaying lower column affinities will wash through with less retention.

Once the sample has been applied, the column is usually washed with several column volumes of the initial mobile phase in order to elute any unbound species. The column is then typically eluted by one of two methods: gradient elution or step elution, each of which involves a binary solvent system. For illustrative purposes, consider that Buffer A contains 0.05 M NaCl (pH 6.5), while Buffer B contains 1.0 M NaCl (pH 8.0). In gradient elution, a linearly increasing concentration of the Buffer B is used (e.g., a constant increase in the ionic strength and pH: 10 to 60 % B in 20 minutes). Step elution involves discrete increases in Buffer B such that after washing the column with the starting buffer (Buffer A), the chromatographer may next wash with buffer containing 0.1 M NaCl (pH 7.0), followed by 0.5 M NaCl (pH 7.5) and so on. In general, gradient elution provides for better total resolution of complex mixtures, although step elution will be effective in many instances.

Column Regeneration and Storage

Regeneration of the ion-exchange resin is a critical step at the conclusion of a chromatographic run in IEC, since incomplete re-equilibration can adversely affect the reproducibility of future separations. The column may be considered re-equilibrated only when the ionic strength and pH of the mobile phase eluting from the column is the same as that at the pump head. These two indices are easily monitored by using conductivity and pH meters. Ion-exchange columns typically require long re-equilibration times, and many column volumes to return to the original (pre-chromatography) conditions. Thus, re-generation is typically carried out at higher flow rates than during a chromatographic run, although care should be taken not to exceed the pressure and flow limits of a particular column solid support. Most manufacturers will supply information regarding appropriate regeneration and clean-up procedures (for removal of strongly adsorbed proteins, lipids or detergents) for a given column.

Proper storage of ion-exchange columns is also important in order to maintain column reproducibility as well as limiting the frequency of column replacement. All IEC columns should be stored in a suitable buffer containing an antimicrobial agent (e.g., 0.002 % chlorohexidane for anion-exchangers or 0.02 % sodium azide for cation-exchangers).

C. Reversed Phase Chromatography

Section 3C contains:
- **Stationary Phase and Choice of Column**
- **Theory and Mechanism**
- **Standard RPC Conditions**
- **Ion-Pairing Reagents and pH effects**
- **Effect of Varying Gradient, Flow Rate on Separation**
- **Temperature Influences**
- **Recovery of Biological Activity**
- **Column Cleaning and Regeneration**

Reversed phase chromatography (RPC) is currently the most widely used HPLC technique in the separation, purification and study of peptides. The popularity of the method can be attributed to the speed and efficiencies typically achieved in its use. Moreover, the mobile phases typically include volatile organic solvents (e.g., acetonitrile) which facilitate both sample concentration and solvent removal prior to further solute analysis.

Although it has been known for over a decade that RPC could be utilized in the separation of proteins, its use in such applications has been somewhat limited until recently. Much of this reluctance by researchers was fueled by the misconception that denaturation and loss of biological activity are unavoidable consequences of the elution process. Thus, in most of the early published reports involving proteins, the macromolecules were either unusually stable or the retention of bioactivity was not a primary concern. However, as will be described below, common strategies can be developed that should allow for the successful use of RPC in the analysis of proteins without the irreversible loss of bioactivity.

Stationary Phases and Choice of Column

The majority of packings in RPC are silica-based and contain covalently bonded alkyl chains of different lengths. These hydrophobic groups are responsible for imparting specific chromatographic character to the column. The most commonly used moieties are *n*-octadecyl (C18), *n*-butyl (C4) and the phenyl group. During the manufacturing process, steric hindrance problems limit the initial bonding reaction such that not all of the silica hydroxyl groups are derivatized. Column manufacturers typically follow with a second reaction involving a small reagent (e.g. trimethylchlorosilane) to block the residual silanol groups, and thus avoid the potential non-specific adsorption complications of these unmodified hydroxyl groups on chromatographic separation. These RPC supports are described as being fully “endcapped”.

It is well known that silica-based supports are unstable in aqueous buffers at alkaline pHs where hydrolysis of the siloxane bonds can occur. This limitation has spurred an interest in the use of polymer-based reversed phase resins such as polystyrene-divinylbenzene copolymer supports. These polymer-based resins offer the advantage of increased stability over a wide range of pH values (pH 2 to pH 12). However, as a class polymer-based columns show lower efficiencies than silica-based columns. Thus, for use at pH values greater than 7.0, one of these non-silica based packings may be chosen. Phenomenex currently offers silica-based columns: the Jupiter® 300 series, which can withstand highly alkaline buffers (up to pH 10), while offering exceptionally high efficiencies (see Section 6, p. 63).

Some other useful suggestions for selecting a particular column packing can be made. The retention of peptides and proteins on reversed-phase columns often correlates well with their increasing relative hydrophobicities. The shorter alkyl chain matrices (e.g., C4, C5) typically perform better with “sticky" hydrophobic samples while their longer alkyl chain counterparts are more suited to hydrophilic samples. Large, especially hydrophobic proteins are ideal candidates for either C4 or C5 phases. Peptide maps, natural and synthetic peptides and small, hydrophilic proteins are best chromatographed on a 4 µm 90 Å C12 column like Jupiter® Proteo. Finally, C18 columns are often chosen for the analysis of hydrophilic proteins. It is important to note that as the alkyl chain size increases, so does the strength of hydrophobic interaction with proteins, a property that can result in significant losses of bioactivity. It is therefore advisable to limit protein separations where there are bioactivity concerns to C4 and C5 bonded silicas.

Other important considerations for optimum RPC results include the length of the column as well as the pore and particle size of the packing. Most analytical reversed phase columns have particle sizes between 5 and 8 µm, although preparative work sometimes employs particles up to 40 µm in diameter. The pore size of the support particles must be chosen according to the size of the solutes of interest in order to prevent restricted access to the stationary phase (i.e., the larger the polypeptide, the larger the optimum pore size required). Peptides (1 to 10 kilodaltons in length) can be used with pore sizes of only 90 Å, whereas very large proteins (e.g., 500

kDa) may require pore sizes approaching 4000 Å. A pore size of 300 Å is a popular choice when working with most proteins.

For most smaller peptides and proteins, little is gained in terms of efficiencies with longer columns (greater than 100 mm) unless the application requires the resolution of extremely complex mixtures (e.g., tryptic digests). Protein chromatography should be attempted with as short of a column as possible (50 to 100 mm), since longer columns result in increased transit times in a harsh environment that may permanently denature some of the more labile samples. Once the best column length has been chosen, the selection of column diameter is based on sample capacity and sensitivity requirements.

A wide range of sample mass capacities have been reported for reversed phase columns, generally in the low milligram range for standard 4.6 mm (ID) columns. Since almost all RPC methods are carried out using gradient elution, large sample volumes can be accommodated as compounds can be effectively concentrated on the head of the column provided the initial organic solvent strength is low enough.

Theory and Mechanism

As described above, the stationary phases normally employed in RPC are silica-based supports modified with alkyl chains which provide the hydrophobic surface where separation can take place. Thus, in this mode of HPLC the retention of peptides and proteins has traditionally been considered to be a function of their relative hydrophobicities.

Currently there is still debate over the mechanism(s) responsible for separations in RPC; however, most researchers agree that the process can be thought of as either the adsorption of the solute at the stationary phase or, alternatively, as the partition of the solute between the mobile and stationary phases. In the adsorption model (the most widely evoked model), the solute and mobile phase compete for binding sites at an interfacial surface on the solid support. Retention is, therefore, a function of the strength of the interaction between the peptide or protein and the hydrophobic groups of the bonded phase. In this model, the polypeptide adsorbs onto the hydrophobic surface and remains bound until a sufficiently high concentration of organic solvent comes along and displaces it from the solid support. After displacement, interaction of the peptide or protein with the hydrophobic stationary phase is negligible. This mechanism accounts well for one of the striking features of RPC: that the elution of solutes from the column is extremely sensitive to minute changes in organic solvent strengths. Thus, shallow gradients represent powerful resolving tools in polypeptide separations. In fact, isocratic elution is almost never used in this RPC, since exact solvent conditions for elution are difficult to ascertain, and protein peaks are usually broad with tailing.

In the partition model of RPC, the solute is said to partition between the mobile phase and the solid support. In this case, the stationary phase is treated as the hydrophobic bulk phase and the system considered analogous to an n-octanol: water system where partition coefficients (logP) are determined. Thus, the more hydrophobic solutes (i.e., higher logP values in octanol:water system) would show increased affinity for the solid support, leading to longer retention times.

Most likely, the separation mechanism of RPC probably involves a mixture of the two above processes. Indeed, a third mechanism is sometimes discussed whereby the organic solvent in the mobile phase adsorbs onto the solid support surface, creating a new stationary phase into which solute molecules partition. Furthermore, one should not presume that the phenomena responsible for the binding of small polypeptides are necessarily the same as those for larger protein structures. Evidence in support of this hypothesis comes from studies that show that the correlation of amino acid hydrophobicity to retention time holds true only for the smaller peptides and is inaccurate above 10-15 residues primarily due to effects of tertiary structure in larger molecules.

Standard RPC Conditions

The most common mobile phase system used in the RPC of peptides and proteins involves an aqueous acetonitrile solution including 0.1 % (20 mM) trifluoroacetic acid (TFA). The organic modifier is used in a gradient that promotes elution of the solutes. Trifluoroacetic acid is a weak hydrophobic ion-pairing reagent (see below) that also serves to maintain a low pH (~2), thereby minimizing ionic interactions between the peptide/protein and the stationary phase. Additional advantages of TFA include its high volatility (allowing for rapid solvent removal) and some compatibility with mass-spectrometry analysis. With the increased use of mass spectrometry detection for the analysis of proteins and peptides, many researchers use buffers like 0.1 % formic acid or 0.1 % acetic acid to improve MS sensitivity. Others reduce TFA concentrations to 0.01 % to achieve the same result. The gradients should initially include 3-5 % organic solvent and not exceed 95 % at the end of the run. At higher organic solvent compositions, water may be completely removed from the stationary phase, making re-equilibration difficult.

Although acetonitrile is the most widely used organic in RPC, mainly due to its properties of being highly volatile and relatively UV transparent, alcohols such as methanol or isopropanol are sometimes employed for more hydrophobic and/or large proteins (Figure 10). The order of solvent strength for the commonly used organics is: methanol < ethanol < acetonitrile < isopropanol. Although isopropanol has the greatest eluting power, it is much more viscous than acetonitrile, and high column backpressures are limitations for its use. These backpressure problems can be overcome by either reducing the flow rate or using a mixture of acetonitrile and isopropanol.

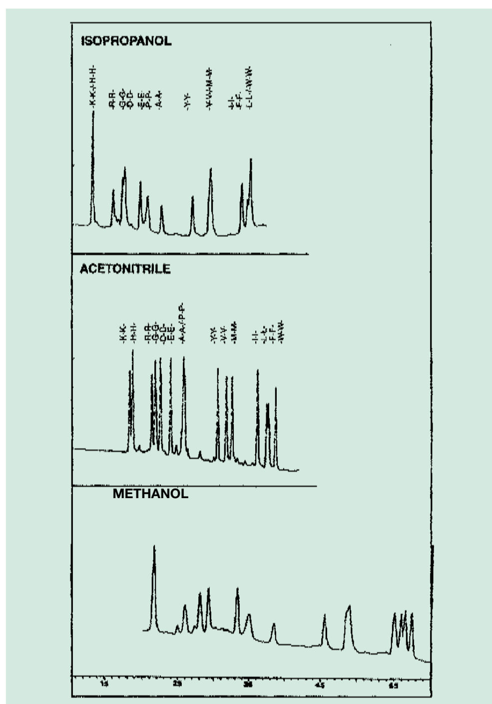


Figure 10. *Effect of organic modifier on RPC of an identical mixture of synthetic model peptides at pH 2.0. Conditions: linear AB gradient (1 % B/min), where Eluent A is 0.1 % aq. TFA and Eluent B is 0.1 % TFA in isopropanol (top), acetonitrile (middle), or methanol (bottom); flow-rate, 1 mL/min; 26 ºC. Reprinted with permission from Reference* 2.

One key to successful use of RPC is that all mobile phase reagents, including the TFA, organic modifier and water, must be of the highest quality, as small amounts of impurities can result in ghost peaks appearing in the chromatogram even during blank runs.

A standard set of suggested first run conditions for a reversed-phase (100 x 4.6 mm ID) column at 40 °C would include the following binary mobile phase system: Buffer A= 5 % acetonitrile in water, 0.1 % TFA; Buffer B= 95 % acetonitrile in water, 0.1 % TFA. Flow rate= 1.0 mL/min. This researcher prefers to maintain these initial solvent conditions for several minutes (especially when loading larger volumes) before beginning a gradient of 0 to 100 % B in 20 minutes (a change in organic of 5 %/min). The section below describes how manipulation of flow and gradient rates can then be applied for method optimization.

Ion-Pairing Reagents and pH Effects

Today a large number of different reversed phase matrices are available, and changing the type of HPLC column may seem an obvious choice for attempting to improve separations, however, such moves often prove unpredictable and frustrating. Many researchers are now realizing the benefits of manipulating the ion-pairing reagent and the pH in optimization of separation protocols.

We saw earlier that all peptides and proteins exhibit a characteristic charge at any given pH. At pH values typically associated with the use of silica-based columns (pH 2 to 7), all basic residues, as well as the free amino terminus, will exist as cations. Hydrophobic, anionic ion-pairing reagents such as trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), and heptafluorobutyric acid (HFBA) complex with these positively-charged groups and effect significant differences in chromatography (Figure 11).

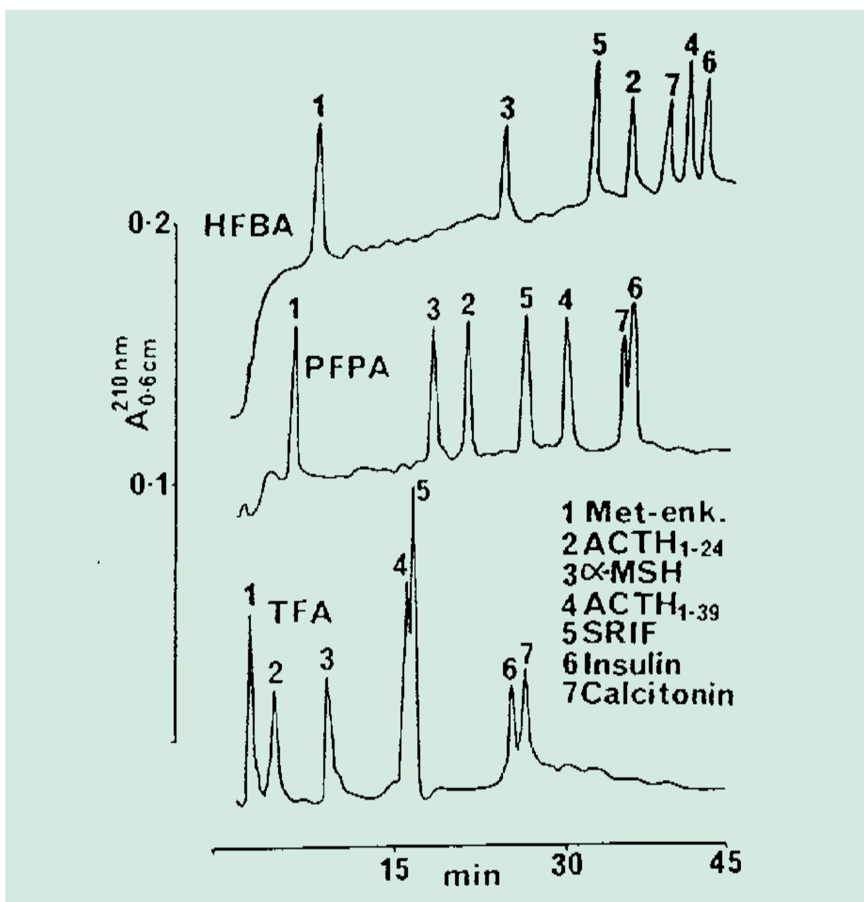


__Figure 11.__ Affect of Ion-pairing reagents on resolution and retention (details on following pages).

Comparison of the effectiveness of trifluoraecetic acid (TFA), pentafluoropropionic acid (PFPA) and heptafluorobutyric acid (HFBA) as hydrophobic ion-pairing reagents in RPC of seven natural and synthetic peptides. Peptides were eluted from a C18 10 µm 150 Å column (300 x 3.9 ID) with a linear AB gradient of 20 to 40 % acetonitrile containing 0.01 M concentrations of each acid over 1 hr (0.33 % acetronitrile/min) at a flow-rate of 1.5 mL/min. The upper panel shows the elution behavior of the peptides with 0.01 M 90.13 % v/v) HFBA, the middle panel shows the behavior of the (0.01 % v/v) PFPA; and the lower panel shows the behavior with 0.01 M (0.07 % v/v) TFA. Samples (2 µg) of the following peptides were injected onto the column (number of basic residues at pH 2, including histidine, is shown in brackets): (1) methionine enkephalin, (1), (2) $ACTH_{1-24}$ (9), (3) α-MSH, (3), (4) human $ACTH_{1-39}$, (9), (5) somatostatin, (3), (6) bovine insulin, (6), (7) human calcitonin (3). Reprinted with permission from Reference 2.

Similarly, the ionization properties of acidic amino acids can also be manipulated into providing changes in solute retention and resolution. At pH values significantly above the pK_a for carboxylic acids (pH 5 or higher), these groups, along with the free carboxyl terminus, will be negatively charged. Triethylamine acetate (TEA) is a volatile, weakly hydrophobic cationic ion-pairing reagent. Utilization of a 10 mM TEA buffering system (pH 5.5), in combination with the usual organic modifier, promotes significant changes in solute retention when compared to the standard TFA system, primarily due to the effects of the increased pH (i.e., the ionization of glutamic acid, aspartic acid and the carboxy terminus). Lastly, TFA and TEA are sometimes used together as an effective mobile phase modifier (e.g., 3:1, TFA:TEA).

In general, polypeptides appear more hydrophilic in TEA-based mobile phases and have decreased retention times. The acidic nature of polypeptides can also be exploited by using the hydrophobic ion-pairing reagent, tetrabutylammonium phosphate, at pH 7. At this pH value the ion-pairing reagent complexes with the free carboxyl groups and increases retention according to the relative number of such moieties on the polypeptides in the sample.

In contrast to the standard TFA/acetonitrile mobile phase system where separation is dictated primarily by the relative hydrophobicities of the sample components, the examples offered in this section offer alternative methods for exploiting the acidic and basic properties of polypeptides, thus providing additional approaches to RPC separations.

Effect of Varying Gradient, Flow Rate on Separations

We have described useful suggestions for method development in RPC including information on the important choices of column packing and ion-pairing reagents (and pH) which represent perhaps the most powerful means of optimizing separations. If, however, after implementation of these selections adequate resolution remains elusive, there are two other mechanisms for manipulating the chromatographic behavior of peptides and proteins: varying the flow rate and/or gradient rate.

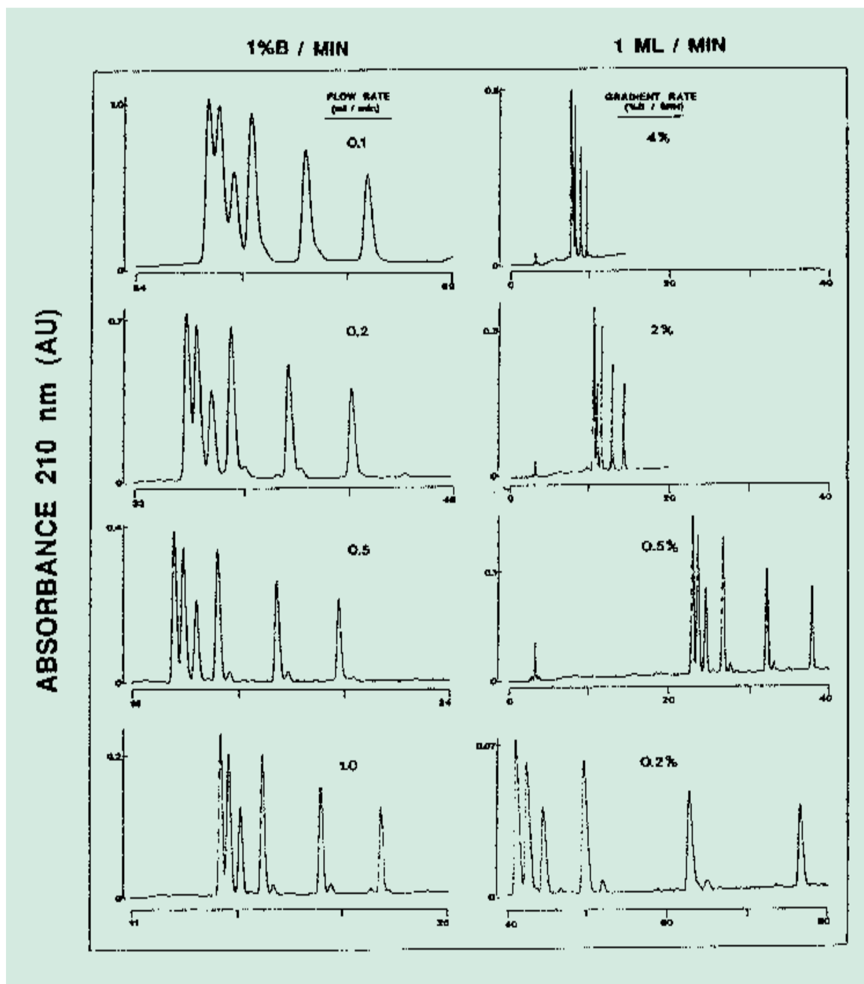


Figure 12. *Effect of varying flow-rate or gradient-rate on separation of peptides in RPC. Conditions: flow-rates and linear AB gradients as shown. Left profiles: effect of varying flow-rate (0.1, 0.2, 0.5, and 1.0 mL/min) at constant gradient-rate (1 % B/min). Right profiles: effect of varying gradient-rate (4, 2, 0.5. and 2 % M/min) at constant flow-rate (1 mL/min). Reprinted with permission from Reference 2.*

The effect of varying either the flow rate or the gradient rate on peptide resolution is shown in Figure 12. Immediately obvious is the general improvement of resolution achievable through either an increase in flow rate (left panel) or a decrease in gradient rate (right panel). Changes in gradient rate typically have a greater effect on resolution than does altering the flow rate. Furthermore, the benefit to resolution attributed to decreases in gradient rates has been shown to be larger at lower flow rates.

Changes in gradient and flow rates can also have profound effects on sensitivity. Thus, a 10-fold increase in flow-rate (from 0.1 to 1.0 mL/min) results in a 4- to 5-fold decrease in peak height and, due to the detector response time and sample dilution effects, a corresponding decrease in peak area. A similar loss of sensitivity is observed upon slowing the gradient rate; however, for those peptide peaks that are integratable, there is no effect on peak area, since the peak widths are increased proportionately.

Prior to utilizing these tools for method optimization, the researcher must carefully assess what the most critical objectives are because definite trade-offs exist when altering flow or gradient rates. Changes in gradient rates, while improving resolution, also result in loss of sensitivity and longer run times. With modern auto-injectors, longer runs may not be a problem. Thus, if sample size is not limited, one should attempt to maximize resolution through decreases in gradient rate until the required sensitivity is lost.

Temperature Influences

Column temperature can also be used to modulate the chromatography, although the influence of this parameter is less significant than those mentioned above. Higher column temperatures increase the mass transfer rate, thereby improving column efficiency and, in general, peak shapes are sharpened and resolution improved. However, this may not always be the case (depending on the system), and higher temperatures usually lead to losses of bioactivity with proteins, which should be considered when important to separation goals.

Recovery of Biological Activity

With the relatively strong binding interactions and harsh elution conditions involved in the separation of compounds via RPC, the perception that proteins will not survive the procedure with their biological activity intact is not surprising. Indeed, many reversed-phase methods for proteins have employed C18 columns with mobile phases containing acetonitrile in low pH buffers. Furthermore, little attention was paid to the critical step of fraction collection as a means for recovering biological activity. Naturally, the amount of structural change will be a function of both the intrinsic stability of the protein as well as the chromatographic conditions employed. Oligomeric proteins are particularly susceptible to denaturation under RPC conditions.

There are several straightforward approaches, however, that make reversed phase separations of proteins with excellent recovery of bioactivity a realistic expectation. Acidic environments, including the standard TFA-based buffers, are notoriously destructive to the activities of many enzymes. Similarly, acetonitrile almost always (depending on concentration and length of exposure) leads to total denaturation of proteins. Thus, these two conditions should be avoided when working with most proteins. A section above describes alternatives for selecting mobile phase buffers more compatible (i.e., closer to physiological pH 7.4) with these macromolecules. With respect to organic modifiers, both ethanol and isopropanol are superior for elution of active species. A critical determinant for the recovery of activity in RPC is the length of time the macromolecule spends in the column. Protein chromatography should therefore be attempted on short length (50 to 100 mm) C4 or C5 columns, which will lead to both weaker binding interactions and shorter transit times through the harsh column environment. Finally, fractions containing the protein(s) of interest should be collected directly into buffer with subsequent removal of organic solvents before lyophilization.

Column Cleaning and Regeneration

During the chromatography of peptide and protein samples, it is possible for some of the more hydrophobic components to become strongly adsorbed to the stationary phase, resulting in loss of column performance (e.g., high backpressures, loss of resolution). A recommended procedure for removing bound protein is to wash the column with one part 0.1 N nitric acid to four parts isopropanol at reduced flow rates (~20 % normal) overnight. For more hydrophobic contaminants, the following two column-cleaning protocols are suggested: (1) either wash the column with 10-20 column volumes of a high concentration (up to 95 %) of the organic solvent component, or make several blank gradient runs (no sample); if this step fails to restore performance, then (2) wash the column with several volumes of methanol, followed by chloroform, followed by methanol before returning to initial chromatography conditions. After cleaning and between normal injection runs, adequate column re-equilibration time must be allowed in order to prevent changes in retention time and/or loss of resolution.

D. Hydrophobic Interaction Chromatography

Section D contains:

- **Stationary Phases**
- **Theory**
- **Typical Starting or First-Run Conditions**
- **Factors Affecting Protein Retention**
- **Column Capacities and Exclusion Limits**
- **Column Cleaning, Regeneration and Storage**

In recent years, hydrophobic interaction chromatography (HIC) has become an increasingly important HPLC technique for the separation of proteins without the problem of denaturation often observed with the use of the reversed phase mode. Although the mechanisms involved in HIC and RPC are similar, with resolution based on the relative strength of interactions between the non-polar solid support and hydrophobic regions on the protein, there are a number of important distinctions. For instance, the bonded ligands found in RPC are more hydrophobic than those used in HIC. The density of the HIC groups is also much less than that of reversed phase columns, such that the solid support interacts primarily with hydrophobic patches on the surface of the intact protein. Perhaps the most significant difference with HIC is in its use of aqueous mobile phases of high ionic strength and neutral pH in contrast to the harsh solvents and acidic pH typically employed in RPC.

It should be noted that although HIC has traditionally been used more for protein work while peptide studies were performed using reversed phase chromatography, hydrophobic peptide mixtures may be better suited to the former technique.

Stationary Phases

The overall hydrophobicity of HIC stationary phases is a combination of both the non-polar character of the bonded ligands as well as their density in the column. Most HIC columns are made of silica-based supports modified aryl groups, diol derivatives, or short alkyl chains. The hydrophobic effects of several common functional groups are illustrated in Figure 13.

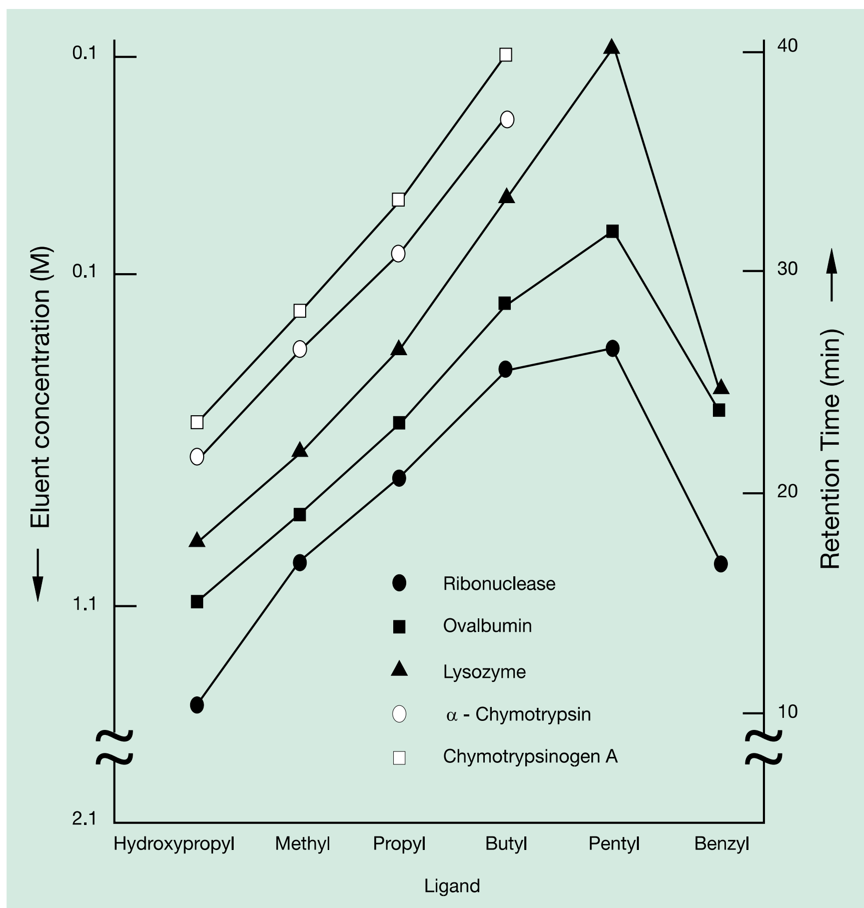


Figure 13. *Effect of ligand arm on protein retention by HIC. Reprinted with permission from Reference 2.*

This figure demonstrates that protein retention times increase as the hydrophobicity of the functional group increases: hydroxypropyl < methyl < benzyl = propyl < phenyl < pentyl. Although the more hydrophobic solid supports may be employed in the chromatography of some hydrophilic proteins, one must use caution to avoid strong column-solute interactions that could lead to denaturation even under the relatively mild elution conditions of HIC.

Theory

Resolution in HIC is based on weak hydrophobic interactions of the protein with the bonded ligands of the stationary phase. This adsorption is achieved through the use of high ionic strength mobile phases that include salts, such as sodium sulfate, ammonium sulfate, sodium chloride, and sodium citrate. There are two hypotheses typically evoked to account for solute binding. The first involves the idea that ordered water molecules surrounding the hydrophobic patches of the polypeptide are displaced such that hydrophobic ligand-protein interactions are thermodynamically favorable. The other hypothesis concerns the “salting out" of proteins onto the solid support due to an increase in the surface tension of water as a result of salts present in the mobile phase. Most likely, the actual mechanism involves a combination of both of the above concepts. Unlike the technique of RPC, HIC is conducted under non-denaturing conditions such that peptides and proteins are separated in their native, folded states. Thus, retention in HIC is highly dependent on exposed hydrophobic regions of the intact molecule, in stark contrast to RPC, where retention is more dependent on the overall amino acid composition.

After binding in the presence of high salt concentrations (e.g., 1-3 M), proteins are eluted with a descending salt gradient that allows the polypeptides to resolvate (with water), thereby making association with the hydrophobic matrix thermodynamically unfavorable. Because these two processes (binding and displacement) take place in an aqueous environment that includes structure-stabilizing salts, HIC allows for the retention of biological activity. This elution scheme is drastically different from those of RPC where organic solvents are utilized to neutralize hydrophobic interactions between solute and column.

Typical Starting or First-Run Conditions

Standard starting conditions for HIC might include a Phenomenex Jupiter 5 µm 300 Å C4 column (100 x 4.6 mm) at 40 °C and a binary mobile phase system consisting of the following: Buffer A= 0.1 M K_2HPO_4 (pH 7.4) containing 1.5 M ammonium sulfate salt; Buffer B= 0.1 M K_2PO_4 (pH 7.4) alone. Flow rate= 1.0 mL/min. After loading the sample onto the column using these initial conditions, elution is achieved by employing a linear, decreasing ammonium sulfate gradient (e.g., 100 to 0 % A in 20 min). As was the case in RPC, this researcher prefers to maintain these initial solvent conditions for several minutes (especially when loading larger volumes) before beginning the gradient.

Practical considerations have also made $(NH_4)_2SO_4$ a popular choice for a salt. $(NH_4)_2SO_4$ has a high aqueous solubility (approx. 4 M at 25 °C), a low UV absorbance, and seems to retard microbial growth.

Factors Affecting Protein Retention

Besides column selection, the major parameters frequently manipulated to evoke changes in selectivity and retention include the type and concentration of salt, mobile phase pH, and temperature.

Salt. It has been demonstrated that both the type and concentration of salt are critical parameters in the HIC technique. The most commonly used salts include those that are antichaotropic (or structure-forming) and are known for their ability to salt out proteins. Table 3 shows a number of such salts along with their molal surface tension values. The listing of these values is based on the idea that surface tension, as well as exposed protein/peptide hydrophobic surface area, is a primary determinant in solute adsorption in HIC. According to this concept, an increase in mobile phase surface tension should lead to an increase in solute retention. Furthermore, as the concentration of such salts increases, the amount of bound protein increases almost linearly.

Table 3. *Molal surface tension increments of selected salts. Reprinted with permission from Reference 2.*

Salt	(σ x 10_3 dyn-g/cm-mol)
KCl	1.50
NaCl	1.64
Na_2HPO_4	2.03
Mg_2SO_4	2.06
$(NH_4)_2SO_4$	2.17
Na_2SO_4	2.74
Na_3PO_4	2.88
Sodium citrate	3.12

Interestingly, column capacities for most proteins continue to increase even beyond their precipitation points, an effect attributed to the salting out of the molecules on the solid support. It should be noted that this phenomenon has a negative effect on the selectivity of the column.

It is currently believed that more hydrophobic proteins should be chromatographed in the presence of salts with higher surface tensions, while salt concentrations (typically 1-3 M) must be chosen so as to maximize either selectivity or column capacity depending on the particular application. Obviously, proteins are eluted

earlier when lower salt concentrations are employed and, as long as resolution is not adversely affected, such concentrations are advocated, since they can lead to dramatically shorter run times.

pH. The aqueous mobile phases in HIC are typically in the neutral pH range of 5-8. As discussed in an earlier section, the isoelectric point of a protein or peptide is the pH where the net charge of the molecule is zero. At this pH, the peptide/protein is also in its least soluble state. Thus, it has been suggested that solute retention would be increased as mobile phase pH is either moved away from or towards the isoelectric point. However, a number of careful studies have shown that there is no straightforward correlation between isoelectric point and pH effects on retention time.

The effect of pH on resolution also appears to be more dramatic with some column and salt combinations. In general, an increase in pH reduces hydrophobic interactions, thereby decreasing retention time. The determination of optimal chromatography pH is usually made empirically and is used to alter resolution/selectivity only when attempts at changing salt composition for such purposes fails. One must bear in mind that some proteins are more susceptible to denaturation with changing pH than are others.

Temperature. Since the localized hydrophobic regions on native proteins play such an important role in the binding interactions of HIC, any factor that influences tertiary structure would be expected to influence retention and resolution. Hence, temperature changes are known to produce sometimes profound effects in this chromatographic mode, although the extent to which changes are observed varies widely between proteins.

It is recognized that hydrophobic interactions are strengthened by increasing temperatures and that, in general, proteins are retained in the column longer. Furthermore, by changing the chromatography temperature in HIC one may promote better resolution as well as the sharpening of individual peaks. However, the effect of temperature is complex, and some proteins that elute as broad peaks at 25 °C may sharpen as temperature is increased while other proteins show the opposite effect. The increased stability of proteins at lower temperatures suggests that, unless otherwise indicated (i.e., due to poor resolution or insufficient binding to the column), HIC should be conducted at the lowest temperatures possible.

Column Capacities and Exclusion Limits

As with other chromatographic modes employing gradients, column capacities are sample mass rather than sample volume limited. Although mass capacities vary widely among columns, a typical HIC column with an ID of 8.0 mm can usually accommodate 10-20 mg protein. Large volumes can be applied to the column with starting conditions chosen so as to concentrate solute molecules onto the head of the support bed. However, these theoretical volumes become important if the salt concentration of the sample is lower than that of the equilibration buffer. Under such conditions, sample components may begin premature migration down the column, causing bandspreading.

HIC columns have a low capacity for proteins larger than the exclusion limit. Most HIC columns have pore sizes of 300 Å and will retain proteins up to one million daltons.

Column Cleaning, Regeneration and Storage

HIC columns are normally cleaned and regenerated after each run by washing with several volumes of deionized water. Strongly adsorbed lipids or proteins can usually be eluted by washing polymer-based columns with several column volumes of 0.5 M NaOH (**do not** use with silica-based columns). Alternatively, washes with either 70 % ethanol or 30 % isopropanol followed by deionized water are often employed for cleaning purposes. As was the case with other types of chromatography, long-term storage of HIC columns must include an anti-microbial agent such as those discussed previously. The manufacturer’s instructions should always be followed closely.

E. Affinity Chromatography

Section E contains:

- **Ligands and Stationary Phases**
- **Principles and Mechanism of Separation**
- **Carrying out the Run**
- **Elution of Bound Species**
- **Mobile Phase Effects**
- **Column Capacity**
- **Column Regeneration, Care and Storage**

The last mode of HPLC to be discussed in this guidebook is affinity chromatography. It has been hailed as the most important advance in the purification of biomolecules in the last twenty years. Today most laboratories involved with protein isolation employ affinity chromatography in their purification schemes, often as a single step. Purification may be up to several thousand-fold and activity recoveries are extremely high.

The reversible, specific binding of one biomolecule to another forms the basis for affinity chromatography, with separation dependent on the native conformations and the relative binding affinities of the solute of interest for the immobilized ligand. Whereas some affinity interactions are extremely specific (an antibody binding to its antigen), others are less restrictive (concanavalin A binding to glycoproteins). The choice of ligand is obviously critical for the successful use of this type of chromatography.

Affinity chromatography is generally considered to be the most specific technique for biomolecule purification, and in addition has proven to be a powerful tool for investigating protein-protein and protein-peptide interactions. The preponderance of early affinity HPLC methods involved the purification of enzymes using their substrates or analogs of their substrates as the stationary phase (i.e., immobilized) ligands. However, today the use of immobilized proteins as affinity ligands is widespread. For example, antibodies are often used to isolate their specific antigens from complex mixtures, while ligands such as Protein A and Protein G are used to purify the antibodies from plasma samples. Where the chemical association rate is great enough, immobilized proteins may also be used to purify molecules capable of binding to their active sites.

Ligands and Stationary Phases

Until recently only silica-based columns were available for affinity HPLC applications; however, synthetic hydrophilic gels may now be purchased from most suppliers. Usually the gel-matrix is coated with a hydrophilic layer before activating with a covalently linked functional group that will bind amino, hydroxyl or sulfhydryl residues on peptide or protein ligands. The majority of columns used today for peptide and protein work contain particles with a minimum 300 Å pore size. These large pore sizes are required to accommodate both the ligand and the peptide/protein solute as well as the resulting complex.

Columns come either pre-derivatized with the stationary phase and a suitable ligand (usually multifunctional) in place, or “activated" with chemically reactive groups ready for covalent coupling with a ligand fitting the experimental design and criteria described below. Table 4 lists a number of affinity resins that are commercially available from Shodex®.

Table 4. *Shodex® HPAC (AFC) separation for proteins and peptides.*

Column Type	ID x Length (mm)	Ligand	Ligand Load/g	Compound Capacity/ Column	Applications
AFpak AAB-894	8 x 50	Aminobenzamidine	100 µmol		Serine proteases
AFpak AAF-894	8 x 50	Acriflavine	10 µmol	ATP Na 1.8 mg	RNA, DNA, vitamins
AFpak AAM-894	8 x 50	5'AMP	10 µmol	Lactic dehydrogenase 1.5 mg	NAD, ATP enzymes
AFpak AAP-894	8 x 50	Aprotinin	5 mg	Trypsin 3 mg	Serine proteases
AFpak AAV-894	8 x 50	Avidin	5 mg	Biotin 8µg	Biotin derivatives
AFpak ABA-894	8 x 50	Bovine serum albumin	20 mg		Optical resolution
AFpak ABA-894L	8 x 150	Bovine serum albumin	20 mg		Optical resolution
AFpak ABT-894	8 x 50	Biotin		Avidin 9 mg	Avidin
AFpak ACA-894	8 x 50	Concanavalin A	15 mg		Glycoproteins, polysaccharides
AFpak ACB-894	8 x 50	Cibacron Blue	40 µmol	BSA 20 mg	Albumin, NAD dependent enzymes
AFpak ADS-894	8 x 50	Dextran sulfate	30 mg	LDL 5 mg	Lipoproteins, blood coagulation factors
AFpak AED-894	8 x 50	Ethylenediaminediacetic acid	30 µmol		Nucleic acids, serum proteins
AFpak AGA-894	8 x 50	N-acetyl-glucosamine		Lysozyme 0.6 mg	Lectins, carbohydrate metabolizing enzymes
AFpak AGE-894	8 x 50	Gelatin	30 mg		Fibronectin
AFpak AGT-894	8 x 50	Glutathione	50 µmol		Enzymes related to glutathione
AFpak AHR-894	8 x 50	Heparin	5 mg	Lysozyme 4 mg	Lipoproteins, blood coagulation factors
AFpak AIA-894	8 x 50	Iminodiacetic acid	70 µmol	BSA 70 mg	Interferon, serum proteins
AFpak ALC-894	8 x 50	LCA (Lentil lectin)	7 mg		Glycoproteins, polysaccharides
AFpak ALS-894	8 x 50	Lysine	50 µmol		Plasminogen, polysaccharides
AFpak ANA-894	8 x 50	NAD	10 µmol	Lactic dehydrogenase, 1.4 mg	
AFpak AOV-894	8 x 50	Ovomucoid	10 mg		Trypsin-like protease
AFpak APA-894	8 x 50	Protein A	8 mg	IgG human 20 mg	Human IgG, immune complexes
AFpak APB-894	8 x 50	Aminophenyl boronic acid	800 µmol	Sorbitol	0.2 mg Nucleic acid and catecholamines
AFpak APD-894	8 x 50	Procion red	50 µmol	BSA 72 mg	NAD, NADP, interferon enzymes
AFpak APE-894	8 x 50	Phosphorylethanolamine	5 µmol	C-reactive protein 0.9 mg	C-reactive protein, enzymes
AFpak APG-894	8 x 50	Protein G	5 mg	IgG human 10 mg	IgG immune complex
AFpak APH-894	8 x 50	Phenylalanine	50 µmol	Subtilisin 9 mg	Proteases
AFpak APR-894	8 x 50	Protamine	5 mg	IgM human 1.9 mg	IgM
AFpak APS-894	8 x 50	Pepstatin	7 mg	Pepsin 3.3 mg	Acid protease
AFpak ARC-894	8 x 50	RCA-I	20 mg		Glycoproteins, polysaccharides
AFpak AST-894	8 x 50	Soybean trypsin inhibitor	20 mg		Trypsin-like proteases
AFpak AWG-894	8 x 50	Wheat germ agglutinin	14 mg		Glycoproteins, polysaccharides

There are four major requirements for an affinity column matrix: (1) the ligand must contain functional groups that will allow for covalent coupling to the solid support without adversely altering its binding activity towards the peptide or protein solute; (2) the ligand must be capable of somewhat specific albeit reversible binding to the protein or peptide of interest, and the affinity of the ligand:solute complex must be sufficient to provide for good binding, but not strong enough to necessitate harsh elution conditions that could result in loss of bioactivity; (3) non-specific interactions between the column and unwanted sample components should be minimal; and (4) the covalent linkages used to immobilize the ligand must be stable to all conditions employed during chromatography and column clean-up.

Ligands in affinity chromatography may be classified as either specific or multi-functional. Specific ligands are potent binders of single classes of peptides or proteins, with examples of these ligands including antibodies and antigens and various enzyme substrates/inhibitors. Multi-functional ligands display varying binding activities towards related peptides or proteins. Examples of multifunctional ligands would include concanavalin A, which binds to specific carbohydrate residues and the triazine dyes that are selective for enzymes that use nucleotides as substrates. Nucleotides that bind to enzymes using these molecules as substrates or cofactors are also examples of multifunctional ligands.

The use of proteins as ligands represents a special and challenging aspect of affinity chromatography. With protein ligands it is assumed that coupling to the solid support occurs through externally exposed residues (e.g., lysine) of the native molecule. In most cases there is a stringent requirement for maintaining the structural integrity of the protein for effective binding. Therefore, it is important that immobilization conditions do not cause denaturation of the protein. The molecular size of these ligands usually limits the degree of column derivatization. When the separation involves a peptide and a protein, the protein is usually employed as the ligand with the peptide serving the role of solute, in order to minimize attachment complications. However, when using large molecular weight proteins as ligands, their concentration on the column should be reduced so as to limit steric hindrance problems which would likely decrease the binding capacity of the column. The smaller sizes of peptides create logistical problems for their attachment to solid supports and care must be used to ensure that the point of immobilization is well away from the binding site for the solute of interest. Typically, either the amino or carboxyl terminus is used in the covalent coupling to the column support.

Ultimately, the careful choice of the ligand, the length of the spacer arm and the chemical properties of the matrix are critical considerations in the design of any affinity gel.

Principles and Mechanism of Separation

As discussed above, affinity chromatography requires that an immobilized ligand, covalently coupled to the column's stationary phase, interacts specifically and reversibly with the solute of interest. Figure 14 is a schematic representation that illustrates the processes involved in an affinity column based separation.

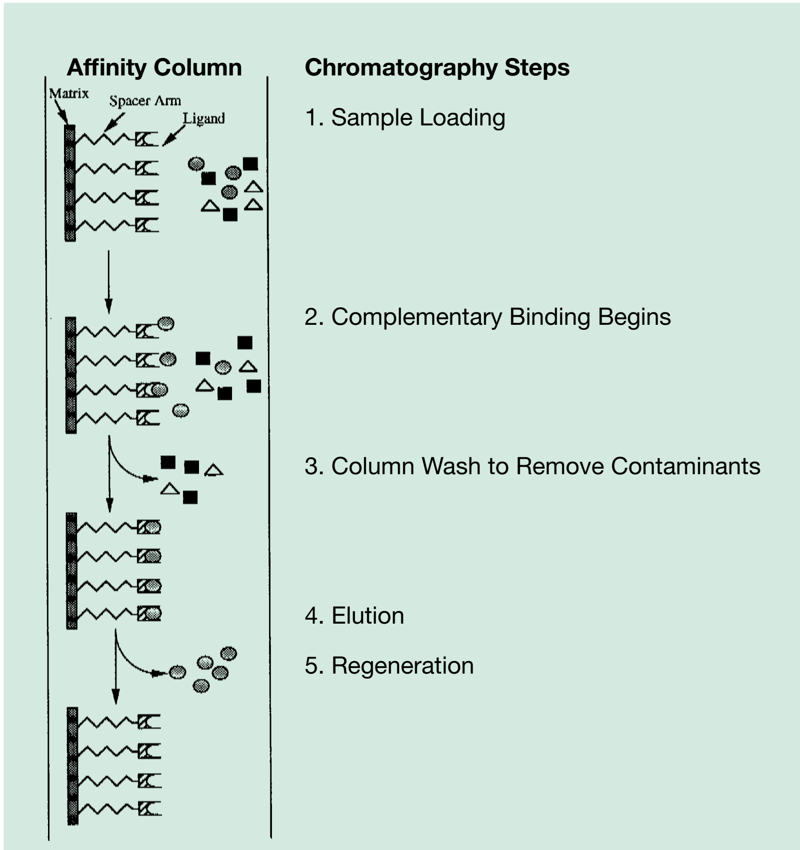


Figure 14. *The affinity chromatography process.*

As the sample passes through the column, the peptide or protein of interest should bind in complementary fashion to the ligand covalently attached to the solid support, while the rest of the solutes in the sample, incapable of bio-specific binding, flow through without direct interaction. After the contaminants wash completely through the column, the peptide/protein is then eluted via a variety of approaches that are discussed below. Elution techniques include ligand:solute complex disruption by inclusion of competitive ligands in the mobile phase or by changes in mobile phase composition such as ionic strength or pH. Finally, the column is re-equilibrated for its next use.

The molecular forces that participate in the binding (and elution) events of affinity chromatography include hydrophobic, ionic and hydrogen-bonding interactions, but the relative contribution of each of these varies with the particular system. Binding of the solute is possibly multivalent for polymeric species like proteins or peptides. Critical determinants of retention time in affinity chromatography are the dissociation constant (K_d) for the ligand:solute complex and the concentration of ligands coupled to the solid support. The other major factors influencing retention time are flow rates and gradient rates; these variables will be discussed in detail below.

It should be emphasized that conditions must be optimized for each step of the chromatographic process and that these conditions will, in general, be limited to the particular separation being performed.

Carrying out a Run

Because of the unique nature of each individual affinity application, there are no standard first run conditions. Initial mobile phase conditions such as ionic strength, temperature and pH should be selected such that strong affinity binding of the solute of interest is obtained without non-specific adsorption of other sample components to the column matrix or spacer arm. When proteins are involved in this mode of chromatography, it is imperative to utilize conditions that keep these molecules stable throughout the course of a run, otherwise the biospecific binding may be compromised with subsequent adverse effects on separation (e.g., loss of resolution and peak shape). Moreover, when the protein is used as the ligand, denaturation may permanently damage the binding ability of the column. Thus, all equilibrating, running and elution buffers must be non-denaturing.

Elution of Bound Species

The elution of bound peptide or protein involves disruption of the same types of binding forces (i.e., ionic, hydrophobic, hydrogen bonding) responsible for maintaining their native structures. In the design of an elution protocol it is therefore essential that conditions be chosen carefully so as not to denature the protein component, whether it be employed as the ligand or as the solute.

The elution techniques available for affinity chromatography may be divided into two broad categories: *competitive* (or affinity) methods and general (or nonspecific) methods. The choice of method will be largely dependent on the nature of the ligand and solute molecules as well as the binding strength of the resultant complex.

As the name implies, competitive agents work by competing directly with the solute of interest for the same specific binding site (i.e., the ligand) on the column. The method is simple and requires only that the competing ligand be included in

the mobile phase during the elution phase of the run. This technique is ideal when dealing with labile proteins as solutes. However, competitive elution can be cost prohibitive with high concentrations of ligands needed when column conditions produce very strong binding interactions between the stationary phase ligand and the solute of interest. One may circumvent this problem by altering the mobile phase ionic strength and/or pH to promote weaker binding. Unfortunately, although increasing the salt concentration may weaken the solid support:solute binding component, it may also lead to weaker interactions between the free, competing ligand and the column. Increased salt concentrations will also increase the effect of the hydrophobic component. Thus, if the hydrophobic interactions are important, addition of a non-ionic detergent may help.

There are several general, or non-specific, elution methods including changes in mobile phase ionic strength or pH as well as the introduction of chaotropic ions into the mobile phase. Elution of peptides and proteins with these agents may be achieved using either a step gradient or a linear gradient, although the latter approach is more common and easier to employ since it is not necessary to have a priori knowledge of the conditions required for elution.

The most common non-specific elution technique involves increasing the mobile phase ionic strength. This method effectively disrupts all non-hydrophobic interactions between the column ligand and the peptide or protein solute. Typically, most affinity running buffers will include, for protein stabilization purposes, low millimolar concentrations of a salt such as KCl or NaCl. Elution can be accomplished by increasing the salt concentration (from 0.2-1.0 M depending on the system). Figure 15 illustrates this approach and the effect of utilizing either a step or linear gradient on compound retention and peak shape. However, as described above, increased salt will tend to reinforce hydrophobic interactions, and as a consequence nonspecific binding may increase.

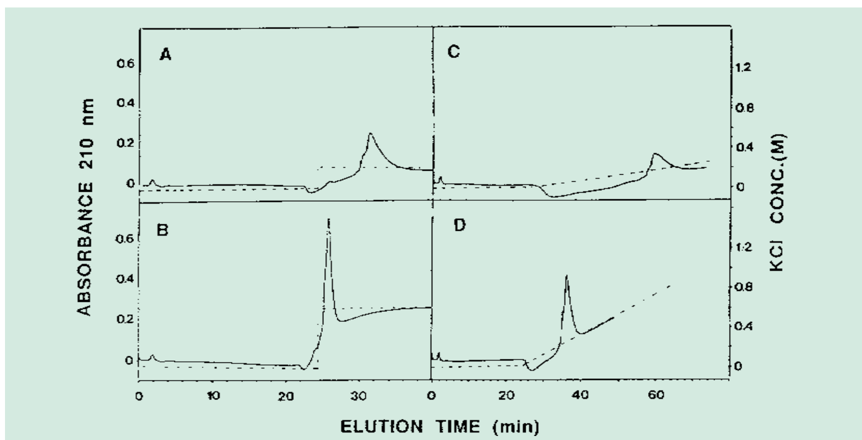


Figure. 15. *Comparison of step-gradient and linear-gradient elution of troponin I peptide from a troponin C HPLC column. The column, HPLC instrument and mobile phases are described in Figure 3. Panels A and B: step-gradient elution using 200 or 400 mM KCL, respectively, as the desorbing agent. Panels C and D: linear- gradient elution at 5 or 15 mM KCL/min, respectively. The dotted line shows the concentration of KCL with time under the various elution conditions. Flow-rate: 0.3 mL/min. All runs had an initial 10-min delay time following sample application (10 nmol peptide) and prior to the start of the linear gradient or step gradient KCL front. Reprinted with permission from Reference 2.*

Another widely used nonspecific elution technique involves reduction of the mobile phase pH. An increase in mobile phase acidity will protonate ionizable groups on peptides and proteins, and as a result weaken the ionic binding component of the interaction between them. One word of caution with this approach is that the pH should not be so low as to denature the protein. Most proteins are stable in the pH range of 6-8.

Chaotropes promote the reordering of water molecules around the peptides and proteins. These agents probably facilitate elution by weakening hydrophobic interactions between the ligand and solute. In contrast to changes in column pH, there is less chance of protein denaturation using chaotropes, except at very high concentrations. The order of elution power for a chaotrope series is:

$$CCl_3COO^- > SCN^- > CF_3COO^- > ClO_4 > I^- > Cl^-.$$

Mobile Phase Effects

As discussed in an earlier section, changes in mobile phase flow rate alter the mass transfer properties of the HPLC column, and thus can have profound influence over both retention time and peak shape (see Figure 8 in Reversed Phase Chromatography section).

When using either linear- or step-gradients, retention time will increase with decreasing flow rates; peak heights typically increase as well due to the eluted molecules spending more time in the detector cell. Peak widths increase with decreasing flow rates during step-gradient elution, but should not change in linear-gradient elutions.

At constant flow rates, decreasing either the gradient rate or the ionic strength of the elution buffer will result in increased retention times for the peptide or protein as well as increased peak widths (with expected decreases in peak height).

Column Capacity

Due to the specific nature of binding with affinity chromatography, columns are relatively insensitive to sample volume. The capacity of these columns is dictated mainly by the number (or density) of ligands bound to the solid support. Since the relationship between flow rate and column capacity is a function of both the diffusional rate of the peptide or protein and the association rate with the complementary ligand, column capacity can be increased by slowing the flow rate.

Column Regeneration, Care and Storage

Column regeneration is typically achieved readily by continued washing with the starting or running buffer. One exception to this rule is if chromatography conditions promote nonspecific binding of sample components to the column matrix, in which case it may become necessary to wash with different concentration salt solutions or with the addition of low levels of detergent. Again, care must be exercised so as not to denature the biological ligands.

Storage should be at 4 ºC in low salt buffers containing an antimicrobial agent such as 0.02 % sodium azide. All storage conditions including pH must ensure ligand stability.

Section 4.

Introduction to Small-Bore HPLC

Section 4 contains:

- **Advantages**
- **Disadvantages**
- **Small-Bore Chromatography Instrumentation Requirements**
- **Special Care Considerations**

Over the last decade, there has been a cocerted effort to develop more sensitive and elegant methods with which to characterize the trace amounts of peptides and proteins typically contained in biological samples. During this time “small-bore" LC has emerged as a powerful tool for these applications. In many cases the separation method is linked directly to an identification technique such as mass spectrometry, allowing for the detailed analysis of sub-picomole amounts of material.

Interest in small-bore HPLC dates back to the mid-70’s when several groups were using silica-packed size exclusion columns with internal diameters of 1 mm which were termed “micro-bore". Since HPLC columns today have internal diameters as small as 50 µm, the use of the “micro" prefix for 1 mm ID columns may seem inappropriate. Indeed, correct nomenclature for describing HPLC columns continues to be quite confusing, however, some generally applicable terms have now gained wide acceptance in the literature. Small-bore columns are now considered to encompass both narrow-bore (1-2 mm ID) and microbore (0.5-1 mm ID) columns, although these latter two terms are sometimes used interchangeably. HPLC columns with internal diameters of 3-500 µm are considered packed capillary. Conventional (or analytical) columns typically refer to those with internal diameters of 3-6 mm, while semi-preparative columns have IDs of 6-20 mm and preparative columns are greater than 20 mm.

Advantages

The most important advantage of small-bore chromatography is considered to be the increased mass sensitivity associated with its use. Mass sensitivity and concentration sensitivity are the two ways of deeming the detection limits of a chromatographic system. The smaller the column ID, the smaller the mass that can be detected. Table 5 shows the theoretical gains in mass sensitivity for a number of

Table 5. *Effect of column internal diameter (ID) on mass sensitivity.*

Column ID (mm)	Cross-sectional area (cm^2)	Flow Rate[a] (μL/min) for a Linear Velocity of 6.02 cm/min	Change in Mass Sensitivity[b] (Compared to a 4.6 ID column)
4.6	1.66×10^{-1}	1000	1.0
2.0	3.14×10^{-2}	189.0	5.3
1.0	7.85×10^{-3}	47.3	21.1
0.05	1.96×10^{-5}	0.12	8469

[a]Flow Rate (mL/min) = Linear Velocity (cm/min) x Cross-sectional area (cm^2)

[b]The change in mass sensitivity is defined as the ratio of cross-sectional areas compared with the conventional 4.6 mm ID column. Assumes injection of same mass while using same linear flow velocity.

different diameter columns as well as flow rates typically used with them. Thus, chromatography on a 1.0 mm ID column should result in a mass sensitivity increase of approximately 20-fold over that of a standard 4.6 mm column, when operating at the same linear velocities. These gains in sensitivity, which are accompanied by proportional reductions in peak volume, are illustrated in Figure 16, where a tryptic digest of apomyoglobin is compared on a 1.0 and 4.6 mm reversed phase column. In this figure, where 100 pmol of total protein was loaded onto each column, we see that the majority of peptide fragments appearing in the microbore chromatogram are not even discernible on the analytical column. It is important to note, however, that the overall efficiency of small-bore columns is no greater than that of their larger diameter counterparts (as long as both are operated at the same linear flow velocities).

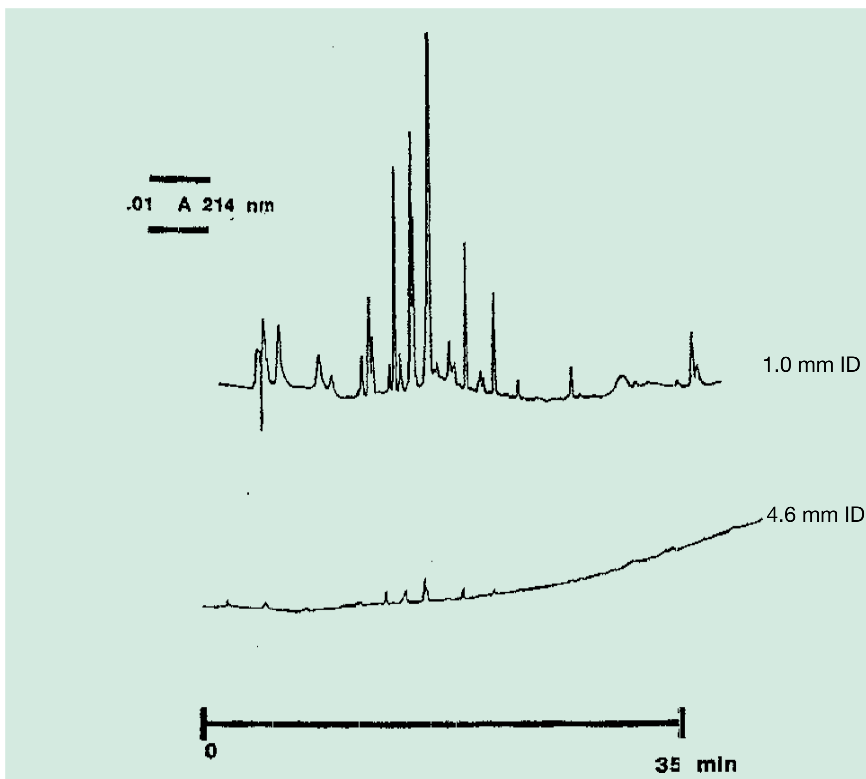


Figure 16. *Comparison peptide mapping of apomyoglobin-tryptic digest on conventional and microbore columns.*

A second advantage of small-bore HPLC concerns the dramatically lower operating costs achievable with these columns. Since the flow rate is equal to the product of the linear flow velocity and the cross-sectional area of the column, mobile phase consumption will decrease proportionally to the square of the column radius (cross-sectional area= πr^2). Some HPLC-grade solvents used today can cost as much as $ 25 per liter. Inspection of the flow rates given in Table 5 shows that substantial savings in solvent costs can be realized for the laboratory using a 1.0 mm ID column as opposed to a standard 4.6 mm column.

Another important benefit involves the extremely accurate and reproducible measurements of retention times possible with small-bore columns. In HPLC analysis, solute identification is often based on retention time measurements which are highly dependent on column temperature. Due to the smaller diameters of these columns, the heat generated internally as mobile phase passes through the packed bed is dissipated very rapidly. As a consequence, small-bore columns can be easily thermostatted to precise and constant temperatures.

Disadvantages

The most obvious disadvantage of small-bore chromatography is, ironically, due to the greatly reduced dimensions of the column. As a consequence of the limited bed volumes (a 150 x 1.0 mm column has a packed bed volume of only about 120 µL), this type of HPLC places high demands on the accompanying instrumentation. The smaller the column volume, the more critical the requirement for special instrument design and construction. Although a thorough examination of these instrument requirements is beyond the scope of this guidebook, it is important to acquaint the potential small-bore column user with some of the basic concepts.

Small-Bore Chromatography: Instrumentation Requirements

For optimal separations, small-bore columns are typically used at the same linear flow velocities as their larger diameter counterparts. Therefore, mobile phase flow rates will be dramatically lower than those employed with conventional columns (see Table 1). The pumps used in these applications must be highly accurate and capable of delivering constant, pulseless flows as low as a few microliters per minute (or less for capillary columns).

Due to the significantly reduced volumes of small-bore columns, the physical band broadening that occurs as solute peaks pass through the chromatographic system becomes a much greater factor in the ultimate success or failure of the separation procedure than it is with conventional columns. Control over this extra-column dispersion can, however, be accomplished through comparable reductions in injector, connecting tubing, and detector volumes.

Sample injection represents an immediate and significant source of extra-column dispersion. In small-bore chromatography, where sample sizes are reduced, special switching valves and injector designs are necessary to limit the band-broadening of the sample plug. Similarly, connecting tubing lengths and internal diameters should be kept at a minimum in order to reduce band-broadening. Most small-bore systems utilize 0.05 in.-0.007 in. ID tubing for this purpose. The use of zero dead volume fittings is also considered essential. Today polyetherether ketone (PEEK) tubing, which is available in 0.005 in. ID's and can be cut easily with a razor blade, is an attractive alternative to stainless steel.

The increase in mass sensitivity associated with small-bore columns is linked, ultimately, to proportional reductions in peak volumes. The emergence from the column of these dramatically smaller solute bands necessitates changes in the detector system. These changes must be designed to limit extra-column dispersion while not adversely affecting chromatography and/or detector performance. The response of a UV detector is proportional to the path length of the flow cell. Thus, a reduction of cell volume as a means of limiting extra-column dispersion must be made while

maintaining adequate path length so as to maintain sufficient sensitivity. Since peak widths are usually reduced in small-bore chromatography, the detector time constant should be decreased to allow for detection of the sharper peaks.

Special Care Considerations

Guard columns are routinely used in conventional chromatography; however, due to the requirement for low system dead volume with small-bore columns, they are not normally used in these applications. Instead, zero dead volume in-line filters (e.g., 0.5 µm porosity) are employed to help prevent plugging of the column. This protection is particularly important for peptide and/or protein applications where aggregation and precipitation are common potential problems. Precautions should be taken in the preparation of the mobile phases as well as the samples, which should appear clear with no visible precipitates. Samples are often either centrifuged or passed through specific filters before injection. The column should also be washed regularly with high concentrations of eluting solvent, and backflushes to remove precipitates on the column frit are often found to help limit backpressure problems.

Section 5.

Choosing the Appropriate Chromatographic Technique and Column

Peptides, Proteins, Glycoproteins

Column	Technique and Features
phenomenex **Jupiter® 300** 300 Å C4, C5, C18	**REVERSED PHASE (RPC)** • For High Molecular Weight Biopolymers • High Selectivity
Jupiter® Proteo 90 Å C12	• For Low Molecular Weight Biopolymers • High Capacity • Peptide Mapping
phenomenex **Jupiter 300** 300 Å C4, C5	**HYDROPHOBIC INTERACTION (HIC)** • Complements Ion-Exchange for Higher MW, More Hydrophobic Biopolymers • Gentle Separation Environment • High Recoveries • High Loading Capacity
phenomenex **BioSep™-SEC-S** Silica-based 2000, 3000, 4000	**SIZE-EXCLUSION (SEC/GFC)** • Sample Characterization by Size and Shape • Fast Separations • High Loading Capacities • Retain Bioactivity • Identify Protein Aggregation
Shodex® AFpak (many ligands)	**AFFINITY (HPAC)** • High Specificity • High Recoveries (Mass and Activity)
phenomenex **BioSep™ DEAE** (weak anion exchange)	**ION-EXCHANGE (IEC)** • For Protein Separations (MW>10 KDa) • High Capacity
Luna® SCX (strong cation exchange) **Luna® NH_2** (weak anion exchange)	• For Peptide Separations (MW<10 KDa) • Extremely High Capacity • High Resolution

Protein/Peptide Chromatography Technique Selection Tree

- Protein/Peptide Separation
  - Size-Exclusion
    - pH 2-7.5
      - MW 1000-2,000,000
        - BioSep-SEC-S
    - pH > 7.5
      - MW < 1,000 / MW > 2,000,000
        - PolySep-GFC-P
  - Reversed-Phase
    - pH > 10
      - PolymerX RP-1
    - pH 1.5-10*
      - MW < 10,000
        - Proteins digest maps/ small peptides
          - Jupiter Proteo
      - MW > 10,000
        - Hydrophobic proteins
          - Jupiter 300 C4, C5
        - Hydrophilic proteins/ large glycopeptides
          - Jupiter 300 C18
  - Ion-Exchange
    - Cation-Exchange
      - Shodex® IEC CM-825 (Weak) / Shodex® IEC SP-825 (Strong)
    - Anion-Exchange
      - BioSep-DEAE (Weak) / Shodex® IEC QA-825 (Strong)
    - MW < 10,000
      - Cation Exchange Luna SCX
      - Anion Exchange Luna NH_2

* This pH range is not representative of all silica-based, reversed-phase columns. Most other silica-based, reversed-phase columns have a pH range of 2-7.

Column Selection Guide

This section outlines the various column parameters that must be taken into consideration when choosing the appropriate reversed-phase, wide-pore HPLC column.

The following parameters must be evaluated:

- Bonded phase
- Dimensions
- Particle size

Bonded Phase

Hydrocarbon chains of varying lengths are the most common bonded phases on the surface of silica-based, wide-pore reversed-phase columns, the common phases include C4, C5, and C18. Below is a table outlining which phase would be most appropriate for different sample types:

Phase	Characteristics	Application
C4 300 Å	Low hydrophobic retention, high surface coverage of bonded phase.	Large proteins, large polypeptides (MW>10,000), highly hydrophobic peptide samples, Hydrophobic Interaction Chromatography.
C5 300 Å	Low hydrophobic retention, high surface coverage of bonded phase. More retentive than C4, offering slightly alternate selectivity.	Large proteins, large polypeptides (MW>10,000), highly hydrophobic peptide samples, Hydrophobic Interaction Chromatography.
C18 300 Å	High hydrophobic retention, medium bonded-phase sur face coverage.	Hydrophilic proteins, glycopeptide separations, peptide mapping ap plications with large peptides
C12 90 Å	Moderate/high hydrophobic retention. High bonded-phase surface coverage. Similar selectivity as a C18 phase.	Peptide separations (MW<10,000), peptide mapping applications.

Column Dimensions

The characteristics of any column are greatly affected by both column internal diameter (ID) and column length. Below you will find a useful guide for selecting the most appropriate column dimension for any given application.

Column ID	When to use	When not to use
0.075 mm 0.3 mm 0.5 mm 1.0 mm 2.0 mm	Excellent for LC/MS, yields greater sensitivity. Requires 80 % lower sample loads than analytical 4.6 mm columns for instances where sample quantities maybe limited. If solvent usage is a concern, this format consumes up to 80 % less mobile phase than 4.6 mm columns.	If analyzing crude sample matrices, the column will tend to clog quickly, resulting in high backpressures and decreased column lifetime. If microbore system with <0.008 in. tubing and capacity to pump accurately at 0.2 mL/min is not available. If sensitivity and/or solvent saving is not a concern.
4.6 mm	All analytical purposes where increased sensitivity and decreased solvent usage are not a major concern.	When sample injection volumes and concentrations are limited. When sensitivity is a concern. When collecting fractions during preparative chromatography.
10 mm	Preparative sample analysis and collection (1-20 mg)	When high resolution analytical chromatography is required.
21.2 mm - 100 mm	Preparative sample analysis and collection (>20 mg)	When high resolution analytical chromatography is required.

Column Length	When to Use	When not to use
150- 250 mm	Standard analytical lengths for virtually any general application. Generally more important for low molecular weight samples (<10,000 Da), since partitioning the continual interaction of the solutes between the solid support and mobile phase is at work to a greater degree.	Rapid analysis assays. LC/MS applications where maximum resolution is not as crucial.
100- 50 mm	Useful for rapid analysis purposes. For analysis of large proteins and peptides where the sample absorbs to the first few mm of column and elutes off the column immediately when a specific organic concentration is reached. For these assays, comparable or superior resolution compared to 250 mm length columns can be achieved. Excellent for LC/MS when maximum resolution is not essential.	When maximum resolution is required for a low molecular weight sample.

Particle Size

Selecting the proper particle size for your HPLC column has a tremendous effect on the resolution, loading capacity, flow rate and backpressure experienced while running your HPLC assay. The table below outlines parameters to evaluate when selecting the proper particle size for your HPLC column:

Particle Size	Use
4 µm, 5 µm	Designed for analytical size columns (1.0-4.6 mm ID). High efficiency (60,000 - 100,000 plates/meter) yields greater resolution for most assays.
10 µm	Designed for small-scale preparative separations (10.0-21.2 mm column ID). Medium efficiency range (50,000-70,000 plates/meter) yields good resolution for most assays. Results in lower backpressures and higher sample injection volumes and concentrations compared to 5 µm materials.
15 µm +	Designed for large-scale preparative separations (21.2-100 mm column ID). Low efficiency range (40,000-60,000 plates/meter) yields fair resolution for most assays. Requires greater flow rates and permits larger sample injection volumes and concentrations.

Section 6.

Column Technologies for Biochromatography

Section 6 contains:

- **Column Technologies for Biochromatography**
  - **Jupiter for Reversed Phase, Hydrophobic Interaction**
  - **BioSep for Gel Filtration Chromatography**

Jupiter for Reversed Phase and Hydrophobic Interaction Chromatography (HIC)

Reversed phase chromatography is a powerful tool for the separation of proteins and peptides. The separation is based on both adsorption and partition mechanisms (Koyama et al. J. Chromatogr. 625, 217, 192). Depending on the presence of silanol groups on the bonded phase, secondary mechanisms, such as ionic and hydrogen bonding interactions, may also contribute to the overall separations of the biomolecules. A second aspect of the silica-based bonded phases is the presence of metals. Many proteins are capable of metal chelation, leading to a loss of recovery and sometimes loss of biological activity. Such secondary interactions may lead to tailing peaks and low recovery of protein mass and biological activity. Phenomenex Jupiter columns were developed with a proprietary bonding and end-capping chemistry to provide an inert and hydrolytically stable bonded phase. Pure silica (5 µm 300 Å, 99.99 % pure) was used to prepare C4, C5 and C18 bonded phases. Figure 17 shows the stability of Jupiter C4, C5 and C18 phases after exposure to acidic (pH 1.5) and basic (pH 10.0) buffer conditions. The columns showed no sign of deterioration after more than 2,500 hours of continuous exposure under these conditions. Such extended pH stabilities can be used with advantage for effecting better separations of proteins and peptides.

Figure 17. *(page 63) Jupiter column stability tested under highly basic conditions (column immersed in 20 mM Na_2HPO_4 in water/acetonitrile (pH 10), 50:50) and highly acidic conditions (column immersed in 0.1 % TFA in water/acetonitrile (pH 1.5), 50:50).*

Acidic pH Stability (pH 1.5) Basic pH Stability (pH 10)

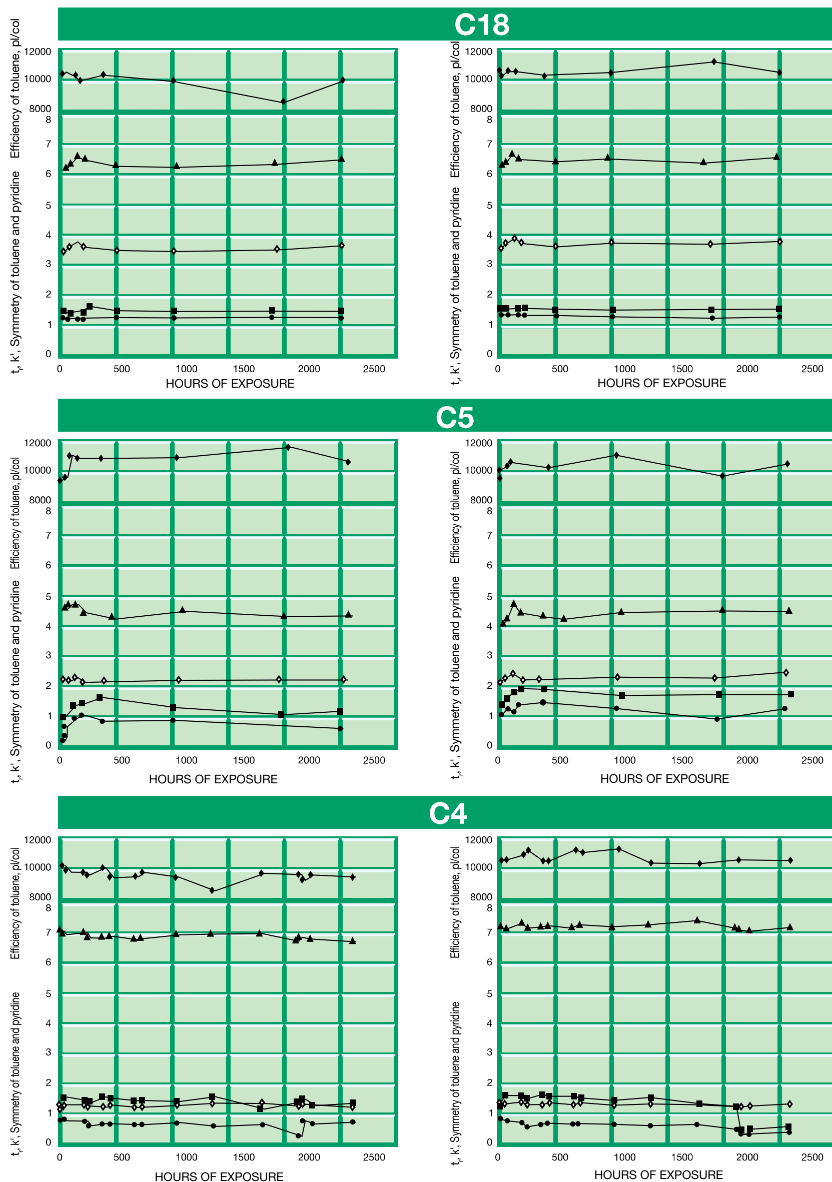


Figure 17. *(Description page 62).*

Test Conditions

Dimensions:	250 x 4.6 mm
Mobile Phase:	Acetonitrile/Water, 50:50
Flow Rate:	1 mL/min
Detection:	UV @ 254 nm
Samples:	1. Pyridine 2. Phenol 3. Toluene

KEY:

- Efficiency, Plates for Toluene
- t_r of Toluene (min)
- k' of Toluene
- Asymmetry of Toluene
- Asymmetry of Pyridine

The presence of residual silanols in the bonded phase can be determined with different test probes. Reversed-phase and cationic peptide standards provide an ideal set of such probes for protein columns. Figures 18 and 19 show the separation of these standards on a Jupiter C18 column. The reversed-phase standards consist of four peptides of increasing hydrophobicity. On an ideal column, the four peptides should elute in the order of S1 to S4 as shown in Figure 18. Residual silanols, when present on the surface of silica bonded phases, can interact ionically with S_1 peptide, leading to its longer retention (Figure 19). The cationic peptides, on the other hand, have increasing charge (+1 to +4) and hydrophobicity. Ionic interactions with the silanols lead to tailing peaks especially for peptides +3 and +4. All four cationic peptide standards show the correct order of elution according to their hydrophobicity (+1 to +4) with excellent peak asymmetry. Inert Test 2, which uses pyridine peak asymmetry as a measure of inertness of the surface (Figure 20), also showed an inert surface chemistry.

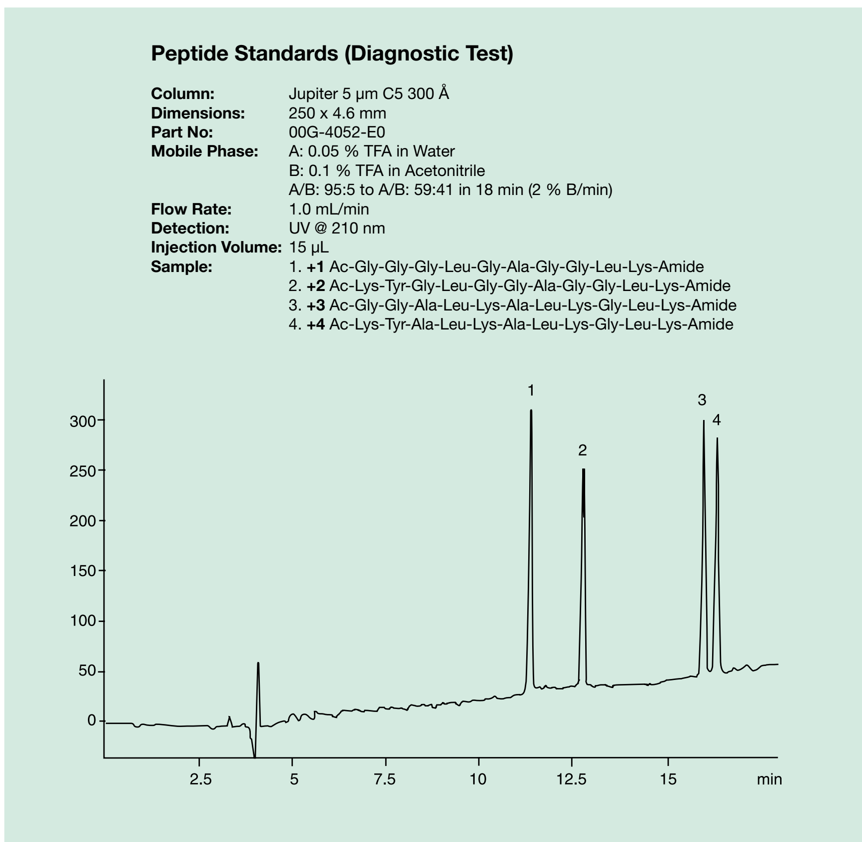


Figure 18.

Cationic Peptide Standards (Diagnostic Test)

Column: Jupiter 5 μm C18 300 Å
Dimensions: 250 x 4.6 mm
Part No: 00G-4053-E0
Mobile Phase: A: 0.1 % TFA in Water
B: 0.1 % TFA in Acetonitrile, A/B: 100:0 to A/B: 60:40 in 20 min (2 % B/min)
Flow Rate: 1 mL/min
Detection: UV @ 210 nm
Sample:
1. **+1**. Ac-Gly-Gly-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-Lys-Amide
2. **+2**. Ac-Lys-Tyr-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-Lys-Amide
3. **+3**. Ac-Gly-Gly-Ala-Leu-Lys-Ala-Leu-Lys-Gly-Leu-Lys-Amide
4. **+4**. Ac-Lys-Tyr-Ala-Leu-Lys-Ala-Leu-Lys-Gly-Leu-Lys-Amide

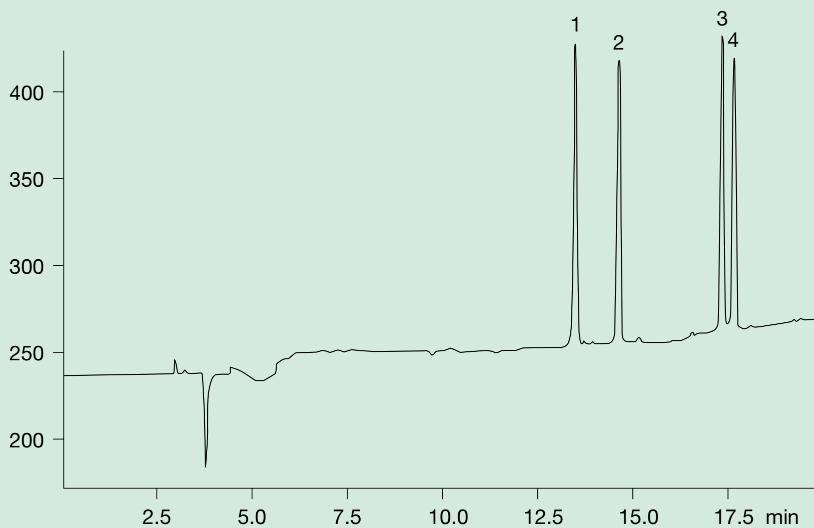


Figure 19.

Pyridine Asymmetry Test

Column: Jupiter 5 µm C18 300 Å
Dimension: 250 x 4.6 mm
Part No.: 00G-4053-E0
Mobile Phase: Water/Acetronitrile 50:50
Flow Rate: 1.0 mL/minute
Detection: 230 nm
Sample: UV @ 254 nm
1. Pyridine
2. Phenol
3. Toluene

Pyridine Asymmetry: 1.47

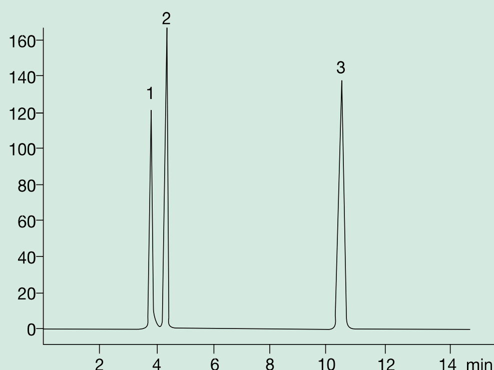


Figure 20.

The presence of metals on Jupiter columns was determined using the dihydroxynaphthalene ratio test (DERT) (Eurby et al, J. Chromatogr A, 705, 229, 1995) The two isomers present in this test mixture, 2,7-dihydroxynaphthalene and 2,3-dihydroxynaphthalene, elute in this order. While the former is not a metal chelating agent, the latter is a strong chelating agent. In a metal-free bonded phase, the two compounds should elute with similar efficiencies and peak asymmetries with their ratios near to one, as shown in Figure 21. In the presence of metals, however, 2,3-dihydroxynaphthalene elutes as a tailing peak or may not elute at all. In such cases, the ratios of peak asymmetries and efficiencies are <1 (Figure 21).

Dihydroxynaphthalene Ratio Test (DERT) on Jupiter 5 µm C18

Column: Jupiter 5 µm C18 300 Å
Dimension: 250 x 4.6 mm
Part No.: 00G-4053-E0
Mobile Phase: $H_2O:CH_3CN$, 50:50
Flow Rate: 1 mL/minute
Detection: 230 nm
Sample: 1. 2, 7-Dihydroxynaphthalene
2. 2, 3-Dihydroxynaphthalene
N_2/N_1 1.02
Sym_2/Sym_1: 0.90

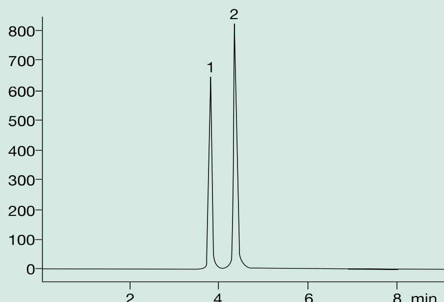


Figure 21.

Protein Standards

Column: Jupiter 5 µm C18 300 Å
Dimensions: 250 x 4.6 mm
Part No: 00G-4053-E0
Mobile Phase: A: 0.1 % TFA in Water
B: 0.1 % TFA in Acetonitrile,
A/B: 75:25 to A/B: 35:65 in
20 min (2 % B/min)
Flow Rate: 1 mL/min
Detection: UV @ 220 nm
Sample: 1. RNAse
2. Insulin
3. An insulin contaminant
4. Lysozyme
5. Bovine serum albumin
6. Myoglobin

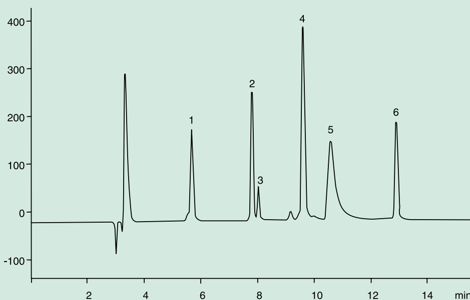


Figure 22.

Cytochrome c Genetic Variants

Column: Jupiter 5 µm C18 300 Å
Dimensions: 250 x 4.6 mm
Part No: 00G-4053-E0
Mobile Phase: A: 0.1 % TFA in Water
B: 0.1 % TFA in Acetonitrile,
A/B: 75:25 to A/B: 45:55 in
15 min (2 % B/min)
Flow Rate: 1.0 mL/min
Detection: UV @ 220 nm
Sample: 1. Equine cytochrome c
2. Bovine cytochrome c
3. Canine cytochrome c

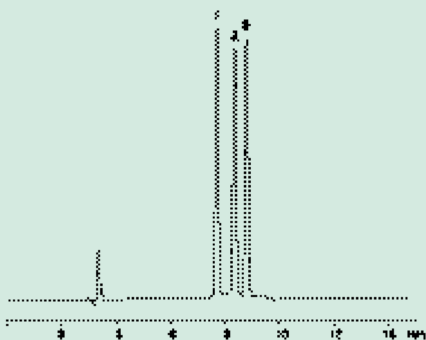


Figure 23.

Figure 22 shows the separation of a test mixture consisting of proteins on Jupiter 5 µm C18. Except for serum albumin, all the proteins show excellent peak asymmetries. Also note the baseline separation between insulin and its contaminant. With many columns, the two components do not separate completely. Reversed-phase chromatography can be used for the separation of closely related proteins, genetic variants of Cytochrome c, as shown in Figure 23. Jupiter columns thus provide for the superior separation of proteins. Traditionally, protein and peptide separations have been carried out in low pH (pH 1.0 to 2.0) eluent, such as 0.1 % TFA, which acts as an ion suppressor and ion-pair agent. Proteins and peptides have different isoelectric points (pI), and changing the eluent pH would provide a powerful means to the separation process. Many reversed-phase packing materials currently available, however, are not stable at higher pH (>7.0), thus limiting the choice of mobile phase. Since Jupiter phases have extended pH stabilities (pH 1.0 to pH 10.0), they provide an entirely new avenue for protein purification using reversed-phase chromatography.

Myoglobin Tryptic Digest

Column: Jupiter Proteo 4 µm C12 90 Å
Dimensions: 250 x 4.6 mm
Part No.: 00G-4396-E0
Mobile Phase: A) 0.012 % TFA in Water
B) 0.01 % TFA in Acetonitrile
Gradient: A/B (95:5) for 5 min, then
to A/B (60:40) in 55 minutes
Flow Rate: 1 mL/min
Detection: UV @ 210 nm
Sample: Myoglobin Tryptic Digest

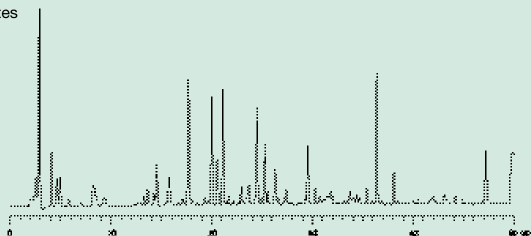


Figure 24.

k-Casein Tryptic Digest Map

Column: Jupiter 4 µm Proteo 90 Å
Dimensions: 250 x 4.6 mm
Part No.: 00G-4396-E0
Mobile Phase: A) 0.012 % TFA in Water
B) 0.01 % TFA in Acetonitrile
Gradient: A/B (95:5) for 5 min, then
to A/B (60:40) in 55 minutes
Flow Rate: 1 mL/min
Temperature: 22°C
Detection: UV @ 210 nm
Sample: k-Casein Tryptic Digest

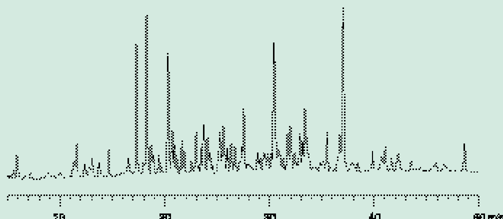


Figure 25.

Figures 24 and 25 show the separation of tryptic peptides on Jupiter 4 µm Proteo columns. Once again, we see excellent resolution for peptides.

References:

Koyama, J., Nomura, J., Shiojima, Y., Ohtsu, Y and Horii, I., J. Chromatogr. 635, 217, 1992.

Eurby, M. R., Johnson . C. M., Rushin, I. D and Tennekoon Sakunthala, D.A.S, J. Chromatogr. A 705, 229, 1995.

BioSep for Gel Filtration Chromatography (SEC/GFC)

In ideal gel filtration chromatography, the protein molecules should pass through the column without non-specific interactions, which are generally ionic or hydrophobic in nature. These interactions are the result of free acidic silanols or hydrophobic sites in the silica-based packing materials. In extreme cases, the non-specific interactions can result in the elution of proteins, peptides or other analytes beyond the total liquid volume (V_T) of the column. Many times, using high salt concentrations and low pH (0.5 M at pH 2.0) are necessary to counter the ionic interactions. However, such conditions may also promote hydrophobic interactions. Non ideal GFC of the proteins can lead to errors in the measurements of their molecular weight and low sample recovery.

Phenomenex BioSep-SEC series of gel filtration columns (BioSep-SEC-S2000, 5 µm 145 Å; BioSep-SEC-S3000, 5 µm 290 Å and BioSep-SEC-S4000, 5 mm 500 Å) have a hydrophilic surface and a proprietary endcapping process that restrict the access of the analytes to free silanols. The columns were tested using a set of diagnostic peptide and protein standards for ionic/hydrophobic interactions. The standards are made of 1-5 repeating units of a polypeptide sequence (Ac-(Gly-Leu-Gly-Ala-**Lys**-Gly-Ala-Gly-Val-Gly) $_{n\,(1\text{-}5)}$, which contains a lysine residue incorporating a positive charge in the sequence. The five polypetide standards thus contain 10-50 residues of increasing hydrophobicity and charges (+1 to +5). In an ideal GFC situation, the peptides should elute in the order of decreasing molecular weights. However, if there are cationic silanol sites, the elution order can reverse or change when the charges on the peptides interact with the bonded phase. As shown in Figure 26, the five cationic peptides along with protein standards elute in the order of their decreasing molecular weights, showing no non-specific interactions, even under low salt buffer conditions. The inset in the Figure shows linearity for the plot of log MW vs. K_D for all the peptides and proteins. These experiments were carried out with BioSep-SEC-S2000, which has a pore size of 145 Å, ideal for the separations of the MW range of proteins and peptides used. However, the peptide standards are not suitable for chromatography on phases having higher pore sizes, such as BioSep-SEC-S3000 and BioSep-SEC-S4000 (290 Å and 500 Å pore sizes respectively). A different approach was used for the study of ionic/ hydrophobic interactions on these columns. Lysozyme is a very basic (pI = 11.0) and, to a certain extent, a hydrophobic protein. Silanol interactions can lead to peak tailing and/or delayed elution of this protein under low ionic strength buffer conditions in the mobile phase. The elution pattern of this protein on BioSep-SEC-S2000, BioSep-SEC-S3000 and BioSep-SEC-S4000 was studied under varying pH conditions. Figure 27 shows, that the K_D does not change drastically when the pH is varied between 2.0 and 7.0. In GFC phases with ionic sites, the K_D values have been shown to increase to nearly a value of 2.0 at pH 7.0 (Pfankoch et. al, J. Chromatogr. Sci., **18**, 430, 1980).

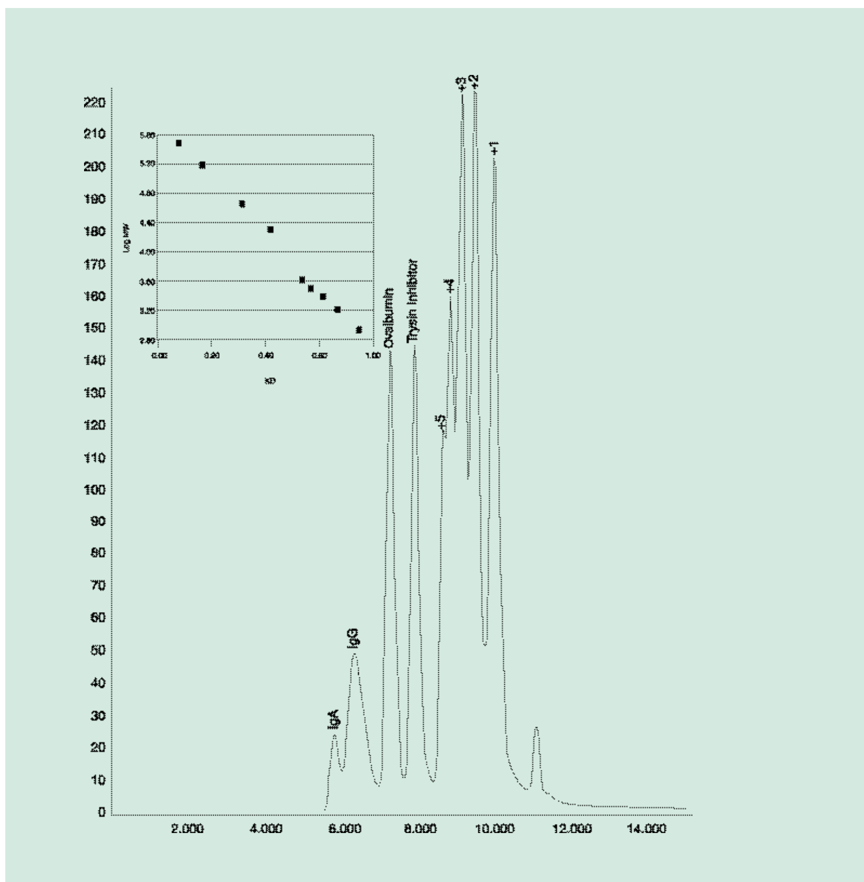


Figure 26. *SEC of proteins and peptides on BioSep-SEC-S2000. A 300 x 7.8 mm ID column was equilibrated with 50 mM NaH_2PO_4 buffer (pH 6.8) at a flow-rate of 1 mL/min. Protein standard was prepared by dissolving 4 mg each of γ-globulin (a mixture of immunoglobulins IgM, IgA and IgG), ovalbumin and trypsin inhibitor in 1 mL of mobile phase. About 4 µL of this mixture were added to 100 µL of a solution of synthetic peptides. A 10 µL volume of the sample was injected onto the column. The elution of proteins and peptides was followed by UV detection at 215 nm. Peaks identified: IgA (300,000 dalton), IgG (150,000 dalton), ovalbumin (44,000 dalton), trypsin inhibitor (20,100 dalton), peptide + 5 (3,897 dalton), peptide + 4 (3,129 dalton), peptide + 3 (2,326 dalton), peptide + 2 (1,595 dalton), and peptide + 1 (826 dalton). The inset is a plot of log molecular weight (MW) vs. K_D for this separation (r^2=0.9936). The K_D values were calculated using the equation $K_D = (V_e - V_0)/(V_T - V_0)$, where V_e is the elution volume and V_T and V_O represent the total liquid volume and void volumes of the column, respectively. Time in min.*

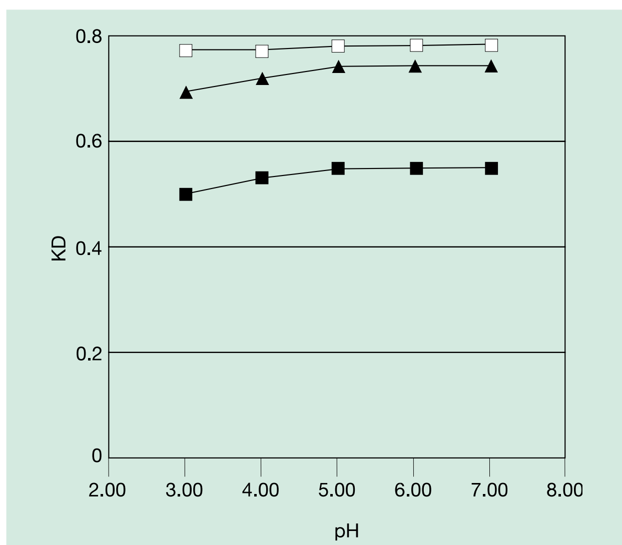


Figure 27. *Effect of mobile phase pH on the K_D of lysozyme. BioSep-SEC-S2000, -S3000 and S4000 columns 300 x 7.75 mm ID) were equilibrated with 0.1 M NaH_2PO_4 buffer at different pH values between 3 and 7. Lysozyme was dissolved in the same buffer as the mobile phase at 10 mg/mL and 20 µL of the solution were injected. The flow-rate was 1 mL/min and detection was at 280 nm. Columns:*

□ = BioSep-SECS2000; ▲ = BioSep-SEC-S3000; ■ = BioSep-SEC-S4000.

A lack of non-specific binding of proteins and peptides to BioSep-SEC columns also means that the recovery of the proteins should be high. Like lysozyme, trypsin is a basic protein (pI= 10.5) and prone to ionic interactions with silanol groups. A study of the recovery of trypsin under pH 3.0 and pH 7.0 mobile phase conditions showed 69 % to 100 % protein recovery and 86 % to 100 % recovery of specific activity (Table 6). The data show that even very basic proteins do not interact with the BioSep-SEC series of columns.

Table 6. *Recovery of Trypsin from BioSep-SEC-S Columns.*

Column	pH	Protein (%)	Specific activity (%)
BioSep-SEC-S2000	3.0	85-100	86-100
	7.0	70-70	95-100
BioSep-SEC-S3000	3.0	98-101	96-100
	7.0	70-78	96-100
BioSep-SEC-S4000	3.0	92-100	95-101
	7.0	69-80	94-100

Table 6. *The columns were equilibrated with 0.1 MNaH_2PO_4 buffer (pH 3.0 or 7.0). Tryspin was dissolved in NaH_2PO_4 buffer (pH 3.0) at 10 mg/mL. A volume of 20 µL of the solution was injected onto the columns and the peaks were collected. For each pH value, at least five runs were performed. The peaks were assayed for protein concentration and enzyme activity by the N-benzoyl-L-arginine-p-nitroacetanilide as a substrate in Tris- HCl buffer (pH 8.0) containing $CaCl_2$. The hydrolysis was followed by the increase in absorption at 386 nm.*

Size exclusion chromatography of protein standards under native conditions on BioSep-SEC-S3000 showed good linearity on a log MW vs K_D plot (R^2 = 0.977, see Figure 28). Similarly, under denaturing conditions, such as 0.5 % SDS or 6 M guanidine hydrochloride (Figures 29 and 30), the protein standards showed excellent correlation between their log MW and K_D (R^2 = 0.99). The results show that the BioSep-SEC- series of columns are ideal for the GFC of proteins and peptides.

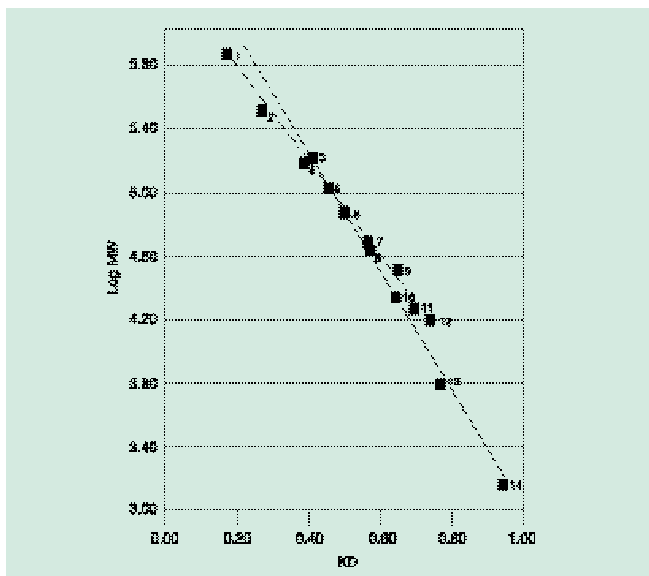


Figure 28. *Chromatography of proteins on BioSep-SEC-S3000. A mixture of different proteins was prepared by dissolving 2 mg each in the mobile phase buffer 50 mM NaH_2PO_4 (pH 6.8). A 10 µL volume of the sample was injected into a 300 x 7.8 mm ID column that was equilibrated with the buffer at 1 mL/min. UV detection at 280 nm was used. Three different runs with mixtures of proteins were performed and the log MW vs. K_D plotted (solid line, for all proteins, R^2 = 0.977; dashed line, excluding insulin and cyanocobalamin, R^2 = IgA (3,000,000 dalton); 5 = phosphorylase b (97,000 dalton); 6 = bovine serum albumin (69,000 dalton); 7 = ovalbumin (44,000 dalton); 8 = horse radish peroxidase (40,000 dalton); 9 = carbonic anydrase (30,000 dalton); 10 = trypsin inhibitor (20,100 dalton); 11 = myoglobin (17,000 dalton); 12 = lysozyme (14,400 dalton); 13 = insulin (5,700 dalton); 14 = cyanocobalamin (1,350 dalton).*

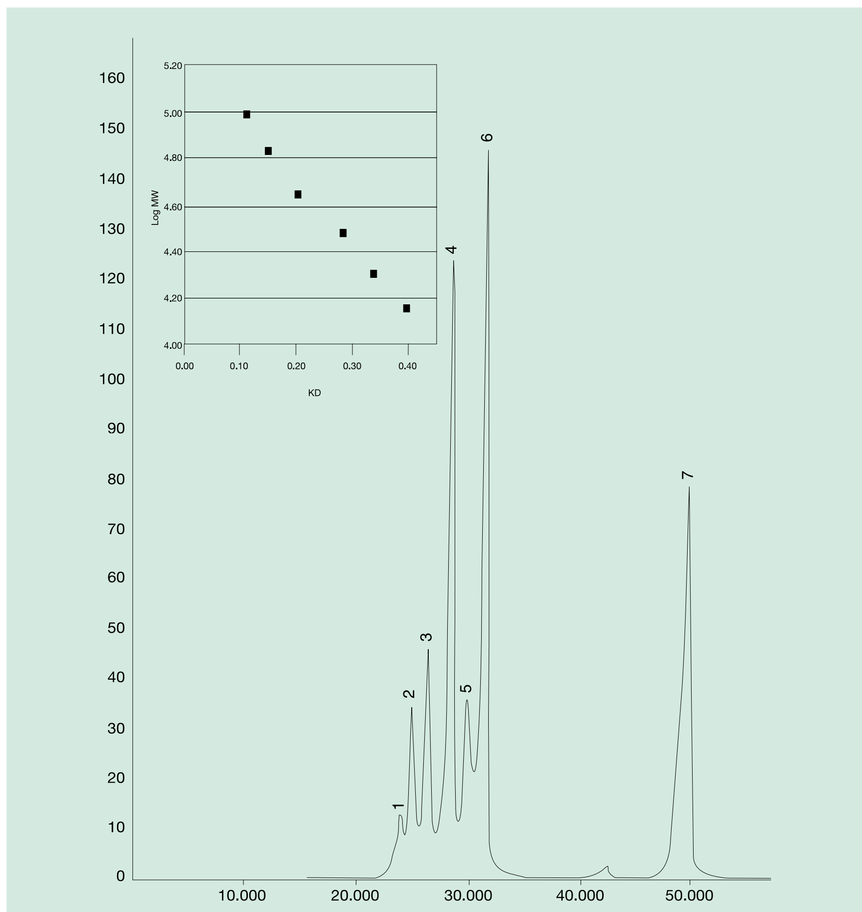


Figure. 29. *SEC of proteins on BioSep-SEC-S3000 using 0.5 % SDS. Protein mixture was prepared by dissolving 2 mg each of (1) phosphorylase b (97,000 dalton), (2) bovine serum albumin (68,000 dalton), (3) ovalbumin (44,000 dalton), (4) carbonic anhydrase (30,000 dalton), (5) trypsin inhibitor (20,100 dalton) and (6) lysozyme (14,400 dalton) in 0.5 mL of 20 mM* NaH_2PO_4 *buffer (pH 6.5) containing 1 % SDS and 1 % ß-mercaptoethanol. The solution was boiled for 3 min and 20 µL were injected on the column (600 x 7.75 ID), equilibrated with 20 mM* NaH_2PO_4 *buffer (pH 6.5) containing 0.5 % SDS at a flow-rate of 10 mL/min. Peak 7 is BME. The inset is a plot of log MW vs.* K_D *with* R^2 *= 0.9908. Time in min.*

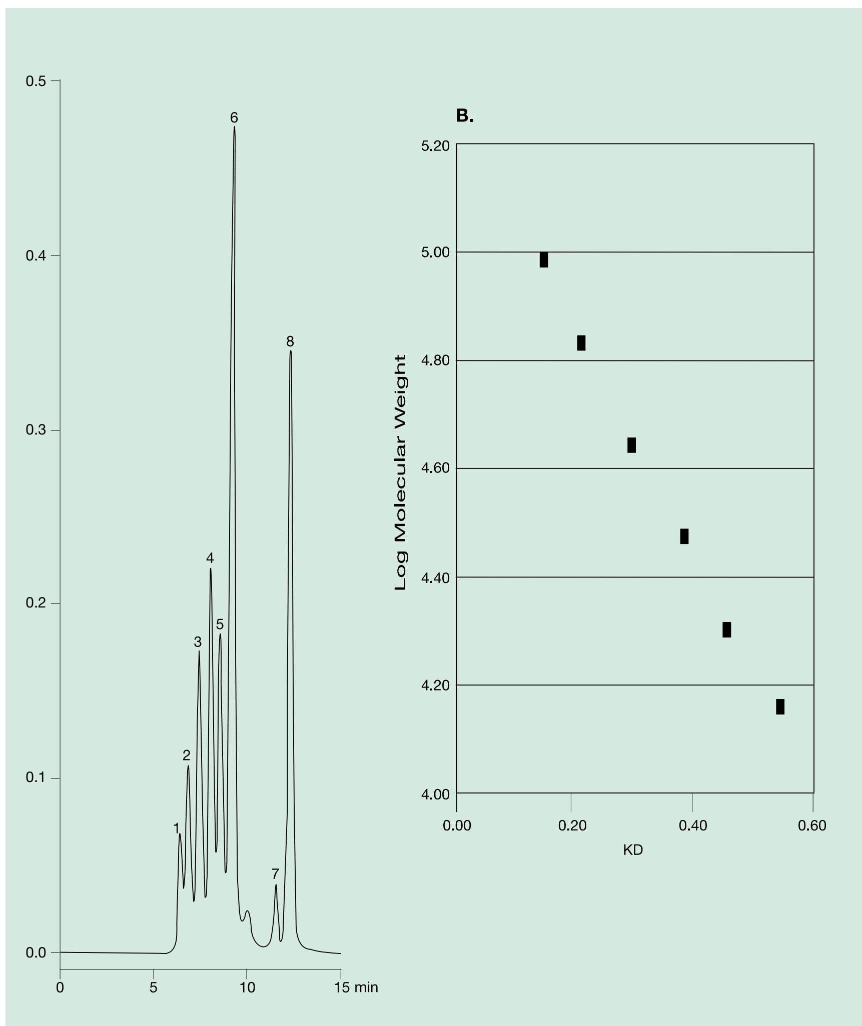


Figure 30. *(A) SEC of proteins on BioSep-SEC-S3000 using 6 M guanidine hydrochloride. The same set of proteins as in Figure 28 was used. The proteins were dissolved in 0.5 mL of 20 mM NaH_2PO_4 buffer (pH 6.5) containing 8 M guanidine hydrochloride and 1 % BME. The solution was boiled for 5 min and 20 µL were injected on to the column, equilibrated with 20 mM NaH_2PO_4 buffer (pH 6.5) containing 6 M GnHCl at a flow-rate of 1 mL/min. The separation profile was followed by UV detection at 280 nm. Peaks: 1= phosphorylase b (97,000 dalton); 2= bovine serum albumin (68,000 dalton); 3= ovalbumin (44,000 dalton); 4= carbonic anhydrase (30,000 dalton); 5= trypsin inhibitor (20,100 dalton); 6= lysozyme (14,400 dalton); 7= a contaminant; 8= ß-mercaptoethanol. (B) Plot of log MW vs. K_D, R^2 = 0.9948*

Appendix A
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APPENDIX B

Glossary of Chromatographic Terms

Activated affinity media - Packing materials for use on affinity chromatography supplied with covalently reactive surface functionality to allow the end user to couple their own affinity ligands.

Activity recovery - The fraction of the biological activity (such as antibody binding activity, enzyme activity, etc.) applied to a chromatographic step that is recovered in the eluted product fraction. Also referred to as activity yield.

Adsorption - A process which may occur at the surface of a liquid or solid as a result of the attractive forces between the adsorbate and solute.

Affinity chromatography - A technique in which a biospecific adsorbent is prepared by coupling a specific ligand (such as an enzyme, antigen or hormone) for the macromolecule of interest to a solid support (or carrier). This immobilized ligand will interact only with molecules that can selectively bind to it. Molecules that will not bind elute unretained. The retained compound can later be released in a purified state.

Alkyl chain length - Refers to the alkyl chain length of the chemically bonded phase of a reversed-phase sorbent. For example, C8 and C18 refer, respectively, to eight carbon (octyl) and the eighteen carbon (octadecyl) n-alkyl chains covalently attached to the support.

Alkysilane - A chemical functional group used to form a reversed-phase bonded phase on silica support matrices, consisting of a chemically reactive silane group coupled to a long alkyl carbon chain (usually C1, C4, C8 or C18).

Alpha (α) - A measure of chromatographic selectivity, equal to the capacity factor (k') of the later eluting molecule, divided by the k' of the earlier eluting molecule. An alpha of 1.0 indicates no resolution.

Analyte - The chemical species being investigated by an analytical method. Usually the identification and amount are to be determined by a separation step followed by quantitation.

Angström (Å) - A unit length commonly used for measurement of pore sizes on chromatographic packings, equal to 10^{-4} µm (10^{-10} meters).

Anion-exchange chromatography - The ion-exchange procedure used for the separation of anions. Both resins and bonded phases are available for this mode. The tetralkylammonium group is a typical strong anion-exchange functional group. An amino group on a bonded or coated stationary phase would be the example of a weak anion exchanger.

Asymmetry (As) - Factor describing the shape of a chromatographic peak. Theory assumes a Gaussian shape and that peaks are symmetrical. The peak asymmetry factor is the ratio (at 10 % of the peak height) of the distance between the peak apex and the back side of the chromatographic curve to the distance between the peak apex and the front side of the chromatographic curve. A value >1 is a tailing peak, while a value <1 is a fronting peak.

Band - Mobile phase zone which contains a sample component; band usually refers to the zone in the column; peak is the band signal at detection.

Band broadening (band spreading) - The dilution of the chromatographic band as it moves down the column. The peak is injected as a slug, and, if not for the process of band broadening, each separated component would be eluted as a narrow slug of pure compound. The measure of band broadening is band width, tw, or more correctly, the number of theoretical plates in the column, N.

Band width (tw) - The width of the chromatographic band (in time units) during elution from a column. It is usually measured at the baseline by drawing tangents to the sides of the Gaussian curve representing the peak. Small band widths usually represent efficient separations. Also referred to as Peak width.

Baseline - A constant signal produced by the background level of the instrument; usually represented by a flat line on the recorder.

Baseline drift - Baseline fluctuations, more prevalent in gradient elution than isocratic elution separations, due either to an imbalance in absorbance characteristics of the two (or more) eluents employed in gradient elution or to UV-active or fluorescent impurities in the mobile phase.

Bed volume -The total volume occupied by the chromatographic packed bed. For a conventional cylindrical column, the bed volume is equal to the cross sectional area (π times the radius of the bed squared) times the bed length. Also called the column volume or CV.

Blank run - The elution of a column with no sample injected; often used to look for system noise or drift.

Bonded phase - The chemical functional groups bonded to the surface of a support matrix of a stationary phase. The bonded phase is responsible for providing the selectivity of the binding interaction.

Buffer exchange - An application of gel filtration chromatography in which a packing is used that completely excludes any proteins from the pores and allows buffer salts and other small molecules to penetrate the pores. On passage through the column, the protein molecules are exchanged from the buffer and salt conditions of the sample to the conditions in which the column was originally equilibrated. Also called "desalting".

Capacity factor (k') - A chromatographic parameter that measures the degree of retention.

Capacity - Volume or mass load of which an HPLC column is capable. Several important capacity measurements include the static or equilibrium binding capacity, dynamic binding capacity, and loadability.

Cartridge column - A type of column that has no endfittings and is held in a cartridge holder. The column is a tube; the packing is contained by frits in each end of the tube. Cartridges are easy to change and are less expensive and more convenient than conventional columns with endfittings.

Cation-exchange chromatography - The form of ion-exchange chromatography that uses resins or packing with functional groups that can separate cations. A sulfonic acid would be an example of a strong cation-exchange group; a carboxylic acid would be a weak cation-exchange group.

Chaotropic agent - Any type of molecule which interferes with hydrophobic interactions by disrupting the ordered structure of water molecules. Common examples include urea, guanidine and thiocyanate salts. Chaotropic agents can be powerful solubilizing agents and can be used to reduce strong hydrophobic adsorption.

Chaotropic Agent - Any salt which enhances denaturation of proteins.

Chromatogram - Plot of detector signal output vs. time or elution volume during the chromatographic process.

Column backpressure - The pressure above gravity at the head of the column, typically expressed in pounds per square inch (psi), bar, atmospheres (atm), or megapascals (MPa); 1 Atm = 14.7 lb/in^2 = 1.013 Bar = 0.1013 MPa.

Counterion - In an ion-exchange process, the ion in solution used to displace the ion of interest from the ionic site. In ion-pairing, it is the ion of opposite charge added to the mobile phase to form a neutral ion pair in solution.

Column efficiency - Column characteristic expressed quantitatively by the number of theoretical plates; efficient columns have many theoretical plates and show only limited band broadening.

Column volume (CV) - The total volume occupied by the chromatographic packed bed. For a conventional cylindrical column, the column volume is equal to the cross sectional area (π times the radius of the bed squared) times the bed length. Also called the "bed volume".

Continuous gradient - Elution- A form of gradient elution in which the mobile phase composition or blend is changed gradually over time or volume. Continuous gradients can range in shape from simple linear profiles to complex functions, sometimes combined with step gradients.

Dead volume (Vd) - The volume outside of the column packing itself. The interstitial volume (intraparticle volume + interparticle volume) plus extracolumn volume (contributed by injector, detector, connecting tubing and endfittings) all combine to create the dead volume. This volume can be determined by injecting an inert compound (i.e., a compound that does not interact with the column packing). Also abbreviated "V_o".

Degassing - The process of removing dissolved gas from the mobile phase before or during use. Dissolved gas may come out of solution in the detector cell and cause baseline spikes and noise. Degassing is carried out by heating the solvent, by vacuum (in a vacuum flask), or by helium sparging (most widely used method).

Denature - Cause a protein molecule to lose its correct three-dimensional folded structure, and thus, usually, its biological activity. Some proteins can be correctly refolded and renatured under the right conditions, but for other proteins the process of denaturation is irreversible.

Desalting - An application of gel filtration chromatography in which a packing is used that completely excludes any proteins from the pores and allows buffer salts and other small molecules to penetrate the pores. On passage through the column, the protein molecules are exchanged from the buffer and salt conditions of the sample to the conditions in which the column was originally equilibrated. Also called "buffer exchange".

Detection limit - The minimum amount of the analyte that can be detected (although not necessarily accurately quantitated) above the background noise under a given set of analytical conditions in a given sample matrix. The standard formal definition is the signal of a blank sample (sample with no analyte) plus two standard deviations, where at least 20 replicates of the blank are run.

Detection limit - Minimum detectable quantity; amount of sample which produces a signal twice the noise level.

Dynamic capacity - The binding capacity of a chromatographic stationary phase in a packed bed with a flowing sample stream. Usually measured by frontal adsorption analysis, and defined based on the volume of a known concentration sample that can be applied at a given flow rate before any measurable amount of the binding molecule breaks through into the eluate. For conventional media, dynamic capacity is usually a function of the flow rate through the bed.

Eddy diffusion term - The contribution to plate height that is due to molecules traveling along different paths through the column; depends on the particle size and geometry of the packing, phenomena occurring in packed columns due to lack of homogeneity of packing; produces peak broadening.

Effective plate number - The number of theoretical plates calculated by using the adjusted retention time instead of the absolute retention time; is considered to be a better measure of the efficiency of capillary columns.

Efficiency (N) - A standardized measure of the bandspreading of a column. Efficiency can be expressed as the number of theoretical plates ($N = 5.54 (V_r / w1/2)^2$, where V_r is the retention volume [time can also be used] and w1/2 is the peak width at half the maximum peak height) or the plates/m (equal to N/L, where L is the column length in meters) or the HETP.

Eluate (eluent) - That which is eluted from the column; designation of the sample when separated and dissolved in the mobile phase.

Elution - The process of passing mobile phase through the column to transport solutes.

Elution volume (V_r) - Refers to the volume of mobile phase required to elute a solute from the column. $V_r = F \times t_r$, where F is the flow-rate and t_r is the retention time for the solute.

Endcapping - A column is said to be endcapped when a small silylating agent (e.g., trimethylchlorosilane) is used to bond residual silanol groups on a packing surface. Most often used with reversed-phase packings. May cut down on undesirable adsorption of basic or ionic compounds.

Exclusion limit - In SEC, the upper limit of molecular weight (or size), beyond which molecules will elute at the same retention volume, called the "exclusion volume". Many SEC packings are referred to by their exclusion limit. For example, a column with an exclusion limit of 100,000 daltons will exclude any compounds with a molecular weight higher than 100,000 daltons.

External standard - Absolute calibration; quantitative analysis method in chromatography where dilutions of pure standard are compared to unknown samples; the standards are the components of interest.

Extracolumn effects - The band-broadening effects of parts of the chromatographic system outside of the column itself. Extracolumn effects must be minimized in order to maintain the efficiency of the column. Areas of band broadening can include the injector, connecting tubing, endfittings, frits, detector cell volume and internal detector tubing. The variances of all of these contributions are additive.

Fronting - Peak shape in which the front part of a peak (before the apex) in a chromatogram tapers in advance of the remainder of the peak. There is an asymmetric distribution with a leading edge. The asymmetry factor for a fronting peak has a value <1. The opposite effect is tailing.

Guard column - A small column placed between the injector and the analytical column. Protects the analytical column against contamination by sample particulates and, perhaps, by strongly retained species. The guard column is usually packed with the same material as the analytical column and is often of the same internal diameter. It is much shorter, costs less, and is usually discarded when it becomes contaminated.

Gel filtration chromatography (GFC) - Size-exclusion chromatography carried out with aqueous mobile phases. Generally refers to separations carried out on soft gels such as polydextrans. Most gel filtration separations involve biopolymers.

Gel permeation chromatography (GPC) - SEC carried out with organic mobile phases. Used for the separation and characterization of polymers. SEC with aqueous mobile phases is referred to as a aqueous GPC, or GFC.

Ghost-peak - Spurious signal due to sample carryover in a syringe or injection valve or possibly from the column itself.

Gradient elution - Technique for decreasing separation time by increasing mobile phase strength (or decreasing mobile phase strength in HIC) over time during the chromatographic separation. Gradients can be continuous or stepwise. Binary, ternary, and quaternary solvent gradients have been used routinely in HPLC.

HETP - Height Equivalent of a Theoretical Plate, a measure of chromatographic efficiency, equal to the length of a column divided by the plate count (see "efficiency"). The lower the HETP, the more efficient the column. Also called the "plate height".

HIC - Hydrophobic Interaction Chromatography.

Hydrophilic - The opposite of hydrophobic; a tendency to bind or include water. Also called "polar".

Hydrophobic - A molecular property of a functional group which causes water to be repelled or excluded. In aqueous mobile phases, hydrophobic groups tend to bind together. The hydrophobic effect is what causes oil (hydrophobic) and water to separate. Also called "nonpolar".

Hydrophobic interaction chromatography - (HIC) Chromatographic separation based on surface hydrophobic functionality of proteins and peptides. Usually performed using packings with weakly hydrophobic bonded phases, using a starting mobile phase of very high ionic strength (to promote hydrophobic binding) and eluting using decreasing salt concentration.

Immobilized - Bound to a surface, usually through covalent chemical bonds.

In-line filter - A device that prevents particulate matter from damaging the column. Modern in-line filters can be placed between the injector and the column without contributing to band broadening. A filter in this position is used to prevent sample particulates from entering the packed bed or the inlet frit.

Ion exchange chromatography - Chromatographic separation based on binding of opposite ionic charge groups on the stationary phase and the molecules in the sample.

Ionic strength - A measure of the concentration of salt in a solution, which includes a factor for the chemical activity of the salt ions.

Ion-pair chromatography - Form of chromatography in which ions in solution can be “paired" or neutralized and separated as an ion pair on a reversed-phase column. Ion-pairing agents (or reagents) are usually ionic compounds that contain a hydrocarbon chain that imparts hydrophobicity so the ion pair can be retained on a reversed-phase column.

Isocratic elution - Elution of solutes from a column using a mobile phase of constant composition.

Isoelectric point - The pH at which the net or total charge of a molecule is zero (i.e., the total number of positive charges equals the total number of negative charges).

Ligand - In affinity chromatography, refers to the biospecific material (enzyme, antigen or hormone) coupled to the support (carrier) to form the affinity column.

Linear flow velocity (u) - The velocity of the mobile phase moving through the column. Expressed in cm/s. Related to flow rate by the cross-sectional area of the column. Sometimes expressed as "v".

Linearity - The measure of the correlation between the assay response and the concentration of an analyte. The correlation may be obtained through a well-defined mathematical transformation (e.g., semi-log or log-log). Note that over a broad enough range, all immunoassays require a transformation for linearity, due to the equilibrium nature of the binding.

Loadability - The maximum amount of sample that can be run in a given separation and still maintain the required chromatographic resolution.

Mass transfer - The process of solute movement into and out of the stationary phase or mobile phase. The faster the process of mass transfer, the better the efficiency of the column. In HPLC, mass transfer is the most important factor affecting column efficiency. It is increased by the use of small-particle packings, thin layers of stationary phase, low-viscosity mobile phases and higher temperatures.

Mass recovery - The mass of sample material recovered in the eluate divided by the mass injected on the column.

Mixed mode - Any chromatographic separation in which there is more than one mode of binding interaction at work at the same time. Note that virtually all chromatographic packings have some mixed mode effects.

Mobile phase - The fluid pumped through the column to elute the sample.

Mode - The class of binding interaction between the molecules in the sample and the bonded phase.

Multidimensional chromatography - The use of two or more columns or chromatographic techniques to effect a better separation. This may be useful for sample cleanup, increased resolution and increased throughput. Also called multicolumn chromatography.

Noise - Random fluctuation of the chromatographic signal; short-term noise (less than 1 sec) is often electrical in nature; long-term noise can be due to flow rate changes, temperature changes, or column “bleed".

Nonspecific binding - Binding of a protein or other solute to an HPLC column by some mechanism other than that of the column design.

Normal-phase chromatography - A mode of chromatography carried out with a polar stationary phase and a nonpolar mobile phase. Adsorption on silica gel using hexane as a mobile phase would be a typical normal-phase system. Also refers to the use of polar bonded phases, such as CN or NH_2.

Packing - The adsorbent, gel or solid support used in the HPLC column. Most HPLC packings are <10 µm in average diameter.

Particle size or particle diameter (dp) - The average particle size of the packing in an LC column. A 5 µm column would be packed with particles having definite particle size distribution; packings are never monodisperse.

Partition chromatography - Separation process in which one of the liquid phases is held stationary on a solid support while the other is allowed to flow freely down the column. Solutes partition themselves between the two phases based on their individual partition coefficients. Liquid-liquid chromatography is an example.

Partition coefficient (K) - The amount of solute in the stationary phase relative to the amount of solute in the mobile phase. Can be the distribution coefficient, K_D.

Peaks - Bands of individual, separated molecules in the eluate stream during a separation, which cause concentration peaks to show up on the chromatogram.

Peak shape - Describes the profile of a chromatographic peak. Theory assumes a Gaussian peak shape (perfectly symmetrical). The peak asymmetry factor describes shape as a ratio.

Polymeric packings - Packings based on polymeric materials, usually in the form of spherical beads. Common polymers used in LC are polystyrene divinylbenzene, polyacrylamide, polymethylacrylate, polyethyleneoxide, polydextran, and polysaccharide.

Post-column derivatization - Post-HPLC derivatization of amino acids or peptides for the purpose of detection.

Preparative chromatography - The process of using liquid chromatography to isolate a sufficient amount of material for other experimental or functional purposes. For pharmaceutical or biotechnological purifications, columns several feet in diameter can be used for multiple grams of material. For isolating just a few micrograms of a valuable natural product, an analytical column can be used.

Pressure drop - The difference in pressure between the inlet and outlet of a column during flow caused by the hydrodynamic resistance of the packed bed.

Reduced plate height (h) - Used to measure efficiencies of columns. A reversed phase column with h value greater than or equal to 6 is considered to be well packed. h= H/dp, where dp is the particle diameter and H is the height equivalent to a theoretical plate.

Regeneration - Returning the packing in the column to its initial state after gradient elution. Mobile phase is passed through the column stepwise or in a gradient. The stationary phase is solvated to its original condition. In ion exchange chromatography, regeneration involves replacing ions taken up in the exchange process with the original ions that occupied the ion exchange sites. Regeneration can also refer to bringing back any column to its original state (e.g., the removal of impurities with a strong solvent).

Reproducibility - Run-to-run consistency of an elution profile of an identical sample on the same column and under the same condition.

Resolution - A measurement of the full degree of separation between two chromatographic peaks, defined as the difference in retention divided by the average of the peak widths.

Retention - the time or volume between sample injection and the elusion of a given peak.

Retention volume (V_r) - The volume of mobile phase required to elute a substance from the column. $V_r = t_r$ (F) where F is the flow-rate.

Reversed-phase chromatography (RPC) - The most common HPLC mode. Uses hydrophobic packings such as octadecyl or octylsilane phases bonded to silica or neutral polymeric beads. Mobile phase is usually water and a water-miscible organic solvent such as methanol or acetonitrile. There are many variations of RPC in which various mobile phase additives are used to impart a different selectivity. For example, for the RPC of anions, the addition of a buffer and tetraalkylammonium salt would allow ion pairing to occur and effect separations that rival ion-exchange chromatography.

Ruggedness - The ability of an assay to withstand small changes in particular operating parameters without affecting the performance. A critical component of precision.

Sample capacity - Refers to the amount of sample that can be injected onto an LC column without overload. Often expressed as grams of sample per gram of packing. Overload is defined as the sample mass injected at which the column efficiency falls to 90 % of its normal value.

Sample matrix - All of the molecular components in a sample aside from the analyte itself. Changes in the sample matrix may affect the outcome of an assay in a range of different ways.

Sample recovery - Amount of desired (pure) product obtained from a sample mixture following preparative HPLC. Often expressed as a percentage of original sample load.

Selectivity (μ) - The selectivity of a sorbent in all chromatographic modes can be defined as the relative separation achieved between adjacent solute peaks and will be reflected in the overall performance of the chromatographic system. Fixed by a certain stationary phase and mobile phase composition, the selectivity is given by the ratio of capacity factors for adjacent peaks. a= k'l/k'2. As the value of a approaches 1, selectivity declines.

Sensitivity - The ability of an assay to detect very small amounts of the target analyte. Often expressed as the detection limit.

Size-exclusion chromatography (SEC) - A major LC mode in which samples are separated by virtue of their size in solution. Also known as size-exclusion, gel permeation, gel filtration, or gel chromatography.

Silanol - The Si-OH group found on the surface of silica gel. There are different strengths of silanols, depending on their location and relationship to each other. The strongest silanols are acidic and often lead to undesirable interactions with basic compounds during chromatography.

Silica gel - The most commonly used packing in liquid chromatography. It has an amorphous structure, is porous, and consists of siloxane and silanol groups. It is used as a bare packing for adsorption, as the support in liquid-liquid chromatography or for chemically bonded phases, and, with various pore sizes, as packing in size-exclusion chromatography. Microparticulate silicas of 5- and 10-μm average particle diameter are used in HPLC.

Solute - The dissolved component of a mixture that is to be separated in the chromatographic column; also referred to as the analyte.

Solvent strength - Refers to the ability of a solvent to elute a particular solute or compound from a column.

Specificity - The ability of an assay to measure only the target analyte without interference from other sample matrix (see above) components or undesired molecules closely related to the target. In the case of immunoassays, the cross-reactivity of the antibody is a major element of the specificity.

Stationary phase - The immobile phase involved in the chromatographic process. The stationary phase in liquid chromatography can be a solid, a bonded or coated phase on a solid support, or a wall-coated phase. The stationary phase used often characterizes the LC mode. For example, silica gel is used in adsorption chromatography, an octadecylsilane bonded phase in reversed-phase chromatography, etc.

Stepwise (Step gradient) elution - Use of eluents of different compositions during the chromatographic run. These eluents are added in a stepwise manner with a pump, or by a selector valve. Gradient elution incorporates continuous changing of solvent composition.

Suitability - The ability of an assay to meet the required performance criteria and produce data with acceptable accuracy and precision for a given type of sample.

Tailing - The phenomenon in which the normal Gaussian peak has an asymmetry factor >1. The peak will have skew in the trailing edge. Tailing is caused by sites on the packing that have a stronger-than-normal retention for the solute. A typical example of a tailing phenomenon is the strong adsorption of amines on the residual silanol groups of a low-coverage reversed-phase packing.

TFA - Trifluoroacetic acid, often used as a mobile phase additive in reversed-phase chromatography of peptides and proteins.

Theoretical plate - A concept described by Martin and Synge. Relates chromatographic separation to the theory of distillation. Measure of column efficiency. Length of column relating to this concept is called height equivalent to a theoretical plate (HETP).

Total permeation volume (Vp) - The retention volume on an SEC packing in which all molecules smaller than the smallest pore will be eluted. In other words, at Vp, all molecules totally permeate all of the pores and are eluted together.

Void - The formation of a space, usually at the head of the column, caused by a settling or dissolution of the packing. A void in the column leads to decreased efficiency and loss of resolution. Even a small void can be disastrous for small microparticulate columns. The void can sometimes be removed by filling it with glass beads or porous packing.

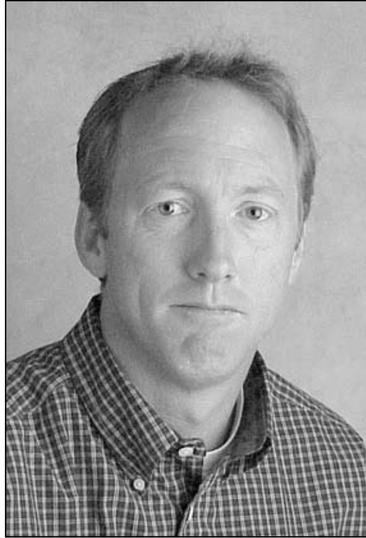
Validation - The establishment of documented evidence that an analytical test or process system will consistently produce results of sufficient quality to meet its intended purpose.

Void volume (V_o) - The total volume of mobile phase in the column; the remainder of the column is taken up by packing material. Can be determined by injecting an unretained substance that measures void volume plus extracolumn volume. Also referred to as "interstitial volume". V_i or V_m are sometimes used as symbols.

Weak ion-exchange chromatography - A form of ion exchange bonded phase functionality in which the charge density changes with mobile phase pH.

Yield - The amount of material or activity recovered from a purification step divided by the amount applied to the step, often expressed as a percent.

About the Author



Dr. Timothy Bradshaw's interest in the area of peptides and proteins dates back to his years at the University of South Carolina, where his research involved thymidylate synthase, the enzyme target for the chemotherapeutic, 5-fluorouracil. Since obtaining his doctoral degree from Carolina in 1989, he has maintained his interest in the relationship between protein function and disease, spending three years as a post-doctoral fellow at the Medical University of South Carolina, where he used HPLC to study the physical changes in red cell membrane proteins that result in hemolytic anemia. For the past eight years, Tim has worked as a scientist at Glaxo Wellcome in Research Triangle Park, North Carolina. As a member of the company's Biopharmaceutical Development, Division, he has focused on the generation of macromolecular-based therapeutics, using HPLC as a tool with which to measure peptide structure, cellular transport and stability.

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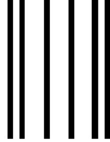

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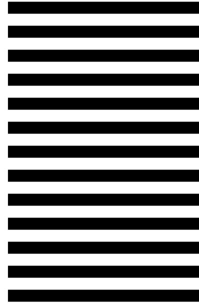

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