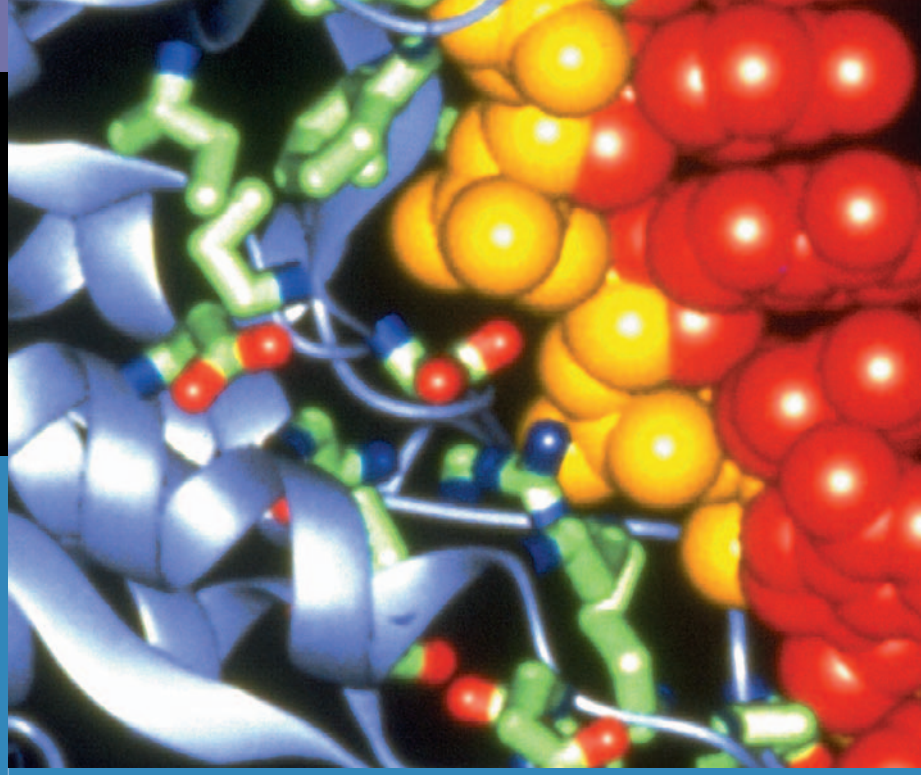
CHROMATOGRAPHY

HPLC Analysis of Biomolecules Technical Guide



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HPLC Modes for Biomolecule Separations

Introduction

Proteins, peptides, carbohydrates, lipids, amino acids, vitamins, coenzymes, and nucleic acids are a few of the members of the diverse collection of compounds involved in biochemical processes, or biomolecules. The structural and chemical diversity of this group along with the need to consider biological activity and complex matrices demands an arsenal of sample preparation, separation modes, column chemistries, column configurations, and detection techniques to effectively characterize the biomolecule under study. There are dozens of techniques or separation modes bioanalytical chemists use to analyze biomolecules. Typically, in any given separation multiple modes exist; it is rare for a separation to be purely ion exchange or purely size exclusion, for example. This is especially true for large biomolecules that may possess many different functional groups and exist in multiple conformations. The focus of this guide is on three of the most commonly encountered separation modes: reversed phase, ion exchange, and size exclusion.

Reversed Phase (page 6)

- separation is based on hydrophobicity

Ion Exchange (page 23)

- separation is based on molecular charge

Size Exclusion (page 28)

- separation is based on molecular weight and molecular shape

Regardless of the separation mode, consistent particle chemistry and efficient column packing are the keys to achieving reliable, reproducible separations of biomolecules. Table 1 presents several Thermo Scientific HPLC products for biomolecular separations.

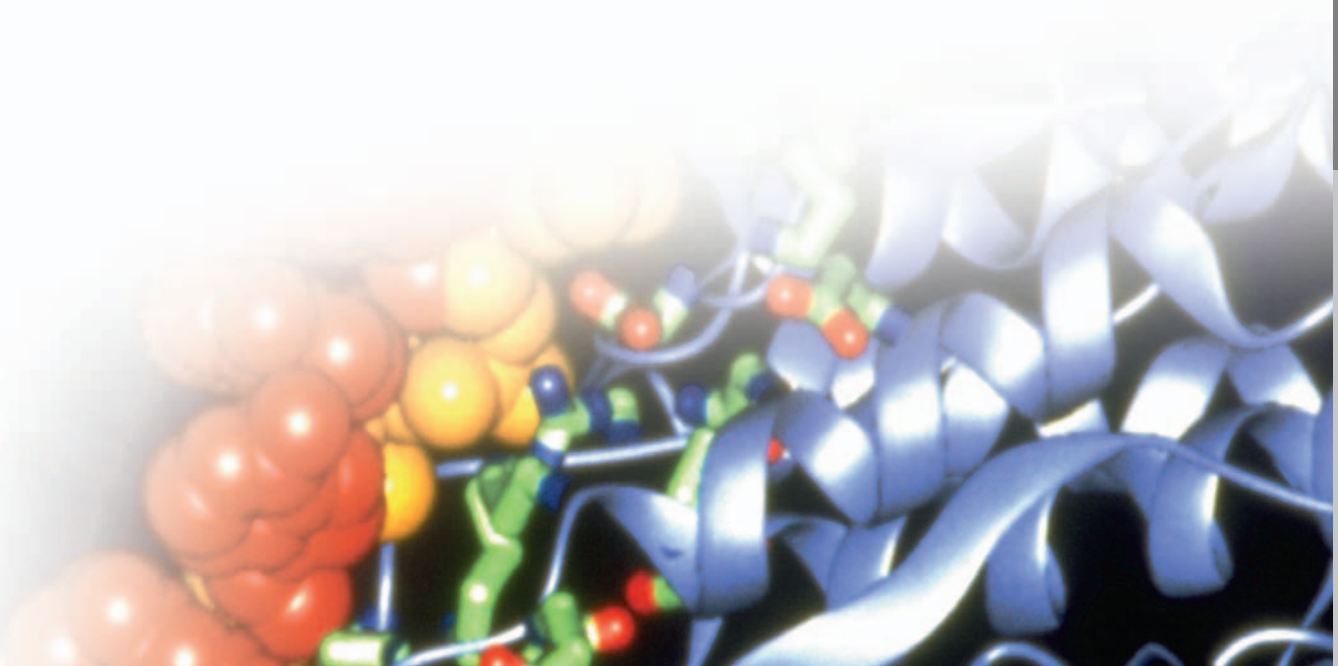
Separation Modes	Thermo Scientific HPLC Columns
Reversed phase	BetaBasic™ C18, C8, C4, CN, Phenyl BioBasic™ C18, C8, C4, CN, Phenyl Hypercarb™
Ion exchange	BioBasic AX and SCX
Size exclusion	BioBasic SEC 60, 120, 300, and 1000

Table 1: Overview of Thermo Scientific columns for biomolecule separation

BioBasic Columns:
300Å silica-based particles with superb efficiency, reproducibility, and stability for HPLC and LC/MS separations of proteins, peptides, and nucleic acids.

BetaBasic Columns:
150Å silica-based particles in a wide array of reversed phase bonded phase chemistry with superb efficiency, reproducibility, and stability for separations of small molecules, nucleotides, nucleosides, peptides, and protein digests.

Hypercarb Columns:
pH-stable carbon particles provide separations based on both hydrophobicity and molecular geometry. Truly unique selectivity compared to silica-based reversed phases with enhanced retention of polar compounds.



Reversed Phase Chromatography

Reversed Phase HPLC of Biomolecules

Reversed phase HPLC (RP-HPLC) is a very powerful and widely-used technique for separating biomolecules, both large and small. Its primary advantages over other separation modes are high efficiency and the ability to distinguish between compounds that are chemically very similar. For example, RP-HPLC can separate large peptides whose primary sequences differ by only one amino acid residue. For analysts using LC/MS in proteomics applications, it is an indispensable technique for separating the peptides in enzymatic digests of proteins.

Mechanism of RP-HPLC Separation

The term "reversed phase" is applied because the first HPLC separations employed a polar support particle and a non-polar eluent. In a typical RP-HPLC separation, these are reversed. The support particle is non-polar; either a resin or carbon particle or a silica particle which has been bonded with a non-polar molecule such as alkyl chains or phenyl rings. Common RP-HPLC eluents are polar solvents such as water, methanol, or acetonitrile.

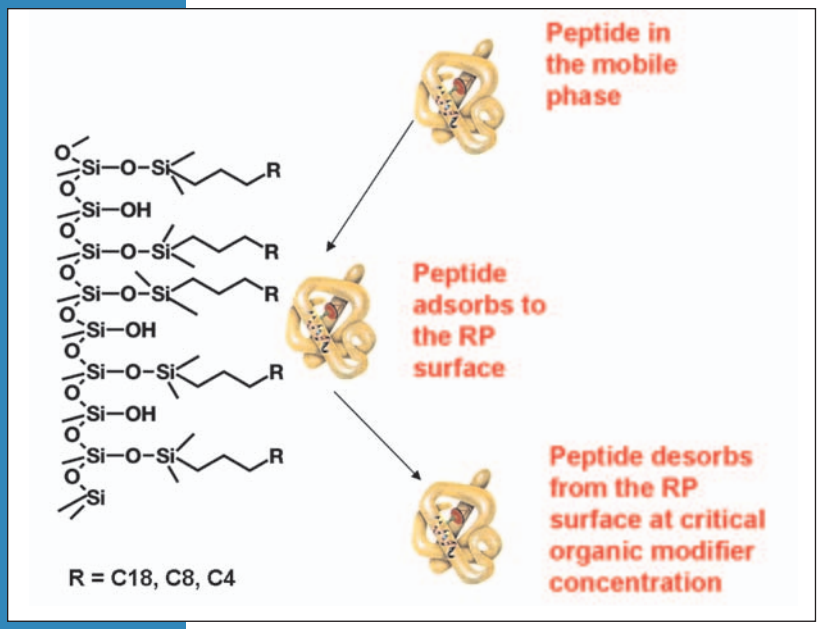


Figure 1: Interaction mechanism with RP-HPLC stationary phase

Retention of proteins and peptides by RP-HPLC usually occurs through a combination of adsorption/desorption and a partitioning type mechanism, compared to small molecules which usually separate strictly by a partitioning type mechanism. The hydrophobic "footprint" of the protein reversibly adsorbs to the bonded phase at the head or inlet of the column. As mobile phase conditions are changed, usually by increasing the concentration of organic modifier, the protein is desorbed and eluted from the column. Figure 1 provides a simplified schematic of this interaction.

Properties of the RP-HPLC column that affect the resolution are: particle size, pore size, bonded phase (functionality and chain length), and column length. Each of these parameters influences one or more variables of the chromatographic resolution equation:

[1] $$R_S = (1/4)\{(\alpha - 1)/\alpha\} N^{1/2} \{k/(1 + k)\}$$

where α is the chromatographic selectivity, N is the column efficiency, and k is the capacity factor, which is a measure of retention. Important system parameters to consider are mobile phase composition (acid, buffer, organic, *etc.*), gradient profile (rate of change, delay time, *etc.*), and the length and internal diameter of connecting tubing.

Particle Size

The diameter of the particles in the HPLC column affects column efficiency and pressure. Efficiency is important because it affects peak height/width ratio and consequently the mass of compound that can be detected above baseline, or the sensitivity of the separation. Efficiency is higher with smaller particles because they pack together more densely than larger particles thereby reducing the amount of empty space between the particles where sample dispersion could occur. A column packed with 3 µm particles will typically have about 1.5-times the efficiency per unit length as the corresponding 5 µm column. The effects of particle size are not as dramatic in gradient separations as they are with isocratic separations because of the concentration effect of the gradient. This tighter packing also produces higher pressures which can reduce system lifetime. A 3 µm column exhibits about twice the pressure as a 5 µm column when run at the same linear velocity.

The chromatograms in Figure 2 show that resolution and efficiency on the BetaBasic 18 column packed with 3 µm particles was higher than on the column packed with 5 µm particles. Higher

Column Packing	Particle	Bonded Phase*	Pore Size (Å)	Particle Size (µm)	% Carbon
BioBasic 18	Spherical, porous, highly pure silica	C18	300	5, 10	9
BioBasic 8	Spherical, porous, highly pure silica	C8	300	5	5
BioBasic 4	Spherical, porous, highly pure silica	C4	300	5	4
BioBasic CN	Spherical, porous, highly pure silica	Cyano	300	5	3.5
BioBasic Phenyl	Spherical, porous, highly pure silica	Phenyl	300	5	3
BetaBasic 18	Spherical, porous, highly pure silica	C18	150	3, 5	13
BetaBasic 8	Spherical, porous, highly pure silica	C8	150	3, 5	7
BetaBasic 4	Spherical, porous, highly pure silica	C4	150	3, 5	6
BetaBasic CN	Spherical, porous, highly pure silica	Cyano	150	3, 5	5
BetaBasic Phenyl	Spherical, porous, highly pure silica	Phenyl	150	3, 5	7
Hypercarb	Porous, graphitic carbon	None	250	3, 5, 7	100

** All BioBasic and BetaBasic phases have secondary end-capping*

Table 2: Thermo Scientific columns for RP-HPLC of biomolecules

efficiency is observed as the larger height/width ratio (and consequently better sensitivity) on the peak eluting near 10 minutes, and the better resolution of the group of peptides eluting around 12 minutes. Note also that the 3 µm particles generated nearly twice the pressure of the 5 µm particles.

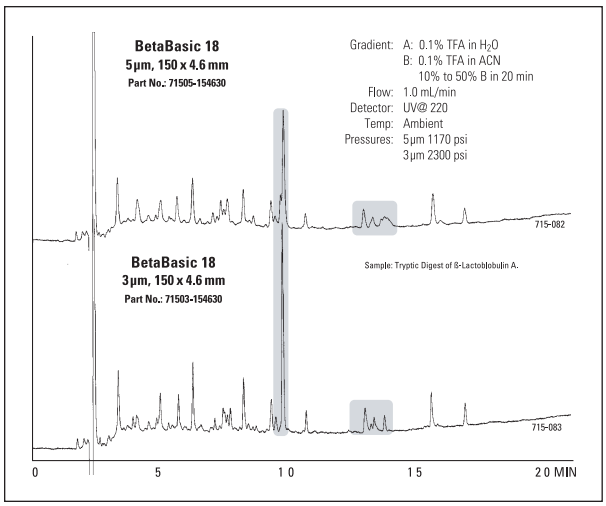


Figure 2: Effect of particle size on RP-HPLC separation of protein digest

Pore Size

Most particles used in HPLC are porous. Porous particles are more permeable and have higher surface area than non-porous particles. Most interactions between the analyte and the HPLC particle occur at the surface of the particle. With the majority of the surface area of a porous particle contained inside the pores, the pores must be large enough to provide access to the interior of the pores. Because biomolecules have a wide range of molecular weights, the pore size of the particle used in the separation is very important. Large molecules typically require particles with large pores. Small molecules do not require large pores and can therefore take advantage of the benefits of smaller pore size particles. Smaller pore size particles have higher surface area which increases the sample mass loading capacity of the column, an important consideration for preparative separations.

An example of the effect of pore size on the RP-HPLC separation of peptides is shown in Figure 3. Note the group of peaks that elute around 40 and 45 minutes on the 300Å BioBasic 18 column are better resolved than the 150Å BetaBasic 18 or the 190Å HyPURITY C18 columns.

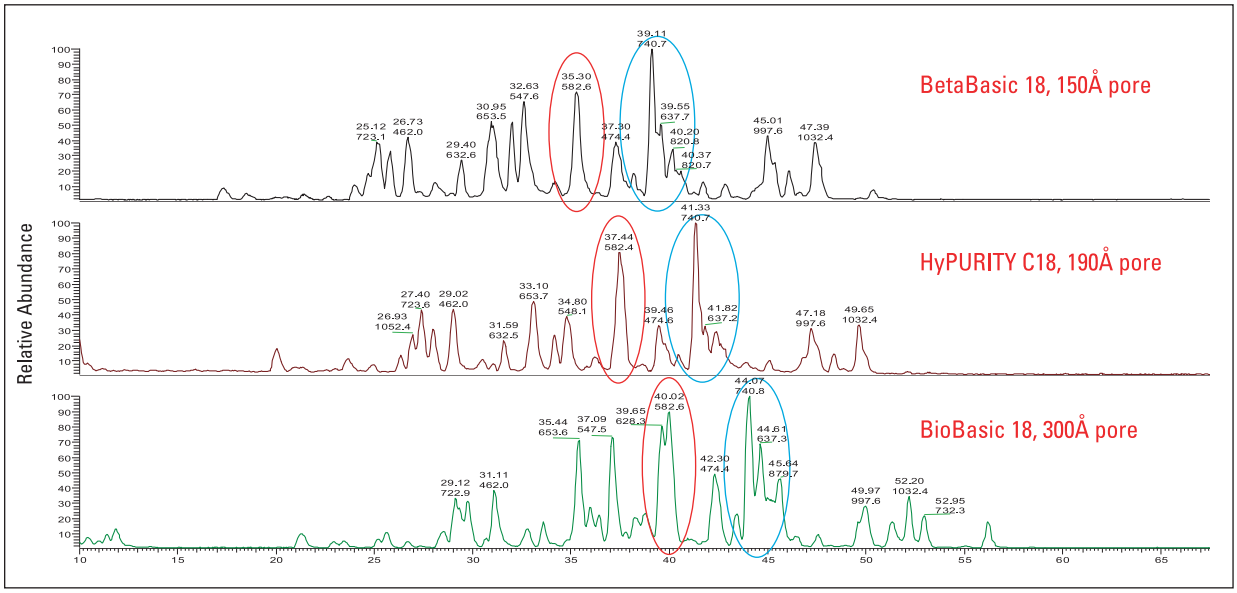


Figure 3: Effect of pore size on RP-HPLC separation of protein digest

Column Length

Retention is generally proportional to column length in reversed phase HPLC. However, protein separations tend to be an exception to this rule. Figure 4 compares 150 mm and 50 mm C8 columns for the separation of proteins. When the same 30 minute gradient program is used for both columns, the retention of the last peak, ovalbumin, is only slightly shorter for the 50 mm column. An explanation for this result is that reversed phase gradient protein separations occur primarily by an adsorption/desorption type mechanism, rather than the typical partitioning mechanism. The adsorption and desorption of proteins occurs at the head of the column with the proteins interacting very little with the majority of the column length that they encounter after desorption. If shorter columns are used with the goal of reducing analysis time, it is necessary to reduce the gradient length in proportion to column length, as shown in Figure 5.

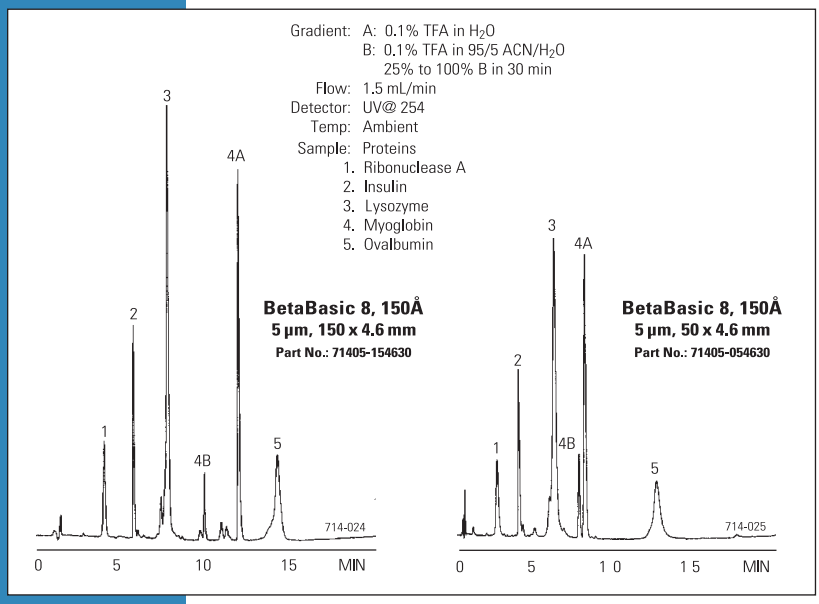


Figure 4: Effect of column length on RP-HPLC separation of proteins

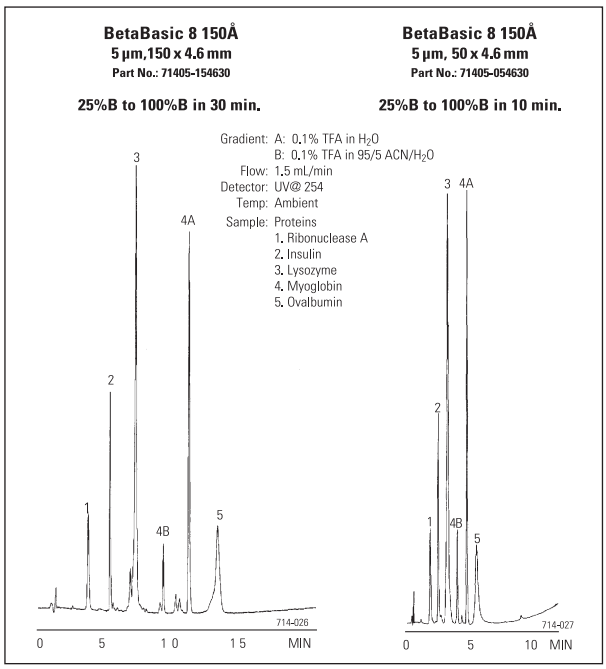


Figure 5: Column length comparison with gradient proportional to column length

Flow Rate

Increasing flow rate is the easiest way to decrease the analysis time. However, flow rate also affects column efficiency, which in turn affects resolution and pressure. Figure 6 demonstrates the relationship between column efficiency and flow rate. Note that the flow rate for optimum efficiency is dependent on the particle size of the column packing material. The optimum flow rate for a 3 µm column is approximately 1.5 times greater than that of a 5 µm column of the same ID This means a 3 µm column will not only provide more efficiency, but can do so in less time than the same length of a 5 µm column. The graph in Figure 6 could be generalized to columns of any ID by converting the values on the x-axis from flow rates to linear velocities. Linear velocity (cm/min) is the rate at which mobile phase moves through the column and can be estimated by dividing column length by the retention time of an unretained peak.

The main disadvantage of increasing flow rate is higher pressure. Elevated pressures will shorten column lifetime as well as increase wear on the HPLC system. Increasing flow rate above the optimum will also reduce column efficiency and resolution. Decreasing the flow rate reduces system wear. However, it also increases analysis time. If the flow rate is reduced below optimum for the particle size, it can reduce efficiency and resolution due to increased band dispersion.

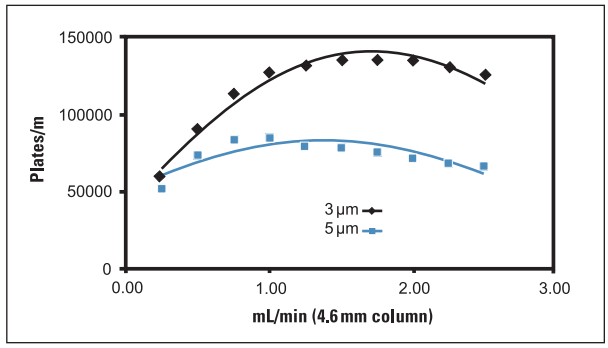


Figure 6: Effect of flow rate on RP-HPLC column efficiency

For isocratic separations, the relationship between flow rate and retention time is generally a straightforward inverse correlation; doubling the flow rate will reduce retention by one half. However, this relationship does not hold for gradient separations of proteins and peptides. As mentioned earlier, reversed phase gradient protein separations occur primarily by an adsorption/desorption type mechanism, rather than the classical partitioning mechanism. Because of the adsorption/desorption mechanism, flow rate has less effect on retention than might be expected.

Figure 7 shows a fast protein separation performed at flow rates of 0.5, 1.0 and 1.5 mL/min with other conditions held constant. Note that increasing flow rate threefold decreases retention by only about a third because the rate of gradient formation determines rate of protein migration. The benefits of reducing flow rate are decreased pressure and lowered consumption of mobile phase. Figure 7 also shows that flow rate can affect selectivity (elution order) possibly due to the different partitioning rates.

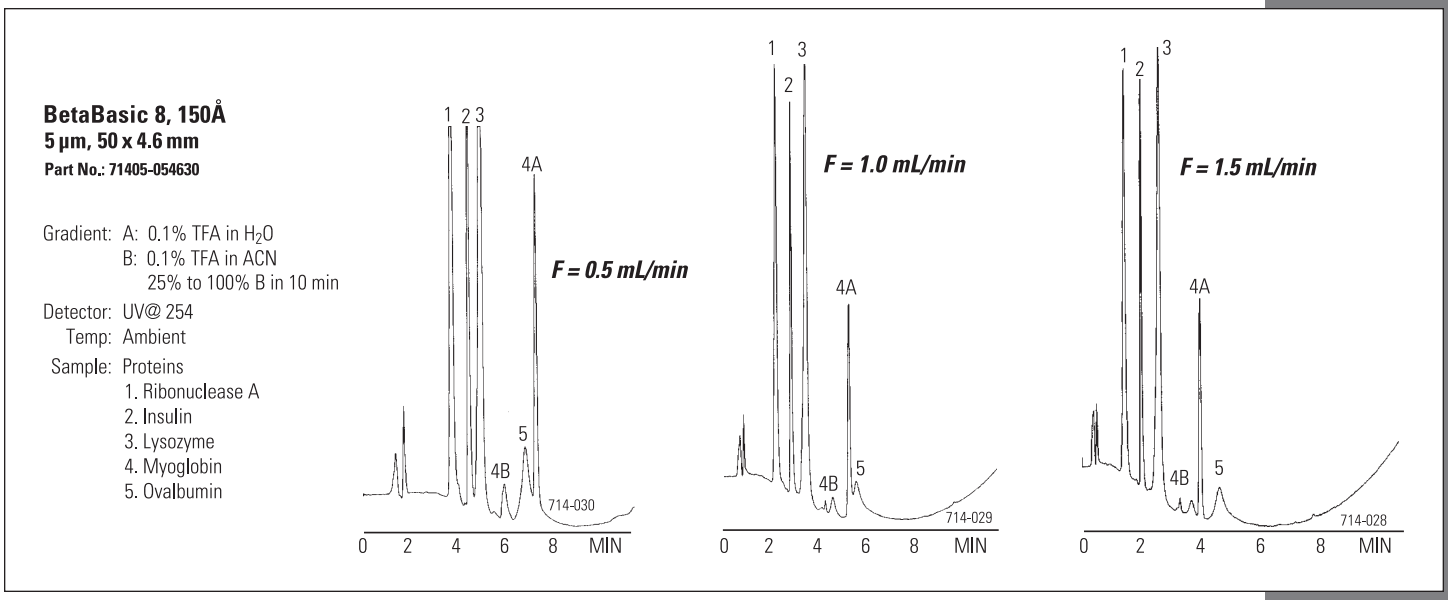


Figure 7: Effect of flow rate on gradient RP-HPLC of proteins

Bonded Phase Functionality and Alkyl Chain Length

Because the interactions between sample and the RP-HPLC particle occur at the surface, the chemistry of the particle's surface is extremely important in determining the extent to which sample components are retained by the column and separated from other components in the sample. The molecules on the particle surface are either covalently bonded to the surface, as in bonded silicas, or make up the particle itself, as in porous graphitic carbon and some resins. Surface chemistry influences resolution by affecting both the selectivity and capacity factor terms of the resolution equation.

Bioanalysts have a relatively large choice in RP-HPLC bonded phase chemistries. RP-HPLC separations of intact proteins are usually performed on wide-pore silica bonded with short alkyl chains, like the BioBasic 4 column. The short alkyl chains reduce the likelihood of protein denaturation or irreversible binding that can occur with longer alkyl chains. Different bonded phase functionalities on regular pore size silica (100 – 200Å) should be studied for separating peptides and small molecules since each phase offers different selectivity.

Most RP-HPLC phases interact with analytes via weak van der Waals or dispersive interactions, which are due to an overlap of the outer electron clouds between the analyte and bonded phase. The phases that comprise unmodified alkyl chains, C18, C8, and C4, interact solely via this mechanism. However, the other RP-HPLC phases, cyano, phenyl, and carbon, can undergo other types of interactions with certain analytes. This often leads to dramatic changes in elution patterns, even peak order reversal, compared to alkyl-only phases.

RP-HPLC Phase	Types of Interactions
C18, C8, C4	van der Waals (dispersive)
Cyano	van der Waals (dispersive), H-bond, dipole-dipole interactions
Phenyl	van der Waals (dispersive), π-π interactions
Carbon (Hypercarb, PGC)	van der Waals (dispersive), charge-induced dipole, π-π interactions

Table 3: Interaction mechanisms for different RP-HPLC phases

An example of the effect that different RP-HPLC bonded phase functionality can have on a separation is shown in Figure 8. Here, a tryptic digest of ß-lactoglobulin A was run on three different bonded phases, C8, cyano, and phenyl under the same mobile phase gradient conditions. The different elution pattern attests to the different selectivity offered by each phase. Running the same sample on columns of different bonded phase chemistry can maximize the identification of the components of a complex mixture, like a protein digest.

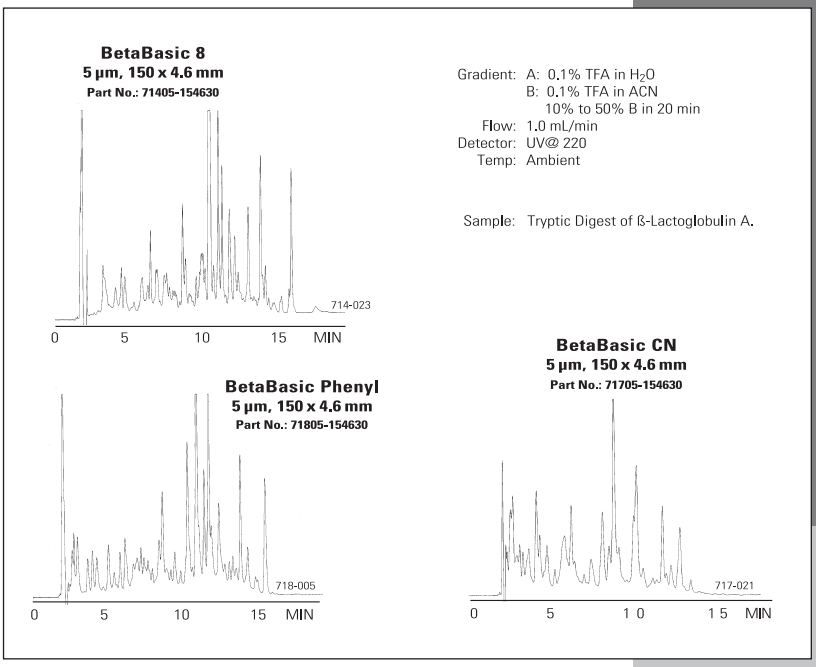


Figure 8: Effect of bonded phase functionality on RP-HPLC selectivity of protein digest

The length of the bonded phase alkyl chain can also influence the selectivity of the separation, however not to the same extent as bonded phase functionality. For small molecules (<1000MW), there is a positive linear correlation between log k (capacity factor) and bonded phase chain length; the longer the chain, the longer the retention. However, proteins and peptides are too large to access the entire alkyl chain and interact primarily with the outer surface of the bonded phase. Therefore, to a large molecule a C4 "looks" like a C8 or even a C18. However, subtle differences in selectivity can still be observed, as shown in Figure 9. Here, careful comparison of the myoglobin peaks 4A and 4B show different elution order between the C18, C8, and C4 bonded phases.

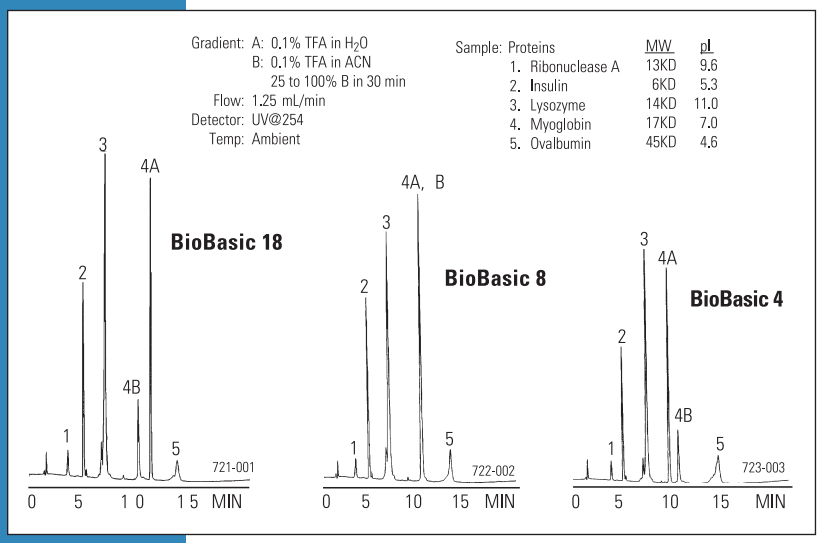


Figure 9: Effect of bonded phase chain length on RP-HPLC selectivity

Porous Graphitic Carbon (PGC)

Other media alternatives to separating proteins and peptides do exist outside of silica. One such alternative is porous graphitic carbon, or PGC. The PGC mechanism of interaction is very dependent of both the polarity and shape of the molecule approaching the graphite surface. As a RP-HPLC particle, PGC has two distinct differences compared to silica-based particles for biomolecule separations. First, because it is composed entirely of carbon, it is not susceptible to acidic or basic hydrolysis and can therefore be used from pH 0 to pH 14 without degradation. Silica-based phases can lose bonded phase under acidic conditions, and the silica can dissolve under basic conditions. Figure 10 shows a mixture of hydrophilic peptides run under basic conditions (pH 10.5) on a Hypercarb PGC column. Under these conditions, most silica-based columns would rapidly deteriorate.

The second difference between carbon and silica is that carbon particles exhibit longer retention of polar compounds, entities that are difficult to retain using silica-based RP-HPLC phases. Many biomolecules are too polar to be retained on C18 silica particles. Examples include carbohydrates (oligosaccharides), nucleosides and nucleotides, underivatized amino acids, and water-soluble vitamins. Several of these compounds classes are shown on PGC in Figures 11, 12, and 13. Figures 11 and 12 show polar, low molecular weight compounds that typically are unretained on a C18-silica column without derivatization. However, they are retained and resolved by a PGC column. Most polar compounds require derivatization to increase their hydrophobicity in order to be retained by silica-based RP-HPLC phases. However, the retention of polar compounds is strong enough that derivatization is usually unnecessary. In fact, the retention is so strong on PGC that it is not recommended for non-polar compounds, like fused-ring systems and hydrophobic peptides and proteins. The oligosaccharides shown in Figure 13 would normally require derivatization and the use of several types of column chemistries for retention. However, because the oligosaccharides are well retained on the PGC column, the separation and characterization of diverse, underivatized oligosaccharides in a glycoprotein is possible in a single analysis.

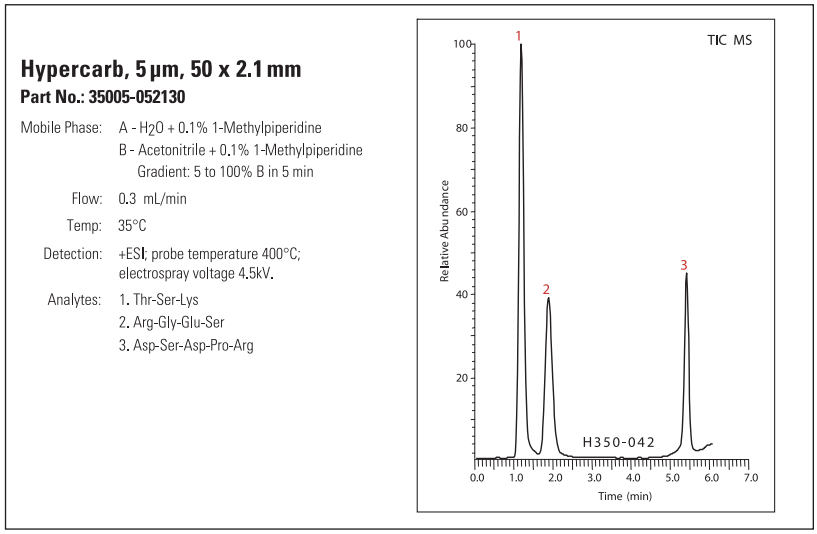


Figure 10: Hydrophilic peptides at high pH on PGC

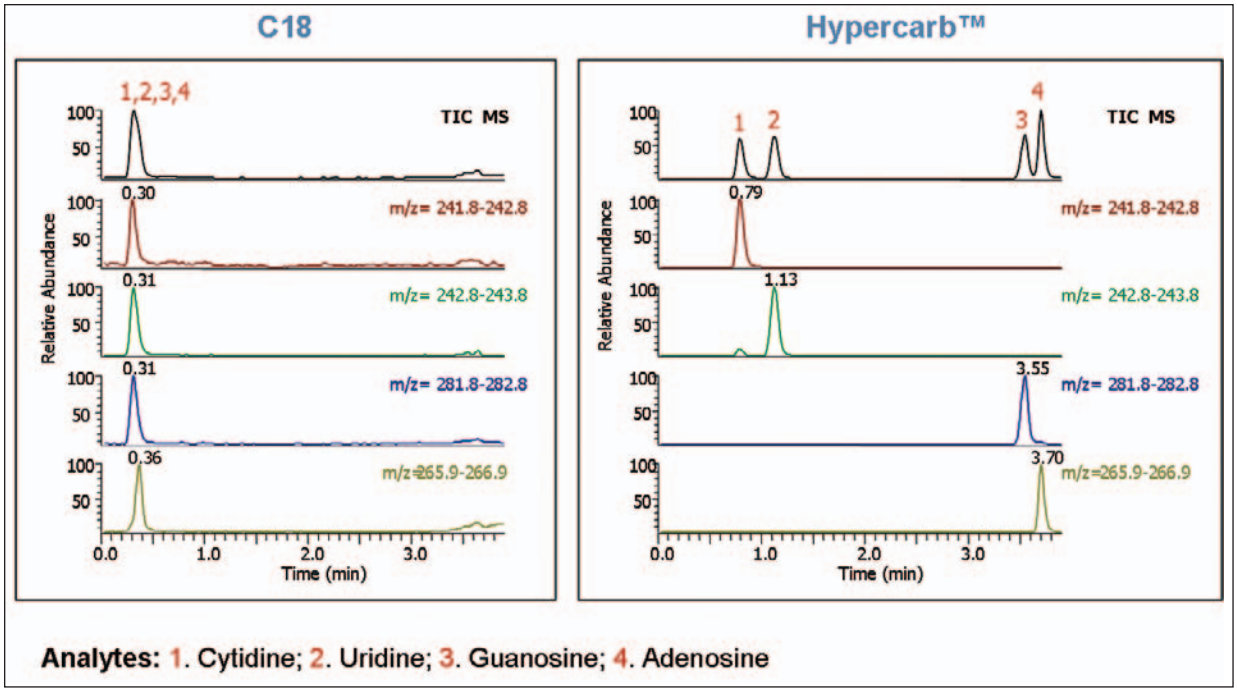


Figure 11: Retention of nucleosides on C18 vs. PGC

Hypercarb, 5 µm, 100 x 0.32 mm

Part No.: 35005-100365

Mobile Phase: A - H_2O + 0.1% + 0.1% formic acid
B - Acetonitrile + 0.1% formic acid
Gradient: 7 to 100% B in 5 min

Flow: 7µL/min

Temp: 35°C

Detection: +ESI; probe temperature 400°C; electrospray voltage 4.5kV.

Analytes: 1. Ornithine
2. Methionine
3. Phenylalanine
4. Tryptophan

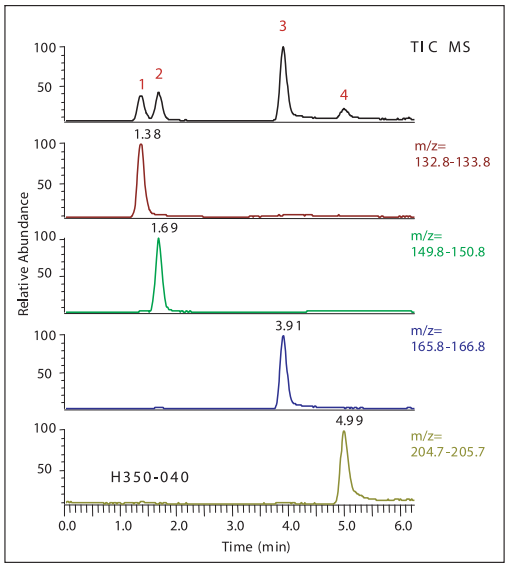


Figure 12: Amino acids on PGC

Hypercarb, 5 µm, 100 x 1.0 mm

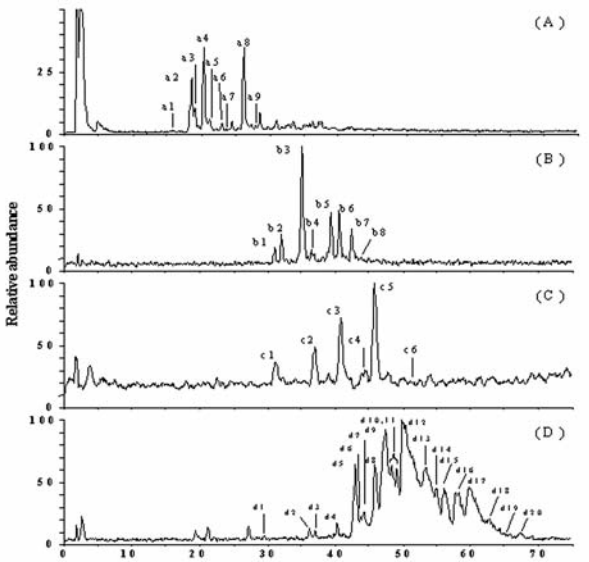
Part No.: 35005-101030

Eluent: A: Ammonium acetate 5 mM, pH 9.6 + 2% ACN
B: Ammonium acetate 5 mM, pH 9.6 + 80% ACN

Gradient: 5 to 40% B in 80 min

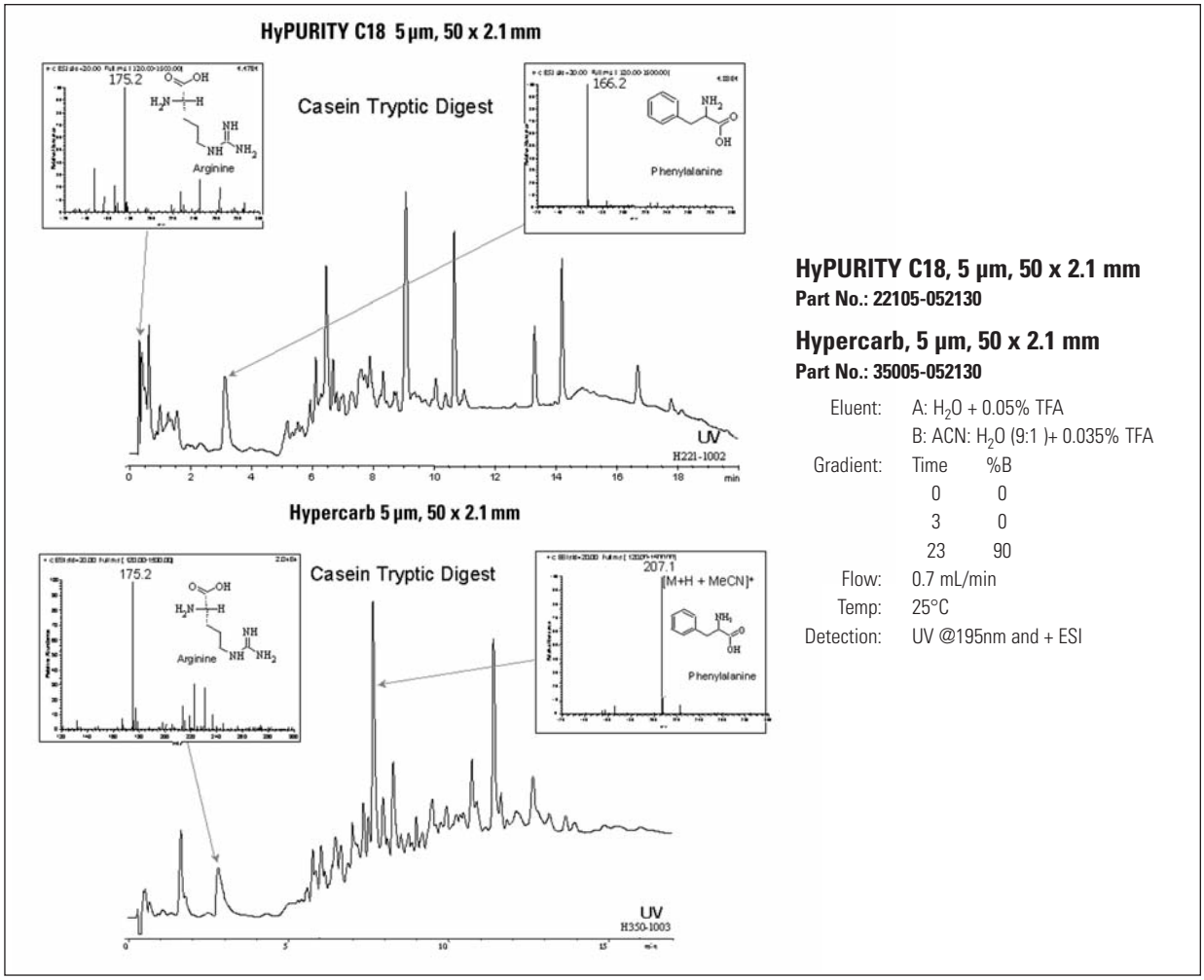
Flow Rate: 50 µL/min

Detection: +ESI



Data courtesy of Nana Kawasaki, National Institute of Health Science, Tokyo, Japan

Figure 13: Oligosaccharides from a glycoprotein on PGC



HyPURITY C18, 5 µm, 50 x 2.1 mm
Part No.: 22105-052130

Hypercarb, 5 µm, 50 x 2.1 mm
Part No.: 35005-052130

Eluent:	A: H_2O + 0.05% TFA
	B: ACN: H_2O (9:1)+ 0.035% TFA
Gradient:	Time %B
	0 0
	3 0
	23 90
Flow:	0.7 mL/min
Temp:	25°C
Detection:	UV @195nm and + ESI

Figure 14: Comparison of tryptic digest on C18-silica and PGC columns

PGC can be used for peptide maps as well, and serve as a demonstration of its unique selectivity and ability to retain polar compounds better than a C18-silica column. Note in Figure 14, a tryptic digest of casein, the longer retention of amino acids arginine and phenylalanine and the overall different elution pattern provided by the PGC column compared to the C18-silica column.

Guidelines for Choosing an RP-HPLC Column

This list serves as a starting point for choosing a RP-HPLC phase based on the properties of the biomolecule. However, when developing a method one should try many different particle chemistries in order to optimize the separation.

Proteins	C4 on wide-pore (>300Å) silica
Hydrophobic peptides	C4-silica, C8-silica, cyano-silica
Hydrophilic peptides	C18-silica, Hypercarb
Peptides	C18-silica, phenyl-silica
Amino acids (derivatized)	C18-silica
Amino acids (underivatized)	Hypercarb PGC
Nucleosides, nucleotides	Hypercarb PGC
Small molecules	C18-silica, cyano-silica, Hypercarb PGC
Carbohydrates, oligosaccharides	Hypercarb PGC
Lipids	C18-silica with 100% organic mobile phase

Table 4: Phase choice based on sample type

Mobile Phases for RP-HPLC

Most biomolecules exist naturally in highly aqueous environments stabilized with ions and other water-soluble compounds that help maintain their biological activity. The ability to use aqueous mobile phases that contain salts and buffers is a main reason why liquid chromatography techniques, including reversed phase, ion exchange, and size exclusion, are so ubiquitous in biomolecule analysis. Rarely does one encounter normal phase liquid chromatography or gas chromatography where water and salts are not compatible with instruments and high organic mobile phases.

RP-HPLC mobile phases for protein and peptide separations are usually gradients of an organic modifier (acetonitrile or methanol) in water, with an ionic modifier that controls the pH and ionization state or acts as an ion pair agent. Trifluoroacetic acid (TFA) is a common ionic additive because it solubilizes most proteins by forming ion pairs; however it has limitations for LC/MS. For proteins and peptides, gradient elution is preferred because proteins elute over a wide percentage of organic modifier. Isocratic elution results in very broad peaks. For small molecule separations, gradient elution is used when the compounds differ widely in hydrophobicity. A list of commonly used organic modifiers and ionic additives is found in the Appendices. The rationale behind the major mobile phase variables is described in the paragraphs below.

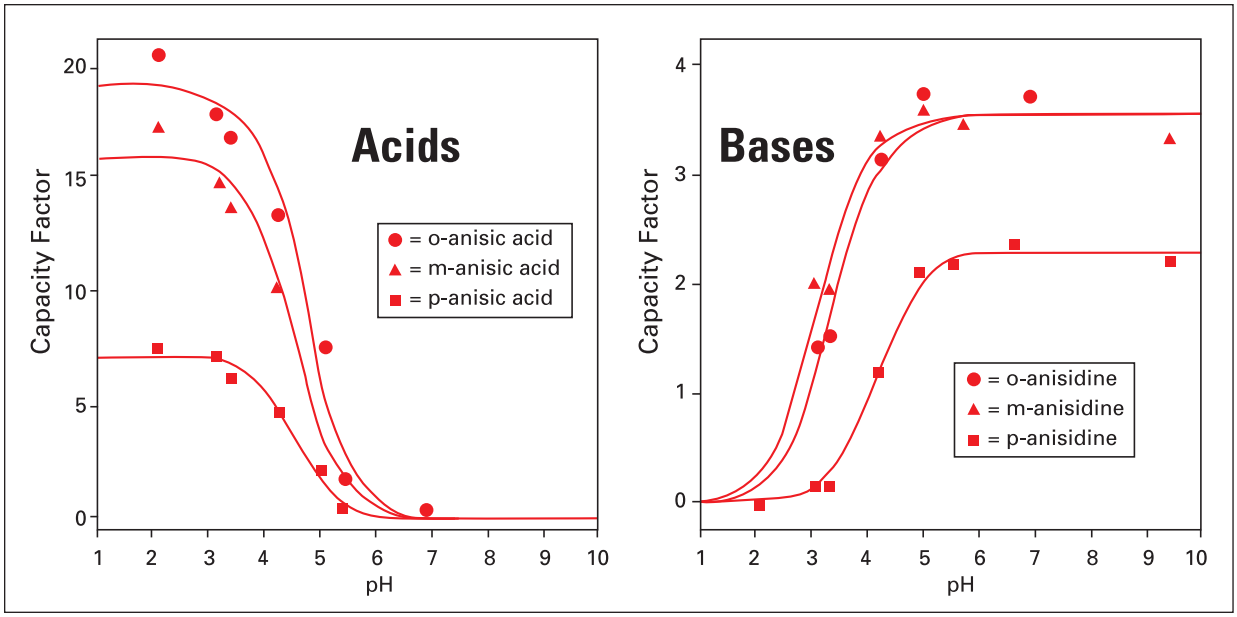


Figure 15: Effect of pH on RP-HPLC retention of acids and bases

Effect of pH on RP-HPLC Analyses

Because pH dictates the degree of ionization of compounds that possess ionizable groups, the pH of the mobile phase influences both RP-HPLC retention and ionization efficiency in LC/MS. Proteins and peptides have carboxylate (-COOH) and amine ($-NH_2$) groups at their C and N termini, respectively. They also have various R groups each with its own pK_a value. The list of each amino acid and the associated pK_a values is found in the Appendices. Since reversed phase retention is based upon the molecule's hydrophobicity, the pH where it is least ionized will provide the longest retention. An acidic group will be ionized (deprotonated) at pH above its pK_a while a basic group will be ionized (protonated) at pH below its pK_a. Because most biomolecules possess more than one ionizable group, the overall hydrophobicity and retention is a result of the sum of number of ionized and non-ionized functional groups. This relationship is shown in Figure 15 which plots the capacity factor (k) versus the pH for three acids and three bases each possessing a single ionizable group. Note at low pH the acidic compounds are retained longer, while high pH is needed to achieve retention of basic compounds.

When performing LC/MS analyses there is a balance between ionization efficiency (sensitivity) and chromatographic performance. The pH of the mobile phase will dictate whether analytes are positively or negatively charged, and the overall extent of charge. More discussion on the influence of pH on LC/MS is found in the LC/MS section of this guide.

Effect of Ionic Modifier on RP-HPLC Analyses

RP-HPLC mobile phases are typically mixtures of water and acetonitrile or methanol. The water usually contains ionic additives (buffers, salts, surfactants, ion pair agents, *etc.*) that serve to adjust or control the pH, control the ionization of the analyte, enhance retention by forming ion pairs, reduce interactions between the analyte and the silica surface, maintain the analyte's solubility or biological activity, or a combination of these functions. A list of commonly encountered ionic additives for HPLC of biomolecules appears in the Appendices. The additive may also alter the selectivity of the separation as well. Figure 16 shows the different elution pattern of angiotensin peptides on a C8 column using either 0.01% TFA or 1.0% acetic acid ionic additives.

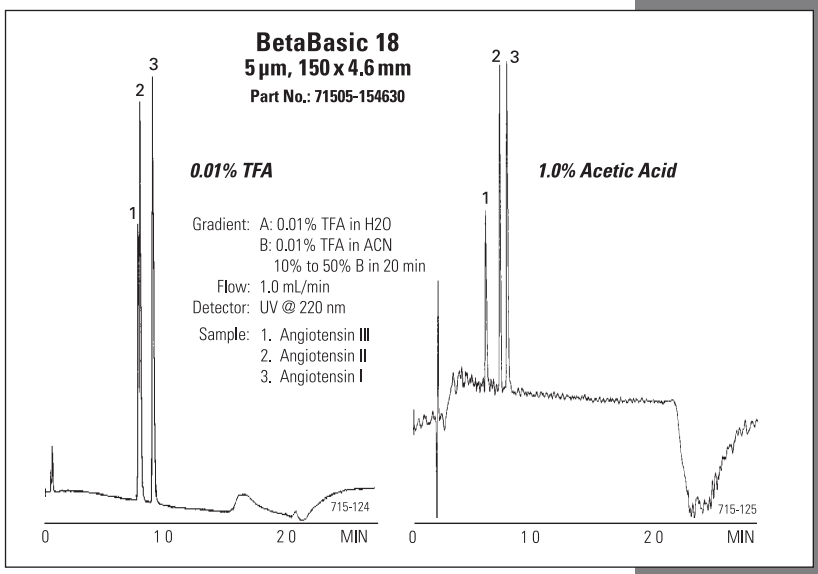


Figure 16: Effect of acidic modifier on selectivity

There are several factors that must be taken into account when choosing an ionic additive for HPLC. First, it must be compatible with the detection method. Many biological buffers have UV absorbance and give high background reducing the sensitivity of UV analyses. If various purity grades of the additive are available, a grade suitable for high sensitivity use is necessary. Most buffers are supplied in HPLC grade. LC/MS compatibility is very important and will be dealt with in the LC/MS section of this guide. Second, the additive must remain in solution when combined with the organic modifier or upon any other changes in the system. Ionic additives may precipitate over time or after refrigeration, are prone to microbial growth, and must be filtered to remove particulate matter initially and during extended periods of use to prevent fouling of the system.

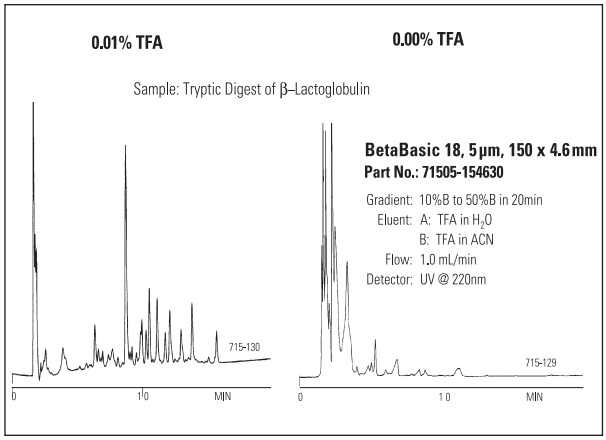


Figure 17: Peptide retention by RP-HPLC with and without TFA in the mobile phase

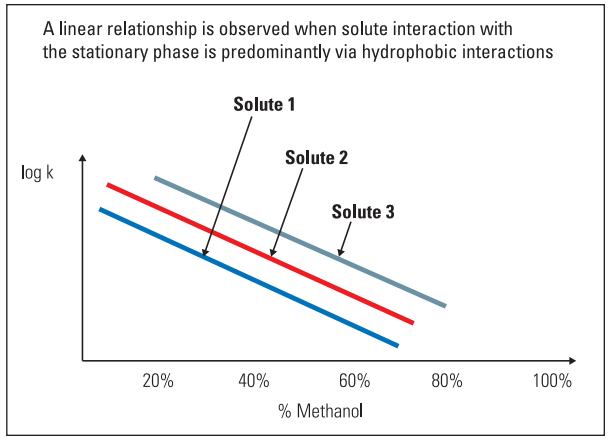


Figure 18: Effect of organic modifier on isocratic retention

Bioanalysts performing RP-HPLC of proteins and peptides will undoubtedly encounter trifluoroacetic acid (TFA) as a mobile phase additive. Typical TFA concentrations are 0.05 – 0.1% by volume added to both the aqueous and organic portions of the mobile phase. TFA acts as an ion pair agent, associating with positively charged functional groups on peptides and proteins, which increase reversed phase retention. An example of this is shown in Figure 17. A small amount, 0.01% v/v, of TFA in the mobile phase had a dramatic effect on the retention of peptides on the C18-silica column.

TFA has several advantages over other ion pairing reagents and buffers. Because it is a very strong acid, it effectively suppresses the ionization of carboxyl groups and keeps amine groups fully protonated. By lowering the pH, TFA also insures that any residual silanol groups on the silica will be protonated, thus eliminating any possible ion exchange interaction with basic solutes. Also, because it is volatile it can be easily removed from solution following preparative purification without the need for dialysis. In addition, it has very little absorbance even at low UV wavelengths and solubilizes very hydrophobic peptides. For these reasons and because it has an extensive history of proven applications, TFA remains the most common first choice for ion pairing of proteins and peptides. TFA has limitations for LC/MS, however. The high surface tension of a 0.1% TFA solution makes spray formation (nebulization) difficult. Also, because TFA forms strong ion pair complexes with the protein's amine groups it reduces the ionizability of the protein and thereby reduces LC/MS sensitivity.

Effect of Organic Modifier Type and Concentration on RP-HPLC Retention

RP-HPLC organic mobile phase modifiers are almost exclusively acetonitrile or methanol. The role of the organic modifier is to compete against the bonded phase molecule for attraction of the analyte molecules. Generally speaking, the higher the organic concentration the stronger the mobile phase and the shorter the retention. Very polar analytes require low concentrations of organic to elute from the column, while non-polar compounds require higher concentrations. In gradient elution, analyte molecules remain associated with the bonded phase until the concentration of organic modifier in the mobile phase reaches a point where they are released. In isocratic elution there is a dynamic equilibrium down the length of the column with the analyte partitioning between the mobile phase and the stationary phase. The effect of organic modifier concentration on log k (capacity factor) is linear in a RP mechanism. An example of this relationship is seen in Figure 18.

Important properties of acetonitrile and methanol and other less common modifiers appear in the Appendices. Like ionic additives, the organic modifier must be compatible with the detection method, providing no background interference. The concentration of organic modifier must also not exceed the solubility of any component in the system, including ionic additives and compounds in the sample itself. Proteins are especially prone to precipitation at high organic so extreme care must be taken to prevent system fouling.

As organic modifiers for RP-HPLC, methanol and acetonitrile differ in several ways. First, for very low UV work, methanol is preferred as it has a lower UV-cutoff than acetonitrile. Second, aqueous mixtures of the two solvents give very different viscosity vs. composition curves, as seen in Figure 19. Mobile phases containing 50% methanol have significantly higher viscosity and subsequently higher pressure. This is a concern with high speed (high flow rates) applications, or when using narrow ID columns. For LC/MS, acetonitrile is preferred because its aqueous mixtures have lower surface tension and desolvate more rapidly. Third, methanol and acetonitrile have different selectivity for certain compounds and may give different elution patterns.

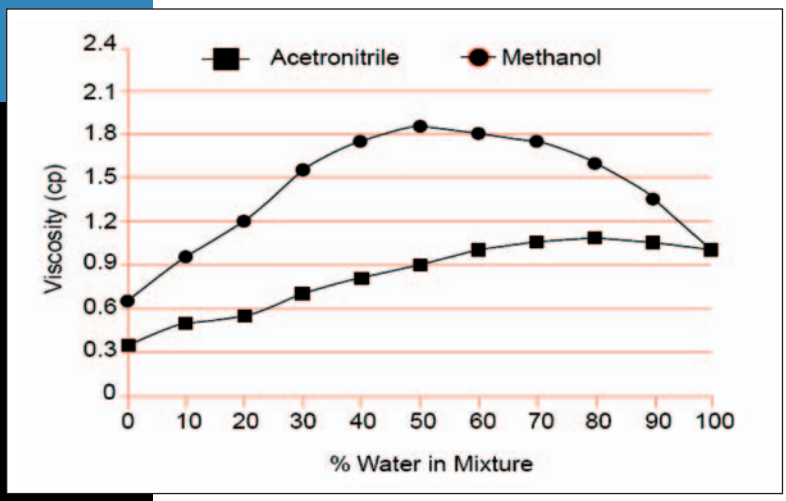


Figure 19: Effect of % organic modifier on viscosity

Effect of Temperature on RP-HPLC Retention

Column temperature can sometimes be used to beneficially alter selectivity. Increasing temperature also reduces overall retention in RP-HPLC. The effects of increasing column temperature are shown for the separation of a tryptic digest in Figure 20. Decreasing retention with increasing temperature is most easily seen by comparing the largest peak of each chromatogram. The highlighted areas of each chromatogram show some changes in selectivity that occurred with temperature. The relative positions of several small peaks near the largest peak appear to change with temperature, especially between ambient and 40°C. It also appears that two late eluting peaks reverse elution order between 40°C and 50°C. Elevated temperatures can somewhat improve efficiency and resolution by lowering mobile phase viscosity and improving mass transfer.

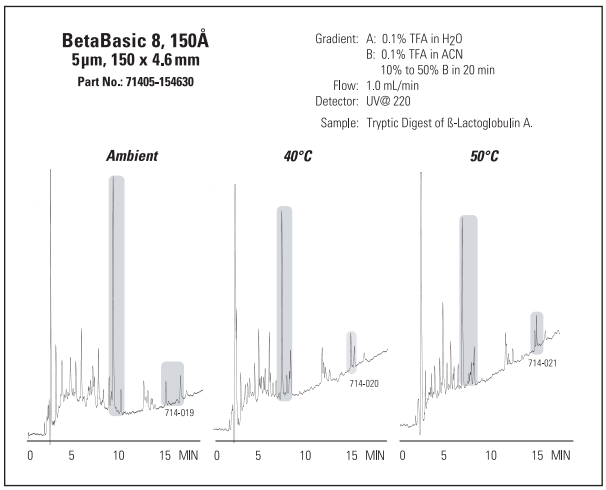


Figure 20: Effect of temperature on RP-HPLC separation of peptides

Gradient vs. Isocratic Elution

For proteins and peptides, gradient elution is preferred because proteins elute over a wide percentage of organic modifier. Isocratic elution results in very broad peaks. Most common are binary gradients comprised of increasing percentages of acetonitrile in water, both containing the same concentration of a suitable ionic modifier. The characteristics of the gradient have a profound effect on the resolution of the system; even more so than the column chemistry in many cases. Some key gradient parameters that must be optimized are:

- Flow rate (dependent on column ID)
- Delay time (time spent at initial % organic)
- Starting % organic
- Ending % organic
- Hold time (time spent at final % organic)
- Rate of change (slope or % organic/time)
- Gradient shape (linear, concave, convex)
- Re-equilibration time (volume needed to reestablish initial conditions)

Length and ID of the tubing connecting the pump to the injector add unnecessary mixing volume to the system. Both length and ID of this tubing should be kept to a minimum. To minimize the baseline change during the gradient, it is good practice to have solvent A contain 10% of the organic modifier and have solvent B contain the final percentage (50% for example).

A good starting point for most peptide separations is a linear gradient of 10% B – 100% B (where solvent A is 10% acetonitrile and B is 50% acetonitrile, both containing the same concentration of the ionic additive). The ionic additive must be soluble in both A and B solvents. For UV analysis, 0.01 – 0.1% TFA is the most common additive. For LC/MS, other volatile additives like acetate, formate, or HFBA are preferred. To optimize the gradient method, the first parameter to reduce is any areas of the chromatogram where no peaks elute. If this empty region is early in the gradient, then start with a higher percentage of organic. If it is late in the gradient then end with a lower percentage of organic. The next parameter to change is the slope, or % organic/time. Generally speaking, flatter slopes provide better resolution. However, this also increases the overall run time. If more resolution is required, increasing the run time and the column length are two viable options. Figure 21 shows how decreasing the rate of change of organic modifier improved the resolution of a tryptic digest on a C18 column.

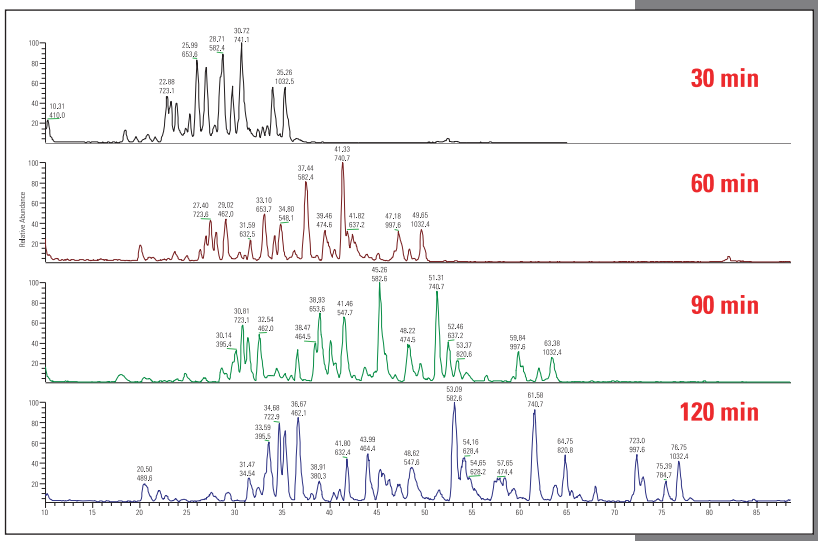


Figure 21: Effect of gradient length on BSA digest analysis.

RP-LC/MS of Biomolecules

HPLC with mass spectrometric detection (LC/MS) is fast becoming the method of choice for many biomolecule analyses. A full discussion of LC/MS, the instruments, ionization techniques, and interfaces, is beyond the scope of this guide. However, the focus of this guide, the HPLC column, is more than a MS filter. The chemistry of the particle and the configuration of the hardware significantly affect the relevance and reliability of the data that is obtained from the LC/MS instrument. In addition to the column, the mobile phase conditions have a profound influence on the quality of LC/MS data. Mass spectrometric detection has high compound specificity and selectivity which lessens the requirements for high selectivity separations; however, it brings limitations to the method development process. For instance, the choice of mobile phase composition is restricted since mobile phase needs to balance the chromatographic requirements with the ionization efficiency.

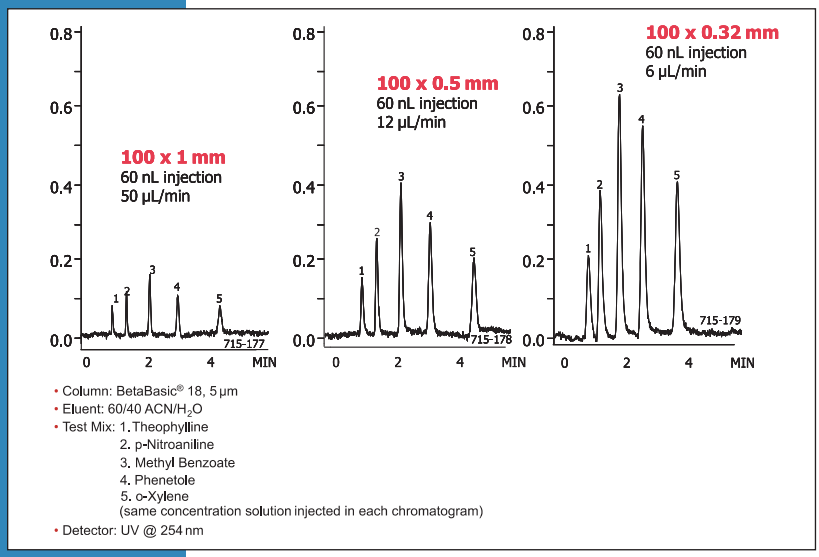


Figure 22: Effect of column ID on sensitivity

Column Selection for RP-LC/MS: Column ID

Usually the aim of an LC/MS experiment is to maximize sensitivity; to be able to see compounds that exist at very low concentration in the sample or that may have adequate concentration but are not easily detected (ionized) by the MS. When samples are limited, as in most proteomics applications, it is essential that maximum LC/MS information is obtained from the smallest injection volume. To increase sensitivity, the concentration of the compound entering the ionization source must be maximized. The more concentrated the analyte, the stronger the signal and the higher the sensitivity. Using concentration to increase sensitivity is especially important for compounds that are not readily ionizable. The ID (internal diameter) of the HPLC column affects the concentration of the sample. Samples are diluted in proportion to the cross-sectional area of the column. Therefore, smaller ID columns yield less dilution. Sensitive LC/MS analyses are typically run on very small ID columns or capillaries.

To demonstrate the effect of column ID on sensitivity, Figure 22 shows the same test solution (same injection volume and mass) on three columns with decreasing IDs (1 mm, 0.5 mm and 0.32 mm). The flow rate was adjusted to obtain the same linear velocity and retention time on each column. The improvement in signal-to-noise ratio obtained with the smaller ID columns is obvious from the normalized chromatograms.

The ideal flow rate in LC/MS varies depending on the ion source type and design. Typical flow rate ranges and the column ID that will be required to match it are shown in Figure 23.

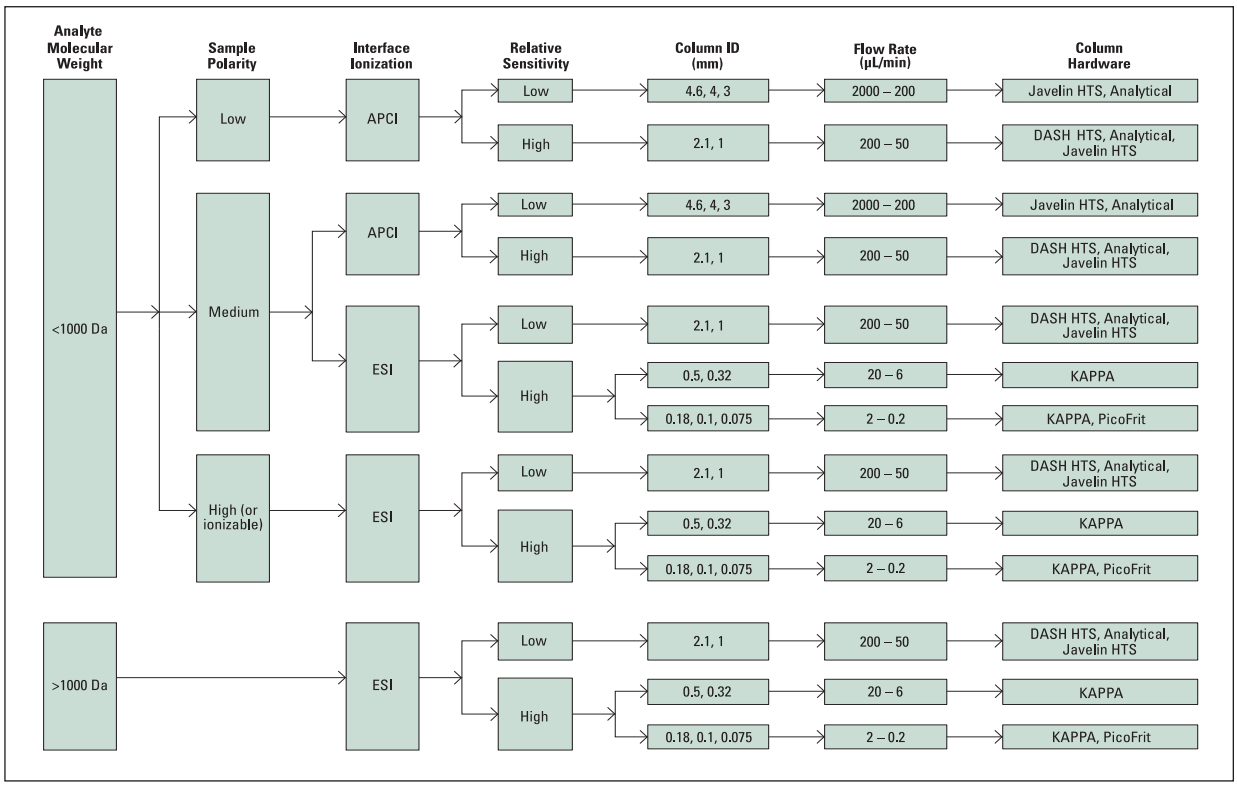


Figure 23: LC/MS method selection guidelines

Maintaining Constant Linear Velocity with Changing Column ID

It is important to use the proper flow rate for the HPLC column and MS interface to which it is connected. The standard flow rate for a 4.6 mm ID column packed with 5 µm particles is 1 mL/min. To achieve the same linear velocity (cm/min) and hence the same retention time when moving from one column ID to another, multiply by the square of the ratios of the column IDs. The equation below presents this calculation for the flow rate on a 2.1 mm ID column (A) when the flow rate on the 4.6 mm ID column (B) is 1 mL/min.

[2] Flow B = Flow A x (ID B/ID A)² = 1 x (2.1/4.6)² = 0.2 mL/min

Flow rates can be changed to achieve different chromatographic goals. Typically columns can be run from one-half to twice the recommended flow rate. However, lower flow rates may give superior chromatography, but result in longer run times. Higher flow rates may result in shorter run times but poorer resolution. The amount of resolution that can be traded for decreased analysis time and faster throughput will depend upon the specific requirements of the analysis.

Electrospray Ionization (ESI)

In addition to choosing the correct column ID for the sample injection volume, the flow rate (sample introduction rate) into the MS interface must is taken into account. Current biomolecule analysis by LC/MS uses electrospray ionization (ESI) because it is more amenable to polar compounds such as amines, peptides, and proteins. Non-polar compounds, such as steroids, are best analyzed using atmospheric pressure chemical ionization (APCI). An example of the output obtained by an ESI analysis of proteins is seen in Figure 24. Here the total ion chromatogram (TIC) and the individual spectra of five whole proteins were obtained using a Thermo Scientific ion trap on a capillary (0.18 mm ID) C18 column. Using the biomass deconvolution module, it is then possible to identify these proteins by their distribution of multiply charged ions, or the ion envelope.

ESI mode typically produces mass spectra consisting of multiply charged ions (for proteins and peptides) depending on the structure of the analyte and the mobile phase. For example, the resulting mass spectrum of a higher molecular weight protein or peptide typically consists of a distribution of multiply charged analyte ions. The resultant mass spectrum can be mathematically manipulated to determine the molecular weight of the sample. The ESI mode transfers ions in solution into the gas phase. It can be used in positive or negative ion polarity mode to analyze any polar compound that makes a preformed ion in solution. Molecular weights greater than 100,000 can be analyzed with ESI due to multiple charging. Since higher ionization efficiencies are achieved at low flow rates, HPLC columns of 2.1 mm ID and smaller are recommended for ESI.

Further reduction in sample consumption and increased sensitivity with ESI is achieved using micro- and nanoelectrospray techniques. Microelectrospray is an ionization method that uses a sheath gas and heat to assist in aerosol formation. Typically this method is used with capillaries ranging from 320 to 180 µm and flow rates from 1 to 10 µL/min. An example of microelectrospray application is illustrated in Figure 25.

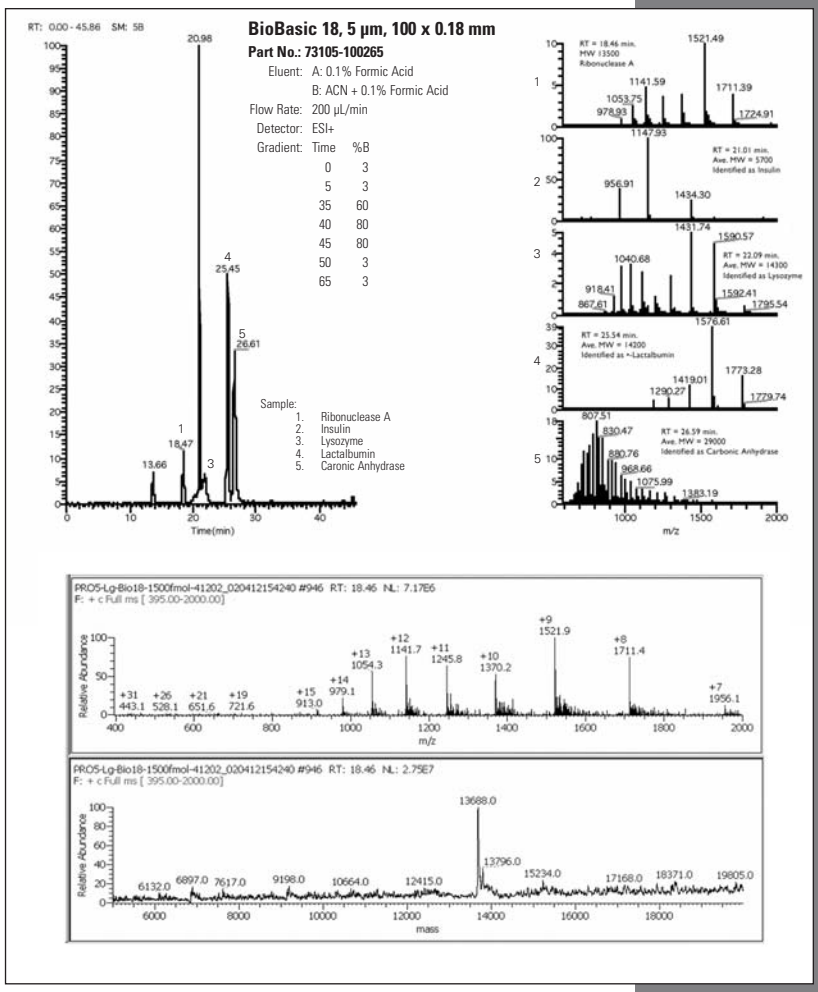


Figure 24: Whole protein RP-LC/MS analysis using ESI with ion trap

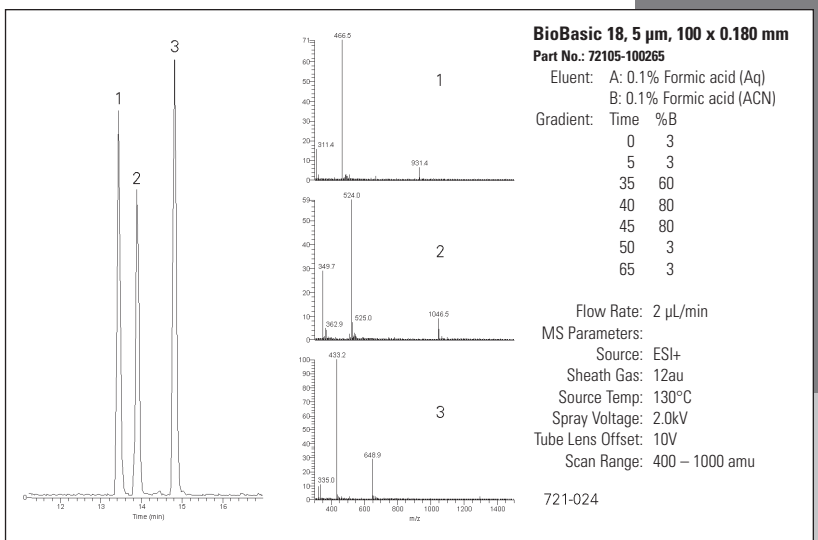


Figure 25: Angiotensins by RP-LC/MS using micro-ESI

Column ID (mm)	Flow Rate Range (µL/min)	Optimum Flow Rate (µL/min)[1]	Injection Volume (µL)[2]	Sample Amount	Ionization Source
2.1	100 – 400	200	5	>25pmole	ESI
1	50 – 100	50	1	>25pmole	ESI
0.50	10 – 25	12	0.35	>25pmole	ESI
0.32	4 – 10	6	0.15	2 – 50pmole	Micro-ESI
0.18	1 – 3	2	0.05	0.1 – 5pmole	Micro-ESI
0.10	0.4 – 1	0.5	0.015	0.05 – 1pmole	Nano-ESI
0.075	0.2 – 0.5	0.3	0.01	<200fmole	Nano-ESI

1. Optimum flow rate recommended for good efficiency and moderate pressure using columns packed with 5um particles. Higher flow rates may lead to column voids. Lower flow rates are recommended for washing column bed or changing solvents.

2. Injection volume estimates based on negligible loss of efficiency and isocratic elution with sample solvent identical to mobile phase. Larger volumes can be introduced under gradient conditions or using weaker sample solvent.

Table 5: Recommended microbore, capillary and nanobore column operation parameters

Nanoelectrospray is an ionization method that utilizes low flow rates in the nL/min range emitted via a spray tip. A major benefit of this technique over micro-ESI is increased sensitivity; femtomole level detection is achieved routinely. The increase in signal is due to the more stable spray formation at the very low flow rates. Figure 26 shows the extreme sensitivity that can be achieved using small ID columns in the separation of a 30 femtomole (10^{-15} mole) phosphorylase B digest on a 75 µm ID capillary packed with C18 particles.

The table above lists some recommended column ID, flow rates, and sample sizes for the various ESI sources.

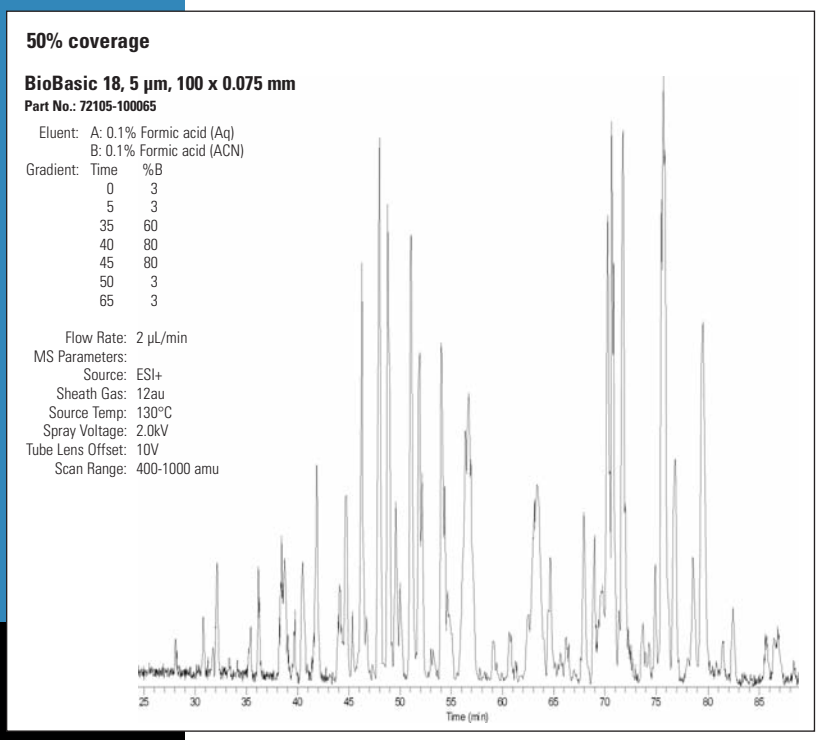


Figure 26: Low level (femtomole) detection of phosphorylase B digest by RP-LC/MS using nano-ESI

Column Packing Materials for RP-LC/MS

The vast majority of peptide RP-HPLC separations of proteins and peptides are columns packed with alkyl-bonded silica particles, primarily C18. The applications shown previously in this section are examples. However, there are realms of use where an alternative to C18-silica particles is desired. Because with LC/MS the choices in mobile phases are restricted to volatile, non-ion pairing acids, there are not as many choices in mobile phases to adjust selectivity as there are in RP-HPLC with UV detection. The bonded phase becomes a valuable tool to adjust selectivity. When developing an LC/MS method, the bioanalyst should not overlook the advantages provided by C4, cyano, and phenyl bonded phases compared to the commonly employed C18.

In addition to the different silica-based phases, porous graphitic carbon (PGC) is a viable alternative phase. It has longer retention of polar compounds, can be run at high pH, and has different selectivity than C18-silica. PGC is also ideal for LC/MS. The separation of oligosaccharides shown in Figure 13 (page 11) was run on 1.0 mm ID PGC (Hypercarb) column in positive ion ESI mode at pH 9.6, too high for most silica-based particles. Figure 14 (page 12) shows ESI of PGC run under acidic conditions with very different selectivity than the C18-silica column. Polar compounds are problematic on RP-HPLC, and PGC provides valuable retention. The ESI-MS analysis of ribonucleotides, very polar biomolecules, is shown in Figure 27. These compounds are usually analyzed using ion exchange or ion pairing on C18-silica columns. However, the PGC column provides retention and resolution under MS-compatible conditions using a simple ammonium acetate mobile phase.

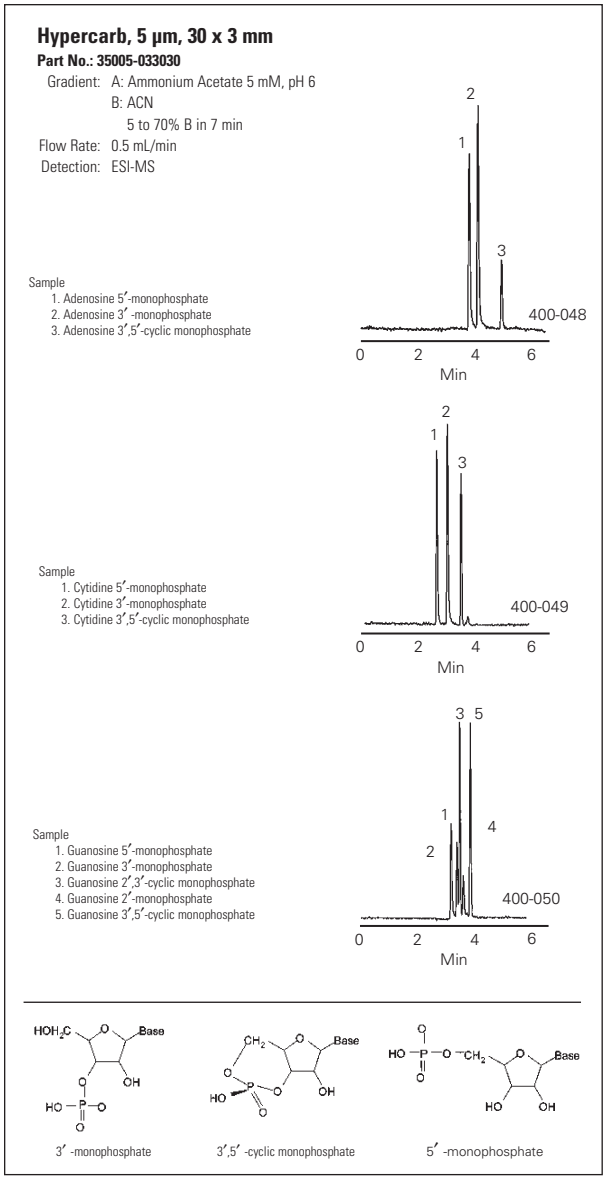


Figure 27: LC/ESI-MS of polar compounds (ribonucleotides) on a PGC column

Column Stability

The LC/MS conditions of low pH can be detrimental to the HPLC column. At low pH, bonded phase can be lost through hydrolytic cleavage, and exacerbated by gradient conditions which mobilize the excised ligands. Bonded phase that has been stripped from the column can contaminate preps, foul instruments, and reduce sensitivity by causing high background. Also, analytes can adsorb to the exposed silica surface causing low recovery, poor peak shape, and irreproducible retention. Using a non-silica based column (like PGC) is an alternative. If silica is preferred, using a stable bonded phase designed for LC/MS, like BioBasic and BetaBasic columns, prevents the problems associated with loss of bonded phase. Figure 28 demonstrates the stability of BetaBasic columns under repeated gradient cycles of acetonitrile in water containing 0.1% TFA.

A demonstration that all HPLC columns *are not created equal* is shown in Figure 29. The BioBasic column withstood repeated gradients containing TFA much better than the competitive phases.

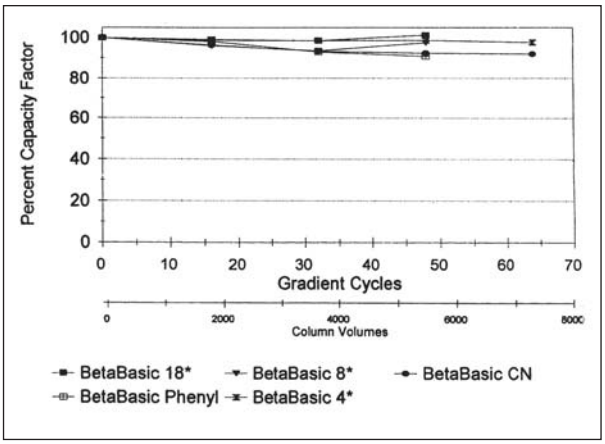


Figure 28: Stability of BetaBasic columns toward acid hydrolysis

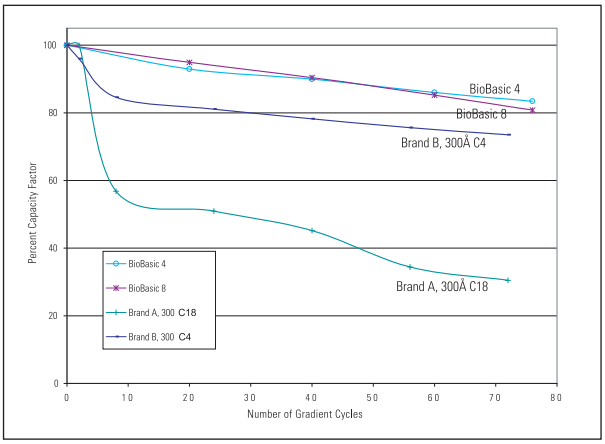


Figure 29: Stability of BioBasic columns toward acid hydrolysis

Mobile Phases for RP-LC/MS

The composition of the mobile phase entering the LC/MS interface plays a large role in the sensitivity and usefulness of the data obtained from the system. The pH of the mobile phase influences the charge state of the analytes, and the ions from salts and other additives dictate the energy with which the analytes enter the mass analyzer. Some of the common LC/MS mobile phase additives and their role in the LC/MS system appear in Table 6.

Additive	Role in the LC/MS System
Acetic	Proton donors
Formic	Proton donors
Ammonia	Proton acceptors
Trichloroacetic	Chromatographic separation
Trifluoroacetic	Chromatographic separation
Isopropyl alcohol (10% of organic phase)	Negative ion formation
Ammonium Acetate	Buffering
Ammonium Formate	Buffering

Table 6: Roles of mobile phase additives in an LC/MS system

An example of how the mobile phase composition influences the sensitivity of LC/MS analysis of a peptide appears in Figure 30. Here, the ion counts were reported for +ESI using different buffers while keeping the analyte constant. The 50/50 acetonitrile/1% acetic acid gave the highest ion count. Note that mobile phases without ions (water only) had very poor sensitivity. Note also that the TFA-containing mobile phases gave lower sensitivity than the formate and acetate mobile phases attesting to the ion-suppressing characteristic of TFA.

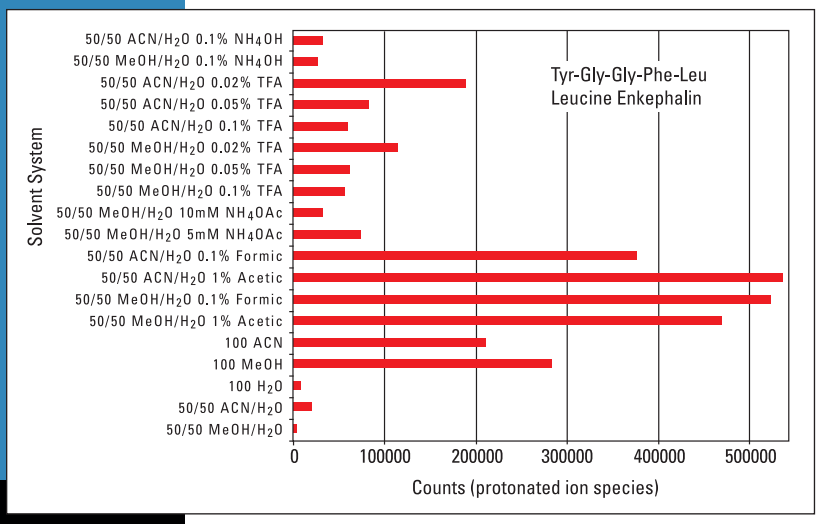


Figure 30: Effect of solvents and additives on ESI-LC/MS

Ionic Additives for RP-LC/MS

The electrospray ionization process is affected by droplet size, surface charge and tension, solvent volatility and ion solvation strength. Large droplets with high surface tension, low volatility, strong ion solvation, low surface charge, and high conductivity prevent good electrospray. The importance of volatility is shown in Figure 31. When comparing formate, acetate, and ammonia to phosphate buffers across the pH range, generally the volatile buffers result in an increase in overall sensitivity (measured here in peak area).

Mobile phases chosen for RP-LC/MS should take into account their effect on the ESI ionization efficiency. Ionic compounds used in the mobile phase should be volatile, and salt concentrations above 50 mM are not recommended. Salts introduced into the MS reduce the sensitivity and may foul the instrument. As discussed previously, TFA is a very common mobile phase additive for RP-HPLC of proteins and peptides. However, it has limitations for LC/MS. The high surface tension of a 0.1% TFA solution makes spray formation (nebulization) difficult. Also, because TFA forms strong ion pair complexes with the protein's amine groups it reduces the ionizability of the protein and thereby reduces LC/MS sensitivity. The preferred additives are formic and acetic acid at 0.01 – 1% v/v. The most compatible ESI buffer systems are shown in Table 7 below.

Buffer or Additive	pK_a	pH Range	Concentration for ESI
Formic acid / ammonium formate	3.8	2.8 – 4.8	10 – 50 mM
Acetic acid / ammonium acetate	4.8	3.8 – 5.8	10 – 50 mM
Ammonium hydroxide / ammonia	9.2	8.2 – 10.2	10 – 50 mM

Table 7: Properties of buffers and additives for ESI-LC/MS

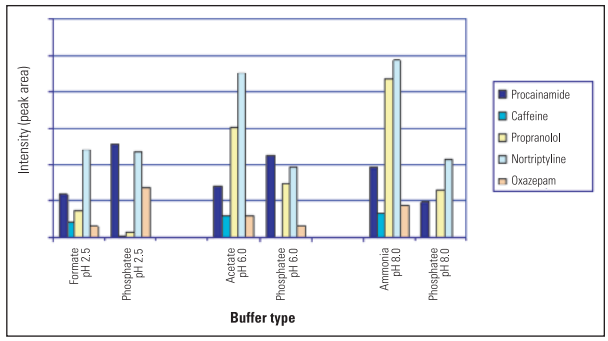


Figure 31: Improved sensitivity with increased buffer volatility

Organic/Aqueous Solvent Ratio for RP-LC/MS

The most common solvents used in LC/ESI-MS are water, methanol, acetonitrile, and mixtures of these. The solvent composition (organic/water ratio) is particularly important in the electrospray nebulization and ionization process, since it determines surface tension of the droplets formed and the vaporization efficiency. Because the surface tension of water is much higher than the surface tension of methanol or acetonitrile, the sensitivity is reduced when using more than 70 to 80% of aqueous mobile phases. The organic/water ratio is more significant when working at high flow rates since there is more solvent to be nebulized and vaporized. The effect of the percentage of acetonitrile on the electrospray signal obtained for two small molecules is shown in Figure 32.

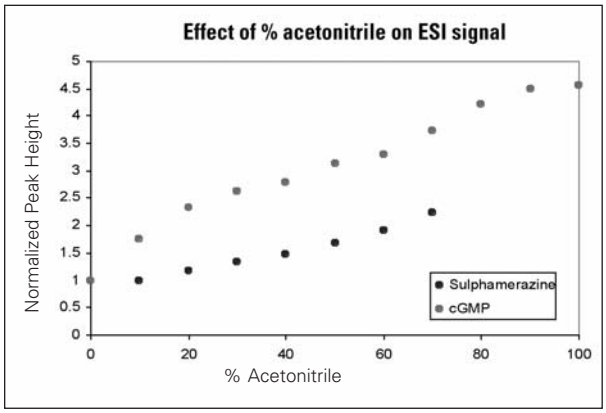


Figure 32: Effect of % acetonitrile on ESI signal

Effect of Connecting Tubing on LC/MS Efficiency

In addition to the capillary column properties, the volume of the tubing between the capillary column and the interface must also be minimized. Length and ID must be considered. Figure 33 shows the flow profiles in a packed bed and an open tube. Note the laminar flow profile in the open tube that causes band dispersion and mixing. In an open tube the contribution to band dispersion is related exponentially to the inner diameter of the tubing. For LC/MS methods, excess post-column tubing volume can result in decreased sensitivity (lower signal to noise ratios) and less confident protein/peptide identification scores. Dispersion in an open tube is dependent on the inner diameter of the connective tube (d_t), the length of the connection (L_t), the flow rate (F) and the diffusion coefficient (D). For gradient methods, this effect is crucial post-column (the connection between the column and the ESI interface).

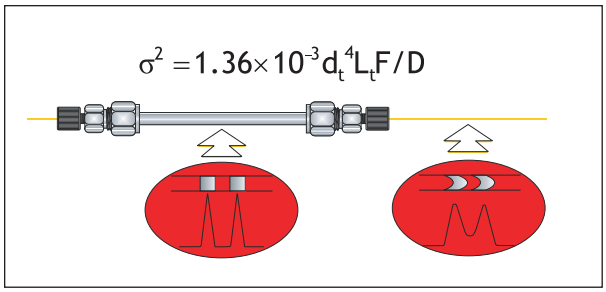


Figure 33: Flow through a packed bed vs. an open tube

The effect of having the optimum tubing length on the resulting chromatography is seen by comparing Figures 34 and 35. In the system with 0.874 µL post-column volume, 39% of the myoglobin sequence was covered. However, in the system with 0.0975 µL post-column volume, 62% of the sequence was covered. This represents almost a 2-fold increase in peptide detection by reducing the post-column tubing volume.

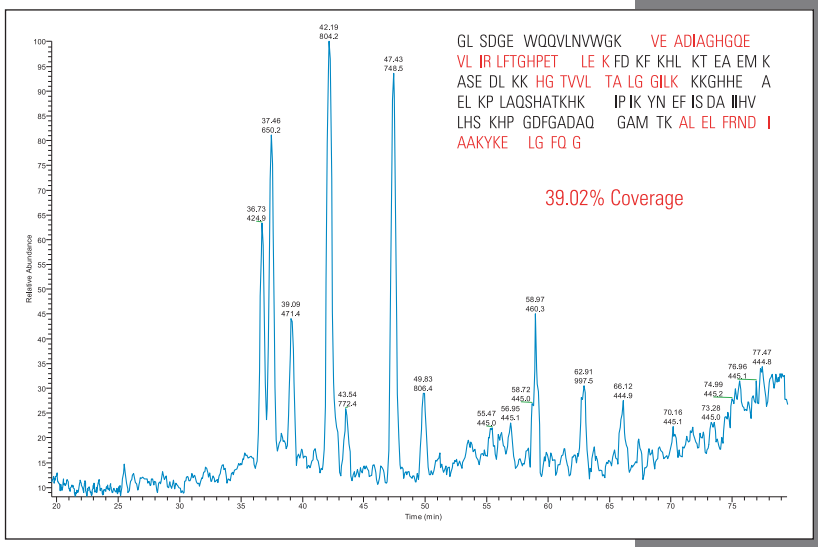


Figure 34: Excess tubing volume – Myoglobin tryptic digest with 0.874 µL post-column volume

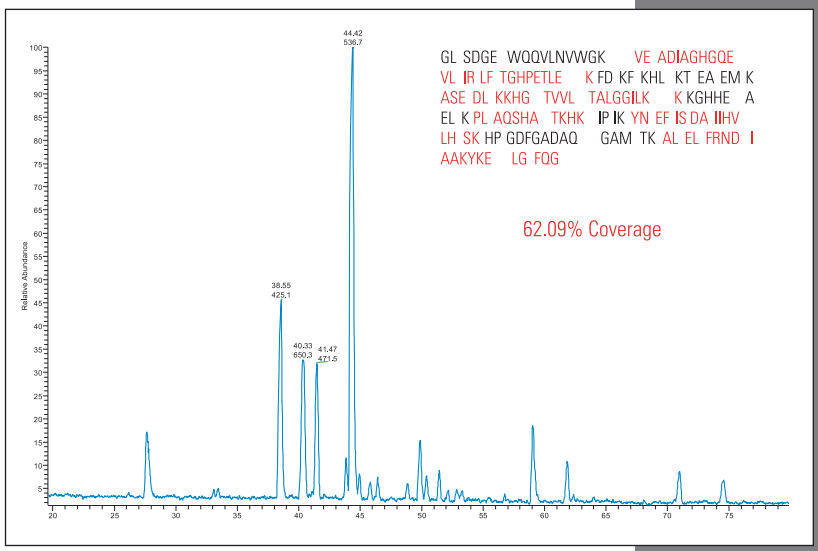


Figure 35: Ideal tubing volume – Myoglobin tryptic digest with 0.0975 µL post-column volume

Column			0.01" ID Tube		0.005" ID Tube		0.003" ID Tube	
ID (mm)	Volume (µL)	Flow (mL/min)	Max L (cm)	Max Vol (µL)	Max L (cm)	Max Vol (µL)	Max L (cm)	Max Vol (µL)
4.60	1080	1.0000	35.314	17.894	565.020	71.575	4359.726	197.820
4.00	817	0.8000	28.051	14.214	403.815	51.154	3115.859	142.095
3.00	459	0.5000	12.777	6.474	204.432	25.897	1577.404	71.935
2.10	204	0.2000	6.310	3.197	100.954	12.789	778.965	35.524
1.00	51.1	0.0500	1.577	0.799	25.238	3.197	194.741	8.881
0.50	12.8	0.0120	0.411	0.208	6.573	0.833	50.714	2.313
0.30	4.59	0.0040	0.160	0.081	2.555	0.324	19.718	0.899
0.18	1.65	0.0014	0.057	0.029	0.920	0.117	7.098	0.324
0.10	0.511	0.0004	0.020	0.010	0.315	0.040	2.434	0.111

Calculations based on 100 mm columns, N = 10,000, Porosity = 0.65, k' = 1. Instrumental variance << Column variance

Figure 36: Maximum allowable tubing length with various ID columns to maintain 95% of the column's efficiency

Figure 36 gives some guidelines for the maximum allowable tubing length of various inner diameters of connecting tubing that will cause a 5% loss in efficiency. (Note that the shaded area in Figure 36 represents unusable lengths for connection. The Appendices in the back of this guide contains a table that calculates the volume per unit length for tubing of various internal diameters.

Using optimized system and chromatographic conditions, a 25fmol Phosphorylase B sample was analyzed on a 100 x 0.075 mm BioBasic 18 column, 9 cm post column tubing (25 µm ID), 25 µm ID PicoTip™ emitter with a 90 minute gradient (Figure 37).

Sensitive capillary LC/MS methods using standard column configurations can be performed if the following steps are taken to optimize the total LC/MS system:

- For low-flow operating ranges, insure adequate gradient delay times are programmed for maximum sample focusing.
- Use adequate gradient lengths for the optimum column packing (as well as sample characteristics).
- Minimize post column volume that can cause band dispersion and result in losses of sensitivity (including nanospray emitter tips).
- Insure tubing is cut squarely and all fittings are snug and leak free.
- Use high efficiency capillary columns such as the BioBasic and BetaBasic columns.

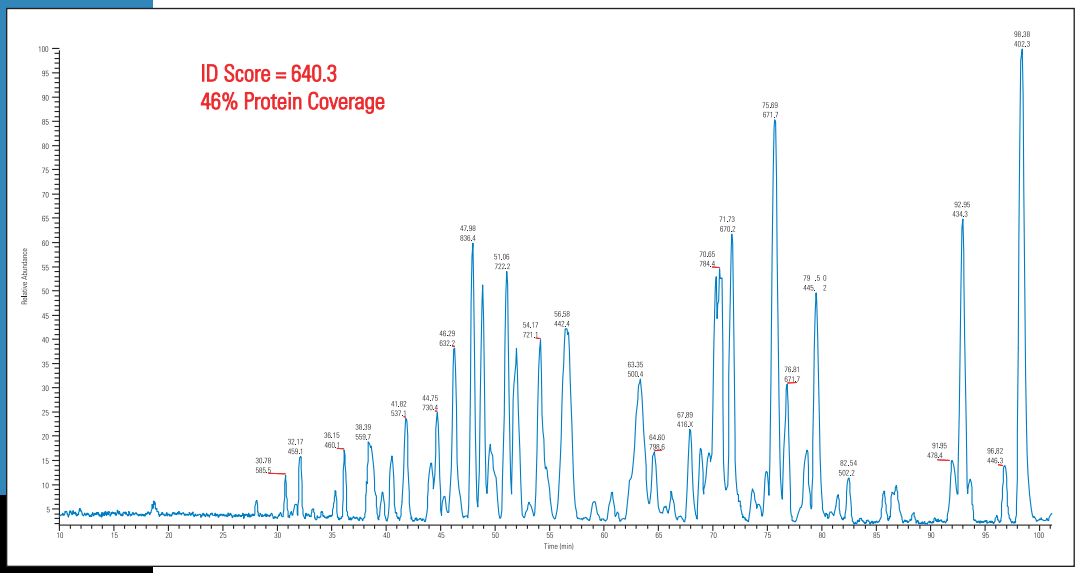


Figure 37: Base peak chromatogram of 25 fmol Phosphorylase B on a 100 x 0.075 mm ID BioBasic 18 column

Ion Exchange Chromatography

Basic Principles of Ion Exchange Chromatography

In ion exchange chromatography, molecules bind by the reversible interaction of electrostatic charges located on the outer surface of the solute molecule with clusters of groups with an opposite charge on the ion exchanger. To maintain neutrality, the charges on both the molecules of interest and the ion exchanger are associated with ions of opposite charge, termed counterions. Because a solute must displace the counterions on the stationary phase to attach to it, the technique is termed "ion exchange."

Ion exchange mobile phase conditions are chosen that permit differential migration of the sample components through the column. The higher the net charge of the analyte, the higher the ionic strength (salt concentration) needed to bring about desorption from the bonded phase. At a certain high level of ionic strength, all the sample components are fully desorbed and move down the column with the same speed as the mobile phase. Conditions that lie somewhere in between total adsorption and total desorption will provide the optimal selectivity for a given pH value of the mobile phase.

Because all proteins and peptides have the ability to exist as a charged species, ion exchange is an excellent choice for protein and peptide characterization. An example of cation and anion exchange separation of a mixture of proteins is seen in Figures 38 and 39, respectively.

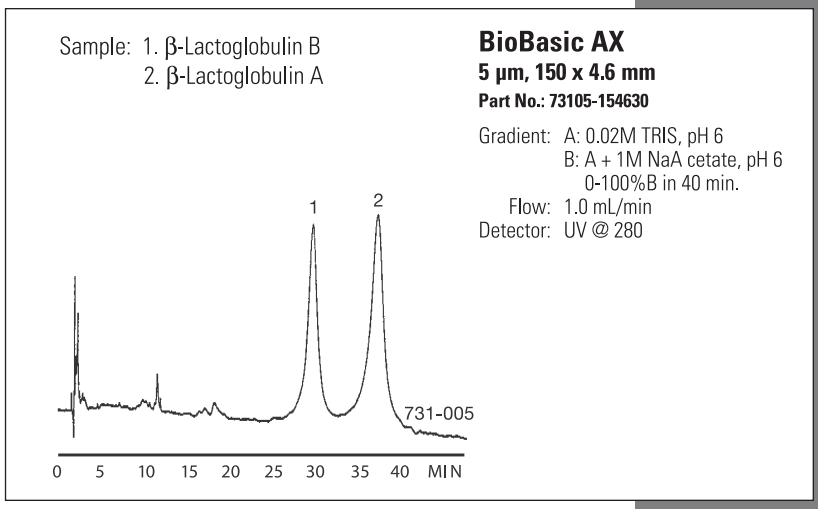


Figure 39: Separation of proteins by anion exchange HPLC

Ion exchange can be used for small biomolecules as well as proteins. The separation of nucleotides on a weak anion exchange column in Figure 40 demonstrates this utility.

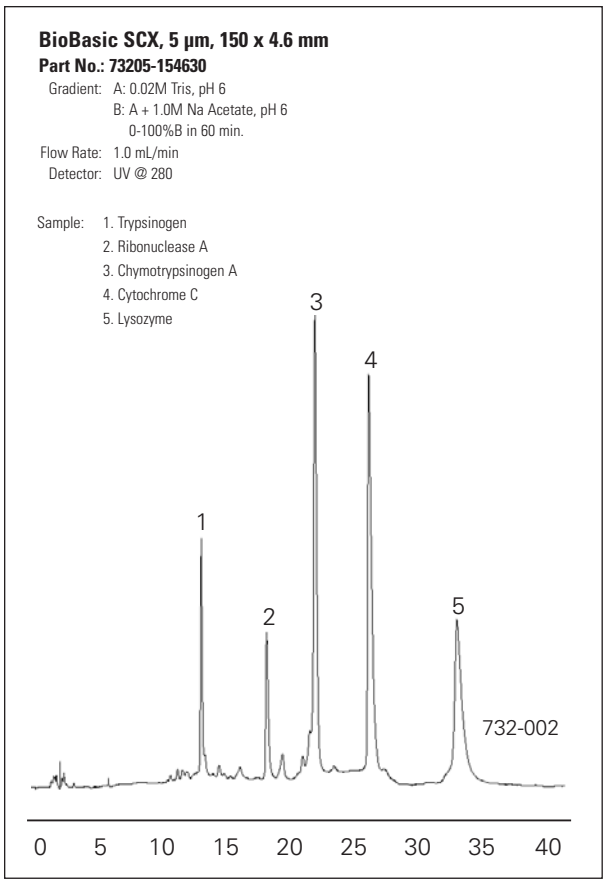


Figure 38: Separation of proteins by cation exchange HPLC

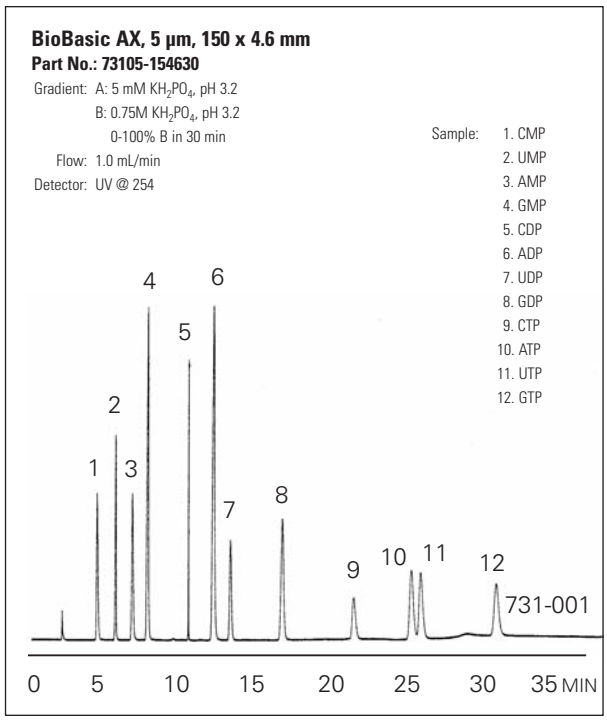


Figure 40: Separation of nucleotides by anion exchange HPLC

The anion exchange process may be represented by the equilibrium [3]:

[3] $$\sim NH^+C^- + X^- = \sim NH^+X^- + C^-$$

where C^- is the counter ion to the fixed ion exchange site, $\sim NH^+$, present in the eluent. Analyte X^- can then displace C^- to give the ion pair NH^+X^-. For effective chromatography, this displacement reaction must be at equilibrium. Retention is therefore controlled by the equilibrium [3] for which the equilibrium constant K_{IE} is given by equation [4]:

[4] $$K_{IE} = \frac{[C^-]\,[\sim NH^+X^-]}{[X^-]\,[\sim NH^+C^-]}$$

The capacity factor, k, of an analyte (X^-) is a more useful chromatographic parameter and is proportional to the distribution coefficient, D_{IE}. Its relationship to the equilibrium constant, K_{IE}, is given by equation [5]:

[5] $$K \propto D_{IE} = \frac{[\sim NH^+X^-]}{[X^-]} = K_{IE} \cdot \frac{[\sim NH^+C^-]}{[C^-]}$$

Since the concentration of the ion exchange sites, $[\sim NH^+X^-]$, is constant and fixed by the rigid structure of the matrix, k is inversely proportional to the concentration of the counter ion (ionic strength) in the eluent: Desorption is then brought about by increasing the salt concentration as shown by equation [6].

[6] $$K \propto [C^-]^{-1}$$

Particles for Ion Exchange

Both polymer- and silica-based particles are available for ion exchange separations of biomolecules. Polymer-based particles have the advantage of extended pH range (chemical stability), while silica-based particles have higher efficiency and better mechanical strength. However, there are hybrid particles that offer the benefits of both. An example is the BioBasic ion exchangers that comprise high purity silica with a polymeric coating. The polymeric coating extends the usable pH range, and also shields proteins from adsorbing to the silica surface.

Particle Size, Pore Size, and Column Length

The effects of these variables in ion exchange are largely the same as for RP-HPLC, discussed previously. Efficiency (resolution) increases with decreasing particle size and increasing column length, but at the expense of higher back pressure for smaller particles and longer columns. Longer columns also increase analysis time. Pore size should be larger, 300Å or greater, for proteins and large peptides to ensure access to the interior of the pores where the majority of the surface area resides.

Flow Rate

High column efficiency (number of theoretical plates) is an important factor to consider when maximizing the resolution of a separation. In ion exchange chromatography the efficiency (sharpness) of a peak is governed not only by how well the column is packed but also by the mass transfer characteristics of the ion exchange mechanism itself. The term mass transfer used here refers to the efficient migration of the analyte from the mobile phase on to the stationary phase ion exchange site and then back into the mobile phase from the ion exchange site. If this process is not very rapid, broad peaks can result with a possible loss in analyte resolution. Figure 41 illustrates how the column efficiency increases as the flow rate is reduced. Depending on the complexity of the analysis, a tradeoff is required between time and efficiency.

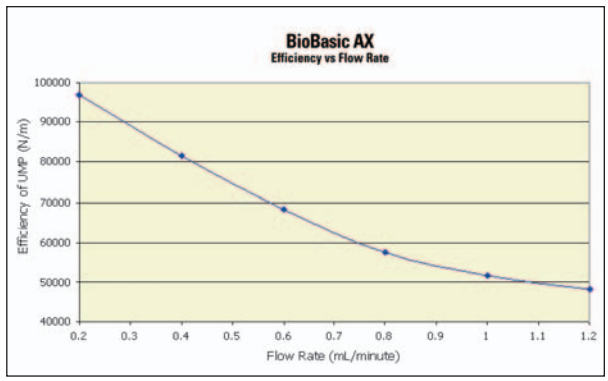


Figure 41: Effect of flow rate on efficiency in ion exchange

Temperature

The mass transfer component of the Van Deemter equation can often be improved through temperature control. The mass transfer can be increased by increasing the temperature at which the separation takes place. The way in which column temperature is achieved is also of significant importance.

Figure 42 shows the effect that increasing column temperature has on efficiency. It is important to note the role of preheating the mobile phase on the separation. For example, heating only the column itself and not heating the solvent supplied to it can actually increase band spreading and reduce column efficiency as shown in Figure 42. Heating the solvent prior to its arrival at the column prevents this band spreading and improves the mass transfer associated with the ion exchange mechanism. The result is an increase in the column efficiency for any given flow rate.

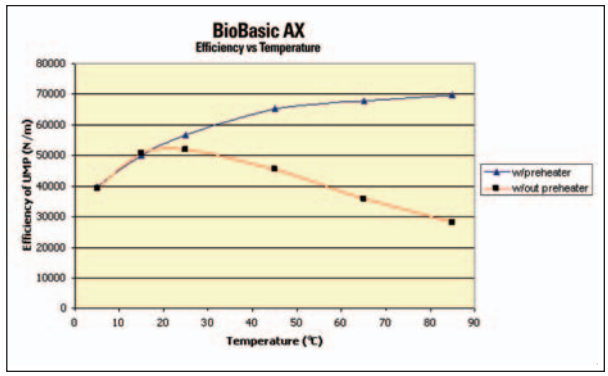


Figure 42: Effect of T on efficiency in ion exchange

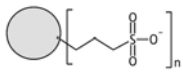
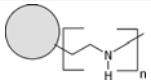
Column packing	Type of exchanger	Particle	Structure	Ion exchange ligand	Pore size (Å)	Particle size (µm)	Ion exchange capacity
BioBasic SCX	Strong cation	Spherical, porous, highly pure silica with polymeric coating		Sulfonic acid	300	5	0.07meq/g
BioBasic AX	Weak anion	Spherical, porous, highly pure silica with polymeric coating		PEI (polyethylenimine)	300	5	0.22meq/g

Table 8: Thermo columns for ion exchange of biomolecules

Ion Exchange Bonded Phases

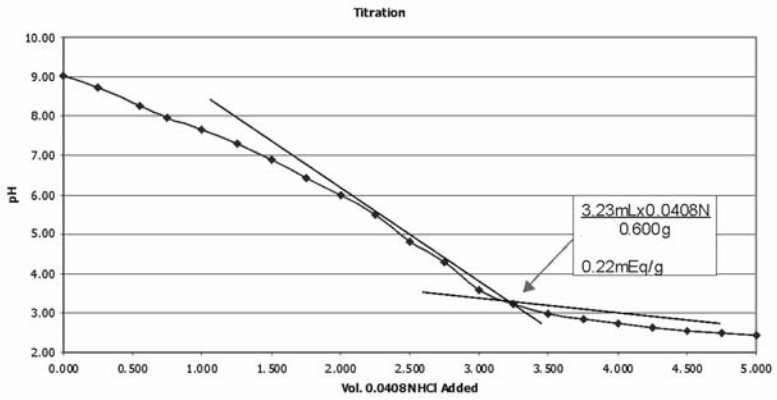
Most ion exchange packings fall into two groups, cation exchangers and anion exchangers. Cation exchangers contain acidic groups, such as sulfonic acid or carboxylic acid, and are used to separate cationic (positively-charged) compounds. Strong cation exchangers commonly comprise sulfonate groups ($-SO_3^-$). These are strong acids and their ion exchange character is relatively unaffected by mobile phase pH. Carboxylate ($-COO^-$) ions are common weak cation exchangers. With pK_a values in the 4 – 6 range, they are ionized and effective as a cation exchange media above pH 6. Anion exchangers contain basic groups, such as secondary, tertiary, or quaternary amines, and are used to separate anionic (negatively-charged) compounds. Quaternary amines ($-NH_4^+$) are strong bases and their exchange character is relatively unaffected by mobile phase pH. Secondary and tertiary amines are weak bases with pK_a values between 8 and 11. A weakly basic anion exchanger should be used below pH 8.

It is important to buffer the mobile phase in this mode of chromatography to control the ionization of both the analyte and the packing, since the ionic state of both affects the acid-base equilibria between analyte and ion exchange packing. A strong anion exchange media will be ionized over most of the pH range 2 – 8. A weak anion exchange media can be turned "on" and "off" within that range through changes in the pH of the mobile phase. It is important to emphasize that while the ion exchange capacity of a weak ion exchange media will be maximized at low pH, it will still have some ionic character and function as an ion exchanger even at pH 8, giving it good versatility and making pH a powerful tool for modifying selectivity.

Measuring Ion Exchange Capacity

Ion exchange capacity is a measure of the concentration of ion exchange groups on the surface of the particle. The units are generally milliequivalents of ion per gram packing material (meq/gram). Exchange capacity does not have a strong effect on overall retention, but it does influence the ionic strength needed to elute analytes from the column. It also affects the total mass of compound that can be loaded onto the column in a preparative mode.

An example of an ion exchange capacity determination is shown in Figure 43. A known amount of the ion exchange media is suspended in a salt solution. The pH of the solution is measured upon addition of acid solution of known molality. The point where additional acid results in no change in pH is the equivalence point. The concentration of ionic groups on the surface (the ion exchange capacity) is determined by dividing the equivalents of acid added at the equivalence point by the weight of the media.



Purpose:
To determine the fundamental ion exchange capacity and demonstrate the effective weak anion exchange pH range for BioBasic AX.

Reference:
A.J. Alpert and F. E. Regnier J. Chrom 185 (1979) pgs 375-392

Method:
0.600g of lot R0J17 was suspended in 20 mL of 0.5M sodium chloride. The stirred suspension was then titrated using 0.0408N HCL. The pH was monitored using a pH meter and probe rather than a visual indicator.

Figure 43: Exchange capacity determination by titration

Mobile Phases for Ion Exchange HPLC

Mobile Phase pH

The pH of the mobile phase can have considerable effect on retention and selectivity. This is because:

- A shift in pH that causes the analyte to change from its ionized state to its neutral state prevents the analyte from taking part in the ion exchange process and consequently retention is reduced.
- A shift in pH that causes the ion exchange site to change from its ionized state to a neutral state essentially eliminates sites available for ion exchange and retention is lost.

The effect of pH on the cation exchange separation of a protein mixture is seen in Figure 44. Retention of the basic peptides increases with decreasing pH, primarily because the carboxylic acid groups of the peptides becomes protonated, or neutral, making the overall peptides more cationic (positively charged). The strong sulfonic acid group on the cation exchange particle is fully deprotonated (negatively charged) throughout this pH range.

Ionic Strength (Salt Concentration)

Mobile phase ions compete with the analyte ions of the same charge for adsorption with ionic groups in the stationary phase. Obviously, the more mobile phase ions (the higher the ionic strength) the better they compete. The result is that ion exchange retention decreases with increasing ionic strength of ions of the same charge as the analytes. The term adsorption used here in a general sense refers to the attraction of an analyte ion for the ionic sites of the packing. It should not be confused with the same term used for adsorption chromatography where traditional hydrogen bonding is the mechanism by which analytes are retained at the surface.

The effect of ionic strength on analyte retention is demonstrated in Figures 45 and 46. Note that as the concentration of salt (ammonium acetate) is decreased the retention of the ionic analytes increases, without significant changes in selectivity or column efficiency. Differential migration and resolution of sample components occurs because of their different degrees to which they adsorb to the stationary phase ion exchange sites.

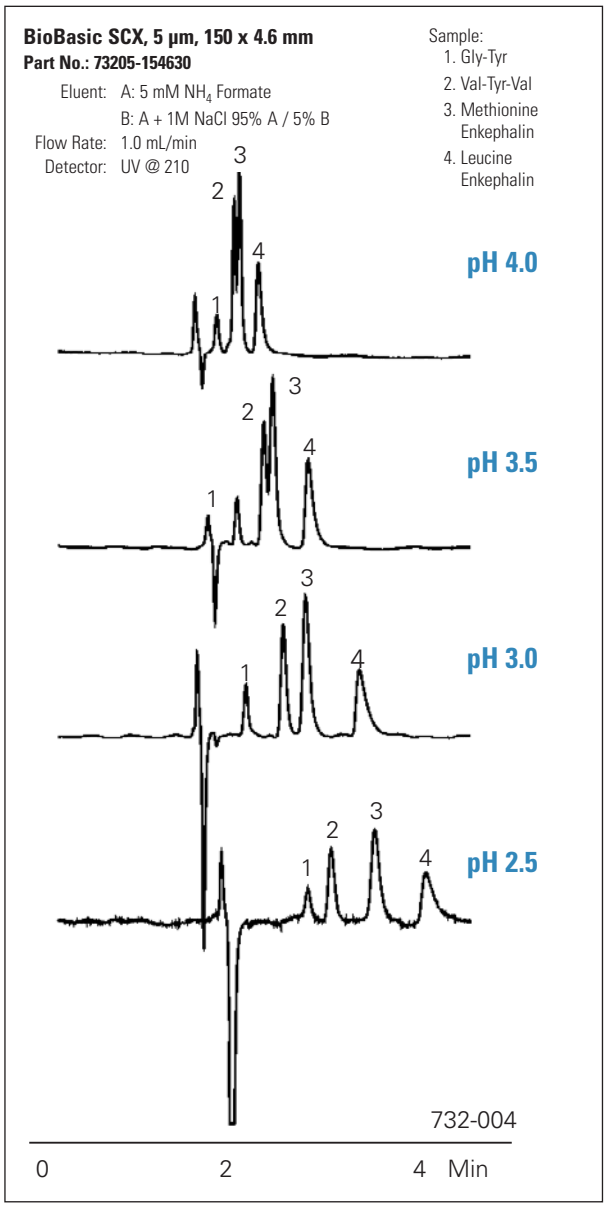


Figure 44: Effect of pH on cation exchange separation of proteins

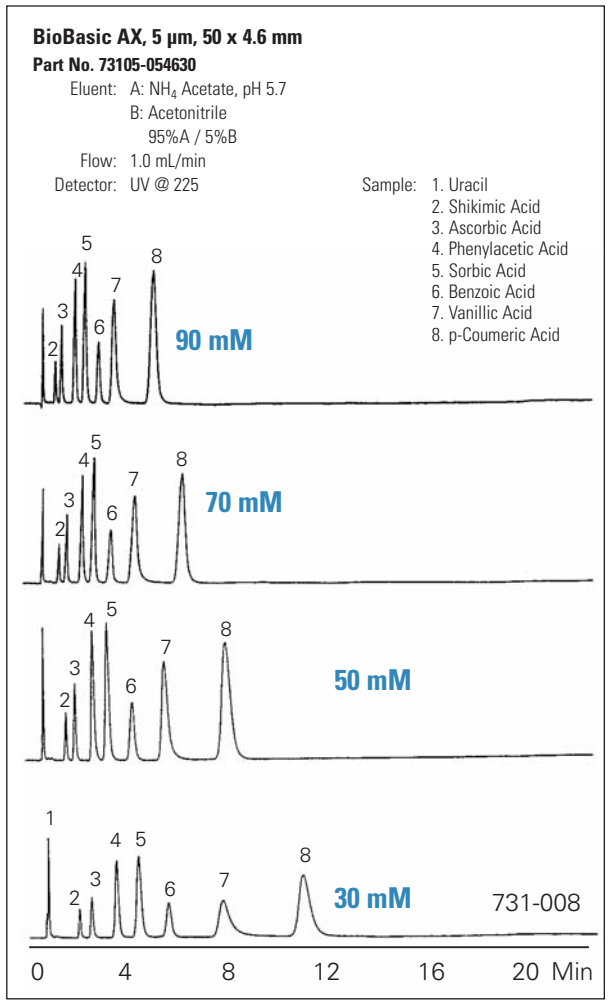


Figure 45: Effect of decreasing ionic strength on anion exchange retention

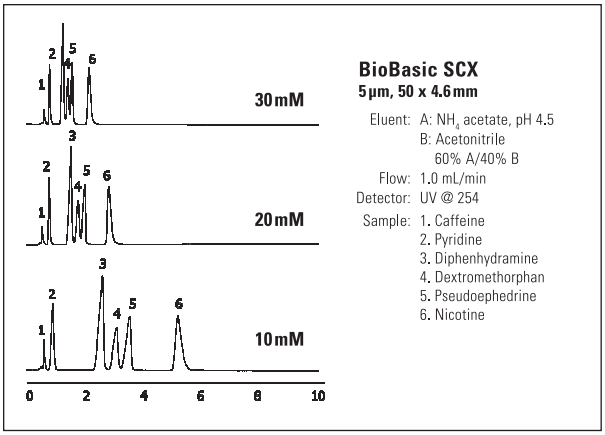


Figure 46: Effect of decreasing ionic strength on cation exchange retention

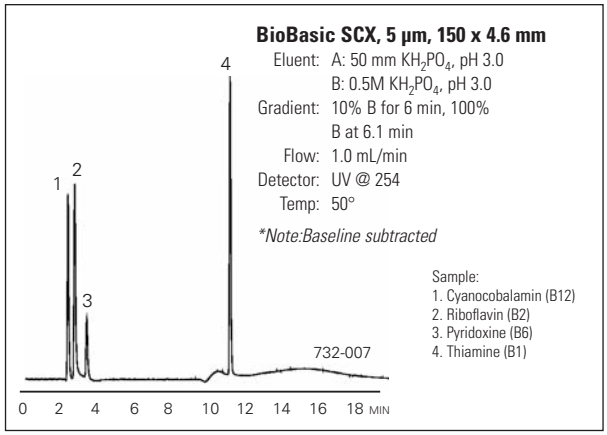


Figure 47: Cation exchange separation of vitamins using potassium phosphate gradient

The competition between analyte and salt for the ion exchange site is largely controlled by the concentration and nature of the salt ion. Choice of the appropriate counterion is therefore of significant importance in adjusting retention. One usually employs NH_4^+, H^+, Na^+, K^+, or Ca^{2+} for cation and acetate$^-$, Cl^-, NO_3^-, SO_4^{2-}, or PO_4^{3-} for anion exchangers. The eluting strength of the counterion correlates directly with its charge. For example, by substituting the eluent ion of a cation exchanger in the series H^+, Na^+, K^+, Ca^{2+} the relative strength between counterion and fixed ion increases and retention of a given cation (analyte) decreases.

Controlling Retention with Salt and pH Gradients

When a single ionic strength is insufficient to elute all analytes (both strongly and weakly retained) from the column in a reasonable time, salt or pH gradients can be applied. Salt gradients employ a gradual increase in ionic strength in the mobile phase. This gradient gradually desorbs the sample components in the order of increasing net charge, so that components are desorbed off one at a time from the surface to provide separation of the mixture. Thus the salt gradient compresses a chromatogram so as to elute components with widely different adsorptive properties within a reasonable time. The gradient profile can be linear or stepwise.

pH gradients affect retention in the same manner, by taking advantage of the different pK_a values of analytes and stationary phase ions. In a typical pH gradient, the mobile phase pH is changed from low pH to high pH (for anion exchange) or high to low (for cation exchange), bracketing the pK_a range of the analytes.

Two examples of an ion exchange separation of biomolecules using salt gradients are found in Figures 47 and 48. Note the two different approaches to forming the gradients. The separation of vitamins in Figure 47 uses two different concentrations of the same salt, potassium phosphate. The concentration of the 0.5M solution is increased to form the gradient. In the separation of peptides in Figure 48, both mobile phase components contain potassium phosphate, but the B solution also contains 0.5M NaCl. The gradient thus formed is of increasing NaCl concentration. In both cases, it is important to consider the changes in the pH upon changes in salt concentration, if different salts are combined.

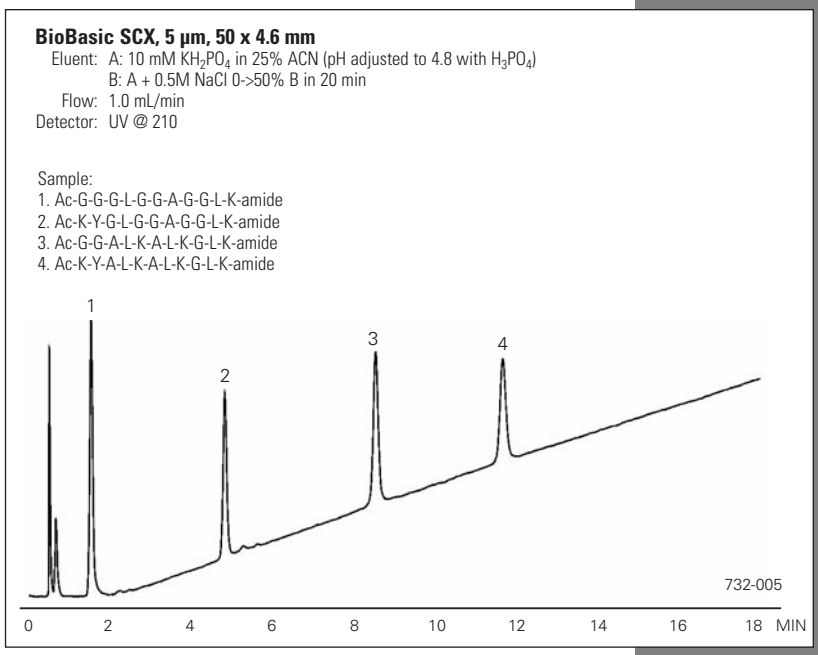


Figure 48: Cation exchange separation of peptides using NaCl gradient by anion exchange

Ion Exchange for LC/MS of Biomolecules

The same factors that govern reversed phase LC/MS analysis also apply to ion exchange analysis. Ion exchange is widely used as a fractionation technique in 2D-HPLC for proteomics applications. This will be discussed in a later section of this guide.

The most important aspect of ion exchange in the realm of LC/MS is the mobile phase. Any salts used in LC/MS should be volatile and used in low concentration to maintain sensitivity and prevent fouling of the instrument. For ion exchange LC/MS applications, ammonium salts of acetic or formic acid are commonly used. Note that many of the applications shown in this section use these and other MS-compatible salts in the mobile phase.

The exchange capacity of the particle should also be considered for LC/MS applications. When using ion exchange as a preparative material where high sample capacity is desirable, a particle with high ion exchange capacity should be used. However, with LC/MS the need for low salt concentration to maintain high sensitivity drives the choice toward an ion exchange particle with lower exchange capacity.

Size Exclusion Chromatography

Basic Principles of Size Exclusion Chromatography

Size exclusion chromatography (SEC) is a noninteractive technique that separates solutes according to their molecular size in solution. It is often used as the first step in isolation of a protein from a crude sample. When used with calibration standards it is possible to determine the molecular mass of compounds with similar molecular shape to the standards, such as proteins and oligosaccharides.

SEC retention is determined by the accessibility of the sample molecule to the pores. Maximum retention (maximum elution volume) occurs if the sample can fully access the pores. Minimal retention (minimum elution volume) occurs if the sample is larger than the pores and elutes with the solvent. Hence, samples elute in order of size, with the highest molecular weight (or largest molecule size) samples eluting first. Since molecules are eluted based on their size in solution, linear or rod-like molecules will elute before globular molecules of the same molecular weight. A schematic of an SEC separation is shown in Figure 49.

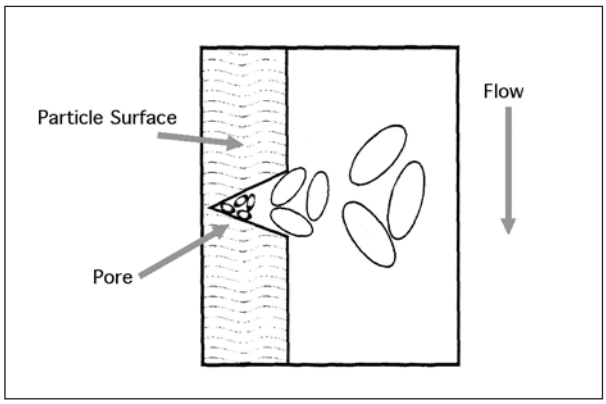


Figure 49: Schematic of an SEC separation

Elution order is based on whether or not the analyte can enter the pores. If the analyte cannot enter the pores it passes through the column in the channels between the particles. Analytes that can enter the pores, either partially or completely, elute later. In a packed column the interstitial volume outside the particles (*i.e.* the volume associated with the channels between the particles) is given as V_o. The volume inside the particles (inside the pores) is denoted as V_i.

The total volume of the mobile phase inside the column (V_m) is then [7]:

[7] $$V_m = V_o + V_i$$

The degree of permeation of the analyte is denoted by K_D (the distribution coefficient). The retention volume of any analyte (V_R) is expressed as [8]:

[8] $$V_R = V_o + K_D V_i$$

For completely excluded analytes $K_D = 0$, while for totally permeating analytes $K_D = 1$. [9]:

[9] $$K_D = (V_R - V_o)/(V_m - V_o)$$

Particles for Size Exclusion

Both silica- and polymer-based particles are popular for size exclusion separations. However, polymer particles can be compressed during the flow of mobile phase through the column, and can shrink or swell with changes in solvent composition. These characteristics limit their operating pressure and can reduce separation efficiency and column lifetime. In contrast, silica particles are mechanically rigid and do not swell or shrink with changes in solvent and shows higher efficiencies than polymer-based columns (minimum 70,000 plates/meter).

The physical properties of the silica particles require tight quality control, as it is the volume of pores with diameters between the inclusion and exclusion limits of the analytes that determine resolution. Some of the physical properties of Thermo SEC columns are shown in Table 9.

To provide accurate data, size exclusion columns must separate sample molecules strictly on the basis of their size in solution. Secondary ionic or hydrophobic interactions must be minimized, as they will degrade the size-based separation. An example of a high efficiency SEC separation is shown in Figure 50. Note the elution in order of decreasing molecular weight.

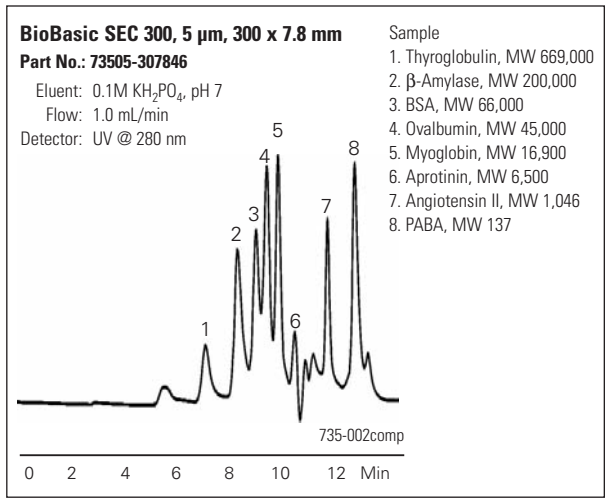


Figure 50: SEC separation of proteins

Column	Particle Chemistry	Surface Chemistry	Particle Size	Pore Size	Pore Volume	V_i/V_o
BioBasic SEC 60	Spherical, high purity silica	Hydrophilic polymer coating	5 µm	60Å	0.7mL/g	1.40
BioBasic SEC 120	Spherical, high purity silica	Hydrophilic polymer coating	5 µm	120Å	1.0mL/g	1.77
BioBasic SEC 300	Spherical, high purity silica	Hydrophilic polymer coating	5 µm	300Å	0.9mL/g	1.16
BioBasic SEC 1000	Spherical, high purity silica	Hydrophilic polymer coating	5 µm	1000Å	0.9mL/g	0.96

Table 9: Thermo columns for SEC

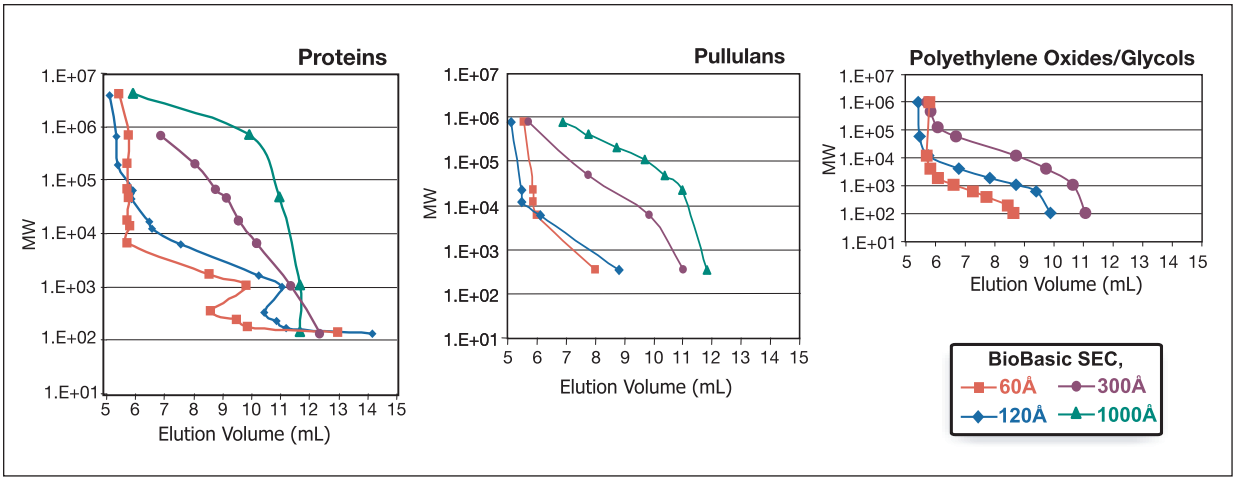


Figure 51: Molecular weight calibration curves

Pore Size and V_i/V_o Ratio

Equation [7] illustrates that pore volume is a very important variable of retention in SEC. A measure of porosity is the ratio of pore volume to void volume (V_i/V_0). The larger the ratio, the more volume available for the separation to occur, giving better resolution of more peaks. However, supports with a large V_i/V_0 ratio are more fragile, which may compromise efficiency. The V_i/V_0 ratio for BioBasic SEC columns appears in Table 9.

Molecular Weight Calibration

A calibration curve allows the analyst to choose the proper pore size SEC particle for a particular biomolecule separation. To create a calibration curve, log molecular weight of the analyte is plotted against elution volume to create a distribution coefficient (calibration curve). The curve is usually linear for distribution coefficients between 0.2 and 0.8. The gradient of the curve is sharp near the exclusion limit of large molecules and near total permeation of small molecules. To determine the molecular weights of unknown samples, the elution volume of the sample can be compared directly with that of standards of known molecular weight. New SEC columns should always be calibrated before injecting unknown samples. Performing a calibration ensures accurate quantification, as some solvent conditions may encourage secondary interactions between the sample and the surface, which may alter molecular weight calculations.

Because SEC separates by molecular size in solution, and not strictly molecular weight, it is important to calibrate the column using standards that approximate the shape of the molecules of interest. Aqueous, synthetic polymers can be obtained that have a uniform, defined shape. Proteins often assume a roughly spherical shape, especially those with globular morphology. Pullalans are naturally-occurring polysaccharides that have a linear shape in solution. Figure 51 shows the SEC separation or calibration curves of these types of molecules on columns packed with particles that range from 60Å to 1000Å. A smooth calibration curve is achieved when the separation is based only on sample mass injected. Secondary interactions of the sample with the silica surface will cause sample retention (higher elution volume) to be greater than otherwise expected from molecular weight calculations.

Choosing a Size Exclusion Column Based on Molecular Weight

Method development consists of selecting a mobile phase compatible with the sample type, and a column or columns with pore sizes that provide resolution for the molecular weight range of the sample. Column selection should be lead by sample molecular weight, as the elution volume has a linear relationship to the log of molecular weight for a series of molecules of similar shape. Figure 52 shows the effect of various pore sizes in separating a set of proteins with a wide range of molecular weights. The smallest pore size (60Å) gives the highest resolution between the smallest pair of peptides, but fails to resolve the largest two proteins. The larger pore size (1000Å) resolves the large proteins, while giving less resolution for the smaller molecules. Table 10 gives a general guideline for the recommend molecular weight range for each BioBasic SEC pore size.

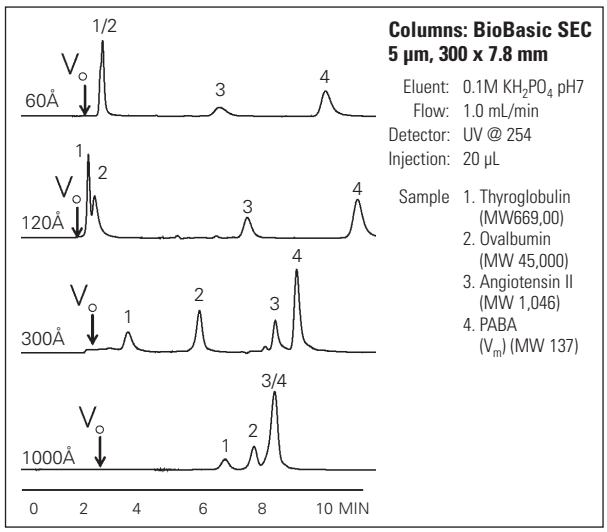


Figure 52: Effect of pore size on SEC separation

	Molecular Weight (kDaltons)		
Column	**Proteins**	**Pullulans (linear dextrans)**	**Polyethylene Oxides/Glycols**
BioBasic SEC 60	0.1 to 6	0.3 to 6	0.1 to 4
BioBasic SEC 120	0.1 to 50	0.3 to 12	0.4 to 10
BioBasic SEC 300	1 to 500	1 to 100	2 to 100
BioBasic SEC 1000	20 to 4,000	20 to >1,000	Not recommended

Table 10: Choosing a BioBasic SEC column based on compound molecular weight

Particle Size and Column Length

The effects of these variables in SEC are largely the same as for RP-HPLC, discussed previously. Efficiency (resolution) increases with decreasing particle size and increasing column length, but at the expense of higher back pressure for smaller particles and longer columns. Longer columns also increase analysis time.

Mobile Phases for Size Exclusion Chromatography

Mobile phases for SEC must maintain the solubility of the analytes. They should also not compromise the biological activity of the biomolecules if that is a requirement for the analysis. Because some biomolecules have strong affinity to the surface of the chromatographic support, mobile phases should be chosen to minimize these interactions. Usually a moderate buffer concentration of 0.05 – 0.2M is used for protein separations.

Effect of Mobile Phase pH

As with ionic strength, the pH of the mobile phase should be chosen that provides ideal analyte solubility and activity, and minimizes the interactions with the silanols on the silica surface. Most silanols are inactive below pH 2. Above pH 8, the dissolution of the silica may occur, leading to shortened column life. Therefore, a pH range of 2 – 8 for most silica-based particles is recommended. Polymer-based particles have higher pH range, but suffer from low efficiency and column lifetime as discussed previously.

Effect of Mobile Phase Ionic Strength (Salt Concentration)

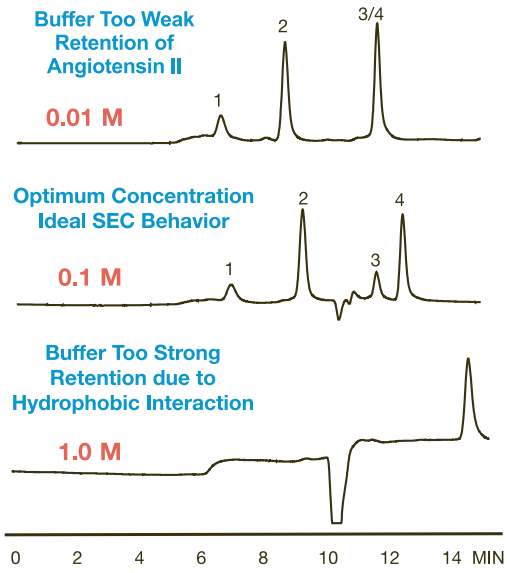
Ionic strength is a key parameter when controlling retention of biomolecules during gel filtration. Mobile phases should be selected to minimize possible interactions that could occur between the protein and surface silanols present in their deprotonated form. This interaction is easily overcome by increasing the ionic strength of the mobile phase. Typically 0.05M salt is sufficient to eliminate this interaction. However, if the ionic strength is too high, hydrophobic interactions are enhanced, leading to increased retention.

Figure 53 shows the effect of potassium phosphate concentration on the SEC separation of two proteins (thyroglobulin and ovalbumin), a peptide (angiotensin), and a small molecule (PABA) that was used to measure the total inclusion volume (V_m). Note that at low ionic strength, the basic peptide angiotensin shows some secondary retention with the acidic silica surface. By increasing the ionic strength to 0.1M, ideal SEC separation is achieved. Higher ionic strength (1M) causes hydrophobic interactions between the analytes and the support leading to excessive retention of all compounds.

Flow Rate and Temperature

Flow rate and temperature affect diffusion rates, and hence affect mass transfer, which is the primary mechanism by which sample molecules move into and out of the pores. The impact of mass transfer is greatest for smaller analytes, as larger proteins have slower kinetics responsible for diffusion and mass transfer. Hence, as seen in Figure 54 greater efficiency is obtained at lower flow rates for larger proteins (thyroglobulin and ovalbumin) than for smaller proteins or peptides (angiotensin).

Protein	MW
Thyroglobulin	669,000
Ovalbumin	45,000
Angiotensin II	1,046
PABA (V_m)	137



Columns: BioBasic SEC 300 5 µm, 300 x 7.8 mm
Eluent: KH_2PO_4, pH7
Flow rate: 1.0 mL/min
Detector: UV @ 254
Injection: 20 µL

Figure 53: Effect of ionic strength on SEC separation

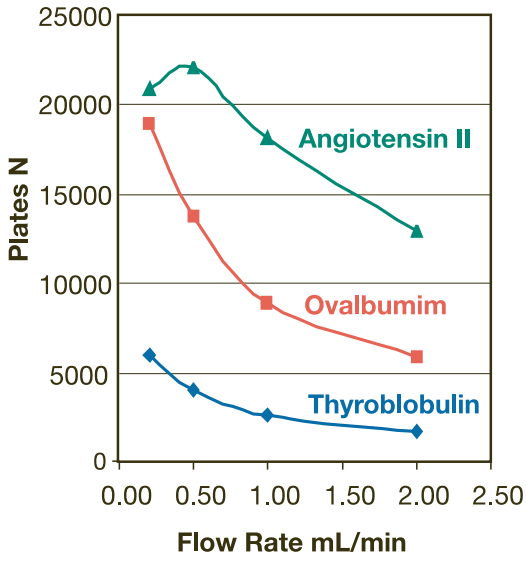


Figure 54: Effect of flow rate on SEC separation

Figure 55 shows how temperature control can increase peak efficiency, as elevated temperatures assist mass transfer. While elevated temperature can be used to increase sensitivity by improving peak efficiency, it should be noted that temperatures above 40°C may denature proteins or compromise other sample types.

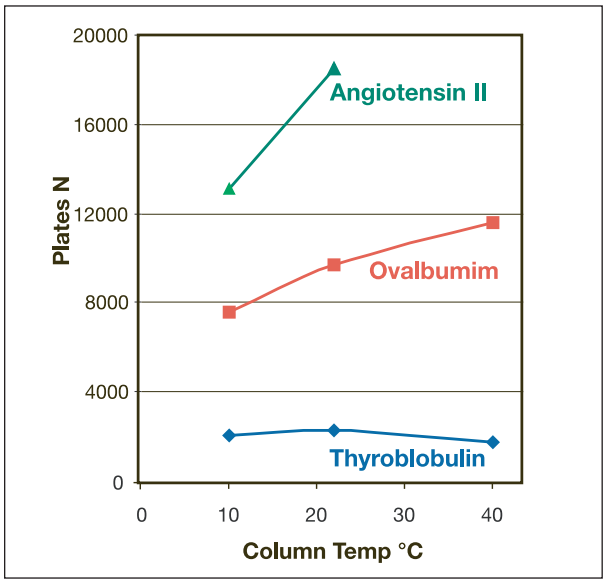


Figure 55: Effect of temperature on SEC separation

Size Exclusion Chromatography for LC/MS of Biomolecules

The same factors that govern reversed phase LC/MS analysis also apply to SEC analysis. As mentioned earlier, SEC is often used as the first step in isolation of a protein from a crude sample (see Figure 56). Its use in the "top-down" proteomics approach will be discussed in a later section of this guide. Like ion exchange, SEC mobile phases must be LC/MS-compatible. Any salts used in LC/MS should be volatile and used in low concentration to maintain sensitivity and prevent fouling of the instrument. Ammonium salts of acetic or formic acid are commonly used.

SEC and Sample Preparation or Fractionation

SEC columns in 60 or 120Å pore sizes can be used to separate proteins from small molecules in biological fluids (serum, urine, *etc.*) in an aqueous mobile phase. The proteins can then be collected for further study, or diverted to waste allowing a protein-free sample to be further separated prior to analysis.

Direct Serum Injection

Often in small molecule analysis of biological fluids, proteins can interfere with quantification or reduce the column lifetime, or both. An alternative to liquid-liquid extraction is the use of small pore size SEC for small molecule analysis with direct serum injection. Figure 56 shows an SEC column application where the slight hydrophobic nature of the polymer coating on the SEC particle and the large surface area of the small pore size combine to retain the small, moderately polar compounds temazepam, diazepam, and lorazepam from a spiked serum sample. LC/MS analysis showed that the large serum proteins cannot enter the 60Å pores of the SEC particles and elute in the unretained fraction.

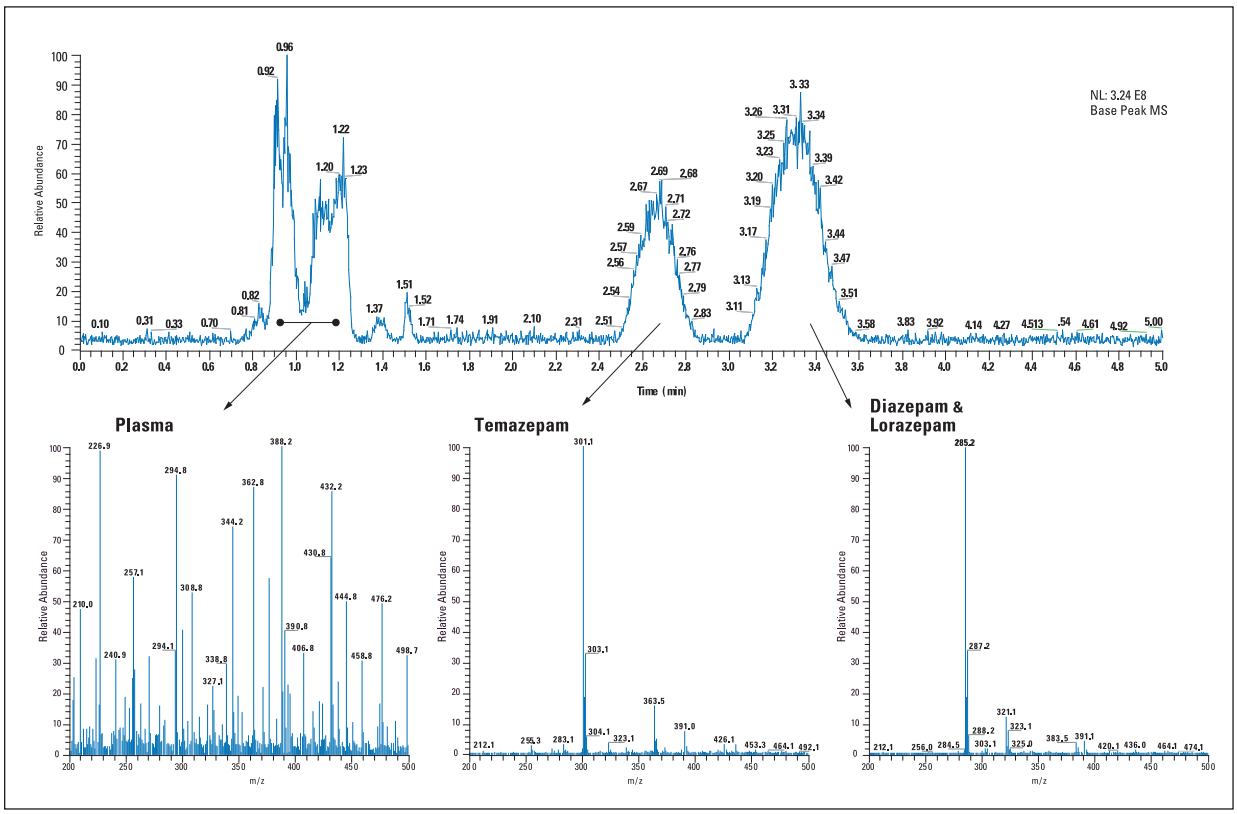


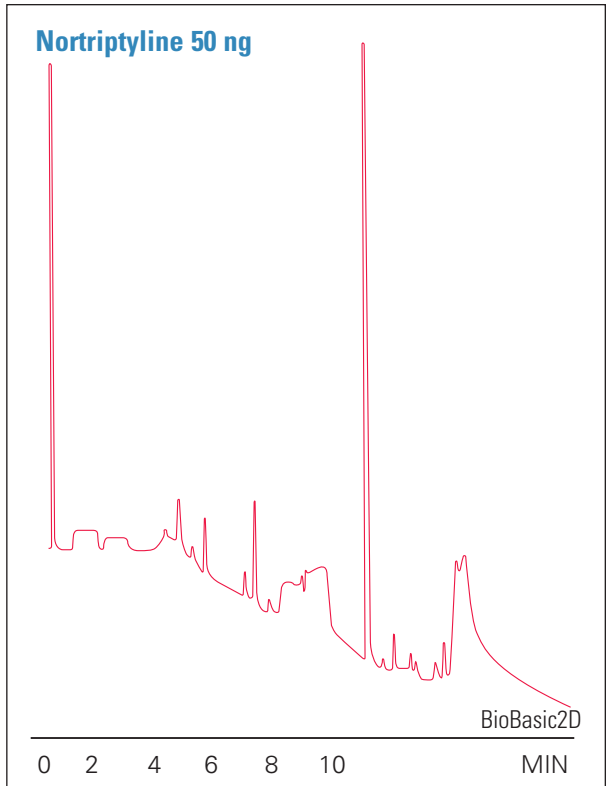
Figure 56: Fractionation of benzodiazepines in plasma using SEC

Two-Dimensional (2-D) Sample Clean-Up

Utilizing a small pore SEC column to separate smaller molecular weight species from serum or other larger molecular weight matrices can be employed in a two-column (two-dimensional) system. This system overcomes the time-consuming sample prep often required for LC/MS analyses of small molecules in serum or other biological matrices. A typical two-dimensional system comprises an SEC column that fractionates the sample by molecular weight, and a second (analytical) column that is appropriate for chromatographic separation of the target analytes (*e.g.* C8, C18, *etc.*).

In Figure 57, column switching was used to transfer the desired small molecule fraction eluted from the 60Å SEC column onto a reversed phase C18 column, where gradient elution was used to analyze the sample. For the more hydrophobic drug (*nortriptyline*), the fraction eluted from the 60Å SEC column with a high aqueous mobile phase is refocused onto a C18 column prior to gradient elution. This combination of size exclusion fractionation and RP-HPLC provides a more sensitive analysis of the nortriptyline as compared to a direct serum injection onto the reversed phase column without prior SEC fractionation.

This 2-D sample fractionation technique can also be used online for mass spectrometer screening of small molecules in proteinaceous matrices. Repeated injections of such samples on most RP-HPLC columns cause rapid column degradation. However, placing an SEC column before the RP-HPLC column and diverting the protein fraction from the SEC column to waste resolves the smaller molecules from serum interferences. Resolution and column stability is exhibited in Figure 58, which shows chromatograms and mass spectra for the first and last injections of the small molecules in a whole serum matrix. Chromatographic resolution and signal intensity are both maintained.



Column 1: BioBasic SEC 60, 5 µm, 150 x 3.0 mm
Part No.:73305-153046
Eluent: 90% 0.05M KH_2PO_4 pH3.5 / 10%ACN
Flow: 0.5 mL/min

Column 2: BetaBasic 18, 3 µm, 100 x 4.6 mm
Part No.:71503-104630
Eluent: A = 90% 0.05M KH_2PO_4 pH3.5/10%ACN
B = 75%ACN/25%Water
Gradient: 0 – 90%B in 10 minutes
Injection: 10 µL Calf Serum x 5µg/mL
Detector: UV @ 215

Figure 57: SEC for sample prep in 2-D mode

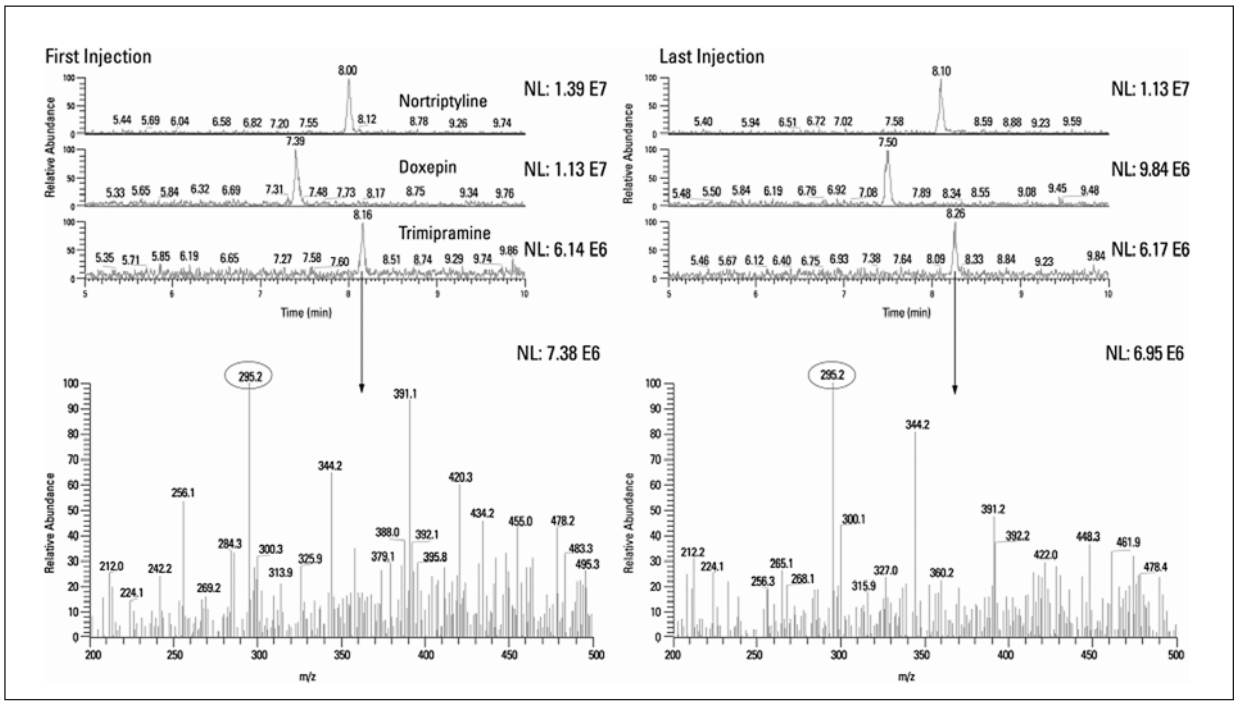


Figure 58: Whole serum full scan LC/MS with 2-D SEC clean-up

Proteomics

Proteomics and LC/MS^n

Proteomics is the study of the proteome, the protein complement expressed by an organism's genome. Whereas the genome is fixed, the proteome it codes for is under constant flux, as a result of daily cycles, diseases, and aging. One of the aims of proteomics research is to accelerate drug discovery, since over 95 percent of all pharmaceuticals target proteins.

Some of the problems facing proteomics researchers are:

- complex, heterogeneous mixtures
- extremely low concentrations of some peptides and proteins
- very small sample volume
- large number of samples to analyze

Now that the humane genome has been sequenced, accelerated protein research is the next logical step, assisted by rapid improvements in the existing technologies. The speed, versatility, resolution, sensitivity, structural information, ability to automate, and the amount of information provided by LC/MS^n compared to traditional protein sequencing methods, makes it an invaluable technique for proteomics researchers.

Top-Down vs. Bottom-Up Approaches

The protein complement of a cell is very complex and variable. Two approaches are currently used to sort through the complexity and generate meaningful data. The bottom-up approach is most common. Here, the entire sample (tissue extract, cell contents, *etc.*) is digested with proteolytic enzymes, separated into peptide fractions, and analyzed by LC/MS^n. Software libraries match the fragmented spectra to known peptides which are in turn used to identify the protein from which the peptide originated. The huge numbers of peptides generated by this approach makes data interpretation difficult, and it is easy to miss peptides that are in very low concentration. The top-down approach, rather than digesting the whole sample, first fractionates the proteins by molecular weight using an SEC column, then digests the fractions prior to LC/MS analysis. This approach has promise in reducing the complexity of the data emerging from the LC/MS system, but it is currently not as routinely used as the bottom-up approach.

HPLC in Proteomics

Much of the work in proteomics is conducted with electrophoresis and 2-D gels; typically isoelectric focusing in one direction and SDS-PAGE in the second direction. However, electrophoresis is difficult to automate and perform online. It also does not permit facile analysis of very large or very small proteins, hydrophobic proteins, and proteins that are at very low concentrations in the sample. The volume of work required calls for methods and tools that offer greater speed and sensitivity to produce rapid breakthroughs. Two recent advances, capillary HPLC and 2-D-chromatography coupled with mass spectroscopy, currently are being applied to solve proteomics challenges.

Capillary LC/MS

Capillary HPLC columns have internal diameters of 500 µm to 25 µm, with smaller ID being an active focus of research. These small ID's require special HPLC systems capable of consistent low flow rates in the µL/min and nL/min range. The main benefit of narrow column ID is that it does not dilute the sample. The more concentrated the sample entering the mass spectrometer, the better the S/N ratio and the lower the analyte levels that can be detected. Another benefit is the very low flow rates enhance production of ions in electrospray ionization (ESI). Table 11 shows the relative concentration effect of the narrow ID columns normalized to a traditional 4.6 mm column. Note that with the same mass of sample injected onto each column, the sensitivity due to the concentration effect of the nanoscale column is over 8000 times that of a standard 4.6 mm ID column. Figure 22 (page 16) also demonstrated the improvement in the S/N ratio of small molecule probes by reducing column ID from 1.0 to 0.5 mm and 0.32 mm.

Column Type	ID	Length	Column Volume	Flow Rate	Injection Volume*	Relative Concentration at Detector
Traditional	4.6 mm	25 cm	4.1 mL	1 mL/min	100 µL	1
Minibore	2.1 mm	25 cm	783 µL	0.2 mL/min	19 µL	5.3
Microbore	1 mm	25 cm	196 µL	47 µL/min	5 µL	21.2
Capillary	100 – 320 µm	25 cm	30 µL	5 µL/min	485 nL	206
Nanoscale	<100 µm	up to 200 cm	490 µL	120 nL/min	12 nL	8459

** Injection volumes are for gradient elution with appropriate gradient delay*

Table 11: Sensitivity provided by narrow ID columns

Online 2-D HPLC

Capillary and nanoscale column dimensions deal effectively with the sensitivity requirement. 2-D HPLC methods use these narrow ID columns to tackle the need to reduce the complexity of the sample. 2-D HPLC combines a fractionation column (ion exchange or SEC) and an analytical column (usually reversed phase) in series to increase the peak capacity and efficiency of protein identification methods. Co-eluting components can be separated into different fractions, in effect removing interferences from more abundant co-eluting species.

The overall aim of the 2-D HPLC system is to increase the peak capacity of the system. Peak capacity is the theoretical number of peaks that can be separated in a given time frame. In a true 2-D HPLC system the total peak capacity is the product (not the sum) of the peak capacities of each dimension. To maximize this product and the resulting peak capacity, the columns should be orthogonal (unrelated) in selectivity.

The generalized steps for a 2-D HPLC experiment are:

Step 1: Load sample onto ion exchange column for low resolution separation/fractionation

- Protein digests
- Protein mixtures
- Size exclusion (whole proteins)

Step 2: Apply series of step gradients to elute fractions from first column onto high resolution reversed phase column

Step 3: Reversed phase separation of each fraction

- Data dependent MS-MS data with dynamic exclusion
- Process fragmentation data
- Repeat steps 2 and 3 until all fractions have been analyzed

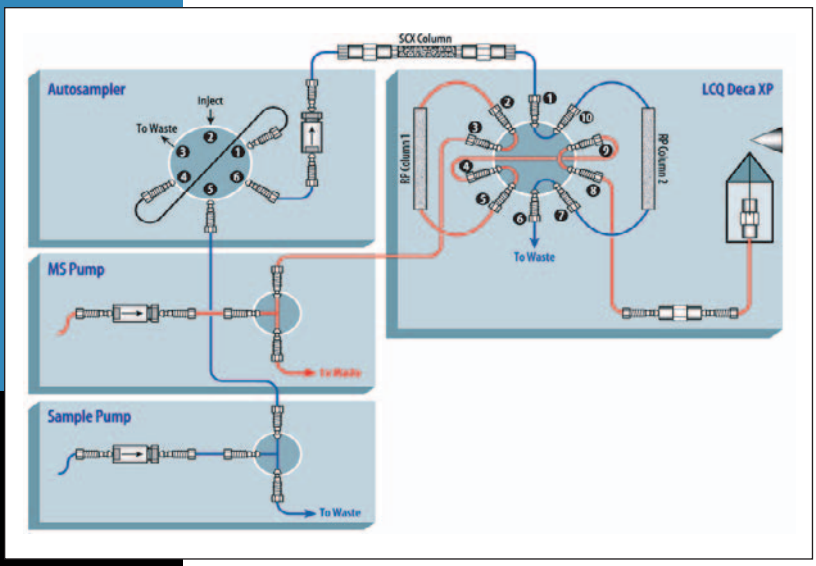


Figure 59: 2-D HPLC set-up

Figure 59 shows a typical 2-D HPLC set-up. An example of an actual 2-D HPLC separation is seen in Figure 60. Peptides were generated by digesting the protein (myoglobin) with trypsin. The protein digest was then loaded onto the SCX ion exchange column at low pH (0.01% or 0.1% formic acid). Note that some peptides elute from the SCX column without salt in the mobile phase; this is considered breakthrough. Fractions of peptides are eluted from the SCX column onto a C18 reversed phase column by 20 µL injections of a salt solution. In the case of Figure pro-x, the salt used was ammonium chloride at 0, 20, 50, 100, 200, and 500 mM concentrations. Each salt aliquot eluted a specific group of peptides from the SCX column. Gradient elution of increasing acetonitrile was applied to the reversed phase column to resolve the peptides in each salt aliquot.

The 2-D HPLC approach yielded 89% sequence coverage, compared to 71% by using the C18 column alone. Another example of a 2-D HPLC method is seen for the protein BSA in Figure 61. Note that the C18 column without prior ion exchange fractionation, resolved approximately fifteen distinct peaks. However, with ion exchange fractionation, the C18 column resolved at least that many peaks from each salt aliquot.

Two aspects of the chromatography columns are worth noting. The ion exchange capillary column used for the initial fractionation should elute analytes with relatively low ionic strength solutions of volatile buffers or salts, particularly for analysis of small organic molecules, nucleotides, peptides and small proteins. High salt concentrations entering the mass spectrometer can reduce the sensitivity. The reversed phase column should also give sharp, symmetrical peaks without TFA or other additives that reduce silanol interactions and also LC/MS sensitivity. The effect of TFA on LC/MS sensitivity was discussed earlier in this guide. It is recommended that both columns be based on 300Å silica to ensure accuracy in quantification. Columns should also be of capillary or nanoscale dimensions to maximize sensitivity. Since the fractionation column sees the entire sample in one injection, it is usually shorter and of larger ID to increase the mass that can be loaded onto it. The analytical (separation) column is often longer than the fractionation column to increase the efficiency and resolution.

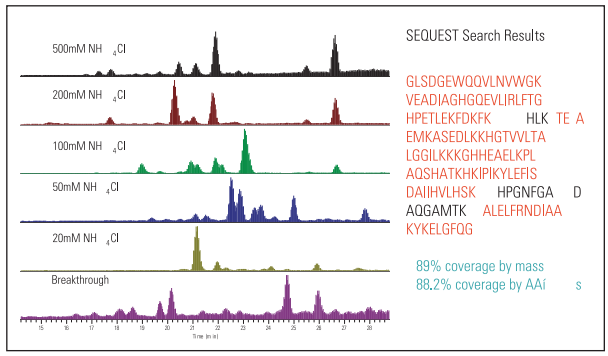
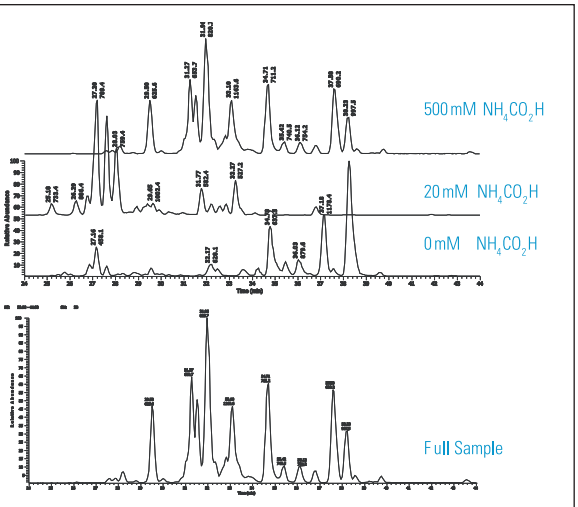


Figure 60: 2-D LC/LC-MS2 tryptic digest of myoglobin



Sample: BSA Digest

Fractionation from BioBasic SCX

Column Dimensions: 100 x 0.32 mm ID
Inj. volume: 3 µL (1500 fmol total digest on column)

Fractions eluted with 20 µL Ammonium Formate (concentrations listed, in 0.1% Formic Acid) onto 100 x 0.180 mm ID BioBasic 18 capillary

Reversed Phase Separation of BSA Digest

Mobile Phase:	A: 0.01% Formic Acid	
	B: ACN + 0.1% Formic Acid	
Flow:	2 µL/min	
Gradient:	Time	%B
	0	0
	3	0
	65	60

Ionization Method:	ESI+
Sheath Gas:	10 arb units
Capillary Temp.:	130°C
Spray Voltage:	2.5 kV
Capillary Voltage:	12.00 kV
Scan Range:	300-2000u

Figure 61: 2-D LC/LC-MS^2 tryptic digest of BSA

Microscale Solid Phase Extraction

Online 2-D HPLC using two (or more) capillary or nanoscale HPLC columns provides high peak capacity and resolution of complex mixtures. However, the online 2-D techniques require specialized column switching hardware and software to control it that are beyond the need and budget of some proteomics applications.

An alternative to 2-D HPLC is solid phase extraction (SPE) followed by 1-D HPLC. The SPE cartridges must minimize sample loss, provide no interferences from the tube or matrix, be able to handle volumes as small as 0.1 µL, and must elute the peptides with MS-compatible eluents. Microscale SPE tips are available packed with many of the same bonded phase materials that reversed phase columns are packed with. The tips are designed to fit directly onto a standard laboratory hand-held pipet.

In a typical microscale SPE experiment, the sample (a protein digest) is loaded onto the microscale SPE tip and washed with water to remove salts. Peptides are removed from the tip with an eluent of low elution strength. The eluting fraction is collected and injected onto an analytical column (usually reversed phase) to separate and identify the peptides. The elution strength is increased until all of the peptides are removed from the microscale SPE tip.

An added benefit of this approach is that they also desalt the sample.

Figure 62a

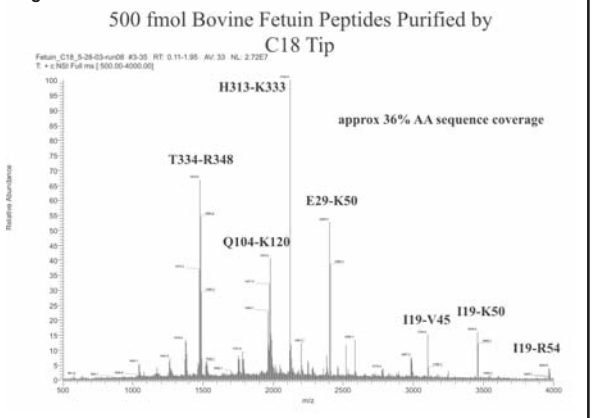


Figure 62b

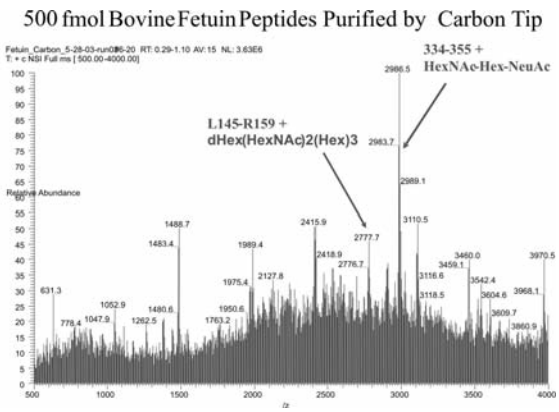


Figure 62c

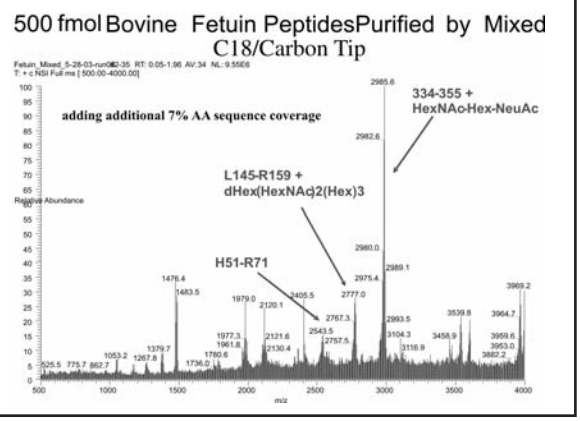


Figure 62: Isolation of bovine fetuin peptides using microscale SPE

An example of a fractionation experiment using microscale SPE is shown in Figure 62. A sample containing peptides from a fetuin digest were loaded onto microscale SPE tips packed with C18, porous graphitic carbon (PGC), or a mixture of both. Both phases work exceptionally well for microscale SPE clean-up of digested proteins, especially in the mixed-mode manner. The mixed mode PCG+C18 phase enabled the detection of additional peptide fragments when compared with using just a single chemistry. This is due to the mixed mode phase's ability to retain both hydrophilic and hydrophobic peptides.

Besides being an alternative to online 2-D HPLC, microscale SPE can be used in desalting, protein purification, MALDI, electrophoresis, or as sample clean-up prior to HPCE, HPLC, and CEC.

Column Care and Maintenance

General Recommendations for Usage

pH: For maximum lifetime, the recommended pH range for silica-based HPLC columns is 2 – 7. The stability of various columns outside this pH range will differ considerably. However, for all columns, lifetime will be progressively shorter as operating pH deviates further from the 2 – 7 range.

Temperature: The typical operating temperature range for silica-based HPLC columns is 5° to 60°C. Higher temperatures may shorten column lifetime.

Buffers: Biological samples often require the chromatographer to include a buffer in the mobile phase to control pH. Care must be taken to ensure that no precipitation occurs in the mobile phase when buffer solutions and organic solvents are mixed. The column and/or HPLC system should never be stored in a mobile phase containing buffer or salt.

Pressure: Column lifetime will generally decrease with increasing operating pressure. A general recommendation is to keep operating pressure below 3000 psi. Pressure is directly proportional to flow rate and column length and inversely proportional to the square of the particle size. Note that a 3 µm column will have approximately twice the pressure of a 5 µm column of the same dimensions. Choice of mobile phase can also have a large effect on pressure. For example, 60/40 methanol/water will generate approximately twice the back pressure of 60/40 acetonitrile/water. A gradual increase in pressure with extended usage is common. A sudden increase in pressure often indicates that the column or connecting tubing has become plugged.

Drop-In Guard Cartridges

Javelin Direct-Connection Guard Columns

UNIGUARD Direct-Connection Guard Cartridge Holder

Javelin Direct-Connection Column Filter

Stand-Alone Guard Cartridge Holder

Column Protection

Using in-line filters and guard columns can extend the lifetime of your column by preventing particles and other impurities from accumulating on the column. For maximum protection install an in-line filter followed by a guard column between the injector and analytical column.

Filters: An in-line filter installed between the injector and analytical column will protect the column from particles that could originate from the mobile phase, pump seals, or sample. For maximum protection the porosity of the in-line frit should be equal or less than that of the inlet frit of the analytical column. A common frit porosity for an in-line filter is 0.5 µm. An increase in system pressure often indicates that it is time to change the in-line filter.

Guards: A guard column is generally a short version of the analytical column it is protecting. The guard protects the analytical column by adsorbing strongly retained impurities that would otherwise foul the analytical column. The frits of the guard can also retain particles as an in-line filter. For maximum protection, and to avoid altering the separation, the guard column should contain packing having the same or similar bonded phase as the analytical column (*i.e.* a C18 guard for a C18 analytical column). The guard packing should also be no more retentive than the packing in the analytical column to avoid significantly increasing overall retention. To minimize the contribution of the guard column to the separation, use a guard packing that is less retentive than that of the analytical column. For example, a wide pore (*e.g.* 300Å) C18 having less surface area, and thus less retention, could be used as a guard for a smaller pore (*e.g.* 150Å) C18 analytical column. A noticeable deterioration of the separation or a significant increase in back pressure can signal that it is time to change the guard column.

Column Cleaning

If some components of the sample are not eluted by the mobile phase, they will gradually accumulate on the column, eventually leading to loss of column performance. For optimum reproducibility and maximum column lifetime, periodically clean the column, rather than waiting until column performance has been affected. For high back pressure, reverse the flow direction if the column design allows. Backflushing at low flow rates should not harm a well-packed silica-based column. Do not backflush into the detector. Clean the column by backflushing at a low flow (*i.e.* half of usual) with 10 – 20 column volumes of an appropriate strong mobile phase. For a reversed phase column the most appropriate cleaning mobile phase is one containing a high proportion of a polar organic solvent, such as isopropanol. Flushing with several gradient cycles of weak to strong mobile phase is sometimes more effective than flushing isocratically with just strong mobile phase, particularly for hydrophobic proteins adsorbed on reversed phase columns.

If the above procedure does not adequately clean the column, alternative flushing mobile phases are a nonpolar organic solvent, or a low concentration surfactant solution. A nonpolar organic solvent like methylene chloride or chloroform may be required to elute very hydrophobic or oily compounds. A surfactant solution, such as 0.5% SDS will be effective at removing precipitated proteins. Denaturing agents, such as 5 – 8M urea or guanidine HCl are sometimes effective at removing strongly adsorbed proteins. In addition, sometimes flushing with a dilute aqueous buffer with pH significantly lower or higher than the chromatographic mobile phase can solubilize precipitated impurities. The most effective cleaning scheme will depend on the conditions and samples to which the column is exposed.

The main purpose of the above procedure is to remove the build-up of compounds that do not elute under the usual mobile phase conditions, but a secondary purpose is to recondition the stationary phase after it has been changed (usually a gradual process) in some way by the mobile phase or one of its components. Before flushing with organic solvents, buffers and additives should first be flushed out of the column with a high aqueous/organic mixture (*i.e.* 90/10 water/acetonitrile) rather than pure water. Elevated temperatures (*e.g.* 50°C) can also enhance the cleaning effect.

Column frits that hold the packing bed in place should be changed only as a last resort to restore the column. Please contact the column manufacturer before removing a column end fitting.

Column Storage

A mixture of acetonitrile and water is a good storage solvent for reversed phase columns. Acetonitrile concentration should be at least 10%. Avoid storage with acidic modifiers such as trifluoroacetic acid. Buffers and additives should be flushed from the system prior to storage to minimize the chance of chemical reaction or residue deposit from inadvertent evaporation. A good storage solvent retards biological growth and minimizes chemical reactions within the column, such as hydrolysis of the stationary phase. Pure water should generally be avoided as storage solvent for silica-based columns as it does not retard microbial growth and can potentially promote hydrolysis. It is desirable to select storage solvents that are miscible with the typical mobile phase employed for separation. Using 10% acetonitrile as your storage solvent will allow you to directly equilibrate the column with almost any buffer containing mobile phase without danger of precipitating salts.

Steel plugs are recommended over plastic for long periods of storage to help prevent evaporation of the storage solvent, although there is no strong evidence that silica-based columns are harmed by dry storage if residues have not formed. When employing alcohol/water mixtures for flushing or storage, use caution because they are more viscous than either water or alcohol alone and can generate high back pressures.

Appendix 1: Quick Look-Up Tables

Protein Structure Defined

The native state of each protein has a characteristic 3-dimensional shape. Protein structure, and hence its shape is classified in the following terms. Protein "conformation" refers to the combined 2°, 3°, and 4° structures.

Primary (1°)	The covalent backbone of the polypeptide chain and its amino acid sequence.
Secondary (2°)	Regular, recurring arrangement in space of the polypeptide chain in one dimension. Includes α-helices, ß-sheets, spirals, coils
Tertiary (3°)	Bending or folding of the polypeptide chain in 3-dimensions, usually via S-S bonds
Quaternary (4°)	Subunit assembly, how the individual polypeptide chains are arranged (not all proteins have 4° structure)

Table 12

Molecular Weight of Some Common Biomolecules

Compound	Approximate MW (Daltons)
Tobacco mosaic virus	40,000,000
Pyruvate dehydrogenase complex	7,000,000
DNA	4,000,000
Thyroglobulin	669,000
Glutamine synthetase	592,000
Ferritin	440,000
Glycogen phosphorylase	370,000
Aspartate transcarbamoylase	310,000
Catalase	232,000
ß-Amylase	200,000
Tryptophan synthetase	159,000
Alcohol deyhdrogenase	150,000
Hexokinase	102,000
BSA	66,000
Hemoglobin	64,500
Ovalbumin	45,000
Carbonic anhydrase	29,000
Chymotrypsinogen	23,200
ß-Lactoglobulin	17,500
Myoglobin	16,900
Lysozyme	13,900
Ribonuclease A	13,700
Cytochrome C	12,500
Aprotinin	6,500
Insulin	5,700
Insulin Chain B	3,496
Neurotensin	1,673
Angiotensin II	1,046
Oxytocin	1,007
5-AMP	347
GL-tyrosine hydrate	238
Triglycine	191
Tyrosine	180
L-Arginine	174
D-Glutamic acid	147
p-Aminobenzoic acid	137
Benzyl alcohol	108

Table 13

Common Amino Acids, Abbreviations, MW, and pK_a

	Abbreviation			pK_a Value		
Name	3 Letter	1 Letter	MW (Daltons)	-COOH	$-NH_3^+$	R Group
Alanine	Ala	A	89	2.34	9.69	
Arginine	Arg	R	174	2.17	9.04	12.48
Asparagine	Asn	N	132	2.01	8.8	
Aspartic Acid	Asp	D	133	1.89	9.6	3.65
Cysteine	Cys	C	121	1.96	8.18	10.29
Glutamine	Gln	Q	146	2.17	9.13	
Glutamic Acid	Glu	E	147	2.19	9.67	4.25
Glycine	Gly	G	75	2.34	9.6	
Histidine	His	H	155	1.8	9.17	6
Isoleucine	Ile	I	131	2.35	9.68	
Leucine	Leu	L	131	2.36	9.6	
Lysine	Lys	K	146	2.18	8.95	10.52
Methionine	Met	M	149	2.28	9.2	
Phenylalanine	Phe	F	165	1.83	9.12	
Proline	Pro	P	115	1.99	10.96	
Serine	Ser	S	105	2.21	9.15	13.6
Threonine	Thr	T	119	2.11	9.62	13.6
Tryptophan	Trp	W	204	2.38	9.39	
Tyrosine	Tyr	Y	181	2.2	9.11	10.06
Valine	Val	V	117	2.32	9.61	

Table 14

Properties of Common HPLC Solvents

Solvent	RI	UV Cutoff	BP (°C)	Polarity Index (Snyder)
Water	1.333	180	100	9
Methanol	1.329	205	64.7	6.6
Acetonitrile	1.344	190	81.6	6.2
Tetrahydrofuran	1.408	212 – 230	66	4.2
Methyl Ethyl Ketone (MEK)	1.379	330	80	4.5
Acetone	1.359	330	56.3	5.4
Ethyl Acetate	1.37	256	77.1	4.3
Dimethyl Sulfoxide	1.478		189	6.5
2-propanol	1.38	210+	82.4 – 117.7	4.3
Ethanol	1.361	205 – 210	78.3	5.2
Acetic Acid	1.372	210	117.9	6.2

Table 15

Common Buffer Systems Used in HPLC of Biomolecules

Buffer		pK_a	Useful pH Range	MS-Compatible?
TFA		0.30		Yes
Phosphate	pK_1	2.1	1.1 – 23.1	No
	pK_2	7.2	6.2 – 28.2	No
	pK_3	12.3	11.3 – 213.3	No
Citrate	pK_1	3.1	2.1 – 24.1	Yes
	pK_2	4.7	3.7 – 25.7	Yes
	pK_3	5.4	4.4 – 26.4	Yes
Formate		3.8	4.4 – 26.4	Yes
Acetate		4.8	3.8 – 25.8	Yes
Tris base (Trizma, THAM)		8.3	7.3 – 29.3	Yes
Ammonia		9.2	8.2 – 210.2	Yes
Borate		9.2	8.2 – 210.2	Yes
Diethylamine		10.5	9.5 – 211.5	Yes
Carbonate	pK_1	6.4		Yes
	pK_2	10.2		Yes
Triethanolamine		7.80		Yes

Table 16

Typical Flow Rates and Injection Volumes Versus HPLC Column ID

ID	Injection Volume	Flow Rate
4.6 mm	30 µL	0.5 – 22 mL/min
4 mm	20 µL	0.8 – 21.2 mL/min
3 mm	10 µL	0.4 – 20.8 mL/min
2.1 mm	5 µL	0.1 – 20.4 mL/min
1 mm	1 µL	50 – 2100 µL/min
0.5 mm	350 nL	10 – 220 µL/min
0.32 mm	150 nL	4 – 210 µL/min
0.18 mm	50 nL	1 – 23 µL/min
75 µm	10 nL	0.2 – 20.5 µL/min
50 µm	5 nL	0.1 – 20.2 µL/min
25 µm	1 nL	<0.1 µL/min

Table 17

Preparative Scale-Up Calculations

Flow rate and sample mass load scaling are required only when changing the column ID. Scaling of flow rates allows peak retention times to remain constant between columns of differing ID. Assuming column length is a constant, the scale factor can be calculated using the following formula:

scale factor = [(ID of column A)/(ID of column B)]

Column ID (mm) Scaling Factor	Flow Rate Capacity (mg)	Load Rates (mL/min)	Typical Flow
4.6	1	1	0.5 – 2.0
10	4.7	4.7	4 – 15
21.2	19.5	19.5	10 – 50
30	42.5	42.5	40 – 100
40	75.6	75.6	60 – 160
50	118	118	100 – 300
100	473	473	400 – 1,000

Table 18

Tubing Internal Diameters and Volumes

The ID and total volume of the capillary tubing connecting the columns to other components of system influences chromatographic efficiency and the sensitivity of the system. This table shows the total volume per length for capillary tubing of various internal diameters.

Internal Diameter			Volume	
Inches	Millimeters	Microns	µL/in	µL/cm
0.001	0.025	25	0.013	0.005
0.002	0.051	51	0.051	0.020
0.003	0.076	76	0.116	0.046
0.004	0.102	102	0.206	0.081
0.005	0.127	127	0.322	0.127
0.006	0.152	152	0.463	0.182
0.007	0.178	178	0.631	0.248
0.010	0.254	254	1.287	0.507
0.015	0.381	381	2.896	1.140
0.020	0.508	508	5.148	2.027
0.030	0.762	762	11.583	4.560
0.040	1.016	1016	20.592	8.107
0.062	1.575	1575	49.472	19.477

Table 19

Appendix 2: Columns for HPLC of Biomolecules

5 µm BioBasic SEC Columns

Chemistry	Pore Size	Dimensions (mm)	Part Number
BioBasic SEC 60	60 Å	300 x 7.8	73305-307846
	60 Å	150 x 7.8	73305-157846
	60 Å	30 x 7.8 (guard)	73305-037821
BioBasic SEC 120	120 Å	300 x 7.8	73405-307846
	120 Å	150 x 7.8	73405-157846
	120 Å	30 x 7.8 (guard)	73405-037821
BioBasic SEC 300	300 Å	300 x 7.8	73505-307846
	300 Å	150 x 7.8	73505-157846
	300 Å	30 x 7.8 (guard)	73505-037821
BioBasic SEC 1000	1000 Å	300 x 7.8	73605-307846
	1000 Å	150 x 7.8	73605-157846
	1000 Å	30 x 7.8 (guard)	73605-037821

Other column dimensions are available. Please call Customer Service for more information regarding semi-prep sizes and sample clean-up dimensions. For information on bulk quantities, please inquire.

5 µm BioBasic Reversed Phase Columns

Description	Length (mm)	4.6 mm ID	4.0 mm ID	3.0 mm ID	2.1 mm ID	1.0 mm ID
BioBasic 18	30	72105-034630	72105-034030	72105-033030	72105-032130	72105-031030
	50	72105-054630	72105-054030	72105-053030	72105-052130	72105-051030
	100	72105-104630	72105-104030	72105-103030	72105-102130	72105-101030
	150	72105-154630	72105-154030	72105-153030	72105-152130	72105-151030
	250	72105-254630	72105-254030	72105-253030	72105-252130	72105-251030
BioBasic 8	50	72205-054630	72205-054030	72205-053030	72205-052130	72205-051030
	100	72205-104630	72205-104030	72205-103030	72205-102130	72205-101030
	150	72205-154630	72205-154030	72205-153030	72205-152130	72205-151030
	250	72205-254630	72205-254030	72205-253030	72205-252130	72205-251030
BioBasic 4	50	72305-054630	72305-054030	72305-053030	72305-052130	72305-051030
	100	72305-104630	72305-104030	72305-103030	72305-102130	72305-101030
	150	72305-154630	72305-154030	72305-153030	72305-152130	72305-151030
	250	72305-254630	72305-254030	72305-253030	72305-252130	72305-251030
BioBasic CN	50	72905-054630	72905-054030	72905-053030	72905-052130	72905-051030
	100	72905-104630	72905-104030	72905-103030	72905-102130	72905-101030
	150	72905-154630	72905-154030	72905-153030	72905-152130	72905-151030
	250	72905-254630	72905-254030	72905-253030	72905-252130	72905-251030
BioBasic Phenyl	50	72405-054630	72405-054030	72405-053030	72405-052130	72405-051030
	100	72405-104630	72405-104030	72405-103030	72405-102130	72405-101030
	150	72405-154630	72405-154030	72405-153030	72405-152130	72405-151030
	250	72405-254630	72405-254030	72405-253030	72405-252130	72405-251030

Other column dimensions are available, including preparative columns. BioBasic 18 is available in a 10 µm particle size. Please call Customer Service for more information.

5 µm BioBasic Reversed Phase Drop-In Guard Cartridges

Description	Length (mm)	4.6 mm ID	4.0 mm ID	3.0 mm ID	2.1 mm ID	1.0 mm ID
BioBasic 18	10	72105-014001	72105-014001	72105-013001	72105-012101	72105-011001
BioBasic 8	10	72205-014001	72205-014001	72205-013001	72205-012101	72205-011001
BioBasic 4	10	72305-014001	72305-014001	72305-013001	72305-012101	72305-011001
BioBasic CN	10	72905-014001	72905-014001	72905-013001	72905-012101	72905-011001
BioBasic Phenyl	10	72405-014001	72405-014001	72405-013001	72405-012101	72405-011001
UNIGUARD Direct-Connect Drop-inGuard Cartridge Holder		850-00	850-00	852-00	852-00	851-00

5 µm BioBasic PEEK™ Columns

Description	Length (mm)	4.6 mm ID	2.1 mm ID
BioBasic 18	100	72105-104668	72105-102168
	150	72105-154668	72105-152168
	250	72105-254668	72105-252168
BioBasic 8	100	72205-104668	72205-102168
	150	72205-154668	72205-152168
	250	72205-254668	72205-252168
BioBasic 4	100	72685-104668	72685-102168
	150	72685-154668	72685-152168
	250	72685-254668	72685-252168

Other phases and column dimensions are available in bio-inert column hardware. Please call Customer Service for more information.

5 µm BioBasic PEEK Guard Cartridges

Description	Length (mm)	4.6 mm ID	2.1 mm ID
BioBasic 18	10	72105-014003	72105-012103
BioBasic 8	10	72205-014003	72205-012103
BioBasic 4	10	72305-014003	72305-012103
Bio-inert Guard Holder	10	C270-01	enquire

5 µm BioBasic Ion Exchange Columns

Particle Size	Length (mm)	4.6 mm ID	4.0 mm ID	3.0 mm ID	2.1 mm ID	1.0 mm ID
BioBasic AX	50	73105-054630	73105-054030	73105-053030	73105-052130	73105-051030
	100	73105-104630	73105-104030	73105-103030	73105-102130	73105-101030
	150	73105-154630	73105-154030	73105-153030	73105-152130	73105-151030
	250	73105-254630	73105-254030	73105-253030	73105-252130	73105-251030
BioBasic SCX	50	73205-054630	73205-054030	73205-053030	73205-052130	73205-051030
	100	73205-104630	73205-104030	73205-103030	73205-102130	73205-101030
	150	73205-154630	73205-154030	73205-153030	73205-152130	73205-151030
	250	73205-254630	73205-254030	73205-253030	73205-252130	73205-2510

Other column dimensions are available. Please call Customer Service for more information.

5 µm BioBasic Ion Exchange Drop-In Guard Cartridges

Particle Size	Length (mm)	4.6 mm ID	4.0 mm ID	3.0 mm ID	2.1 mm ID	1.0 mm ID
BioBasic AX	10	73105-014001	73105-014001	73105-013001	73105-012101	73105-011001
BioBasic SCX	10	73205-014001	73205-014001	73205-013001	73205-012101	73205-011001
UNIGUARD Direct-Connect Drop-inGuard Cartridge Holder 		850-00	850-00	852-00	852-00	851-00

Other column dimensions are available. Please call Customer Service for more information.

Replacement Columns for Thermo Scientific ProteomeX LCQ™ System

Description	Dimensions (mm x µm)	Thermo Scientific ProteomeX LCQ Part Number	Replacement Part Number
5 µm BioBasic 18 Flexible KAPPA	100 x 180	00109-00508	72105-100266
5 µm BioBasic SCX KAPPA	100 x 320	00109-00510	73205-100365

Replacement Columns for Thermo Scientific ProteomeX LTQ™ System

Description	Dimensions (mm x µm)	Therm Scientific ProteomeX LTQ Part Number
5 µm BioBasic 18 KAPPA	100 x 180	72105-100267
5 µm BioBasic SCX KAPPA	100 x 320	73205-100367

3 µm & 5 µm BetaBasic™ Columns

Description	Length (mm)	4.6 mm ID	4.0 mm ID	3.0 mm ID	2.1 mm ID	1.0 mm ID
3 µm BetaBasic 18	30	71503-034630	71503-034030	71503-033030	71503-032130	71503-031030
	50	71503-054630	71503-054030	71503-053030	71503-052130	71503-051030
	100	71503-104630	71503-104030	71503-103030	71503-102130	71503-101030
	150	71503-154630	71503-154030	71503-153030	71503-152130	71503-151030
5 µm BetaBasic 18	30	71505-034630	71505-034030	71505-033030	71505-032130	71505-031030
	50	71505-054630	71505-054030	71505-053030	71505-052130	71505-051030
	100	71505-104630	71505-104030	71505-103030	71505-102130	71505-101030
	150	71505-154630	71505-154030	71505-153030	71505-152130	71505-151030
	250	71505-254630	71505-254030	71505-253030	71505-252130	71505-251030
3 µm BetaBasic 8	50	71403-054630	71403-054030	71403-053030	71403-052130	71403-051030
	150	71403-154630	71403-154030	71403-153030	71403-152130	71403-151030
5 µm BetaBasic 8	50	71405-054630	71405-054030	71405-053030	71405-052130	71405-051030
	100	71405-104630	71405-104030	71405-103030	71405-102130	71405-101030
	150	71405-154630	71405-154030	71405-153030	71405-152130	71405-151030
3 µm BetaBasic 4	50	71603-054630	71603-054030	71603-053030	71603-052130	71603-051030
	100	71603-104630	71603-104030	71603-103030	71603-102130	71603-101030
5 µm BetaBasic 4	50	71605-054630	71605-054030	71605-053030	71605-052130	71605-051030
	150	71605-154630	71605-154030	71605-153030	71605-152130	71605-151030
	250	71605-254630	71605-254030	71605-253030	71605-252130	71605-251030
3 µm BetaBasic CN	50	71703-054630	71703-054030	71703-053030	71703-052130	71703-051030
	100	71703-104630	71703-104030	71703-103030	71703-102130	71703-101030
	150	71703-154630	71703-154030	71703-153030	71703-152130	71703-151030
5 µm BetaBasic CN	50	71705-054630	71705-054030	71705-053030	71705-052130	71705-051030
	100	71705-104630	71705-104030	71705-103030	71705-102130	71705-101030
	150	71705-154630	71705-154030	71705-153030	71705-152130	71705-151030
	250	71705-254630	71705-254030	71705-253030	71705-252130	71705-251030
3 µm BetaBasic Phenyl	50	71803-054630	71803-054030	71803-053030	71803-052130	71803-051030
	150	71803-154630	71803-154030	71803-153030	71803-152130	71803-151030
5 µm BetaBasic Phenyl	50	71805-054630	71805-054030	71805-053030	71805-052130	71805-051030
	150	71805-154630	71805-154030	71805-153030	71805-152130	71805-151030
	250	71805-254630	71805-254030	71805-253030	71805-252130	71805-251030

Other column dimensions are also available. Please call Customer Service for more information.

3 µm & 5 µm BetaBasic™ Drop-In Guard Cartridges

Description	Length (mm)	4.6 mm ID	4.0 mm ID	3.0 mm ID	2.1 mm ID	1.0 mm ID
3 µm BetaBasic 18	10	71503-014001	71503-014001	71503-013001	71503-012101	71503-011001
5 µm BetaBasic 18	10	71505-014001	71505-014001	71505-013001	71505-012101	71505-011001
3 µm BetaBasic 8	10	71403-014001	71403-014001	71403-013001	71403-012101	71403-011001
5 µm BetaBasic 8	10	71405-014001	71405-014001	71405-013001	71405-012101	71405-011001
3 µm BetaBasic 4	10	71603-014001	71603-014001	71603-013001	71603-012101	71603-011001
5 µm BetaBasic 4	10	71605-014001	71605-014001	71605-013001	71605-012101	71605-011001
3 µm BetaBasic CN	10	71703-014001	71703-014001	71703-013001	71703-012101	71703-011001
5 µm BetaBasic CN	10	71705-014001	71705-014001	71705-013001	71705-012101	71705-011001
3 µm BetaBasic Phenyl	10	71803-014001	71803-014001	71803-013001	71803-012101	71803-011001
5 µm BetaBasic Phenyl	10	71805-014001	71805-014001	71805-013001	71805-012101	71805-011001
UNIGUARD Direct-Connect Drop-inGuard Cartridge Holder 		850-00	852-00	852-00	851-00	

3 µm & 5 µm Hypercarb Columns

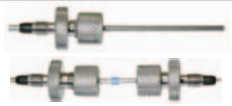
Description	Length (mm)	4.6 mm ID	3.0 mm ID	2.1 mm ID	1.0 mm ID
3 µm Hypercarb	30	35003-034630	35003-033030	35003-032130	35003-031030
	50	35003-054630	35003-053030	35003-052130	35003-051030
	100	35003-104630	35003-103030	35003-102130	35003-101030
5 µm Hypercarb	30	35005-034630	35005-033030	35005-032130	35005-031030
	50	35005-054630	35005-053030	35005-052130	35005-051030
	100	35005-104630	35005-103030	35005-102130	35005-101030

Other column dimensions are also available. Please call Customer Service for more information.

3 µm & 5 µm Hypercarb Drop-In Guard Cartridges

Description	Length (mm)	4.6 mm ID	3.0 mm ID	2.1 mm ID	1.0 mm ID
3 µm	10	35003-014001	35003-013001	35003-012101	35003-011001
5 µm	10	35005-014001	35005-013001	35005-012101	35005-011001
UNIGUARD Direct-Connect Drop-inGuard Cartridge Holder 		850-00	852-00	852-00	851-00

Other Hardware Designs for Thermo Columns

KAPPA™ Columns		Highly efficient capillary columns
PicoFrit™ Columns		Nanobore fused silica columns for LC/MS
Javelin™ Guard Columns		Economical, non-tested analytical columns
Preparative Columns		Available in 10, 21.2, 30, 40, 50 and 100 mm ID
SLIPFREE™ Column Connectors		Easy to use, void and leak free connectors

Call Customer Service for availability of phases in these unique designs.

Appendix 3: Sample Preparation Products for Biomolecules

HyperSep Tip is a revolutionary micro pipette tip for sample preparation and protein purification. The chromatographic material is attached to the inner wall of the tip without using polymers or glue. This maximises the surface area in contact with the sample and avoids potential contamination problems from a supporting matrix. HyperSep Tips are available in two sizes, 1-10 µL and 10-200 µL and permit separation in volumes as low as 100 nL.

HyperSep SpinTip is a unique concept in sample preparation. HyperSep SpinTip is a pipette tip with a fine slit at the bottom (1-2 µL) which permits liquids to pass through, but retains the chromatographic media (20-30µm) in the tip. This eliminates the need for a filter and, therefore, dead volume. HyperSep SpinTips are availabel in two sizes, 1-10µL and 10-200µL and permit separation in volumes as low as 100nL. HyperSep SpinTips have the additional benefit in that they can be used as a spin column to process samples via centrifugation.

HyperSep Tips and SpinTips can be used for several different applications, such as protein purification, mass spectrometry and MALDI. In many of these applications, the volume of sample is small. The benefit of using HyperSep Tips and SpinTips here is that the danger of losing your sample is eliminated. For example, the most commonly used analytical method for the structural analysis of proteins is mass spectrometry. In this method, the protein is first digested by a proteolytic enzyme, such as trypsin, and the resulting peptide mixtures are purified and then analysed. The purification process can be carried out using a HyperSep Tip or SpinTip product.

HyperSep Tip and SpinTip products overcome the problems associated with handling small volumes of sample, for example, small volumes associated with gel-isolated proteins used in proteolytic digests. HyperSep Tip and SpinTip are available in a wide range of materials, including the unique Hypercarb material for the retention of polar compounds. HyperSep Tip and SpinTip are compatible with a range of different pipettes, including the Thermo Scientific range of Finnpipettes.

Ordering Information

HyperSep Tip, 1 - 10 µL Volume

Description	Quantity	Part Number
BioBasic™ C18	96	60109-201
BioBasic C8	96	60109-202
BioBasic C4	96	60109-203
Hypercarb	96	60109-204
Hypercarb + C18 (mix mode)	96	60109-205
HILIC	96	60109-206
Trypsin	96	60109-207
Titanium Dioxide	96	60109-208

HyperSep Tip, 10 - 200 µL Volume

Description	Quantity	Part Number
BioBasic C18	96	60109-209
BioBasic C8	96	60109-210
BioBasic C4	96	60109-211
Hypercarb	96	60109-212
Hypercarb + C18 (mix mode)	96	60109-213
HILIC	96	60109-214
Trypsin	96	60109-215
Titanium Dioxide	96	60109-216

Ordering Information

HyperSep SpinTip, 1 - 10 µL Volume

Description	Quantity	Part Number
BioBasic C18	96	60109-401
BioBasic C8	96	60109-402
BioBasic C4	96	60109-403
Hypercarb	96	60109-404
Hypercarb + C18 (mix mode)	96	60109-405
HILIC	96	60109-406
Trypsin	96	60109-407
POROS™ Weak Anion Exchanger	96	60109-408
POROS Strong Anion Exchanger	96	60109-409
POROS Strong Cation Exchanger	96	60109-410
Titanium Dioxide	96	60109-411

HyperSep SpinTip, 10 - 200 µL Volume

Description	Quantity	Part Number
BioBasic C18	96	60109-412
BioBasic C8	96	60109-413
BioBasic C4	96	60109-414
Hypercarb	96	60109-415
Hypercarb + C18 (mix mode)	96	60109-416
HILIC	96	60109-417
Trypsin	96	60109-418
POROS Weak Anion Exchanger	96	60109-419
POROS Strong Anion Exchanger	96	60109-420
POROS Strong Cation Exchanger	96	60109-421
Titanium Dioxide	96	60109-422

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Appendix 4: Index

Appendix 5: References

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