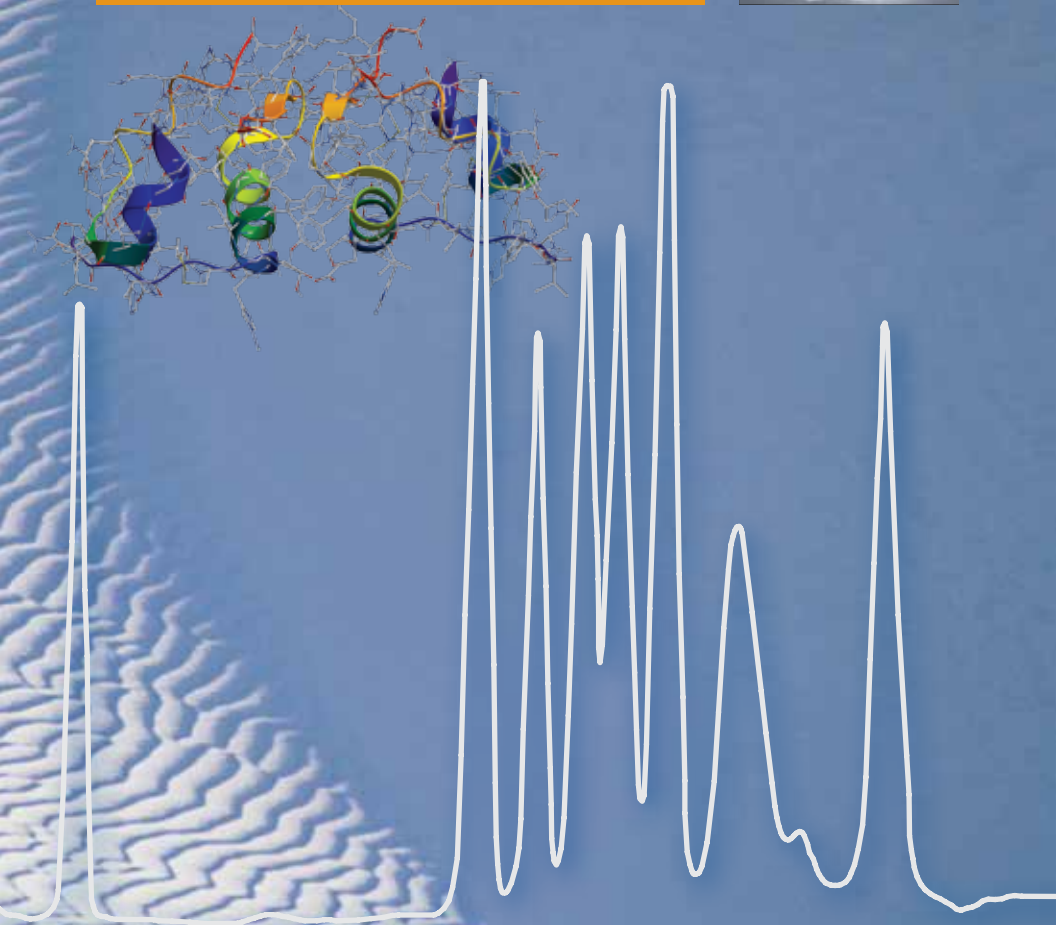# Protein and Peptide Analysis and Purification

GRACE

Fifth Edition, 2013

This handbook presents the basic principles of reversed-phase HPLC for the analysis and purification of polypeptides. The fifth edition offers new, exciting information on:

- New approaches to improve polypeptide separations, including modification of the solvent system
- Application of new column technologies, including ultra-fast techniques
- Mass Spec optimization for nano, capillary, and microbore columns
- Scaling considerations for preparative purification
- New high capacity Vydac® 150 media for peptide purification

Table of Contents

Introduction: Analysis and Purification of Proteins and Peptides by Reversed-Phase HPLC

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) has become a widely used, well-established tool for the analysis and purification of biomolecules. The reason for the central role that RP-HPLC now plays in analyzing and purifying proteins and peptides is **Resolution:** RP-HPLC is able to separate polypeptides of nearly identical sequences, not only for small peptides such as those obtained through trypsin digestion, but even for much larger proteins. Polypeptides which differ by a single amino acid residue can often be separated by RP-HPLC as illustrated in **Figure 1** showing the separation of insulin variants.[1] Insulin variants have molecular weights of around 5,300 with only slightly different amino acid sequences, yet most variants can be separated by RP-HPLC. In particular, reversed-phase chromatography is able to separate human and rabbit insulin **which only differ by a methylene group—rabbit insulin has a threonine where human insulin has a serine!**

The scientific literature has many examples where RP-HPLC has been used to separate similar polypeptides. Insulin-like growth factor with an oxidized methionine has been separated from its non-oxidized analogue[2] and interleukin-2 muteins have been separated from each other.[3] In the latter paper, Kunitani and colleagues proposed that RP-HPLC retention could provide information on the conformation of retained proteins on the reversed-phase surface. They studied thirty interleukin-2 muteins and were able to separate muteins that were nearly identical. Interleukin in which a methionine was oxidized was separated from the native form and in other cases single amino acid substitutions were separated from native forms. They concluded that protein conformation was very important in reversed-phase separations and that RP-HPLC could be used to study protein conformation.

Separation of Closely Related Insulin Variants by RP-HPLC
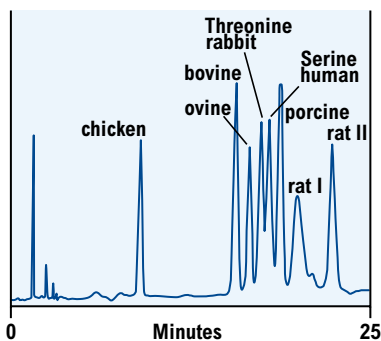
**Figure 1.** RP-HPLC separates rabbit and human insulin that differ by only a single amino acid. **Column:** Vydac® 214TP54 column. **Eluent:** 27–30% acetonitrile (ACN) in 0.1% TFA over 25 minutes at 1.5mL/minute.

In the process they demonstrated the resolving power of the technique for similar polypeptides.

RP-HPLC is used for the separation of peptide fragments from enzymatic digests[10-16] and for purification of natural and synthetic peptides[17]. Preparative RP-HPLC is frequently used to purifiy synthetic peptides in milligram and gram quantities[46-50]. RP-HPLC is used to separate hemoglobin variants[34, 35], identify grain varieties[32], study enzyme subunits[21] and research cell functions[33]. RP-HPLC is used to purify micro-quantities of peptides for sequencing[45] and to purify milligram to kilogram quantities of biotechnology-derived polypeptides for therapeutic use[59-62].

RP-HPLC is widely used in the biopharmaceutical field for analysis of protein therapeutic products. Enzymatic digests of protein therapeutics are analyzed for protein identity and to detect genetic changes and protein degradation (deamidation and oxidation) products. Intact proteins are analyzed by RP-HPLC to verify conformation and to determine degradation products. As the biotechnology revolution has expanded so have the technique's applications. The number of U.S. granted patents and U.S. applications referencing Vydac® reversed-phase columns alone has averaged 546 per year over the last 12 years as illustrated in **Figure 2** (Also see Reference 74).

Number of U.S. Granted Patents and U.S. Applications Using Grace® Vydac® Reversed-Phase HPLC Columns
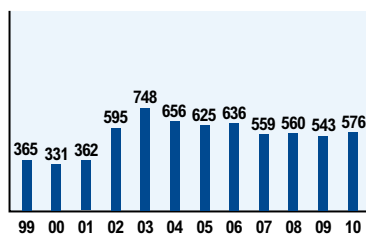
Figure 2. The number of U.S. granted patents and U.S. applications in the years 1999–2010 in which Vydac® Reversed-Phase HPLC columns are referenced.

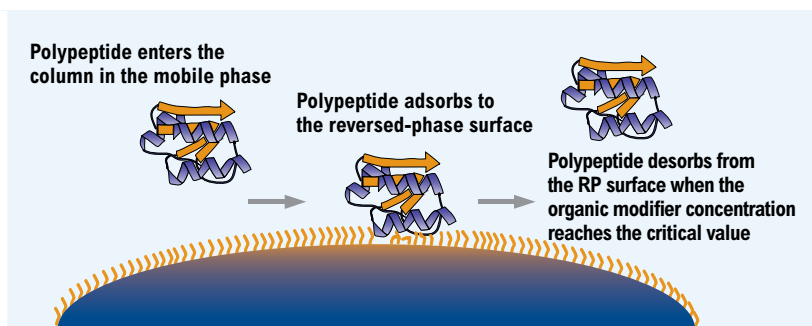# Mechanism of Interaction Between Polypeptides and RP-HLPC Columns

Understanding the mechanism by which polypeptides interact with the reversed-phase surface is important in understanding RP-HPLC polypeptide separations. The separation of small molecules involves continuous partitioning of the molecules between the mobile phase and the hydrophobic stationary phase. Polypeptides, however, are too large to partition into the hydrophobic phase; they adsorb to the hydrophobic surface after entering the column and remain adsorbed until the concentration of organic modifier reaches the critical concentration necessary to cause desorption **(Figure 3)**. They then desorb and interact only slightly with the surface as they elute down the column[4].

Polypeptides may be thought of as "sitting" on the stationary phase, with most of the molecule exposed to the mobile phase and only a part of the molecule—the "hydrophobic foot"— in contact with the RP surface. **RP-HPLC separates polypeptides based on subtle differences in the "hydrophobic foot" of the polypeptide being separated.** Differences in the "hydrophobic foot" result from differences in amino acid sequences and differences in conformation.

**Adsorption/Desorption Model of Polypeptide/
Reversed-Phase Interaction**

Polypeptide enters the column in the mobile phase

Polypeptide adsorbs to the reversed-phase surface

Polypeptide desorbs from the RP surface when the organic modifier concentration reaches the critical value

Figure 3. Polypeptide enters the column in the mobile phase. The hydrophobic "foot" of the polypeptide adsorbs to the hydrophobic surface of the reversed-phase material where it remains until the organic modifier concentration rises to the critical concentration and desorbs the polypeptide.

Important aspects of the adsorption/desorption mechanism of interactions between polypeptides and the hydrophobic phase.

Because the number of organic modifier molecules required to desorb a polypeptide—called the 'Z' number by Geng and Regnier[4]—is very precise, desorption takes place within a very narrow window of organic modifier concentration. This results in complete retention until the critical organic modifier concentration is reached and sudden desorption of the polypeptide takes place **(Figure 4)**. The sensitivity of polypeptide desorption to precise concentrations of organic modifier accounts for the selectivity of RP-HPLC in the separation of polypeptides. The sudden desorption of polypeptides when the critical organic concentration is reached produces sharp peaks. The sensitivity of the 'Z' number to protein conformation[3] and the sudden desorption at the critical modifier concentration give RP-HPLC the ability to separate very closely related polypeptides (see Page 2).

Molecule Retention Versus Organic Modifier Concentration
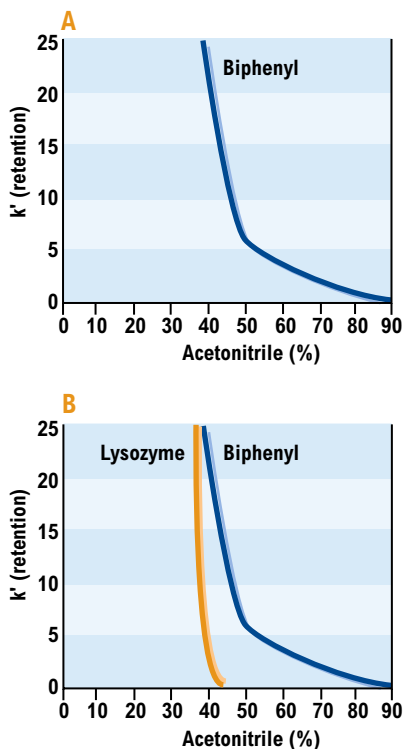
Figure 4. A: The retention of small molecules such as biphenyl decreases gradually as the organic modifier concentration increases because they are retained by partitioning.
B: The retention of polypeptides such as lysozyme changes suddenly and drastically as the organic modifier reaches the critical concentration needed to desorb the polypeptide, evidence of the adsorption/desorption model of polypeptide-reversed-phase surface interactions.

The "hydrophobic foot" of a polypeptide, which is responsible for the separation, is very sensitive to molecular conformation. This sensitivity of RP-HPLC to protein conformation results in the separation of polypeptides that differ not only in the hydrophobic foot but elsewhere in the molecule as well. Kunitani and Johnson[3] found that, due to conformational differences, very similar interleukin-2 muteins could be separated, including those differing in an oxidized methionine or in single amino acid substitutions. Geng and Regnier[4] found that the 'Z' number correlates with molecular weight for denatured proteins, however, proteins with intact tertiary structure elute earlier than expected because only the "hydrophobic foot" is involved in the interaction, while the rest of the protein is in contact with the mobile phase.

The adsorption/desorption step takes place only once while the polypeptide is on the column. After desorption, very little interaction takes place between the polypeptide and the reversed-phase surface and subsequent interactions have little affect on the separation.

A practical consequence of this mechanism of interaction is that polypeptides are very sensitive to organic modifier concentration. The sensitivity of polypeptide elution to the organic modifier concentration is illustrated in **Figure 5**. Large changes

occur in the retention time of lysozyme with relatively small changes in the acetonitrile concentration. The sensitivity of polypeptide retention to subtle changes in the modifier concentration makes isocratic elution difficult because the organic modifier concentration must be maintained very precisely. **Gradient elution is usually preferred for RP-HPLC polypeptide separations, even if the gradient is very shallow—i.e., a small change in organic modifier concentration per unit time.**

Effect of Acetonitrile Concentration on Elution
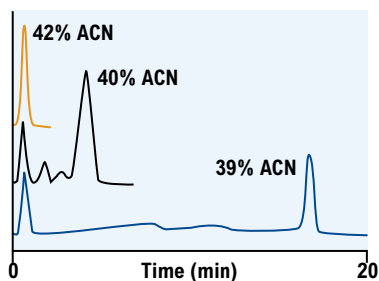
Figure 5. At 39% ACN, the retention time of lysozyme is nearly 18 minutes. Increasing the ACN concentration to 40% reduces the retention time by more than half, to 7.6 minutes. Increasing the ACN concentration to 42% reduces the retention time of lysozyme again by more than half, to 3.1 mintues. **Column:** Vydac® 214TP54 column. **Eluent:** ACN at 39, 40 and 42% in 0.1% aqueous TFA.

Shallow gradients can be used very effectively to separate similar polypeptides where isocratic separation would be impractical.

Small peptides appear to separate by a hybrid of partitioning and adsorption. They desorb more quickly with changes in organic modifier concentration than small molecules which partition, however they desorb more gradually than proteins **(Figure 6)**, suggesting a hybrid separation mechanism. Attempts to correlate peptide retention with side chain hydrophobicity have been partially successful, however tertiary structure in many peptides limit interactions to only a portion of the molecule and cause discrepancies in the predictions of most models. It has been shown that the exact location of hydrophobic residues in a helical peptide is important in predicting peptide retention[5].

Because large polypeptides diffuse slowly, RP-HPLC results in broader peaks than obtained with small molecules. Peak widths of polypeptides eluted isocratically are a function of molecular weight, with large proteins such as myoglobin having column efficiencies only 5–10% of the efficiencies obtained with small molecules such as biphenyl. Gradient elution of polypeptides, even with shallow gradients, is preferred, since it results in much sharper peaks than isocratic elution. Isocratic elution is rarely used for polypeptide separations.

Retention Behavior of Peptides
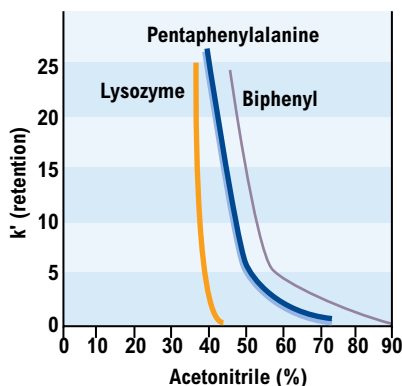
Figure 6. The retention behavior on RP-HPLC of many peptides is midway between that of proteins and of small molecules. Pentaphenylalanine, a small peptide, desorbs more quickly than biphenyl, a small molecule, but more gradually than lysozyme. Small peptides appear to chromatograph by a mixed mechanism.

The Role of the Column in Polypeptide Separations by Reversed-Phase HPLC

The HPLC column provides the hydrophobic surface onto which the polypeptides adsorb.

Adsorbent Pore Diameter

HPLC adsorbents are porous particles and the majority of the interactive surface is inside the pores. Consequently, polypeptides must enter a pore in order to be adsorbed and separated.

For many years, HPLC was performed with small molecules on particles having pores of about 100Å diameter. Polypeptides chromatographed poorly, in part because many polypeptides are too large to enter pores of this diameter. The development by Grace of large pore (~300Å) spherical silica particles for HPLC heralded the beginning of effective separations of polypeptides by RP-HPLC. Today most polypeptide separations are performed on columns with particles with pores of about 300Å, although some peptides (<~2,000 MW) may also be separated on particles of 100Å pores.

Adsorbent Particle Size

The particle size of the adsorbents in the column affect the narrowness of the eluting peaks. Smaller diameter particles generally produce sharper peaks and better resolution.

1.5-5µm materials are recommended for analytical separations. Larger diameter laboratory columns are usually packed with 10µm materials. Process chromatography columns of greater than 22mm i.d. are normally packed with particles of 10µm or greater and have wider particle size distributions than the particles used in analytical columns (see Pages 51–52).

Column Selection and Characteristics of Sample Molecule
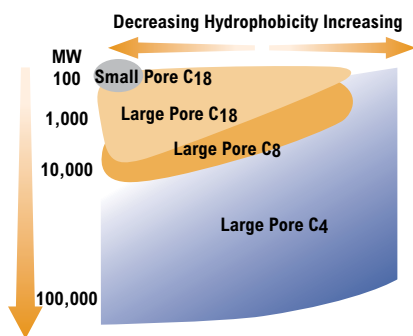
Figure 7. This chart indicates the pore size and bonding recommended for various molecular weights and hydrophobicities.

Adsorbent Phase Type

Reversed-phase HPLC adsorbents are formed by bonding a hydrophobic phase to the silica matrix.

The hydrocarbon group forming the hydrophobic phase is usually a linear aliphatic hydrocarbon of eighteen (C_{18}), eight (C_8) or four (C_4) carbons. The length of the hydrocarbon chain often makes little difference in the effectiveness of protein separations. There are guidelines as to which phase is likely to be most effective in separating polypeptides of a given size and hydrophobicity. These are summarized in **Figure 7**. C18 columns are generally preferred for peptides and small proteins less than about 5,000 daltons. The smallest and most hydrophilic peptides are often best separated on small pore C18 columns. Proteins larger than 5,000 daltons or small polypeptides that are particularly hydrophobic are best chromatographed on C4 columns. C8 columns are similar to C18 columns in their application but sometimes offer a different selectivity or ability to separate particular peptides. Phenyl columns are slightly less hydrophobic than C4 columns and may offer unique selectivity for some polypeptides.

Subtle differences in reversed-phase surfaces sometimes result in differences in RP-HPLC selectivity for peptides and proteins that can be used to optimize specific peptide/protein separations.

As illustrated in **Figure 8**, peptide separation selectivity may be affected by the nature of the hydrophobic surface. Selectivity for the five peptides shown is about the same on the C18 and C4 columns, although the C4 column has slightly shorter retention. The phenyl column exhibits shorter retention times and a different selectivity than the C18 column. Bradykinin, with two phenylalanines, is retained somewhat longer, relative to the other peptides, on the phenyl column than on the C18 column.

### Peptide Separation on Different Reversed-Phase Columns
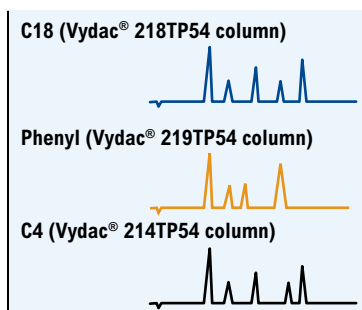
Figure 8. Peptide separation on different reversed-phase columns **Columns:** Vydac® 218TP54 (C18) column; Vydac® 214TP54 (C4) column; Vydac® 219TP54 (phenyl) column; **Eluent:** 15–30% ACN in 0.1% aqueous TFA over 30 minutes at 1.0mL/min. **Sample:** 1. oxytocin, 2. bradykinin, 3. angiotensin II, 4. neurotensin, 5. angiotensin I.

Angiotensin I—with one histidine—and angiotensin II—with two histidines—both elute earlier relative to the other peptides on the phenyl column. When developing peptide separations, such as those resulting from protein digestion, it is best to try several different hydrophobic phases to determine which has the best selectivity for that particular mixture of peptides. RP-HPLC separation of peptides result from subtle interactions of peptides with the reversed-phase surface. Small variations in the reversed-phase surface can affect peptide separations in important ways. The retention behavior of a

### Helical Peptide/Protein Separation on Different Reversed-Phase Columns
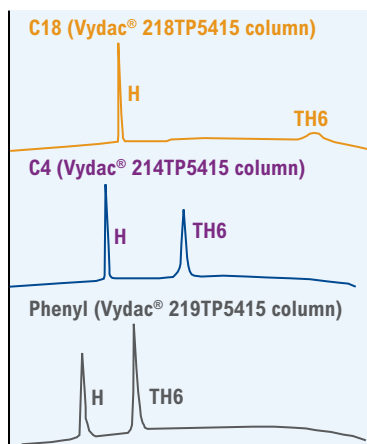
Figure 9. Columns: Vydac® 218TP5415 (C18) column; Vydac® 214TP5415 (C4) column; Vydac® 219TP5415 (phenyl) column - all 5μm, 4.6 x 150mm; **Eluent:** 10-90% ACN in 0.1% aqueous TFA over 30 minutes. **Sample:** 18-residue helical peptide (H) and six-helix template assembled synthetic protein (TH6). Data courtesy of V. Steiner, Ciba-Geigy (now Novartis).

six-helix bundle template-assembled synthetic protein molecule (TH6) and its helical peptide (H) building blocks (sequence KLALKLALKALKLALKLA) was investigated by Steiner et al. at Ciba-Geigy. The TH6 molecule was strongly adsorbed to the C18 column, whereas it was eluted earlier from C4 and phenyl columns as a single sharp peak **(Figure 9)**.

The different reversed-phase adsorbents may offer different selectivity when separating the peptide fragments from enzymatic digestion of a protein. Separation of tryptic digest fragments of β-lactoglobulin A on two RP-HPLC columns illustrates the subtle effects that different phases sometimes have on reversed-phase separations of peptides **(Figure 10)**. The C4 column has slightly less retention and a

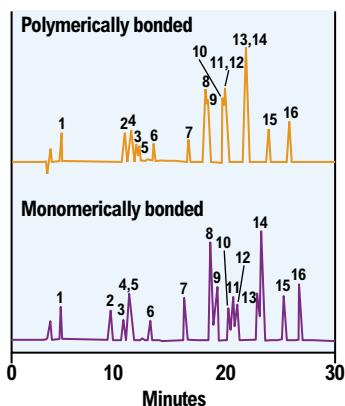### Separation of a Tryptic Digest on Different Reversed-Phase Columns
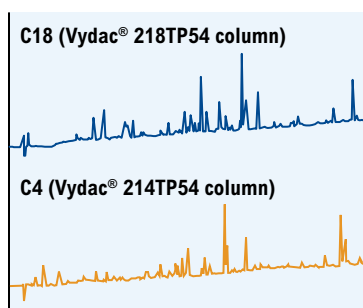
Figure 10. Columns: Vydac® 218TP54 (C18) column; Vydac® 214TP54 (C4) column; **Eluent:** 0–30% ACN in 0.1% aqueous TFA over 60 minutes at 1.0mL/min. **Sample:** tryptic digest of β-lactoglobulin A.

somewhat different peptide fragment elution pattern than the more commonly used C18 column. Testing different columns is the only practical way of determining which column will give the best resolution. Selectivity differences between reversed-phase columns are used in some laboratories to perform two-dimensional peptide separations[11].

What is polymeric bonding and how does it affect peptide selectivity?
Reversed-phase HPLC adsorbents are usually prepared by bonding hydrocarbon chlorosilanes with one reactive chlorine to the silica matrix.

The Separation of Synthetic Peptides on Monomerically Bonded and Polymerically Bonded C18 Reversed-Phase Columns

These form what are called **monomerically** bonded phases, having a single point of attachment to the silica matrix. Chlorosilanes with multiple reactive chlorines can also be used. These form what are called **polymerically** bonded phases, where individual chlorosilanes crosslink and form a silicone polymer on top of the silica matrix with multiple hydrophobic chains attached. Although similar in hydrophobicity and separation characteristics, monomerically bonded and polymerically bonded phases can exhibit different selectivities when separating peptides, particularly those resulting from enzymatic digests of proteins. The different selectivities afford chromatographers additional options for optimizing selectivity and resolution of protein digests and other peptides. An example is given in **Figure 11** where a series of synthetic peptides are separated on a monomerically bonded adsorbent and a polymerically bonded adsorbent. Distinct differences in separation selectivity of the peptides is noted, offering yet another option in column selection when developing peptide separations.

Figure 11. Columns: Vydac® 218TP54 polymeric and Vydac® 238TP54 monomeric (C18, 5µm, 4.6 x 250mm) columns; **Eluent:** 10–40% ACN with 0.1% TFA over 30 min. **Flow Rate:** 1.0mL/min.

Column Dimensions: Length

The adsorption/desorption of proteins responsible for their separation takes place almost entirely near the top of the column. Therefore, column length does not significantly affect separation and resolution of proteins. Consequently, short columns of 5–15cm length are often used for protein separations. Small peptides, such as those from protease digests, are better separated on longer columns and columns of 15–25cm length are often used for the separation of synthetic and natural peptides and enzymatic digest maps. For instance, Stone and Williams found that more peptide fragments from a tryptic digest of carboxymethylated transferrin were separated on a column of 250mm length–104 peaks–than on a column of 150mm–80 peaks–or a column of 50mm–65 peaks[12].

Column length may affect other aspects of the separation.

Sample capacity

Sample capacity is a function of column volume. For columns of equal diameter, longer columns maximize sample capacity.

Column back-pressure

Column back-pressure is directly proportional to the column length. When using more viscous solvents, such as isopropanol, shorter columns will result in more moderate back-pressures.

Column Dimensions: Diameter

The column diameter does not affect peak resolution, but it does affect sample loading, solvent usage and detection sensitivity. As the diameter of an HPLC column is reduced, the flow rate is decreased, thus lowering the amount of solvent used, and the detection sensitivity is increased. Very small diameter HPLC columns are particularly useful when coupling HPLC with mass spectrometry (LC-MS).

The standard diameter of analytical columns suitable for analysis of polypeptide samples of 1–200 micrograms is 4.6mm. Larger diameter columns are used for purification of large amounts of polypeptide (see Pages 51–62 on preparative separations). The use of small diameter columns (0.075mm to 2.1mm) has increased in recent years. Small diameter columns offer:

Reduction in solvent usage

Flow rates of as little as a few microliters per minute are used with capillary and small bore columns (See Appendix A, Page 68). Low flow rates can significantly reduce the amount of solvent needed for polypeptide separations.

Separation of the Tryptic Digest of Hemoglobin on a Microbore (1mm Diameter) Column
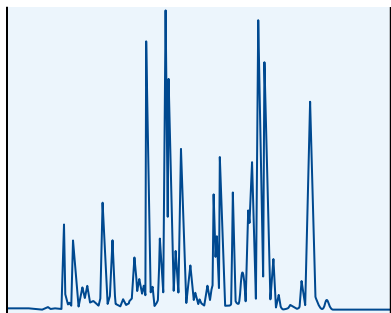
Figure 12. Separation of the tryptic digest of hemoglobin on a microbore RP-HPLC column (Reference 26). **Column:** C18, 1.0 x 250mm (Vydac® 218TP51 column). **Flow Rate:** 50µl/min. **Eluent:** Gradient from 0 to 40% B over 50 minutes, where Solvent A is 0.1% TFA in water and Solvent B is 0.1% TFA in acetonitrile.

Separation of the Tryptic Digest of Myoglobin on a Capillary (75µm Diameter) Column
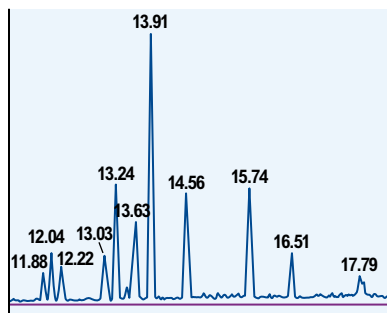
Figure 13. Separation of the tryptic digest of myoglobin on a capillary RP-HPLC column. **Column:** C18 (Vydac® 218MS column), 75µm i.d. capillary. **Flow Rate:** 0.5µl/min. **Eluent:** water/TFA/acetonitrile gradient.

Increased detection sensitivity

Polypeptides elute in smaller volumes of solvent at the reduced flow rates of small bore columns. Detector response increases in proportion to the reduction in flow rate. A narrowbore column with a flow rate of 200 microliters per minute gives a five-fold increase in sensitivity compared with an analytical column run at a flow rate of 1.0mL/min (see **Appendix D**, Pages 74-75).

Ability to work with smaller samples

Increased detection sensitivity means that smaller amounts of polypeptide can be detected. Tryptic digests of as little as five nanomoles of protein have been separated and collected using narrowbore RP-HPLC columns.

Separation of Tryptic Digest of Bovine Serum Albumin on Capillary RP-HPLC Columns
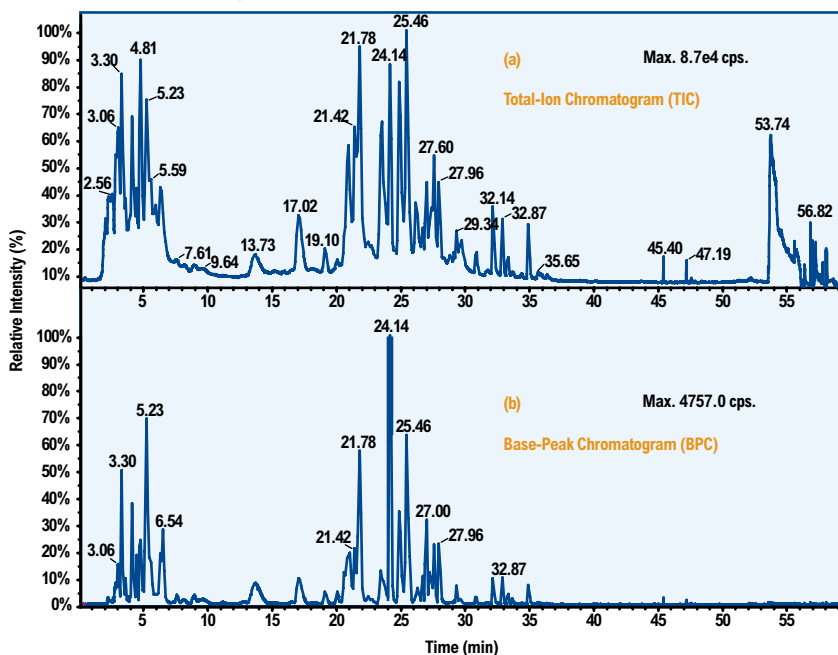
Figure 14. Separation of the tryptic digest of bovine serum albumin (BSA) on a 300µm i.d. capillary RP-HPLC **Sample:** 3 pmole. **Column:** Vydac® 218MS5.305 5µm, 300Å, polymeric-C18 reversed-phase (300µm i.d. x 50mm L) column. **Flow Rate:** 5µl/min. **Mobile Phase: A =** 0.1% formic acid, 98% water, 2% ACN. **B =** 0.1% formic acid, 98% ACN, 2% water. **Gradient:** Hold 3% B from 0 to 5 minutes. Then ramp from 3% B to 50% B at 65 minutes. Final ramp to 75% B at 70 minutes. **Detection:** MS. (a) Total ion count. (b) Base peak intensity. The base peak is defined as the single mass peak with maximum amplitude at each time in the chromatogram. The base peak chromatogram emphasizes peaks containing a single predominant molecular species and deemphasizes heterogeneous peaks and noise. **Data courtesy of Applied Biosystems.**

Interface with mass spectrometry

Direct transfer of the HPLC eluent into the electrospray mass spectrometer interface is possible with small bore columns and attomole (10^{-18}) levels of individual sample are routinely detected using sophisticated MS equipment. (See LC-MS, Pages 36–40).

Current Trends in Small Diameter Columns

Narrowbore columns

Narrowbore columns of 2.1mm i.d. are run at 100–300 microliters per minute. Narrowbore columns are a practical step for most laboratories to take in reducing solvent usage and improving detection sensitivity. Most standard HPLC systems can operate at these low flow rates with little or no modification. Narrow bore columns with flow rates around 200 microliters/minute interface well with pneumatically-assisted electrospray mass spectrometer interfaces.

Microbore and capillary columns

Columns of 1.0mm diameter and less offer significant reductions in solvent usage and increases in detection sensitivity; however, these may require modifications to the HPLC system or the use of instruments specifically designed for this purpose.

Capillary columns can be interfaced with electrospray mass spectrometer interfaces or even nanoelectrospray interfaces after stream splitting.

An article by Davis and Lee provides valuable information for getting the best performance using microbore and capillary columns[44] and is recommended reading for anyone embarking on the use of small bore columns. A number of journal articles detail the use of mass spectrometers with capillary columns[41–43] (Also see Pages 37–40).

Examples

Microbore. Figure 12 illustrates the separation of a tryptic digest of hemoglobin on a microbore (1.0mm i.d.) column.

Capillary. Figure 13 is an example of the separation of a tryptic digest of myoglobin on a 75μm i.d. capillary column.

Capillary sample load. Figure 14 illustrates that three picomoles of a tryptic digest of BSA can be separated on a 300μm i.d. capillary column. Detection was by mass spectrometry.

Analytical Conditions: The Role of the Mobile Phase and Temperature in Reversed-Phase HPLC Polypeptide Separations

The desorption and elution of polypeptides from RP-HPLC columns is accomplished with aqueous solvents containing an organic modifier and an ion-pair reagent or buffer. The organic modifier solubilizes and desorbs the polypeptide from the hydrophobic surface while the ion-pair agent or buffer sets the eluent pH and interacts with the polypeptide to enhance the separation. Elution is accomplished by gradually raising the concentration of organic solvent during the chromatographic run (solvent gradient). When the solvent reaches the precise concentration necessary to cause desorption, the polypeptide is desorbed and elutes from the column.

Organic Modifiers

The purpose of the organic solvent is to desorb polypeptide molecules from the adsorbent hydrophobic surface. This is typically done by slowly raising the concentration of organic solvent (gradient) until the polypeptides of interest desorb and elute.

Acetonitrile (ACN)

Acetonitrile (ACN) is the most commonly used organic modifier because:

- It is volatile and easily removed from collected fractions;
- It has a low viscosity, minimizing column back-pressure;
- It has little UV adsorption at low wavelengths;
- It has a long history of proven reliability in RP-HPLC polypeptide separations.

Isopropanol

Isopropanol is often used for large or very hydrophobic proteins. The major disadvantage of isopropanol is its high viscosity. To reduce the viscosity of isopropanol while retaining its hydrophobic characteristics, we recommend using a mixture of 50:50 acetonitrile: isopropanol. Adding 1–3% isopropanol to acetonitrile has been shown to increase protein recovery in some cases[52].

Improved Resolution of Enzyme Subunits Using Low Gradient Slope
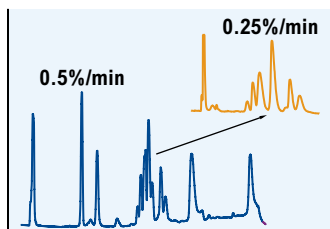
Figure 15. Column: C18 (Vydac® 218TP104 column). **Flow Rate:** 1mL/min. **Eluent:** Gradient slope as shown. Gradient from 25–50% ACN in aqueous TFA. **Sample:** Subunits of cytochrome c oxidase. Data from reference 21.

Ethanol

Ethanol is often used for process scale purifications. Ethanol is a good RP-HPLC solvent, it is readily available at reasonable cost and it is familiar to regulatory agencies such as the FDA. Ethanol has been used to elute hydrophobic, membrane-spanning proteins[33] and is used in process purifications[59].

Methanol or other solvents

Methanol or other solvents offer little advantage over the more commonly used solvents and are not used for polypeptide separations.

Elution Gradients

Solvent gradients are almost always used to elute polypeptides. Slowly raising the concentration of organic solvent results in the sharpest peaks and best resolution.

Gradient elution is generally preferred for polypeptide separations. Peaks tend to be unacceptably broad in isocratic elution and very low gradient slopes are preferred to isocratic elution. A typical solvent gradient has a slope of 0.5 to 1% per minute increase in organic modifier concentration. Extremely shallow gradients, as low as 0.05 to 0.1% per minute, can be used to maximize resolution. The gradient slope used to separate insulin variants in **Figure 1** (Page 2) was only 0.25% per minute.

Figure 15 illustrates that, for proteins, decreasing the slope of the gradient generally improves resolution.

For the best reproducibility and equilibration, avoid extremes in organic modifier composition. We recommend beginning gradients at no less than 3 to 5% organic modifier concentration. Gradients beginning with less organic modifier may cause column equilibration to be long or irreproducible because of the difficulty in "wetting" the surface. We also recommend ending gradients at no more than 95% organic modifier. High organic concentrations may remove all traces of water from the organic phase, also making column equilibration more difficult.

Peptide Separation with Different Gradient Times
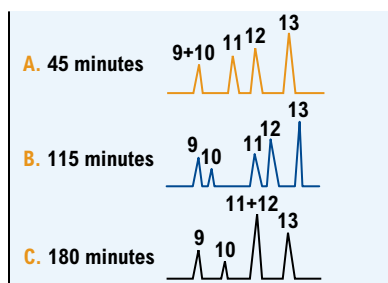
Figure 16. The effect of gradient time (slope) on peptide selectivity. **Column:** C18, 150 x 4.6mm. **Flow Rate:** 1mL/min. **Eluent:** Gradient from 0–60% ACN in aqueous 0.1% TFA in A. 45 min.; B. 115 min.; C. 180 min. **Sample:** tryptic digest of human growth hormone. Fragments 9, 10, 11, 12, 13 from the digest. Data from reference 38.

The Effect of Gradient Slope on Peptide Selectivity

Because of slight differences in the way that some peptides interact with the reversed-phase surface, the slope of the solvent gradient may affect peptide selectivity and, therefore, resolution between peptide pairs.

This effect is best illustrated by the separation of a tryptic digest of human growth hormone at different gradient times with different gradient slopes. **Figure 16** shows the separation of several tryptic digest fragments from human growth hormone at three different gradient slopes (times). As the slope is decreased fragments 9 and 10 behave as expected, that is resolution increases as the gradient slope is decreased (increasing gradient time). Fragments 11 and 12, however, behave differently. Resolution decreases as the gradient slope is decreased, indicating a change in the selectivity with changing gradient slope. This effect should be monitored when changing gradient slope by making only modest changes in the gradient slope when developing a method and examining the effect this has on each peptide pair.

Ion-Pairing Reagents and Buffers

The ion-pairing reagent or buffer sets the eluent pH and interacts with the polypeptide to enhance the separation.

Trifluoroacetic acid

The most common ion-pairing reagent is trifluoroacetic acid (TFA). It is widely used because:

- It is volatile and easily removed from collected fractions;
- It has little UV adsorption at low wavelengths;
- It has a long history of proven reliability in RP-HPLC polypeptide separations.

TFA is normally used at concentrations of about 0.1% (w/v). TFA concentrations up to 0.5% have been useful in solubilizing larger or more hydrophobic proteins and lower concentrations are occasionally used for tryptic digest separations. When chromatographing proteins, using TFA concentrations below 0.1% may degrade peak shape, although new column developments allow the use of much lower TFA concentrations (see Page 35).

Elution gradients with a constant concentration of TFA sometimes result in a drifting baseline when monitoring at 210–220nm.

The change in dielectric constant as the solvent environment goes from aqueous to non-aqueous affects π-π electron interactions which, in turn, affects the adsorption spectrum in the 190 to 250nm region, leading to a baseline shift during many reversed-phase separtions. To reduce or eliminate baseline drift due to TFA spectral adsorption, adjust the wavelength as close to 215nm as possible and put ~15% less TFA in Solvent B than in Solvent A to compensate for the shift. For example, use 0.1% TFA in Solvent A and 0.085% TFA in Solvent B.

It is important to use good quality TFA and to obtain it in small amounts. Poor quality or aged TFA may have impurities that chromatograph in the reversed-phase system, causing spurious peaks to appear (see Appendix B).

The Effect of TFA Concentration on Selectivity

The concentration of trifluoroacetic acid may affect selectivity or resolution of specific peptide pairs.

Although TFA is typically present in the mobile phase at concentrations of 0.05 to 0.1%, varying the concentration of TFA has a subtle affect on peptide selectivity as illustrated in **Figure 17**. This means that, for good reproducibility, it is important to control the TFA concentration very carefully in peptide separation methods. This also provides another tool for optimizing peptide resolution. After the column and gradient conditions have been selected, it is possible to vary the TFA concentration slightly to further optimize resolution between peptide pairs.

The Effect of TFA Concentration on Peptide Selectivity

0.1% TFA

0.3% TFA
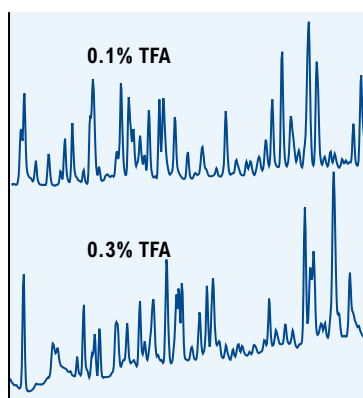
Figure 17. Significant differences in the peptide separation pattern due to differences in TFA concentration are evident. **Column:** C18 (Vydac® 218TP54 column). **Flow Rate:** 1mL/min. **Eluent:** Gradient from 0–50% ACN in aqueous TFA, concentration as indicated. **Sample:** Tryptic digest of apotransferrin. **Note:** Only part of the chromatogram is shown.

Alternate Ion Pairing Agents

Although TFA is widely used as the ion pairing reagent, use of other reagents may result in better resolution or peak shape than TFA. In the separation of five small peptides (**Figure 18**) phosphate gives sharper peaks for some peptides than TFA and causes a reversal in the elution order of oxytocin and bradykinin. The last three peaks are sharper in phosphate than TFA because phosphate interacts with basic side chains, increasing the rigidity of the peptide. Bradykinin elutes earlier in phosphate than TFA because TFA pairs with the two arginines in bradykinin resulting in relatively longer retention. Also, two small impurities, hidden in the TFA separation, were revealed by phosphate (**Figure 18B**). Hydrochloric acid also reverses the elution order of oxytocin and bradykinin and separates impurities not seen in TFA (**Figure 18C**).

Heptafluorobutyric acid (HFBA) is effective in separating basic proteins[20] and triethylamine phosphate (TEAP) has been used for preparative separations[46, 47, 49]. One study found that sample capacity was greater using TEAP than with TFA[32]. Formic acid, in concentrations of 10 to 60%, has been used for the chromatography of very hydrophobic polypeptides. Formic acid is also gaining usage in LC/MS separation of peptides because TFA reduces the ion signal in the electrospray interface and the volatile acid, formic acid, has proven to be effective in the LC/MS of peptides (See Pages 26–40 for a more detailed discussion of LC-MS). Guo and colleagues compared the use of TFA, HFBA and phosphoric acid in the elution of peptides and found that each gave somewhat different selectivity[8].

Comparison of TFA and Alternate Ion-Pairing Agents/Buffers for the Separation of Peptides
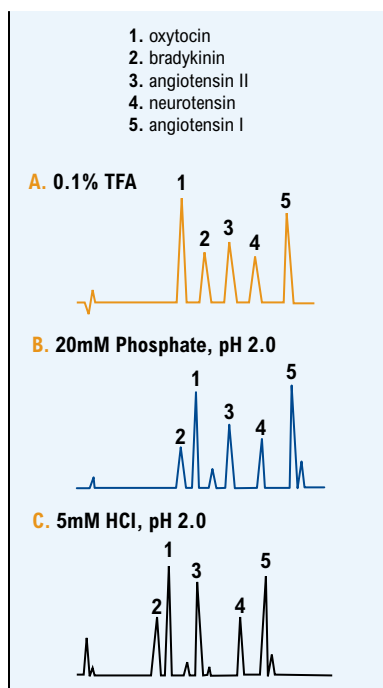
Figure 18. Elution of five peptides using TFA (A), Phosphate (B) or HCl (C) as the buffer/ ion-pairing agent. **Column:** Vydac® 218TP54 (C18, 5µm, 4.6 x 250mm) column. **Eluent:** 15–30% ACN in 30 min at 1.0mL/min; plus A. 0.1% TFA B. 20mm phosphate, pH 2.0 C. 5mm HCl, pH 2.0

The Effect of pH on Peptide Separations

Peptide separations are often sensitive to the eluent pH because of protonation or deprotonation of acidic or basic side-chains, as illustrated in **Figure 19**. All five peptides elute earlier at pH 4.4 (**Figure 19B**) than at pH 2.0 (**Figure 19A**) and the relative retention of peptides changes. This is due to ionization of acidic groups in the peptides. Bradykinin and oxytocin are well separated at pH 2.0 but co-elute at pH 4.4. Peptide retention at pH 6.5 (**Figure 19C**) is greater than at pH 4.4, however the elution order is drastically different.

Angiotensin II, which elutes third at pH 2.0 to 4.4, now elutes first. Neurotensin elutes before oxytocin; bradykinin and neurotensin co-elute. This illustrates that pH can have a dramatic effect on peptide selectivity and can be a useful tool in optimizing peptide separations.

The Effect of pH on Peptide Separations
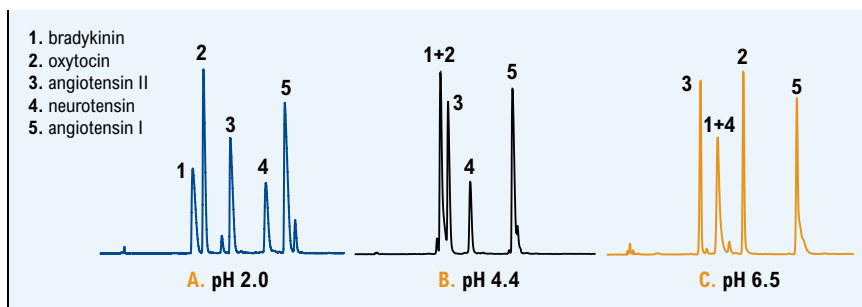
Figure 19. Elution of five peptides at pH 2.0, 4.4 and 6.5 with phosphate as the buffer. **Column:** Vydac® 218TP54 (C18, 5μm, 4.6 x 250mm) column. **Eluent:** 15–30% ACN in 30 min at 1.0mL/min; plus A. 20mM phosphate, pH 2.0 B. 20mM phosphate, pH 4.4 C. 20mM phosphate, pH 6.5

The chromatograms in **Figure 20** represent the extremes of a series of resolution trials that were aimed at finding the best conditions for purification of the major component, a partially purified cyclic peptide with a C-terminal acyl hydrazide. Each chromatogram reveals one large peak of target product surrounded by several much smaller contaminating peaks. In the figures, the vertical axis in the region containing the major peak has been expanded to show resolution in detail.

The effects of buffers and pH are dramatic. In general, separation at mildly acidic pH (6.0 or 6.5) with triethylamine acetate (TEAA) or phosphate buffer appears to produce better peak shape and resolution, or at least a greater number of resolved components, than separation at highly acidic pH with TFA, TEAP or phosphate. This observation is significant in view of the fact that highly acid conditions such as 0.1% TFA tend to be the norm for reversed phase peptide chromatography. The results shown here suggest that pH 6.0 or 6.5 separations are more likely to separate difficult to resolve components. However, it should be noted that these results are specific to the particular peptide sample used. The conclusions may or may not apply to other samples. A series of trial separations can be helpful in choosing optimal conditions for a specific peptide purification.
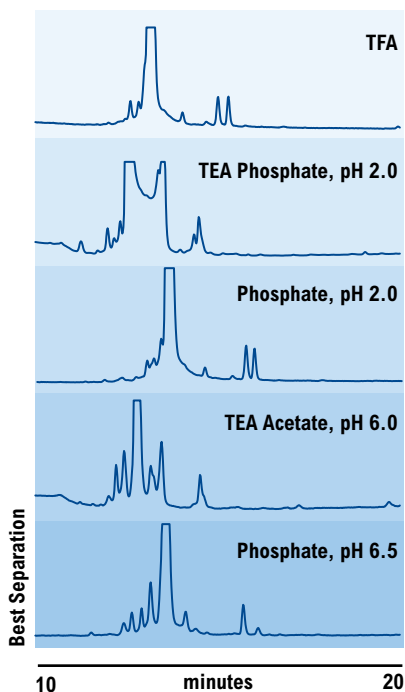
Figure 20. **Sample:** Partially purified cyclic peptide with C-terminal acyl hydrazide. **Column:** Vydac® 218TP54 (C18, 5μm, 4.6 x 250mm) column. **Flow Rate:** 1.0mL/min. Ion pairing reagents/buffers: 0.1% TFA (v/v). 10mM TEA Phosphate, pH 2.0. 10mM NaH_2PO_4, pH 2.0. 10mM TEA acetate, pH 6.0. 10mM NaH_2PO_4, pH 6.5. **Mobile Phase: A:** Buffer as indicated. **B:** Buffer as indicated (except 0.09% for TFA), 90% ACN (except 80% ACN for phosphate buffers). **Gradient:** Initial 5% B to 2 minutes. Then 20 minute linear to 45% B. Then 2 minutes to 100% B. Hold 2 minutes. Return to 5% B in 1 minute. **Detection:** 215nm.

Data courtesy of Tim Malaney, Telios Pharmaceuticals, Inc., now Seattle Genetics

Developing Conditions for HPLC Separation of Peptide Fragments from a Protein Digest

Although most enzymatic maps are performed using 0.1% TFA as the ion-pairing reagent, resolution may sometimes be better using a different ion-pairing agent or a higher pH.

TFA is widely used as an ion-pairing reagent and is the best starting point for peptide separations. However, consider the use of buffers such as phosphate or hydrochloric acid or exploring pH effects to optimize peptide separations. To test pH effects, prepare a 100mM solution of phosphate—about pH 4.4. Adjust one-third of this to pH 2.0 with phosphoric acid and one-third to pH 6.5 with NaOH. Then dilute each to 10–20mM for the eluent buffers. Testing peptide resolution with TFA, each of the three phosphate buffers (pH 2.0, pH 4.4 and pH 6.5) and HCl is an excellent way to find the optimum reagent and pH conditions to develop a good peptide separation.

Mobile Phase Flow Rate

Flow rate has little effect on polypeptide separations. The desorption of polypeptides from the reversed-phase surface, and hence resolution, is not affected by the flow rate.

Polypeptide desorption is the result of reaching a precise organic modifier concentration. Protein resolution, therefore, is relatively independent of mobile phase flow rate.

The resolution of small peptides may be affected by the eluent flow rate because their behavior on RP-HPLC columns is between that of proteins and small molecules (see Page 4-7). Stone and Williams found that the number of peptide fragments separated from a tryptic digest of carboxymethylated transferrin depended on the eluent flow rate[12]. On an analytical HPLC column, fewer than 80 peptide fragments were resolved at a flow rate of 0.2mL/min, compared to 116 fragments being resolved at 0.8mL/min. From flow rates of 0.5mL/min to 1.0mL/min there was little difference in the number of peptide fragments resolved.

It should be noted that, when refining a separation of small peptides where resolution is limited, slight improvements in resolution may be gained through minor changes in the eluent flow rate. The flow rate may also influence other aspects of a separation such as:

Detection sensitivity

Low flow rates elute polypeptides in small volumes of solvent and, consequently, adsorption and sensitivity increase. The major reason that narrowbore HPLC columns increase detection sensitivity is because they are run at low flow rates and polypeptides are eluted in small volumes of solvent.

Sample solubility

High flow rates may improve the solubility of hydrophobic polypeptides although this also increases the amount of solvent to be removed from the purified sample.

Column back-pressure

Column back-pressure is directly related to flow rate. The higher the flow rate the higher the column back-pressure.
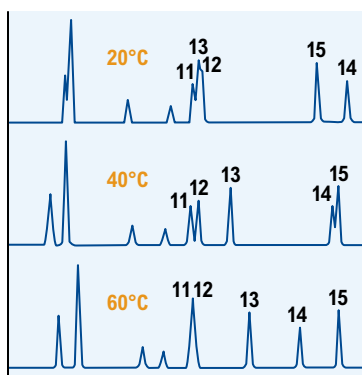
Gradient

Changes in eluent flow rate may subtly affect gradient slope and shape, depending on the hardware configuration used. Since polypeptide separations are sensitive to gradient conditions, flow rate adjustments may change the resolution due to the effects on the gradient shape.

The Effect of Temperature on Peptide Separations

Column temperature affects solvent viscosity, column back pressure and retention times. It may also affect peptide selectivity.

Temperature is an important separation parameter when chromatographing peptides and should be optimized in any HPLC method for the separation of peptides. This is illustrated in **Figure 21** by the separation of fragments from a tryptic digest of human growth hormone[39]. At 20°C fragments 11, 12, and 13 nearly co-elute. As the temperature is raised fragment 13 is more retained than fragments 11 and 12, resulting in good resolution between the three peptides at 40°C. At 60°C, however, fragments 11 and 12 co-elute, showing the change in selectivity as the temperature is raised. At 20°C fragment 15 elutes before fragment 14, at 40°C they nearly co-elute and at 60°C fragment 14 elutes first and the two are well separated. These results illustrate the significant impact that temperature may have on peptide selectivity.

The Effect of Temperature on the Separation of Peptide Fragments

Figure 21. Column: C18, 4.6 x 150mm. **Flow Rate:** 1mL/min. **Eluent:** Gradient from 0–60% ACN in aqueous 0.1% TFA in 60 min. **Temperature:** As indicated. **Sample:** Tryptic digest of human growth hormone. Data from Reference 39.

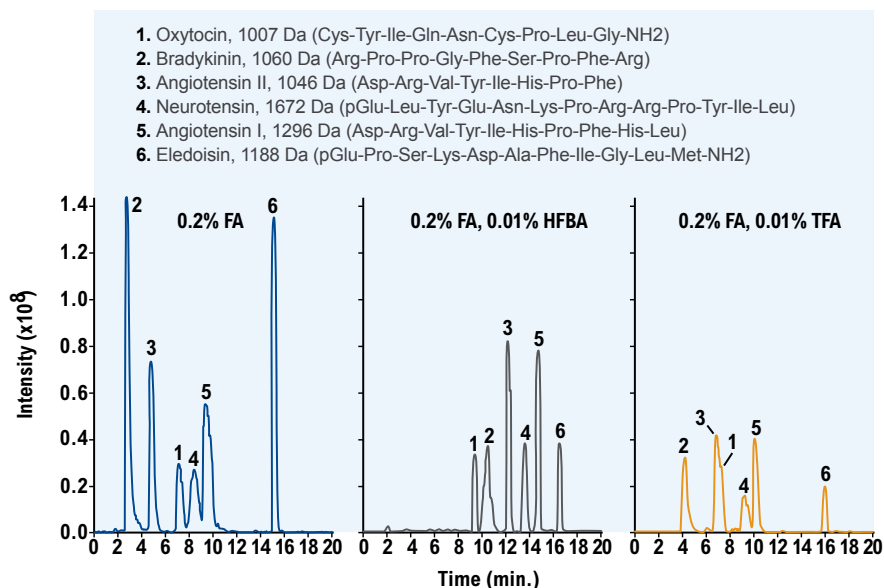# Reversed-Phase HPLC/Mass Spectrometry for the Analysis of Polypeptides

The development of the electrospray interface to couple mass spectrometry with HPLC has caused a virtual explosion in the use of LC-MS in the analysis of polypeptides. RP-HPLC peptide maps are routinely monitored by an on-line mass spectrometer, obtaining peptide molecular weights and causing fragmentation of peptides to obtain sequence information.

The combination of mass spectrometry with HPLC reduces the need for chromatographic resolution because of the resolving capacity of the mass spectrometer. Analysis times are generally short to best utilize the sophisticated mass spectrometer. Detection sensitivity is often much better with mass spectrometry than with UV detection.

Organic acids promote ionization of basic compounds (nitrogen containing), and provide the best sensitivity with positive ion detection. The mechanism of action is the protonation of C-terminal residue and Asp and Glu side chains. Volatile buffers/ion pairing reagents are recommended. These include:

- Ammonium formate or acetate (2 - 10mM optimum; can see suppression effects if > 20mM).

- Acetic Acid at 0.1 – 1.0% v/v.

- Formic Acid (FA) at 0.1 – 0.5% v/v.

- Trifluoroacetic acid (TFA) at up to 0.1% v/v for positive ion mode. Signal suppression at > 0.01% TFA may be significant due to the formation of zwitterions.

- Heptafluorobutyric acid (HFBA) at up to 0.1% v/v for positive ion mode. HFBA dramatically retains peptides containing basic amino acid residues, better than TFA, formic, and acetic acid. As with TFA, signal suppression at > 0.01% HFBA may be significant.

- A combination of weak and strong ion pairing agents. For example, formic acid plus HFBA or TFA may improve retention, peak shape, and selectivity (see **Figures 22-24**).

LC-MS of Peptides — Combining Ion Pairing Reagents

1. Oxytocin, 1007 Da (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH2)
2. Bradykinin, 1060 Da (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)
3. Angiotensin II, 1046 Da (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)
4. Neurotensin, 1672 Da (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu)
5. Angiotensin I, 1296 Da (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)
6. Eledoisin, 1188 Da (pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH2)

Figure 22. LC-MS total ion chromatograms of peptides using different ion pairing reagents. For peptides containing basic amino acid residues, the FA plus HFBA combination of ion pairing agents improves peak width and increases retention. **Sample:** 0.4µg (200 to 400 pmol) of each peptide. **Column:** C18, 300Å, 5µm, 1.0 x 150mm (Vydac® Everest® 238EV5115 column). **Flow Rate:** 50µl/min. **Mobile Phase: A:** 95:5 water:ACN with ion pairing agent(s); **B:** 20:80 water:ACN with ion pairing agent(s). **Gradient:** (%B, min.): (12.5, 0), (50, 30), (75, 35), (75, 40). **Detection:** Linear ion trap MS. Data from Reference 64.

Mass Spectra of Peptides Using 0.2% FA, 0.01% HFBA
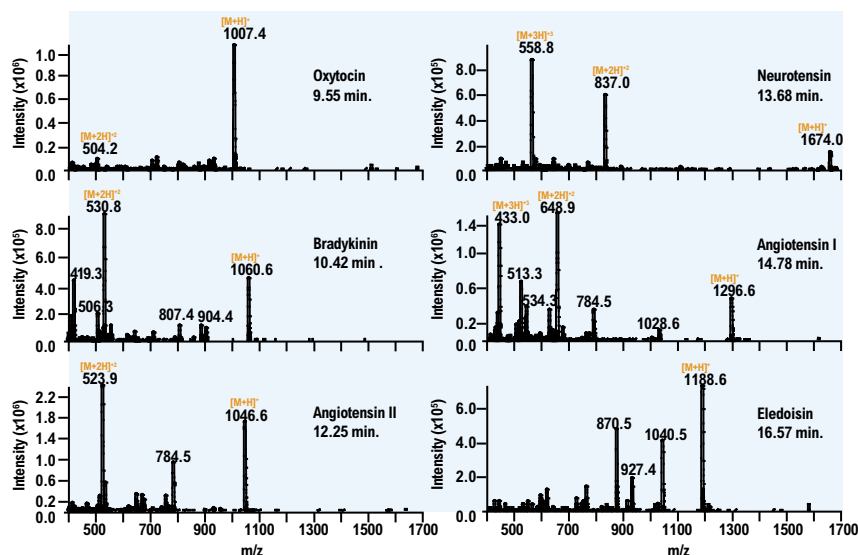
Figure 23. Mass spectra of peptides shown in previous figure. Unambiguous mass spectra are obtained with the FA/HFBA combination.

LC-MS of Proteins, Combining Ion Pairing Reagents

Expected Mass based on the Swiss-Prot sequence information is 22129 Da. The observed mass is 22128 Da.
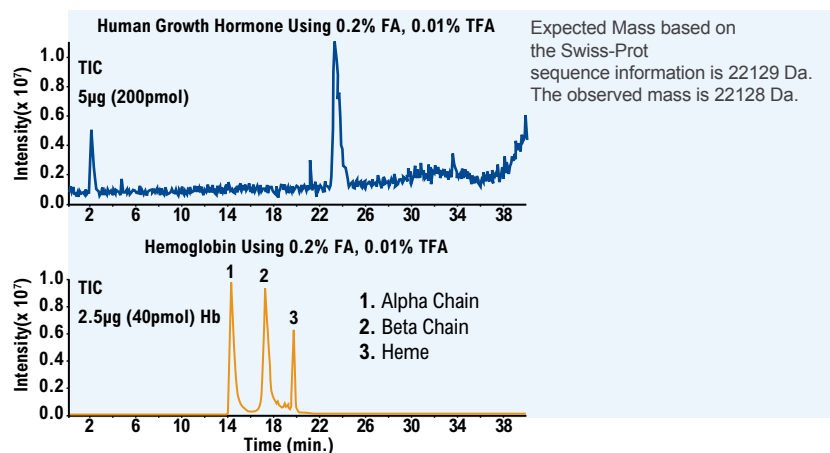
Figure 24. Total ion chromatograms for human growth hormone and hemoglobin using a combination of ion pairing reagents. For proteins, a small amount of TFA may improve peak shape and resolution even on column with high carbon coverage. **Column:** C18, 300Å, 5µm, 1.0mm x 150mm (Vydac® Everest® 238EV5115 column). **Mobile Phase: A:** 95:5 H_2O:ACN with ion pairing agents; **B:** 20:80 H_2O:ACN with ion pairing agents. **Gradient:** (%B, min.): (25, 0), (75, 30), (100, 35), (100, 40). **Flow Rate:** 50µl/min. Data from Reference 64.

Sub-Two Micron Short Columns for Fast HPLC and LC-MS

ProZap™ columns are the perfect tool for fast reversed-phase protein separations.[65, 66, 67] Under gradient conditions, longer columns only increase run time and do not increase resolution to improve the separation. Proteins adsorb at the head of the column and then desorb once the critical mobile phase concentration is reached. Since the proteins do not interact with the full length of the packed bed, short columns are sufficient for full resolution. Proteins of broad molecular weight ranges can be separated with high resolution in less than one minute by taking advantage of the following: 1.5μm particle size, 500Å pore diameter silica; short 10 to 20mm columns; high flow rates; and fast, modified gradients. The low back pressures associated with the short columns make them suitable for conventional LC systems. For best results high-pressure mixing should be used with fast gradients (**Figures 25 to 29**).

High- Versus Low-Pressure Gradient System
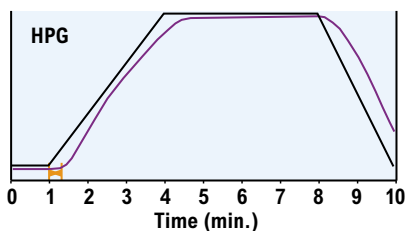
- High-pressure gradient (HPG) binary HPLC system: each partial flow is delivered via separate pump into mixing chamber against pressure of column.
- Short gradient delay time.
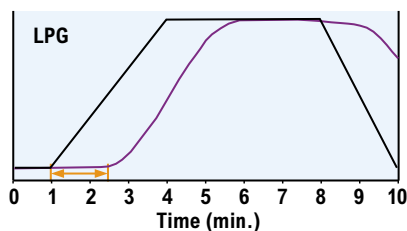
- Low-pressure gradient (LPG) **quaternary** HPLC system: the same pump delivers all partial flows.
- Correct mixing ratio delayed significantly.

Figure 25. To achieve fast gradients and minimize re-equilibration times, the binary pump is recommended instead of the quaternary pump. Extra column volume should be reduced by minimizing tubing length (from injector to column and from column to detector) and internal diameter (use 0.12mm or 0.005" i.d.). Also, use flow cell volume < 5-μL. Black profile = programmed gradient. Green profile = observed gradient. See **Appendix E** for method to measure actual gradient profile.

Fast Separation of Proteins

1. Ribonuclease (13.7 kDa)
2. Bovine insulin (5.7 kDa)
3. Bovine cytochrome c (12.2 kDa)
4. Lysozyme (14.3 kDa)
5. Bovine serum albumin (66.4 kDa)
6. Bovine carbonic anhydrase (29 kDa)
7. Ovalbumin (45.5 kDa)

Flow Rate: 0.2mL/min.
Column Backpressure: 650 psi
Gradient (min.,%B): (0.0, 23), (4.0, 75), (4.5, 75), (4.7, 23)

Flow Rate: 0.4mL/min.
Column Backpressure: 1380 psi
Gradient (min.,%B): (0.0, 23), (4.0, 75), (4.5, 75), (4.7, 23)

Flow Rate: 0.8mL/min.
Column Backpressure: 2680 psi
Gradient (min.,%B): (0.0, 23), (1.0, 75), (1.1, 75), (1.2, 23)
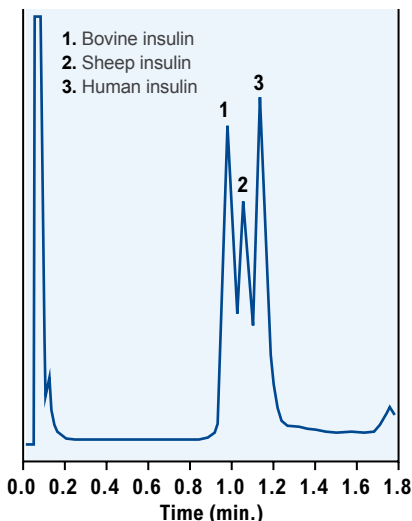

Figure 26. Ultra-fast separations of seven proteins in one minute. **Column:** C18, 500Å, 1.5µm, 2.1 x 10mm (Vydac® ProZap™ column). **HPLC System:** HPG binary pump. **Mobile Phase: A:** 0.1% TFA in Water; **B:** 0.085% TFA in ACN. **Detector:** UV at 280nm. **Injection Amount:** 37ng each protein, except 4ng for lysozyme.

Fast Separation of Closely Related Insulin Variants

1. Bovine insulin
**2.** Sheep insulin
3. Human insulin

Figure 27. Ultra-fast separations of insulin differing in sequence by only one or two amino acids at positions 8, 9 and 10 (out of 51 total amino acids). **Column:** C18, 500Å, 1.5µm, 2.1 x 10mm (Vydac® ProZap™ column). **HPLC System: HPG** binary pump. **Mobile Phase: A:** 0.1% TFA in Water; **B:** 0.085% TFA in ACN. **Gradient:** (min., %B): (0.0, 25), (1.5, 30), (1.6, 50), (1.7, 50), (1.8, 25). **Flow Rate:** 0.8mL/min. **Detector:** UV at 215nm. **Injection Amount:** 300ng each. **Column Backpressure:** 2465 psi.

Fast Separation of Human Proteins

1. Human Insulin (5.8 kDa)
2. Human Serum Albumin (66.5 kDa)
3. Human Growth Hormone (22.1 kDa)
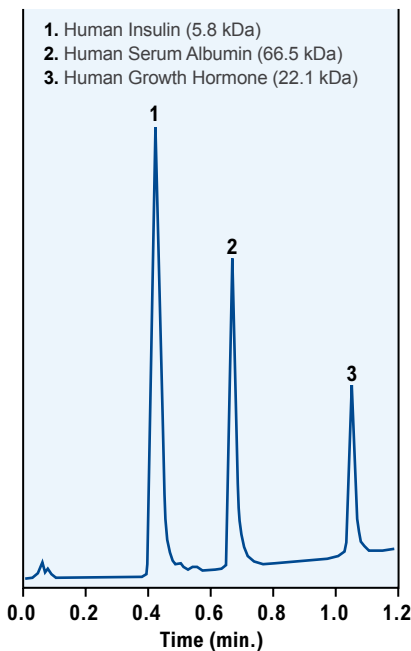
Figure 28. Ultra-fast separations of human proteins in one minute. **Column:** C18, 500Å, 1.5µm, 2.1 x 10mm (Vydac® ProZap™ column). **HPLC System:** HPG binary pump. **Mobile Phase: A:** 0.1% TFA in Water; **B:** 0.085% TFA in ACN. **Gradient:** (min.,%B): (0.0, 25), (1.0, 75), (1.1, 75), (1.2, 25). **Flow Rate:** 0.8mL/min. **Detector:** UV at 280nm. **Injection Amount:** 1.7µg each, except 300ng for growth hormone. **Column Backpressure:** 2680 psi.

Fast Separation of Intact Antibodies
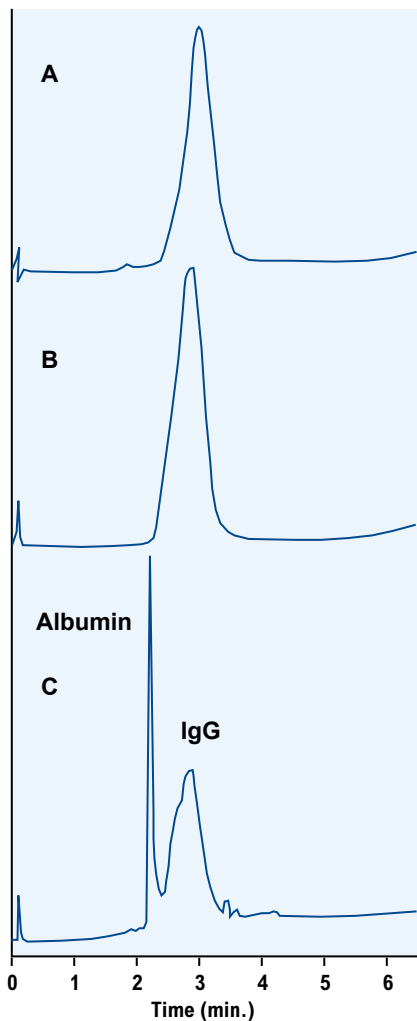
Figure 29. Fast separation of high molecular weight antibody in organic solvent. **A.** Rabbit IgG. **B.** Sheep IgG. **C.** Sheep serum. **Column:** C18, 500Å, 1.5µm, 2.1 x 10mm (Vydac® ProZap™ column). **HPLC System:** HPG binary pump. **Mobile Phase: A:** 0.1% TFA in Water; **B:** 0.085% TFA in 90:10 n-propanol:water. **Gradient:** (min.,%B): (0.0, 5), (6.0, 75), (6.5, 75), (7.0, 5). **Flow Rate:** 0.5mL/min. **Detector:** UV at 280nm. **Column Backpressure:** 2465 psi. **Column Temperature:** 75°C.

Using a LPG System for Protein Separations

1. Ribonuclease (13.7 kDa)
2. Bovine insulin (5.7 kDa)
3. Bovine cytochrome c (12.2 kDa)
4. Lysozyme (14.3 kDa)
5. Bovine serum albumin (66.4 kDa)
6. Bovine carbonic anhydrase (29 kDa)
7. Ovalbumin (45.5 kDa)

Flow Rate: 0.8mL/min.
Column Backpressure: 2680 psi
Gradient (min., %B): (0.0, 23), (1.0, 75), (1.1, 75), (1.2, 23)

Flow Rate: 0.2mL/min.
Column Backpressure: 650 psi
Gradient (min., %B): (0.0, 23), (4.0, 75), (4.5, 75), (4.7, 23)

Flow Rate: 0.4mL/min.
Column Backpressure: 1380 psi
Gradient (min., %B): (0.0, 23), (4.0, 75), (4.5, 75), (4.7, 23)

Flow Rate: 0.8mL/min.
Column Backpressure: 2610 psi
Gradient (min., %B): (0.0, 23), (4.0, 75), (4.5, 75), (4.7, 23)

Figure 30. The significant gradient delay time of a LPG system does not allow for less than one minute separations. Modification to the gradient program still allows for relatively fast separations. **Column:** C18, 500Å, 1.5µm, 2.1 x 10mm (Vydac® ProZap™ column). **HPLC System:** LPG quaternary pump. **Mobile Phase: A:** 0.1% TFA in Water; **B:** 0.085% TFA in ACN. **Detector:** UV at 280nm. **Column Temperature:** 25°C.

Fast LC-MS of Proteins with a LPG Quaternary HPLC

TIC

1. Bovine Cytochrome c, M =12222
2. Horse Apomyoglobin, M = 16941
3. Bovine Carbonic Anhydrase II, M = 28998
4. Chicken Ovalbumin, M = 44455
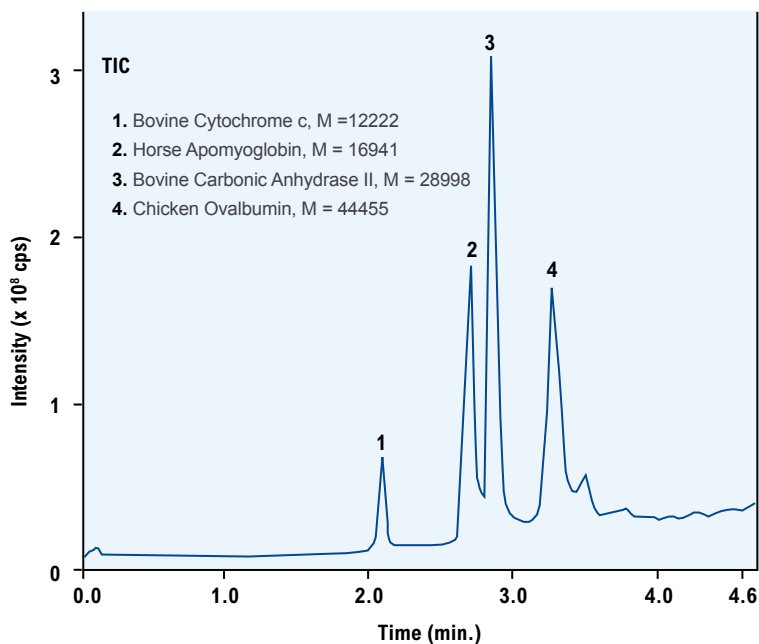
Figure 31. Total ion chromatogram for proteins. **Column:** C18, 500Å, 1.5μm, 2.1 x 10mm (Vydac® ProZap™ column). **Mobile Phase:** **A:** 0.2% formic acid, 0.01% TFA in 5:95 ACN:water; **B:** 0.2% formic acid, 0.01% TFA in 80:20 ACN:water. **Gradient Program:** (min., %B): (0, 20), (4.0, 90), (4.5, 90), (4.7, 20). **Flow Rate:** 0.4mL/min. **Detector:** Linear ion trap MS. **Injection Amount:** 50ng each.

Reducing or Eliminating TFA in the Mobile Phase

TFA forms such strong complexes with polypeptides that electrospray signal, and hence detection sensitivity, is reduced when TFA is present at concentrations typical for polypeptide separations.

The reduction of electrospray signal by TFA has led to the use of ion-pair reagents such as formic acid and acetic acid for polypeptide separations. These ion-pair reagents, however, do not always give good resolution. Recent developments in HPLC columns have resulted in columns with good polypeptide peak shapes using very low concentrations of TFA.

In some cases the TFA may be completely replaced with formic or acetic acid while retaining good resolution.[63] **Figure 32** shows the separation of several peptides on an HPLC column specially developed to allow the use of very low concentrations of TFA. Good peak shapes are maintained on this column with only 0.01% TFA. It should be noted, however, that the TFA concentration does affect peptide selectivity.
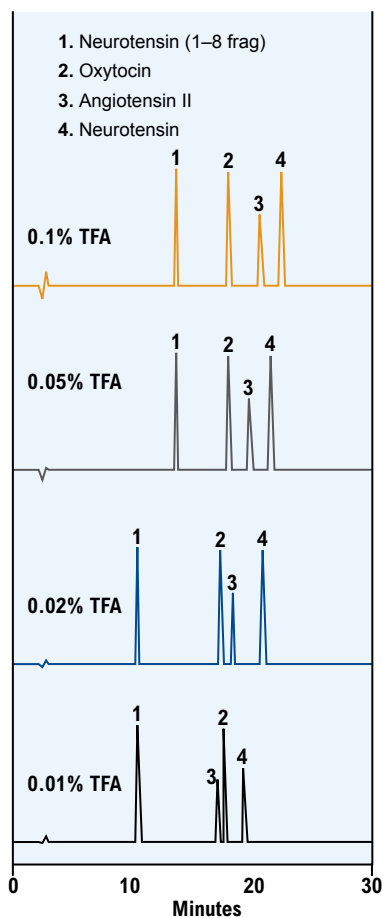
Figure 32. Column: C18, 5μm, 4.6 x 250mm (Vydac® 218MS54 column). Flow Rate: 1.5mL/min. Eluent: Gradient from 5–19% ACN in aqueous 0.1% TFA.

Figure 33 and 34 demonstrate that, with columns developed for use with low concentrations of TFA, it is sometimes possible to eliminate the TFA entirely, relying on ion pair reagents such as acetic acid or formic acid.

HPLC columns developed for low TFA use enable the use of a wider selection of ion-pairing reagents to optimize resolution of peptides. Peptide separations can now be done with acetic acid or formic acid acid replacing the trifluoroacetic acid. Mixtures of ion-pair reagents can also be used to optimize a peptide separation.

### Tryptic Map Replacing TFA with Acetic Acid (No TFA)
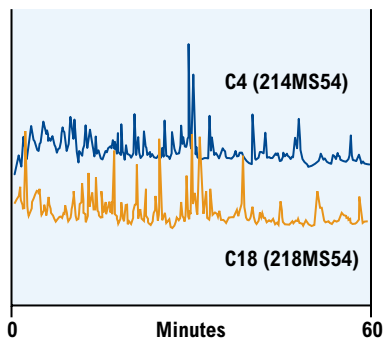
Figure 33. **Columns:** Columns developed for peptide separations in the absence of TFA. **Top:** C4 (Vydac® 214MS54 column); **Bottom:** C18 (Vydac® 218MS54 column); **Flow Rate:** 1mL/min. **Eluent:** Gradient from 0–30% ACN in 5mM HOAc. **Sample:** Tryptic digest of apotransferrin.

Example of Peptide Isolation and Sequencing

Reversed-phase HPLC using capillary columns with very small sample loads coupled with mass spectrometry has become a powerful tool for the isolation and identification of peptide fragments of proteins generated by enzymatic digests. The example in **Figure 35** shows the separation of a tryptic digest of bovine serum albumin followed by mass spectrometric analysis. The eluent from the column was monitored by on-line mass spectrometry, measuring the total ion current (**Figure 35**, top). When the current exceeded a threshold value the mass spectrum was obtained on the eluting peak and its molecular weight was reported. The eluting peak was then fragmented in a triple quadrupole mass analyzer producing product ions of the peptide which were used to generate a sequence of the peptide (**Figure 35**, bottom). The peptide fragments can also be matched with a protein or DNA database to identify the protein.

LC-MS of Crude Cone Snail Venom Peptides using 0.1% FA
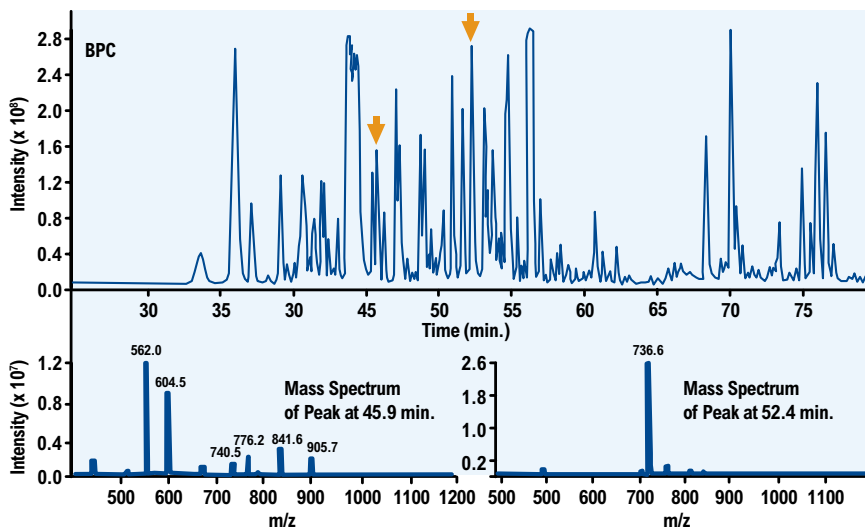
Figure 34. 50,000 to 150,000 polypeptide chains are present in the venom from marine cone snails. The combination of polypeptide chains have the potential to treat chronic pain. Columns developed for peptide separations in the absence of TFA allow for use of formic acid to increase detection sensitivity. **Column:** C18, 300Å, 5µm, 75µm x 250mm (Vydac® Everest® 238EV5.07525 column). **Sample Preparation:** Dissolved in 2% ACN with 0.1% FA. **Mobile Phase: A:** 98:2 H_2O:ACN with 0.1% FA; **B:** 40:60 H_2O:ACN with 0.1% FA. **Gradient:** equilibrate 25 min. at 0% B; 0 to 100% B in 60 min; hold 100% B for 10 min.; 100% to 0% B in 5 min. **Flow Rate:** 250nL/min. Sample and gradient conditions courtesy of Drs. Frank Mari and Victor Asirvatham, Dept. Chem. & Biochem., Florida Atlantic University, Boca Raton, FL 33431. Data from Reference 64.

Identification of Peptide Fragment of Proteins from Enzymatic Digest
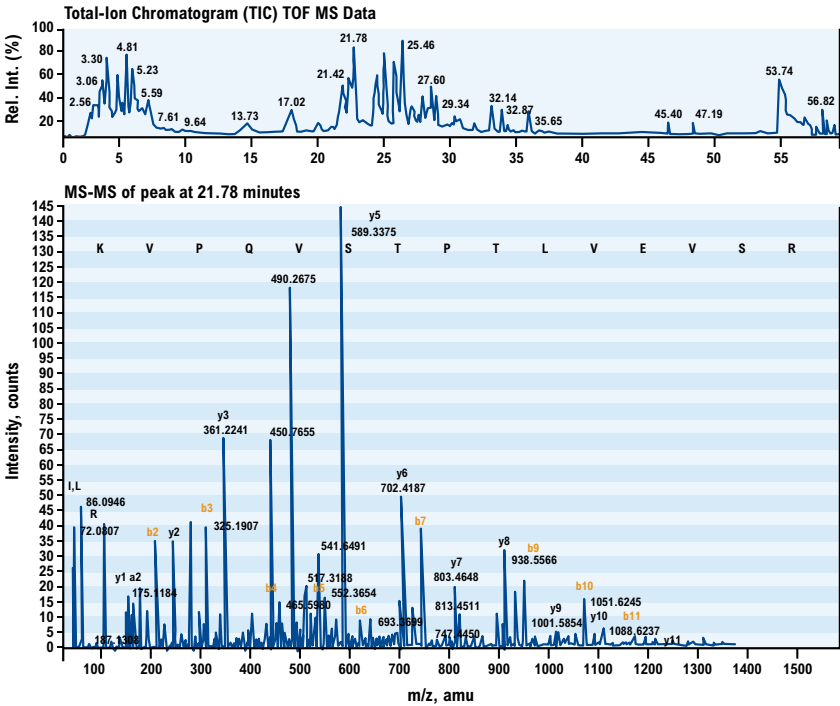
Figure 35. Reversed-phase separation of tryptic digest peptides of bovine serum albumin (BSA) followed by MS determination of molecular weights of each peptide followed in turn by MS fragmentation of each peptide providing data to enable sequencing of the separated peptide. **Sample:** 3 pmole of a tryptic digest of bovine serum albumin. **Column:** Vydac® 218MS5.305, 5μm, 300Å, polymeric-C18 reversed-phase (300μm i.d. x 50mm) column. **Flow Rate:** 5μl/min. **Mobile Phase: A =** 0.1% FA, 98% water, 2% ACN. **B =** 0.1% FA, 98% ACN, 2% water. **Gradient:** Hold 3% B from 0 to 5 minutes. Then ramp from 3% B to 50% B at 65 minutes. Final ramp to 75% B at 70 minutes. **Detection:** Triple quadrupole MS. Data from Reference 75.

The Role of Reversed-Phase HPLC in Proteomic Analysis

Proteomics is the study of cellular processes by identification and quantitation of expressed proteins. Proteomics seeks to catalogue all expressed proteins in prokaryote or differentiated eukaryote cells and is used to compare protein expression in two states, for instance comparing protein expression in normal cells and diseased cells or in diseased cells and cells treated with a therapeutic drug.

Proteomic methodologies have traditionally used two-dimensional gel electrophoresis to separate and isolate cellular proteins. The separated proteins are then protease digested and the resulting peptides are analyzed by Matrix-Assisted Laser Desorption Ionization (MALDI) mass spectrometry. The results are compared to protein and DNA databases for identification of the isolated proteins.

Newer proteomic techniques involve the chromatographic separation of peptide fragments generated by protease digests of whole cell lysates. This approach produces very large numbers of peptide fragments which require high resolution techniques to resolve. Two-dimensional chromatography, consisting of separation of the peptide fragments by ion exchange chromatography followed by separation of the ion exchange fractions by RP-HPLC, has been recently described[43]. The peptide fragments separated by the two chromatography steps are then analyzed by electrospray ionization and tandem mass spectrometry. The MS results are compared to DNA or protein databases for identification (Figure 36).

Proteomic Analysis of Cellular Proteins by Two-dimensional Chromatography and Tandem Mass Spectrometry

Cellular proteins → **Peptide fragments** → **Ion Exchange** **Reversed-phase** → **Tandem Mass spectrometry** → **Mass spectra of peptides and MS fragmentation product ions** → **Database Identification of cellular proteins**
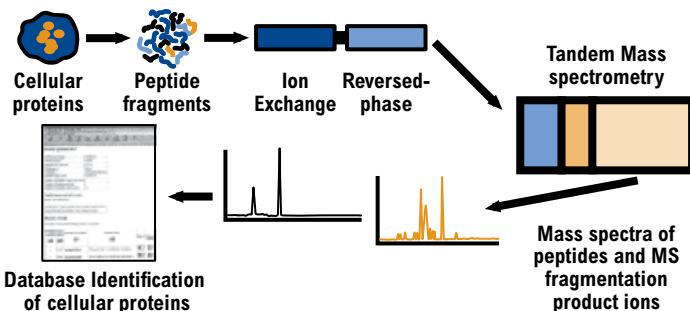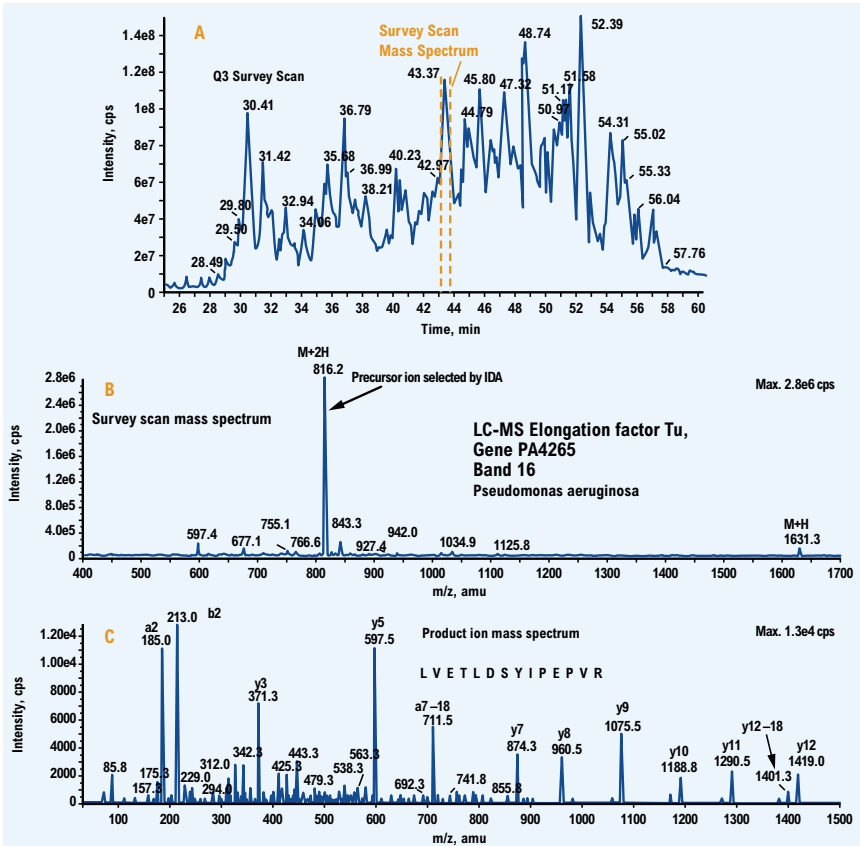
Figure 36.

Scientists from the Protein Characterization and Proteomics Laboratory at the University of Cincinnati College of Medicine reported using a capillary (300µm i.d. x 100mm) reversed-phase column together with a triple-quadrupole mass spectrometer for detection and identification of expressed sequence tags to identify gene products in *Pseudomonas aeruginosa* (Example shown in **Figure 37**). One objective of this work was to identify proteins which could be therapeutic targets for mediation of P. *aeruginosa* biofilms that do not respond to conventional antibiotic therapy and are involved in a number of human diseases including cystic fibrosis. The proteins were first extracted and separated by SDS-PAGE. Bands of interest were digested and subjected to RP-HPLC separation followed by MS and tandem MS to obtain data for protein database searching[76].

Proteomic Analysis of *Pseudomonas aeruginosa*

Figure 37. LC-MS data for tryptic peptides from PAGE band. **Column:** C4, 300Å, 5µm, 300µm x 100mm (Vydac® 214MS5.310 column). **Flow Rate:** 1µL/min. **Mobile Phase A:** 0.1% TFA in water; **B:** 0.085% TFA in 90:10 ACN:water. **Gradient:** Ramp from 3% B to 65% B over 50 min. **Detection:** MS. **A.** Total ion count. **B.** Mass spectrum of selected peptide, precursor ion at 43.37 min. **C.** Product ion spectrum from fragmentation of selected m/z 816.2 precursor.

# Examples of the Use of Reversed-Phase HPLC in the Analysis of Polypeptides

Reversed-phase HPLC has become a principle analytical technique in the separation and analysis of proteins and peptides. It is widely used in research studying natural proteins and peptides and in the analysis of protein therapeutic products in the pharmaceutical industry. This section will focus on a number of applications and uses, with typical specific analytical conditions, to increase understanding of how to put into practice the previous sections which have focused on laying a foundation of theory and practical aspects of the RP-HPLC separation of polypeptides.

Natural and Synthetic Peptides

RP-HPLC has long been important in the separation and isolation of natural and synthetic peptides. C18 columns are most commonly used in the isolation of peptides as illustrated in **Figure 38** in the separation of two naturally occuring cardioacceleratory peptides[17]. Elution conditions are generally gradients from low to moderate concentrations of acetonitrile and use 0.1% TFA.

RP-HPLC was used to separate peptides related to Alzheimer's disease[18] and is widely used to purify synthetic peptides (Page 65).

**RP-HPLC Separation of Natural Peptides**
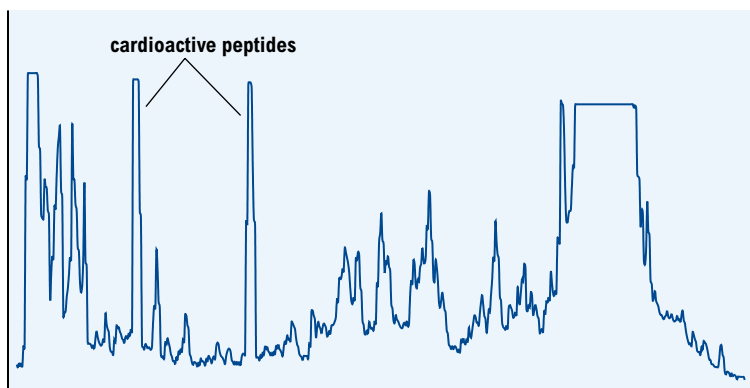
Figure 38. RP-HPLC was used to separate two octapeptides with cardioacceleratory activity from an extract of *Periplaneta americana*—american cockroach. **Column:** Vydac® 218TP54 (C18, 5μm, 4.6 x 250mm) column. **Eluent:** Hold at 18% ACN for 10 min; 18–30% ACN from 10–70 min, 30–60% ACN from 70–100 min; all with 0.1% TFA. Data from Reference 17.

Protein Digests

The study and analysis of proteins have long involved protease digestion to produce small peptide fragments, which can then be sequenced or which provide important information on the character and nature of the protein. Although many proteases have been used, trypsin, which cleaves a polypeptide backbone at the carboxy-terminus of lysine or arginine, has been the most popular protease. Digestion typically involves denaturation of the protein in the presence of high concentrations of a chaotropic agent such as guanidine-HCl (6 M) or urea (8 M) together with the addition of a reducing agent to reduce the disulfide bonds present in the protein. The free cysteines are usually carboxymethylated to prevent reformation of disulfide bonds. Digestion may be performed at room temperatures or higher temperatures which reduce the time required for the digestion. The resulting fragments of the protein, averaging about 10 amino acids each, can be separated by RP-HPLC under conditions such as those shown in **Figure 39**. In this instance a monoclonal antibody was digested and the resulting fragments chromatographed on a C18 column (Vydac® 218TP54) using a gradient from 0 to 40% acetonitrile containing 0.1% TFA. The defect causing sickle cell anemia is the replacement of glutamic acid by valine in position 6 in the hemglobin protein. Tryptic digests can reveal amino acid changes in a protein by the effect the change has on the tryptic fragment containing that position. As illustrated in **Figure 40** comparing the tryptic maps of normal hemoglobin and sickle cell hemoglobin, the substitution of valine for glutamic acid causes the peptide fragment containing position 6 to shift to longer retention because valine is more hydrophobic than glutamic acid[26].

### RP-HPLC Separation of the Tryptic Digest of a Monoclonal Antibody
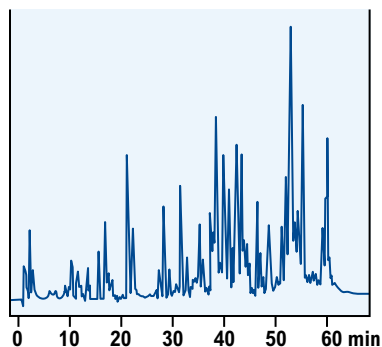
Figure 39. Column: Vydac® 218TP54 (C18, 5µm, 4.6 x 250mm) column. **Eluent:** Gradient from 0–40% acetonitrile with 0.1% TFA over 65 minutes. Data from Reference 27.

One of the most common degradations to occur with protein therapeutics is the conversion of an asparagine residue to either aspartic acid or isoaspartic acid, termed deamidation[14]. Deamidation often results in the loss of biological activity. A common means of determining deamidation is to digest the protein with trypsin and to look for new peptide fragments eluting slightly later than fragments which are known to contain asparagine. Under acidic conditions aspartic acid is slightly more hydrophobic than asparagine, thus a fragment containing the aspartic acid deamidation product will elute slightly later than a fragment containing asparagine (**Figure 41**).

Tryptic Maps of Normal Hemoglobin and Sickle Cell Hemoglobin
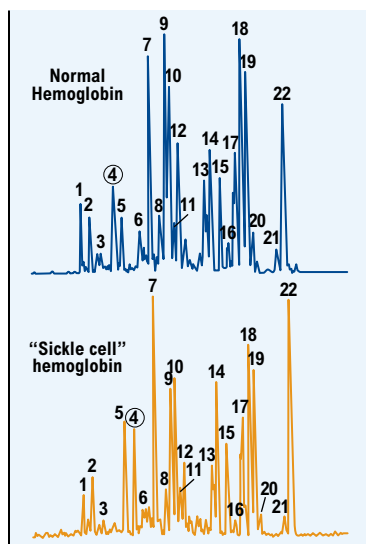
Figure 40. Hemoglobin from normal and sickle cell subjects was tryptic digested and analyzed by RP-HPLC. Peptide 4 contains position six, which is mutated from glutamic acid to valine in sickle cell anemia subjects. **Column:** Vydac® 218TP51 (C18, 5µm 1.0 x 250mm) column. **Eluent:** 0–40% ACN over 50 min, with 0.1% TFA, at 50µl/min. Data from Reference 26.

RP-HPLC Used in the Study of Protein Deamidation
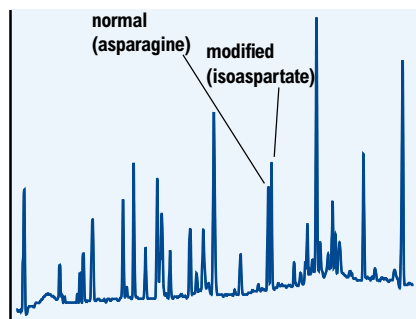
Figure 41. RP-HPLC separation of peptide fragments from tryptic digests of normal bovine somatotropin (BST) with asparagine at position 99 and deamidated BST with the asparagine replaced by isoaspartate. **Column:** Vydac® 218TP54 (C18, 5µm, 4.6 x 250mm) column. **Eluent:** 0–15% ACN over 20 min, 15–21% ACN over 12 min, 21–48% ACN over 27 min, 48–75% ACN over 4 min, all with 0.1% TFA, at 2.0mL/min. Data from Reference 14.

Peptide Maps to Identify Glycopeptides

The LC-MS analysis of a tryptic digest provides information about the structure of a protein. It is possible, among other things, to identify the site of glycosylation (addition of an oligosaccharide) of a protein. During the RP-HPLC separation of the peptide fragments, the mass spectrometer is switching between measurement of the mass (m/z) of the intact peptide and fragmenting the peptide through collisionally-induced dissociation, measuring the mass (m/z) of the resulting fragments of the peptide[40]. In particular if an oligosaccharide is present, certain "diagnostic ions" are produced by fragmentation which have m/z of 168 and 366. By requesting a combined trace of the ion currents produced by these two ions, an "oligosaccharide specific" trace is produced (**Figure 42**). This identifies which peptide the glycan (oligosaccharide) is attached to and the site of attachment can be identified.

Protein Analysis

While peptide digests are often used to study protein structure, intact proteins can be separated and analyzed by RP-HPLC, providing information about the intact protein. RP-HPLC is sensitive to both protein modifications, such as deamidation or oxidation, and to protein conformation.

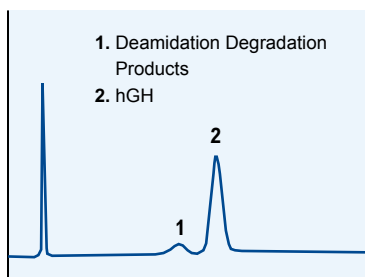### Glycosylated Peptides in a Peptide Map
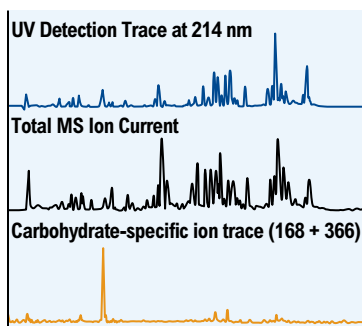
Figure 42. Glycosylated peptides in a peptide map can be identified by the monitoring of "carbohydrate diagnostic ions" by on-line mass spectrometry. **Column:** Vydac® 218TP54 (C18, 5µm 4.6 x 250mm) column. **Eluent:** 0–40% ACN over 65 min, with 0.1% TFA, at 1.0mL/min. Data from Reference 40.

Deamidation and Oxidation

Protein deamidation results in conversion of an asparagine to an aspartic acid (or isoaspartic acid), thus adding an acidic group to the protein. At neutral pH the protein therefore becomes somewhat more hydrophilic. Separating proteins at neutral pH can identify protein degradation deamidation products as illustrated in **Figure 43**. Human growth hormone elutes after the deamidation products because they are less hydrophobic under these conditions[31].

Methionine residues in proteins can oxidize through metal catalysis, oxygen and light. Most proteins lose biological activity when oxidized. Oxidation causes a protein to become more hydrophilic and oxidized proteins elute before the native form in RP-HPLC, as shown in **Figure 44**. In this instance oxidized forms of a coagulation factor are well separated from the native protein[30]. Because reversed-phase HPLC is very sensitive to the "hydrophobic foot" of a protein, even slight changes in protein conformation can result in changes in

Detection of Deamidation by RP-HPLC

Figure 43. The protein therapeutic recombinant protein deamidates during storage. **Column:** Vydac® 214MS54 (C4, 300Å, 5μm, 4.6 x 250mm) column. **Flow Rate:** 0.5 mL/min. **Mobile Phase:** Isocratic, 71% 50mM Tris, pH 7.5, 29% n-propanol. **Absorbance:** 220nm. **Column Temperature:** 45°C. **Injection Volume**: 20μL of a 1mg/mL preparation. Data from Reference 70.

### Separation of Oxidized Forms of Coagulent Factor from Native Protein
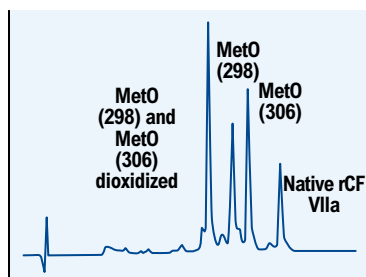
Figure 44. Separation of oxidized forms of coagulant factor VIIa from the native protein. **Column:** Vydac® 214TP54 (C4, 5μm 4.6 x 250mm) column. **Eluent:** 37–47% ACN over 30 min, with 0.1% TFA. Data from Reference 30.

reversed-phase elution. In **Figure 45**, the retention of an insulin-like growth factor is shifted when two adjacent disulfide bonds are switched[37].

In **Figure 46**, RP-HPLC is used to monitor a recombinant protein production process. Aggregates of the protein elute later than the monomer, carbamylated protein (caused by the use of urea) elutes as a shoulder on the native protein peak, oxidized (methionine) protein elutes before the native form, the desGlyPro clipped protein elutes earlier than the native protein and misfolded IGF elutes earlier yet. Reversed-phase is able to identify and quantitate a number of protein modifications[25].

RP-HPLC of Insulin-Like Growth Factor
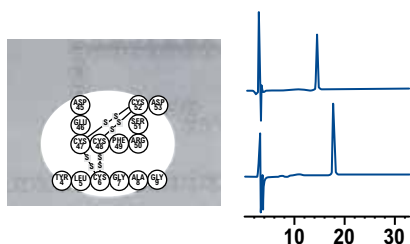
Figure 45. Insulin-like growth factor has two adjacent disulfide bonds which can "switch". This changes the conformation of the protein, which, in turn, affects reversed-phase elution. **Column:** Vydac® 214TP54 (C4, 5μm, 4.6 x 250mm) column. **Eluent:** 20–38% ACN:IPA (88:2) with 0.1% TFA. Data from Reference 37.

RP-HPLC of Modified Insulin-Like Growth Factor
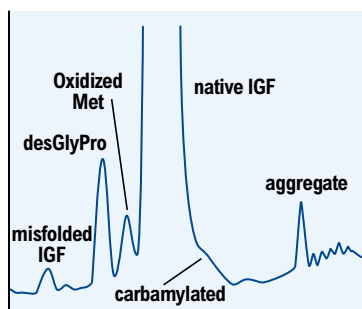
Figure 46. Insulin-like growth factor modified during production was analyzed by RP-HPLC, revealing several modified forms. **Column:** Vydac® 218TP54 (C18, 5μm, 4.6 x 250mm) column. **Eluent:** A. 0.12% TFA in H2O. B. 0.1% TFA in acetonitrile. **Gradient:** 27.5–28.5% B over 9 minutes, followed by 28.5–40% B over 4 min., followed by 40–90% B over 90 minutes at 2mL/min. Data from Reference 25.

Examples of Protein Separations

Proteins as large as 105 kD[33] and 210 kD[19] have been separated using RP-HPLC. Examples include:

Protein subunits

Eleven subunits of bovine cytochrome c oxidase ranging from MW 4962 to 56,993 were separated and analyzed by RP-HPLC[21] (**Figure 47**). The inset in **Figure 47** illustrates the use of shallow gradients to improve resolution for critical proteins.

Histones

Histones are a class of basic nuclear proteins that interact with DNA and may regulate gene activity. They have been separated on C4 RP using heptafluorobutyric acid (HFBA) as the ion-pairing agent[20].

Protein folding

The folding of insulin-like growth factor was studied using RP-HPLC[37]. Oxidative refolding of reduced IGF-1 resulted in two major peaks on RP-HPLC which had identical linear sequences but different disulfide pairing.

RP-HPLC Separation of Bovine Cytochrome c Oxidase Subunits
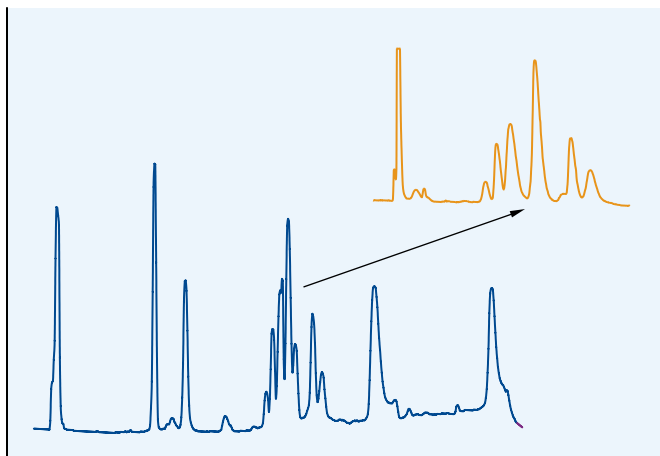
Figure 47. Eleven subunits of bovine cytochrome c oxidase ranging in MW from 4962 to 56,993 are separated by RP-HPLC. **Column:** Vydac® 214TP104 (C4, 10μm, 4.6 x 250mm) column. **Eluent:** 25–50% ACN over 50 min, then 50–85% ACN over 17.5 min; all with 0.1% TFA. **Flow Rate:** 1.0mL/min. Inset: 35–45% ACN with 0.1% TFA over 40 min. Data from Reference 21.

Viral proteins

Water insoluble poliovirus proteins were chromatographed by RP-HPLC[28].

Ribosomal proteins

30S and 50S ribosomal proteins have been separated by RP-HPLC using isopropanol as the organic modifier[29].

Membrane proteins

Many proteins are bound to the cell membrane and are very hydrophobic. Although hydrophobic proteins are particularly difficult to separate, Vydac® MS columns provide excellent selectivity, peak shape, and recovery for these molecules.[68-71]

In **Figure 48**, a hydrophobic reptilian reovirus p14 protein was separated from its myristoylated derivative, another cellular protein, and the surfactant Triton X-100 using an analytical C4 column and TFA as ion pairing reagent. The protein molecular masses were determined by LC-MS using a microbore C4 column and formic acid as ion pairing reagent (**Figure 49** on next page). The p14 transmembrane protein is a component of a potential new vaccine delivery system (R. Duncan and R. de Antueno, Dalhousie University, Halifax, Nova Scotia).

Separation of p14 Transmembrane Proteins

1. 18.8 kD Protein
2. Non-myristoylated p14
3. Myristoylated p14
4. Triton X (surfactant)
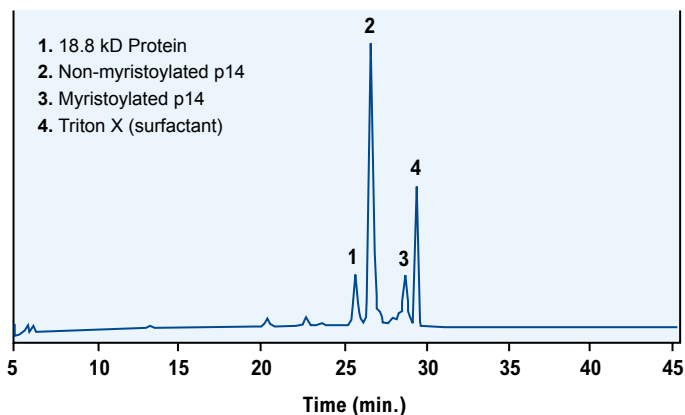
Figure 48. Analytical separation of transmembrane proteins. **Column:** C4, 300Å, 5µm, 4.6 x 250mm (Vydac® 214MS54 column). **Mobile Phase: A:** 0.1% v/v TFA in H_2O; B: 0.085% v/v TFA in ACN. **Gradient (min.,% B):** (0,20), (20,60), (25,80), (45,80). **Flow Rate:** 1.0mL/min. **Column Temperature:** Ambient, 25°C. **Detector:** UV at 215nm. Data from References 69 and 71.

LC-MS of p14 Transmembrane Proteins
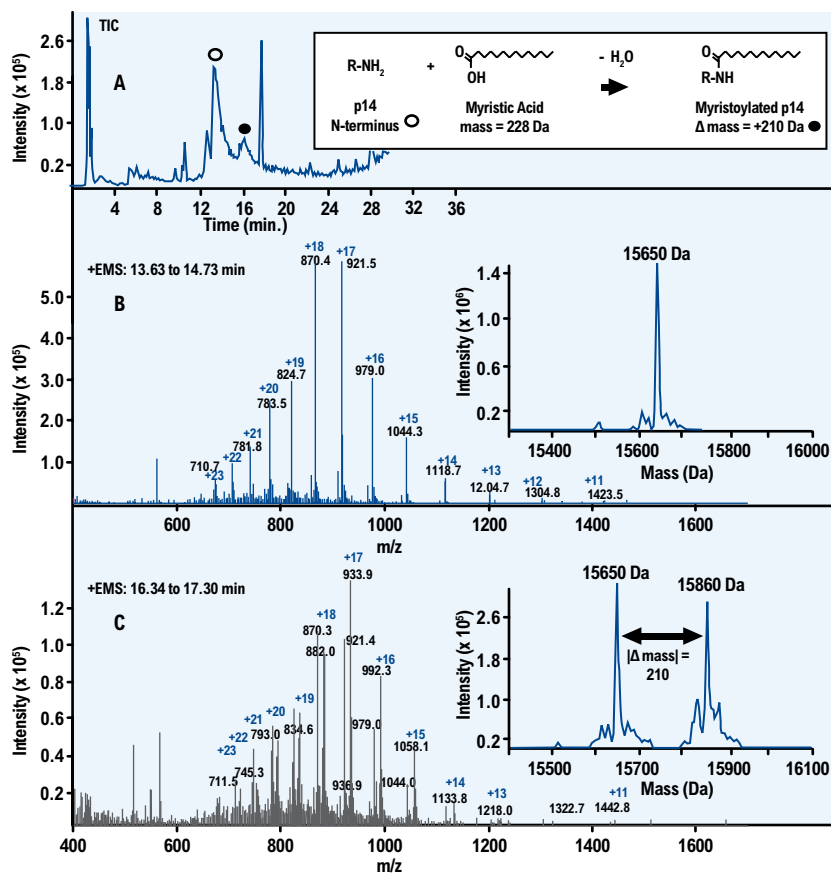
Figure 49. Microbore LC-MS analysis of p14 proteins. **Column:** C4, 300Å, 5µm, 1.0 x 150mm (Vydac® 214MS5115 column). **Mobile Phase: A:** 0.1% formic acid in 5:95 ACN:Water; **B:** 0.1% formic acid in 80:20 ACN:Water. **Flow Rate:** 50µL/min. **Gradient Program (%B, min.):** (25,0), (75,20), (100,25), (100,35), (25, 37). **Detector:** Linear ion trap MS. Data from Reference 69.

A. Total ion chromatogram. **B.** Mass spectrum of non-myristoylated p14; inset represents the deconvoluted mass spectrum corresponding to the chromatographic peak at 13.6 min. **C.** Mass spectrum of non-myristoylated and myristoylated p14; inset represents the deconvoluted mass spectrum corresponding to the chromatographic peak at 16.3 min. Charge series were observed for both non-myristoylated and myristoylated p14, since there was partial coelution of non-myristoylated p14 under the LC conditions using formic acid.

A large, 105 kD, transmembrane protein from *Neurospora crassa* was dissolved in anhydrous TFA and purified by RP-HPLC using a C4 column and a gradient from 60 to 100% ethanol containing 0.1% TFA. These results demonstrate that a crude membrane preparation can be directly applied to RP-HPLC columns to isolate very hydrophobic, integral proteins[33].

Hemoglobin variants

A RP-HPLC method using a C4 column has been developed for the separation of globin chains[27]. This method has been used to study hemoglobin variants in both animals and humans. RP-HPLC has helped to detect at least fourteen abnormal hematological states in humans and was used to study a silent mutant involving substitution of threonine for methionine[34].

Protein characterization

Proteins are routinely purified for sequencing and characterization by RP-HPLC, for example the purification of an acid soluble protein from *Clostridium perfringen spores*[36].

Grain proteins

Grain varieties cannot usually be identified by physical appearance, so methods based on RP-HPLC profiles of soluble proteins have been developed to identify grain varieties. RP-HPLC profiles of alcohol-soluble endosperm proteins—glutelins— were obtained on C4 columns and used to identify varieties of rice[32].

Purification and Isolation of Polypeptides

Growth of Peptide Therapeutics

There has been a growing trend towards peptides as therapeutic agents in recent years. This has been driven by the many inherent benefits peptides provide over small molecule drugs and recent developments that overcome past limitations in manufacturing and delivery of peptide therapeutics.

The failure of combinatorial processes to successfully deliver viable small molecule drug candidates has renewed interest and exploration of biologic therapeutic targets. In addition, peptides often have higher potency and higher efficacy on the desired target, minimal side effects and interactions, offer greater biological and chemical diversity, and have reduced number of side effects.

Challenges of Peptide Purification

In peptide synthesis, many side reactions occur that generate impurities. These impurities often are chemically similar to the target peptide and therefore present challenges in isolation of the peptide of interest. The most powerful method for peptide purification is reversed phase chromatography. For industrial purification, important consideration and selection of particle size, pore size, and stationary phase in relation to the peptide can optimize purification. Efficient column packing and use of dynamic axial compression can further improve results.

RP-HPLC is routinely used in the laboratory to purify microgram to milligram quantities of polypeptides for research purposes. Columns of 50mm i.d. and greater are used to purify up to gram quantities of recombinant proteins for use in clinical trials or for marketed products. Scaling up separations in the laboratory usually involves the use of standard solvents and ion-pairing agents or buffers, choosing column dimensions with the necessary sample load characteristics (see Appendix A), and optimization of the elution gradient.

Scaling up laboratory separations to process scale involves not only increasing the size of the column and the elution flow rate, but may also involve a change in elution solvents, use of different ion-pairing agents or buffers, and a change in gradient conditions.

In all cases, scaling up laboratory separations is simplified by the availability of separation materials for large scale columns that have nearly identical separation characteristics as the columns that are routinely used in laboratory scale separations.

Selecting Separation Materials

Process scale reversed-phase separation materials are available with nearly the same separation characteristics as analytical RP columns.

Vydac® 300Å silica is produced in particle sizes from 3-30µm. Physical sizing procedures are used to isolate fractions of five and ten micrometers particles for use in analytical and laboratory scale preparative separations.

Silica fractions with larger average particle size and broader ranges are

separated for preparative and process scale applications. Process-scale reversed-phase materials based on silica from the same manufacturing process as analytical size silica and bonded by matched chemical procedures have nearly identical protein and peptide selectivity characteristics as analytical scale materials. The separation of several proteins on columns of five, ten and fifteen-to-twenty micrometer particle size materials illustrates this (**Figure 50**). Protein selectivity and retention are the same on all three materials. The only difference between the materials of different particle sizes is that peak widths are broader with the larger particle materials, causing some loss in resolution. Large particle materials—10-to-15, 15-to-20 or 20-to-30µm—are normally used in the large scale purification because they are less costly than small particle materials, they result in lower column back-pressure and they are easier to pack into large diameter columns. In addition, in preparative chromatography, the column is nearly always "overloaded" in order to maximize sample throughput (see Page 59). When columns are "overloaded", large particle materials perform nearly as well as small particle materials, as illustrated in **Figure 51**. Although peak width and resolution are much better (2–3 times) with five or ten micrometer materials than with larger particle materials at low sample loads, at high sample loads using typical "overload" conditions, peak widths are only about 20 to 50% greater on the larger particle materials. The slight resolution advantage of small particles when overloading columns does not compensate for the higher cost and backpressure and practical difficulties of working with small particle materials in process applications.

Separation of Proteins on RP-HPLC Columns of Different Particle Size
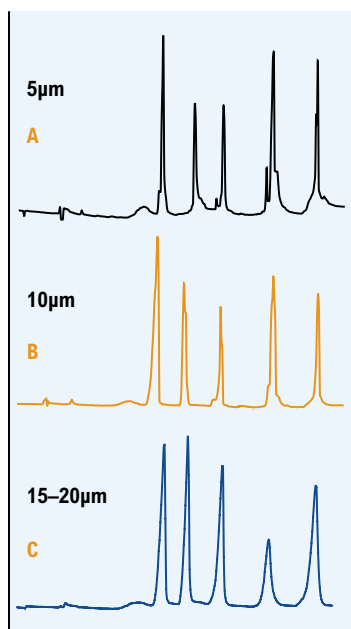
Figure 50. Protein selectivity is the same on RP materials of different particle sizes. The only difference between materials of different particle sizes is that peak width increases and resolution decreases as particle size increases. **Column Materials: A.** Vydac® 214TP, 5µm column. **B.** Vydac® 214TP, 10µm column. **C.** Vydac® 214TP, 15–20µm column. **Mobile Phase:** 24–95% ACN with 0.1% TFA over 30min at 1.5mL/min.

Figure 51. Although peak widths are much narrower with small particle materials at low sample loads, there is little difference in peak widths at high loads, where the column is "overloaded". **Column Materials:** Vydac® 214TP, 5µm column; Vydac® 214TP, 10µm column; Vydac® 214TP, 15–20µm column; Vydac® 214TP, 20–30µm column. **Eluent:** 24–95% ACN in 0.1% aqueous TFA over 30 min at 1.5mL/min; Protein: ribonuclease.

A New High Capacity Media for Peptide Purification

To address the growing demand for effective peptide purification, Grace has developed the new Vydac® 150HC purification media. The new Vydac® 150HC media is highly effective at purifying many small to medium-sized peptides with greater loading capacity and improved productivity compared to competitive media. It has unique selectivity that can reveal peaks masked by other C18 media and improves resolution of closely related peptides and impurities for higher purity target peptides[72].

Vydac® 150HC Media Properties	
Phase	C18
Pore Size	150Å
Pore Volume	1.05mL/g
Surface Area	320m^2/g
Particle Size	10µm and 20µm
Carbon Load	16%

Scaling-up Elution Conditions

The three key factors to consider in scaling up polypeptide separations are the elution solvent, the ion-pairing reagent or buffer, and the gradient characteristics.

Elution solvent

Laboratory scale purifications generally use the same organic modifier, namely acetonitrile, as analytical chromatography.

Ion-pairing agent or buffer

Laboratory scale purifications generally use the same ion-pairing agents or buffers as analytical chromatography.

Gradient characteristics

To retain the resolution obtained on an analytical column while increasing column diameter, the gradient shape must be maintained by keeping the ratio of the gradient volume to the column volume constant. For example, a 22mm diameter column has about 23 times the volume of a 4.6mm diameter column of the same length (22 divided by 4.6, squared). A 1.0mL/min gradient over 30 minutes on an analytical column has a volume

of 30mL. To transfer the method to a 22mm column, the gradient volume should be increased 23 times to 690mL. The flow rate can be increased 23 times while maintaining the gradient time constant or the flow rate can be partially increased while lengthening the gradient time. For instance, a flow rate of 23mL/min for 30 minutes would result in a gradient volume of 690mL. However, a flow rate of 10mL/min for 69 min would give the same gradient volume, hence the same gradient shape and sample resolution. In either case the separation would be comparable to that obtained on an analytical column. In practice the gradient is often made more shallow—i.e., a smaller increase in organic modifier concentration per unit time—to increase resolution, particularly for the main polypeptide to be collected.

Scaling Gradient Volume to Column Volume: A Case Study

In order to maintain the same elution pattern when scaling a separation between columns of the same phase but different inner diameters, gradient volume must be scaled in proportion to column volume. As an example of what can happen if this rule is not followed, observe the two chromatograms (**Figure 52a and Figure 52b**).

The three components of hemoglobin – the α polypeptide, the β polypeptide, and the relatively small-molecular-weight iron binding component, heme, are well separated on a 10mm i.d. by 250mm Vydac® C4 reversed-phase

column using the flow and gradient conditions indicated. When the same separation was attempted on a 22mm i.d. by 250mm Vydac® C4 column of the same chemistry with only a 33% increase in flow rate and the same gradient ramp time, the elution order changed. A similar change in elution order was observed with a 2.5x increase in flow rate to 7.5mL/min and the same gradient time. What is the problem here?

Column Volume

The bed volume of a cylindrical column is given by the formula:

$$V_c = 0.25\pi d^2 L$$
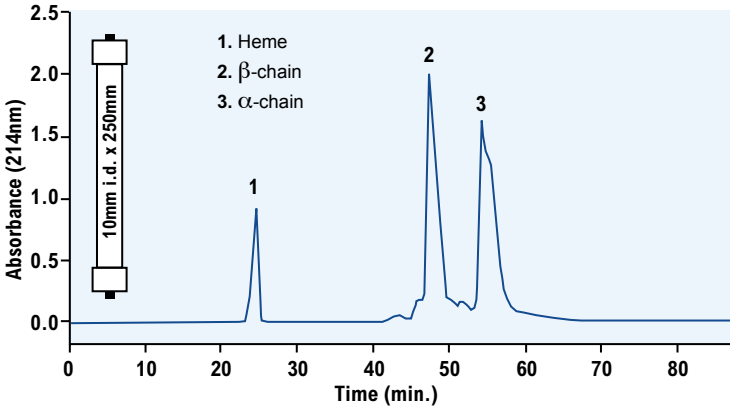
where :
    d = column bed diameter
    L = column bed length

Note that for columns of equal length, column volume is proportional to the square of the diameter. The volume of the 22mm i.d. column is $2.2^2 = 4.84$ times the voume of the 10mm i.d. column.

Separation on Semi-Prep Column

Figure 52a. Separation of hemoglobin components on a 10mm i.d. Vydac® C4 reversed-phase column (Vydac® 214TP1010, C4, 300Å, 10µm, 10mm i.d. x 250mm column). **Flow Rate:** 3mL/min. Gradient: 35% to 50% ACN to water (with 0.1% TFA) in 90 minutes. **Sample:** 10µl of a 15g/dL solution of hemoglobin.

Separation on Prep Column with Flow Rate Too Low
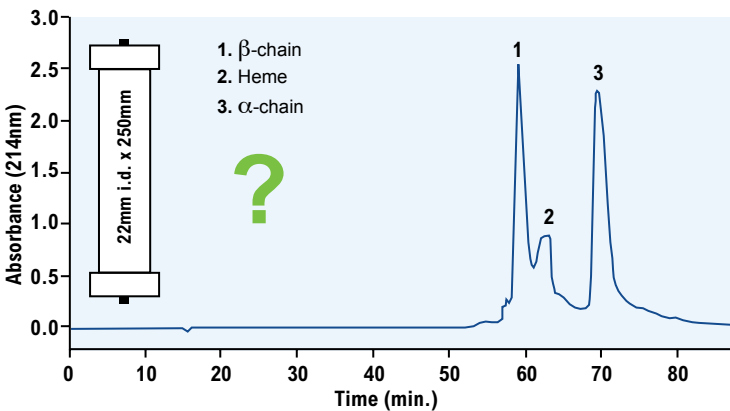
Figure 52b. Separation of hemoglobin components on a 22mm i.d. Vydac® C4 reversed-phase column (Vydac® 214TP1022, C4, 300Å, 10µm, 22mm i.d. x 250mm column). **Flow Rate:** 4mL/min. **Gradient:** 35% to 50% ACN to water (with 0.1% TFA) in 90 minutes. **Sample:** 100µl of a 15g/dL solution of hemoglobin.

Gradient Volume

The volume of a gradient is given by:

$$V_g = T_g F$$

where:

T_g = gradient time
F = mobile-phase flow rate

Note that for equal gradient times, gradient volume is proportional to flow rate. Thus for our example, in proportion to column volume the mobile phase flow rate on the 22mm i.d. column should be:

4.84 x 3mL/min = 14.52mL/min

or approximately 15mL/min.

When the flow rate was increased to 15mL/min (**Figure 53b**), the separation on the 22mm i.d. column appeared as in **Figure 53a**, showing the expected elution order.

Interpretation

In separations on a reversed-phase column, smaller molecules (like heme) tend to partition between the stationary phase and the mobile phase in a ratio that changes gradually with mobile phase composition. Larger biopolymers (like the α and β polypeptides) tend to be retained in an all-or-nothing manner, adsorbing firmly to the stationary phase below a critical mobile phase solvent concentration and releasing rapidly once the critical concentration is attained.

When the gradient develops in too small a volume (as in **Figure 52b**), there is not sufficient mobile-phase flow to sweep the partitioning heme molecules from the column before the critical concentration is reached for releasing the β polypeptide. Hence the elution order changes.

In more complex reversed-phase separations of peptides of various sizes, changes in elution patterns may be more subtle but nonetheless problematic.

Data courtesy of:

Dr. Tim Roach
Department of Chemistry and Biochemistry
University of Maryland, Baltimore County
100 Hilltop Circle, Baltimore, MD 21250

Separation on Semi-Prep Column

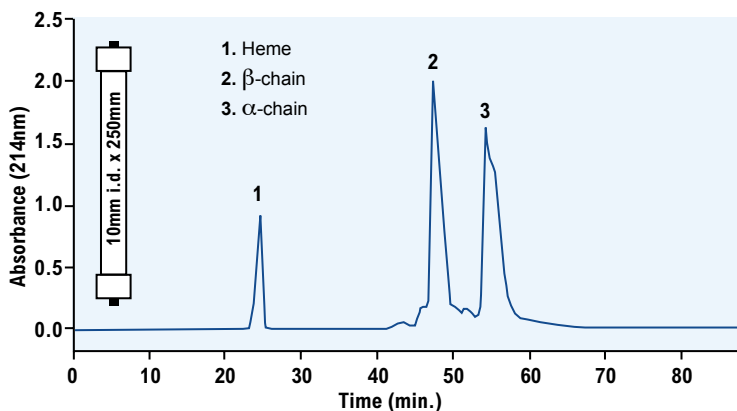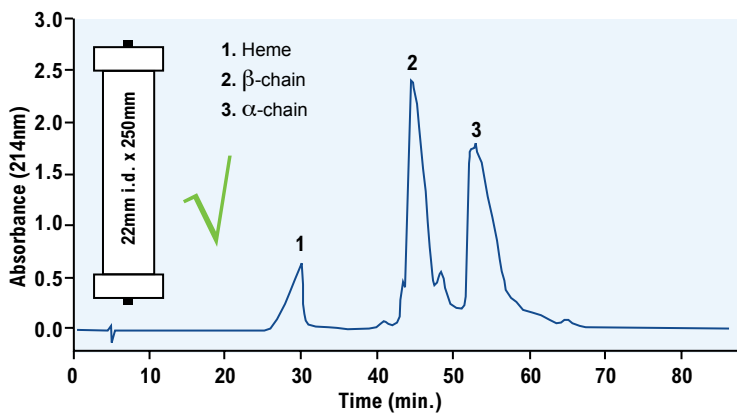**Figure 53a.** Same as **Figure 52a** (for comparison)

Separation on Prep Column with Correct Flow Rate

Figure 53b. Separation of hemoglobin components on a 22mm i.d. Vydac® C4 reversed-phase column (Vydac® 214TP1022, C4, 300Å, 10µm, 22mm i.d. x 250mm column). **Flow Rate:** 15mL/min. **Gradient:** 35% to 50% ACN to water (with 0.1% TFA) in 90 minutes. **Sample:** 100µl of a 15g/dL solution of hemoglobin.

The following equations may be applied for simple scale-up calculations:

Flow Rate (Constant Linear Velocity) for $L_{final} = L_{initial}$

$$\text{Flow Rate}_{final} = \text{Flow Rate}_{initial} * \frac{(D_{final})^2}{(D_{initial})^2}$$

$$= 3\text{mL/min.} * (22\text{mm})^2 / (10\text{mm})^2 = 14.5\text{mL/min.}$$

Sample Load

$$\text{Load}_{final} = \text{Load}_{initial} * \frac{(D_{final})^2 L_{final}}{(D_{initial})^2 L_{initial}}$$

D = diameter
L = length

initial   final

Process-Scale Purification: More Than Five Grams of Peptide

Elution solvent

The organic solvents commonly used in laboratory scale chromatography pose problems of cost, disposal or safety in a process environment. Solvents such as ethanol are more practical for process chromatography. Ethanol is relatively non-toxic, non-flammable when mixed with water, is available at low cost and is known and understood by regulatory agencies such as the FDA. Ethanol is presently used in large scale process purifications[59].

Ion-pairing agent or buffer

Ion pairing agents commonly used for analytical chromatography are less practical for process scale chromatography. Alternate ion-pairing agents or buffers useful for process chromatography include acetic acid—which also converts the polypeptide to the acetate form, useful in formulations—and phosphate. Acetate is presently used in the purification of several biotechnology derived polypeptide therapeutics[61].

Gradient characteristics

The comments in the laboratory scale purification section regarding scaling up elution gradients to larger columns apply to process scale purifications (see above). Very shallow gradients in the region where the polypeptide of interest elutes are common.

How Much Polypeptide Can Be Purified in a Single Chromatographic Run?

When the purpose of the RP-HPLC separation is to collect purified polypeptide for further use, the amount of sample that can be loaded onto a column while maintaining satisfactory purity is very important. The approach to preparative purifications is generally to load the maximum amount of polypeptide that can be loaded while balancing three important factors:

Throughput

The amount of purified polypeptide produced in a given time period. While low sample loads yield maximum resolution, only small quantities are purified per chromatographic run and throughput is low.

Purity

The purity of the polypeptide expressed in percent of total weight of final purified product. Pure polypeptides are obtained by avoiding overlap with adjacent peaks although this may limit the amount of sample that can be loaded onto the column.

Yield

The percent of polypeptide purified as a percent of the total amount of polypeptide present in the original sample. Maximizing resolution enables recovery of most of the loaded polypeptide while removing impurities. If resolution is poor then only the center of a peak is collected, reducing yield.

There are three measures of sample capacity on a RP-HPLC column:

- the loading capacity with optimum resolution;
- the practical sample loading capacity;
- and, the maximum amount of polypeptide the column will bind.

Sample Loading Capacity with Optimum Resolution

In chromatography the loading limit of a column is normally defined as the maximum amount of analyte that can be chromatographed with no more than a 10% increase in peak width.

Peak width and resolution remain constant up to the "overload" point which, for analytical (4.6mm diameter) columns, is about 100 to 200µg for most polypeptides (**Figure 54**). Loading samples greater than this amount results in broadened peaks and decreased resolution.

Practical Loading Capacity

Preparative separations require maximizing throughput by balancing resolution, yield and purity. Often

improving yield comes at a cost of reduced purity or reduced throughput. In practice this generally requires "overloading" the column—that is, injecting polypeptide samples greater than the sample capacity defined by optimum resolution. As the sample load is increased, polypeptide peak widths increase (**Figures 54 and 55**); however, peak shape remains reasonably symmetrical. This often allows the loading of samples 10 to 50 times the nominal sample capacity while still retaining acceptable resolution.

Sample Loading Curve for Ribonuclease on Analytical Column
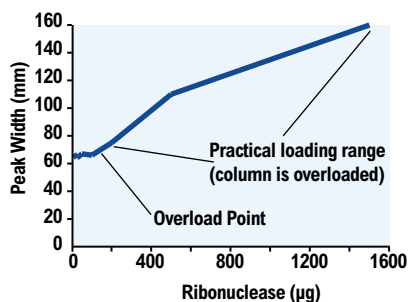
Figure 54. Peak width is constant with sample loads up to 200µg. Above 200µg—the "overload" point—the peak width gradually increases. The practical loading region for ribonuclease is 200 to 5000µg. **Column:** Vydac® 214TP54 (C4, 5µm, 4.6 x 250mm) column. **Eluent:** 24–95% ACN with 0.1% TFA over 30 min. **Sample:** ribonuclease.

In **Figure 55**, injections of 25, 100, 200, 500 and 1,000 micrograms of ribonuclease and lysozyme illustrate the effect on resolution of increasing peak width resulting from increasing sample loads. At 25 and 100µg injections—in the region of optimum

resolution—resolution between ribonuclease and the small impurity preceding it remains constant (**Figure 55-B**).

Resolution begins to decrease between ribonuclease and the impurity above 100µg—the "overload" point. The 200µg load shows a definite increase in peak width and consequent loss of resolution (**Figure 55-C**). At 500µg there is considerable loss in resolution (**Figure 55-D**) and at 1,000µg the impurity peak completely merges with the ribonuclease peak (**Figure 55-E**). Resolution between lysozyme and the preceding impurity peaks remains constant to about 200µg, after which resolution is slowly lost. At 500µg (**Figure 55-D**) the impurity peaks appear only as shoulders on the lysozyme peak and by 1,000µg (**Figure 55-E**) the impurity peaks have completely merged with the lysozyme peak. Resolution between the protein and impurity peak can be improved by running a more shallow gradient.

Since resolution between the two, well separated, major peaks—ribonuclease and lysozyme—remains good even at the 1,000µg sample load and peak shape is not seriously degraded, very high sample loads are possible for well separated peaks.

There are many examples in the literature of practical purification of polypeptides at high loading levels[46–50]. In one case 1.2 grams of a synthetic peptide mixture were purified on a 5 x 30cm column[46]. In a personal communication it was reported that 5 grams of synthetic peptide were purified on a 5 x 25cm column in two steps.

Effect of Sample Load on Protein Peak Shape and Resolution
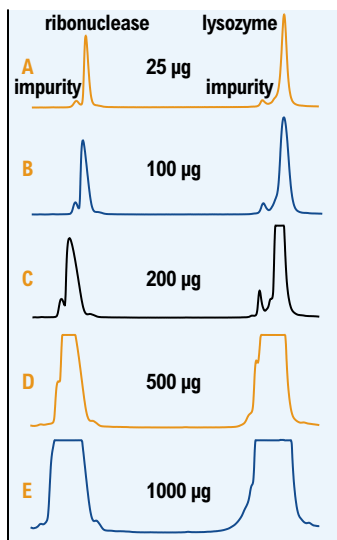
Figure 55. A. 25µg each protein **B.** 100µg each protein **C.** 200µg each protein **D.** 500µg each protein **E.** 1000µg each protein **Column:** Vydac® 214TP54 (C4, 5µm, 4.6 x 250mm) column. **Eluent:** 25–50% ACN in 0.1% TFA over 25 minutes at 1.5mL/min. **Sample:** ribonuclease and lysozyme.

Maximum Polypeptide Binding Capacity

The maximum binding capacity of a polypeptide on a reversed-phase column depends on the size and characteristics of the polypeptide. Small peptides have binding capacities of about 10mg of peptide per gram of separation material— 25mg on a 4.6 x 250mm column. Proteins have slightly higher binding capacities between 10 and 20mg of protein per gram of separation material, depending on the ratio of the area of the hydrophobic foot to the total molecular weight.

Although sample loads near the maximum binding capacity of a column provide little resolution, they are useful for simple, fast desalting of polypeptide samples.

High Loading for Improved Productivity

Loading capacity and purity are essential to improving overall productivity in process purification of peptides. By increasing loading capacity while maintaining or improving purity more sample can be purified in the same amount of time. The unique physical properties of the Vydac® 150HC C18 media increase loading capacity by 2-3X allowing more sample to be loaded on to the column while processing. Ultimately reducing the amount of purification cycles needed to process a batch. Couple this with lower overall pressure and the possibility for increased flow rates, significant time savings can be realized.

Ways to Optimize Throughput and Resolution

Sample concentration

Resolution between closely eluting polypeptides may be affected by sample concentration. Dilute samples appear to spread out over the column surface better than concentrated samples and this results in somewhat better resolution. Recommendation: Use dilute samples to improve resolution and sample loading capacity.

Use shallow gradients

Resolution between closely eluting polypeptides may be improved by using a more shallow gradient slope. This is usually done by lengthening the gradient time. Suggestion: Use longer elution times and shallow gradients to obtain maximum resolution for closely eluting peaks.

Increase the column volume

Since sample capacity is a function of column volume, either column diameter or column length can be increased for increased sample load. It is the volume of the column that is important, not the diameter or the length.

Use large particle adsorbents

When columns are "overloaded", particle size becomes less significant in obtaining resolution (**Figure 51**). Small particle materials give only slightly better resolution than large particle materials under "overload" conditions and the higher cost, higher back-pressure and practical difficulties of column preparation with small particle materials make them impractical for most preparative separations.

Effective loading of the sample

Load the sample in a solvent that will not interfere with adsorption of the polypeptide. This generaly means keeping the organic content well below that required to elute the polypeptide from the column. Some solvent in the sample, however, improves sample loading.

Frontal Loading Tests for the New Vydac® 150HC and Other Media

For nearly 100% aqueous loading experiments, different media are packed

in 2.1 x 50mm hardware and tested with the following conditions: mobile phase including solvent A comprising 0.1% v/v TFA in water; and solvent B comprising 250 mg polypeptide in 5 mL acetonitrile, 2 mL 50% glacial acetic acid and 43 mL deionized (DI) water. This is diluted down 1:5 with 0.1% TFA in DI water to make a 1 mg/mL polypeptide solution. A gradient process is used wherein the column is equilibrated at 100% solvent A; followed by increasing from 0% up to 100% solvent B for 1 min.; holding flow of solvent B at 100% for 100 min.; followed by increasing from 0% up to 100% solvent A (0% solvent B) for 1 min. The flow rate is 0.2 mL/min. The columns are run at a room temperature of 25ºC. UV detection at 276 nm. Loading experiments also are performed with 10% final acetonitrile concentration in solvents A and B. The unique physical properties of the Vydac® 150HC C18 media contributes to increased loading capacity compared to competitive media, especially under nearly 100% aqueous conditions (Figures 56 and 59).

The capacity, Q, of each material is calculated from the following equation:

$$Q = \frac{CF_v\,(t_{10\%} - t_0 - t_{step} - t_e)}{V_c - F_v t_0}$$

C = Polypeptide concentration in mobile phase B

F_v = Flow rate

$t_{10\%}$ = Frontal breakthrough time measured at 10% height

t_0 = Column hold-up based on injection of uracil in 90:10 Acetonitrile/Water

t_e = Extra-column time, or transit time between pump mixer and column inlet, and between column outlet and detector cell

V_c = Column volume.

Vancomycin Frontal Loading

Column	Uracil Retention (min.)	10% Acetonitrile Loading Capacity (mg/mL media)	Vancomycin Loading Capacity ~100% Aqueous (mg/mL media)
Vydac® 150HC, C18 20μm	0.65	49	123
Media C, C18 15μm	0.57	33	26
Media B, C18 10μm	0.51	28	14
Media A, 15-20μm	0.67	35	53

Loading, 10% Acetonitrile
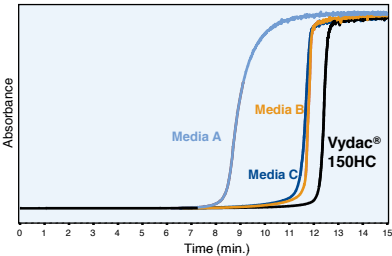
Loading, Nearly 100% Aqueous
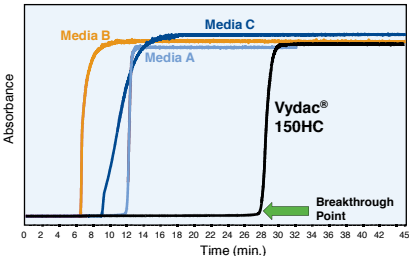
Figure 56. Frontal loading of vancomycin, a glycopeptide antibiotic, using 10% organic solvent and nearly 100% aqueous conditions (Reference 72).

Effect of Increased Vancomycin Loading on Peak Shape and Width

Column: 4.6 x 250mm, 20µm
Injection volume: 500 µL (fixed for all 5 samples)
Diluent: 0.1% TFA in Water
Mobile Phase A: 0.1% TFA in Water
Mobile Phase B: 0.05% TFA in Acetonitrile
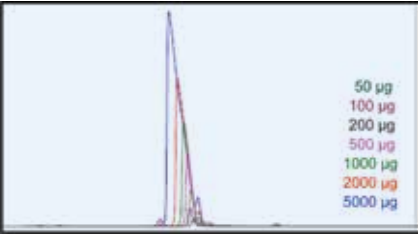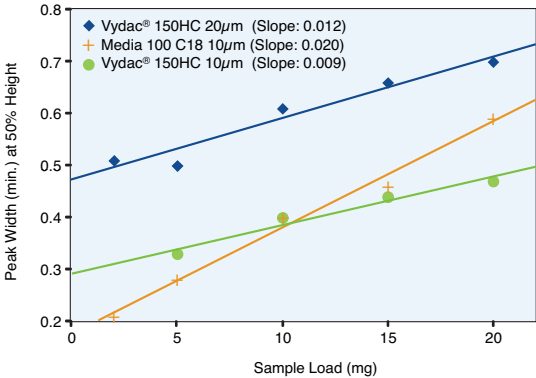Flow Rate: 1 mL/min.

Figure 57. Vancomycin loading from 50µg to 5 mg on an analytical column packed with 20µm Vydac® 150HC media (Reference 72).

Time (min)	%A	%B
0	95	5
25	60	40
27	20	80
30	20	80

Vancomycin (at 300nm)							
Sample (mg)	0.05	0.1	0.2	0.5	1.0	2.0	5.0
Plates/meter	35660	49904	28848	17984	10536	5328	2752
Width (at 50% ht)	0.35	0.29	0.38	0.47	0.60	0.81	1.08

Vancomycin Prep Scale Loading

Figure 58. Plot of peak width at 50% height vs. vancomycin loading for three 22 x 250mm prep columns, each packed with the following C18 media: Vydac® 150HC 10 or 20µm media; competitor 100Å 10µm media. Peptide loading ranged from 2.5 mg to 20 mg (Reference 72).

The slope of the plot (Figure 58) indicates less variation across different sample loads for Vydac® 150HC vs. competitive media. Also, the 20µm Vydac® 150HC demonstrates comparable chromatographic performance with greater loading capacity than the competitive 10µm media and with ~3X lower backpressure. Less backpressure can help extend column lifetimes and also allows for faster flow rates which improves overall productivity.

Insulin Frontal Loading

Column	Loading Capacity (mg/mL media) ~100 Aqueous
Vydac® 150HC C18, 20µm	361
Media C, C18 15µm	180
Media B, C18 10µm	18
Media A, C18 15-20µm	249
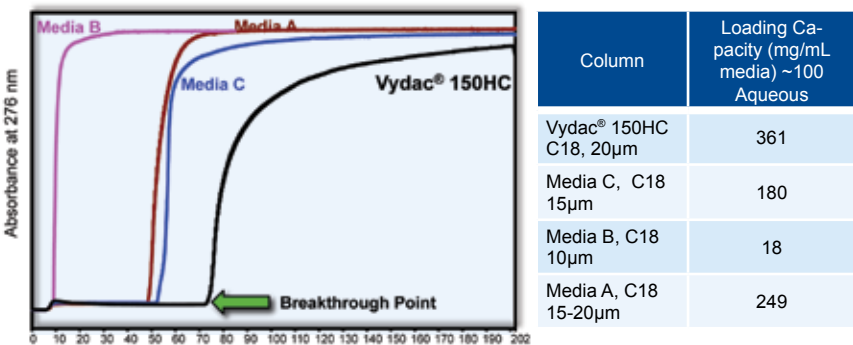
Figure 59. Frontal loading of insulin using nearly 100% aqueous conditions (Reference 72).
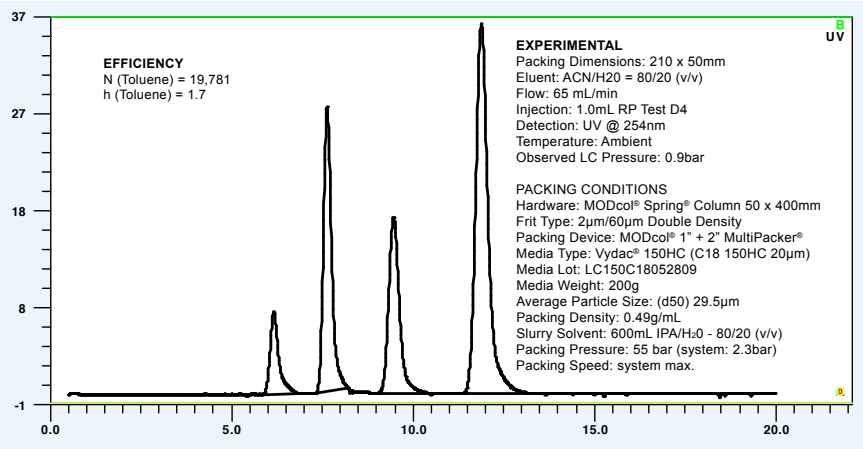
Lifetime and Stability

Selecting a media that is robust and withstands loading of peptide mixtures is critical for long term productivity and consistency. Compared to competitive media the Vydac® 150HC 20µm media exhibits a lower pressure drop and maintains column performance after multiple purification cycles[72]. Starting with a low pressure drop media enables multiple peptide mixtures be processed before column pressures build.

QC Test Mix (Values for Toluene) (at 254 nm)						
Injection #	1	2	3	4	5	6
Column Volumes	0	250	500	750	1000	1250
Retention Time (min.)	12.68	13.1	11.86	11.53	11.41	12.40
Plates/meter	11137	10890	10999	11866	10458	10898
Width (at 50% height)	0.56	0.59	0.53	0.49	0.52	0.55
Pressure (bar)	14	14	15	15	15	16

Vancomycin (at 254 nm)				
Injection #	1	2	3	4
Column Volumes	0	500	1000	1250
Retention Time (min.)	6.43	6.47	6.54	6.52
Plates/meter	10494	10447	9823	10031
Width (at 50% height)	0.29	0.29	0.31	0.30
Pressure (bar)	19	19	20	21

Efficiency maintained over 1250 column volumes for prep column packed with Vydac® 150HC 20µm media.

Vydac® 150HC 20μm Media Maintains Performance at 55 bar Packing Pressure

EFFICIENCY
N (Toluene) = 19,781
h (Toluene) = 1.7

EXPERIMENTAL
Packing Dimensions: 210 x 50mm
Eluent: ACN/H20 = 80/20 (v/v)
Flow: 65 mL/min
Injection: 1.0mL RP Test D4
Detection: UV @ 254nm
Temperature: Ambient
Observed LC Pressure: 0.9bar

PACKING CONDITIONS
Hardware: MODcol® Spring® Column 50 x 400mm
Frit Type: 2μm/60μm Double Density
Packing Device: MODcol® 1" + 2" MultiPacker®
Media Type: Vydac® 150HC (C18 150HC 20μm)
Media Lot: LC150C18052809
Media Weight: 200g
Average Particle Size: (d50) 29.5μm
Packing Density: 0.49g/mL
Slurry Solvent: 600mL IPA/H$_2$0 - 80/20 (v/v)
Packing Pressure: 55 bar (system: 2.3bar)
Packing Speed: system max.

Signal	No.	Name	Retention Time	Peak Height	Peak Area	Plate Count	Asymmetry
UV	1	Uracil	4.85min	8.55	129.41	2073	1.571
	2	Phenol	5.98min	29.14	438.40	3053	1.409
	3	DEET	7.19min	219.16	331.97	3492	1.259
	4	Toluene	9.17min	38.78	822.94	4154	1.250

Stability

Vydac® 150HC Media Remains Stable in DAC Spring® Column Hardware over a Wide Range of Mechanical Packing Pressures

Efficiency

Vydac® 150HC Media is Highly Efficient in DAC Spring® Column Hardware over a Wide Range of Mechanical Packing Pressures
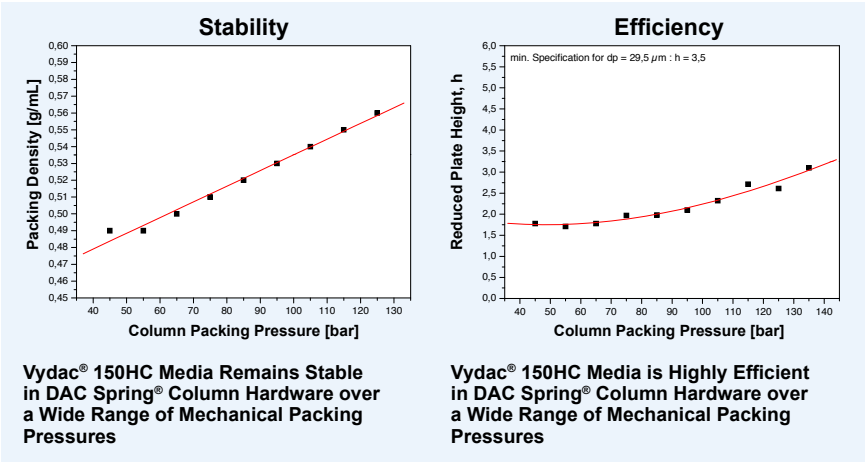
Figure 60. Vydac® 150HC 20μm media packed in a MODcol® Spring® dynamic axial compression column, 50mm i.d. x 210mm bed length. Chromatographic performance data includes packed density and reduced plate height versus column packing pressure (Reference 72).

Biological Activity and Reversed-Phase HPLC

Biological activity of proteins depends on tertiary structure and permanent disruption of tertiary structure eliminates biological activity.

RP-HPLC may disrupt protein tertiary structure because of the hydrophobic solvents used for elution or because of the interaction of the protein with the hydrophobic surface of the material. The amount of biological activity lost depends on the stability of the protein and on the elution conditions used. The loss of biological activity can be minimized by proper post-chromatographic treatment.

Small peptides and very stable proteins are less likely to lose biological activity than large enzymes. Some specific points to keep in mind are:

Protein denaturation

Denaturation of proteins on hydrophobic surfaces is kinetically slow. Reducing the residence time of the protein in the column generally reduces the loss of biological activity.

Solvent effects

Some solvents are less likely to cause a loss of biological activity than others. Isopropanol is the best solvent for retaining biological activity. Ethanol and methanol are slightly worse and acetonitrile causes the greatest loss of biological activity.

Stabilizing factors

Stabilizing factors, such as enzyme cofactors, added to the chromatographic eluent, may stabilize proteins and reduce the loss of biological activity.

The most important factor in maintaining or regaining biological activity is post-column sample treatment. Dissolution of a collected protein in a stabilizing buffer often allows the protein to re-fold. An example is HIV protease (**Figure 61**)[56].

HIV Protease Biological Activity

Dissolution

After lyophilization, dissolve residue at 5–15mg/mL in 50mM sodium acetate, pH 5.5, containing 8M urea, 1mM EDTA and 2.5mM dithiothreitol.

Refolding

Dilute with 9 volumes of 50mM acetate, pH 5.5, containing 1mM EDTA and 2.5mM dithiothreitol, 10% glycerol, 5% ethylene glycol and 0.2% Non-idet P-40 at 4°C.

Result

Specific activity = 1.0 + -0.1mmol substrate hydrolyzed per minute per mg of protein—compared to specific activity of 1.2 for enzyme expressed in E. coli.

Figure 61. Procedure used to regain biological activity of HIV protease after reversed-phase chromatography **(Reference 56)**.

Examples of Biological Activity after RP-HPLC

Trypsin

Reversed-phase chromatography has been used to purify trypsin for use in protein digestion[57].

Polio virus proteins

Polio virus proteins purified by reversed-phase chromatography were able to induce production of specific antibodies in rabbits, indicating a retention of biological activity[23].

Pollen allergens

The main protein allergen of *Parietaria judaica* retained IgE-binding activity even after RP-HPLC purification because it eluted at low acetonitrile concentration[58].

HIV protease

HIV protease regained most of its biological activity after reversed-phase chromatography and post chromatographic treatment to allow refolding (**Figure 61**)[56].

Use of Reversed-Phase HPLC in the Purification of Commercial Polypeptide Therapeutics

Perhaps the most compelling evidence that biological activity is not inevitably lost during reversed-phase chromatography is the fact that several commercial bio-therapeutics use reversed-phase chromatography in the purification of the marketed product.

- Erythropoetin may be purified using reversed-phase chromatography as an integral part of the purification process[59].

- Leukine, a marketed polypeptide therapeutic, uses reversed-phase HPLC as an integral part of its purification procedure[60, 61].

- Human recombinant insulin purification uses reversed-phase chromatography in its production[62].

While the conditions of reversed-phase chromatography may cause some loss of tertiary structure and biological activity, in most cases this loss of biological activity may be moderated or eliminated by use of optimum chromatographic conditions or by post-chromatographic treatment.

Example of Large Scale Purifications

There are a number of examples describing the preparative purification of synthetic peptides by RP-HPLC in the literature[46–50]. In one case[46] 128mg of gonadotropin releasing hormone (GnRH) antagonist was purified from 1.2 grams of synthesis mixture in two RP-HPLC purification steps (**Figure 57**). The procedure involved (see Reference 46 for details):

1 Establishing elution conditions with triethylammonium phosphate and acetonitrile on a five micrometer, 4.6 x 250mm, column;

2 Loading the synthetic peptide onto a 5 x 30cm column packed with 15–20μm adsorbent comparable to the five micron material in the column in Step One and elution with acetonitrile and triethylammonium phosphate;

3 Analysis of collected fractions for purity and yield and combining the best fractions for desalting and final purification;

4 Dilution and re-injection on the same column;

5 Elution using acetonitrile and TFA to remove the non-volatile phosphate salt and improve resolution further;

6 Analysis of collected fractions for purity and yield;

7 Combining the optimum fractions for a final yield of 128 milligrams of GnRH antagonist at a purity of 99.7%.

Removal of Virus Particles During Reversed-Phase HPLC Purification

One of the benfits of incorporating a reversed-phase chromatography separation step into a process to produce large quantities of a therapeutic protein is the removal or clearance of virus from the protein "soup".

### Purification of Synthetic Peptide
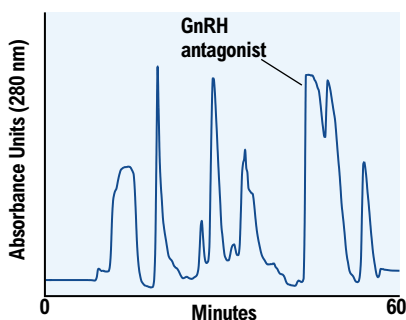
Figure 62. Purification of 128mg of a synthetic peptide, GnRH antagonist. 1.2 grams of synthesis mixture were loaded onto a 5 x 30cm column packed with Vydac® 218TPB1520 adsorbent (C18, 15–20μm) and eluted with a gradient of acetonitrile in water containing triethylammonium phosphate.

Viral Inactivation During Reversed-Phase HPLC Purification

Reversed-phase HPLC usually reduces or eliminates viral activity in protein preparations, making it a valuable step in recombinant protein purification. Viral inactivation occurs through two mechanisms. First, exposure to ethanol inactivates many viruses. Second, viruses can be separated from protein in the chromatographic step (see Figure 63).

The data in the table below illustrates that some viruses are highly inactivated in ethanol (Xenotropic Murie Leukemia Virus and Pseudorabies Virus) while others (Minute Virus of Mice and Human Adenovirus type 5) are less strongly inactivated. The combination of ethanol and chromatographic separation, however, significantly reduces the infectivity level of all four viruses.

Separation of Xenotropic Murine Leukemia Virus
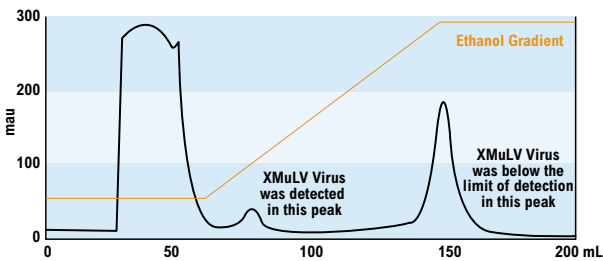
Figure 63. Separation of Xenotropic Murine Leukemia Virus (XMuLV) from target protein during preparative HPLC. **Column:** Vydac® C4, 20-30mm column. **Elution:** Ethanol gradient
Data courtesy of Holly Harker and Marcus Luscher, Amgen, Boulder, Colorado

Viral inactivation by ethanol and chromatographic separation

Log$_{10}$ infectivity reduction by exposure to ethanol for 30 minutes (**1st Row**) or a combination of ethanol and chromatographic separation (**2nd Row**).

	XmuLV	MVM	Adeno 5	PRV
Ethanol exposure	>4.9±.13	.4±.2	0.1±.44	>4.6±.08
RP-HPLC in ethanol	>5.9	2.9±.4	2.4±.44	>5.6±.32

XMuLV–Xenotropic Murine Leukemia Virus
MVM–Minute Virus of Mice
Adeno5–Human adenovirus type 5
PRV–Pseudorabies Virus

Appendix A: Column Characteristics

	Column Diameter (mm)	Typical Flow Rate (1)	Sample Capacity (2)	Maximum Practical Sample Load (3)
Capillary	0.075	0.25µL/min	0.05µg	
	0.15	1µL/min	0.2µg	
	0.30	5µL/min	1µg	
	0.50	10µL/min	2µg	
Microbore	1.0	25–50µL/min	0.05–10µg	
Narrowbore	2.1	100–300µL/min	0.2–50µg	
Analytical	4.6	0.5–1.5mL/min	1–200µg	10mg
Semi-preparative	10	2.5–7.5mL/min	1,000µg	50mg
Preparative	22	10–30mL/min	5mg	200mg
Process	50	50–100mL/min	25mg	1,000mg
	100	150–300mL/min	125mg	5,000mg

Figure A-1.

1. **Actual Flow Rates** can be a factor of two higher or lower depending on the method.
2. **Sample Capacity** is the quantity of polypeptide that can be loaded onto the column without reducing resolution.
3. **Maximum Practical Sample Load** is approximately the maximum quantity of sample that can be purified with reasonable yield and purity on the column.

Appendix B: The Care and Maintenance of Reversed-Phase Columns

Reversed-phase HPLC columns, if properly cared for, may give good performance for over a thousand sample injections, depending on sample preparation and elution conditions. Although the following ideas are specifically applicable to Vydac® RP-HPLC columns, they also apply to most other columns.

Column Protection

Column lifetime can be extended by filtering all solvents and samples and using an eluent filter and a guard column. We recommend using an eluent filter between the solvent delivery system and the injector to trap debris from the solvents, pumps or mixing chamber. We also recommend using a guard column between the injector and the column if samples contain insoluble components or compounds that strongly adsorb to the material.

Column Conditioning

Because of the nature of the reversed-phase surface, column performance (resolution, retention) may change slightly during the first few injections of proteins. A column can be conditioned by repeated injections of the protein until the column characteristics remain constant (requires injection of about 100µg of protein) or by injection of 100µg of a commonly available protein, such as ribonuclease, followed by running an acetonitrile/0.1% TFA gradient.

Column Storage

RP-HPLC columns can be stored in organic solvent and water. For long term storage the ion-pairing agent or buffer should be rinsed from the column and the organic content should be at least 50%.

Chemical Stability

Reversed-phase HPLC columns are stable in all common organic solvents including acetonitrile, ethanol, isopropanol and dichloromethane. When switching solvents it is important to only use mutually miscible solvents in sequence. Silica-based RP-HPLC columns are stable up to pH 6.5 to 7 and are not harmed by common protein detergents such as sodium dodecylsulfate (SDS).

Pressure and Temperature Limits

RP-HPLC columns are generally stable to 60°C and up to 5,000 psi (335 bar) back-pressure. Typical back-pressures for RP-HPLC columns are shown in **Figure B-1**.

RP-HPLC Column Trouble-Shooting

The performance of RP-HPLC columns may deteriorate for a number of reasons including use of improper eluents, such as high pH, contamination by strongly adsorbed sample constituents, insoluble materials from the solvent or sample or simply age or extensive use. Here are some suggestions to restore the performance of a RP-HPLC column.

High back-pressure

Disconnect the column from the injector and run the pumps to ensure that the back-pressure is due to the column and not the HPLC system.

If the column back-pressure is high, most HPLC columns can be reversed and rinsed to try to flush contaminants from the inlet frit. Begin the reverse rinse at a low flow rate—10 to 20% of normal— for 10–15 minutes and then increase to the normal flow rate.

Contaminated column

Wash the column either with 10–20 column volumes of a strong eluent or run 2–3 'blank' gradients (without sample injection) to remove less strongly adsorbed contaminants.

Typical Back-Pressures of RP-HPLC Columns

	Column Size (mm)	Flow Rate (mL/min)	Typical Back-pressure (with 50:50 ACN:Water)
5µm Media	2.1 x 250	0.20	1000–1800 psi
	4.6 x 250	1.0	1000–1800 psi
	4.6 x 150	1.0	600–1200 psi
	10 x 250	5.0	1000–1800 psi
10µm Media	4.6 x 250	1.0	500–1000 psi
	10 x 250	5.0	500–1000 psi
	22 x 250	25.0	500–1000 psi

Figure B-1.

Protein contamination

If the loss in column performance appears to be due to adsorbed protein rinse any of the polymeric-bonded columns (i.e., 214, 218, 219 series) with a mixture of one part 0.1 N nitric acid and four parts isopropanol. Rinsing at a low flow rate—20% of normal—overnight is most effective.

Lipids or other very hydrophobic contaminants

If lipids or very hydrophobic small molecules are causing the change in column performance, we recommend rinsing the column with several column volumes of dichloromethane or chloroform. When changing from water to chloroform or dichloromethane or back again it is important to rinse the column with a mutually miscible, intermediate solvent such as isopropanol or acetone between the two less miscible solvents.

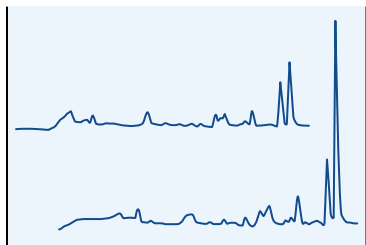**Evidence of Solvent Contaminants as Source of Ghost Peaks**
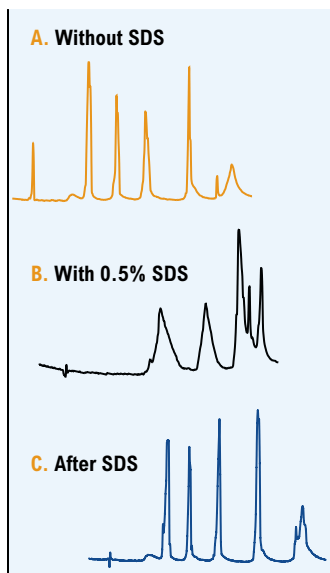

Figure B-2.

Spurious–"ghost"–peaks

Unexpected peaks sometimes appear in HPLC chromatograms. These are usually caused by contaminants in the solvents used. Hydrophobic contaminants in Solvent A-contaminants may be present in the water or the ion-pairing agent or buffer—accumulate on the column during equilibration and at low solvent concentrations and elute as "ghost" peaks during the gradient. This can be easily diagnosed by making two gradient runs, the first with a relatively long equilibration time–30 minutes—and the second with a short equilibration time— 10 minutes (example, **Figure B-2**). The short equilibration will have smaller peaks than the long equilibration if the "ghost peaks" are due to contaminants in the "A" solvent because less contaminants will adsorb onto the column with the short equilibration. To correct the problem use higher purity or fresh water or ion-pairing agent or buffer.

Appendix C: The Effect of Surfactants On Reversed-Phase Separations

Polypeptide samples sometimes contain surfactants. To determine the effect of surfactants on RP-HPLC polypeptide separations and on the columns themselves, five proteins—ribonuclease, insulin, lysozyme, myoglobin and ovalbumin—were chromatographed with and without 0.5% sodium dodecyl sulphate (SDS) in the sample (**Figures C-1, C-2**).

The separation on a C18 column of the protein sample with SDS was much worse (**Figure C-1B**) than the separation of the same sample without SDS (**Figure C-1A**). Subsequent chromatography of the sample without SDS, however, showed no deterioration (**Figure C-1C**), confirming that the SDS was removed in the gradient and did not harm the column or affect subsequent separations.

Effect of Surfactants on C18 RP-HPLC of Polypeptides

Effect of Surfactants on C4 RP-HPLC of Polypeptides

Figure C-1. Surfactants affect chromatography (B) but do not harm column or subsequent separations (C). **Column:** Vydac® 218TP54 column. **Eluent:** 24–95% ACN in 0.1% TFA over 30 min at 1.5mL/min **Sample:** ribonuclease, insulin, lysozyme, myoglobin and ovalbumin.
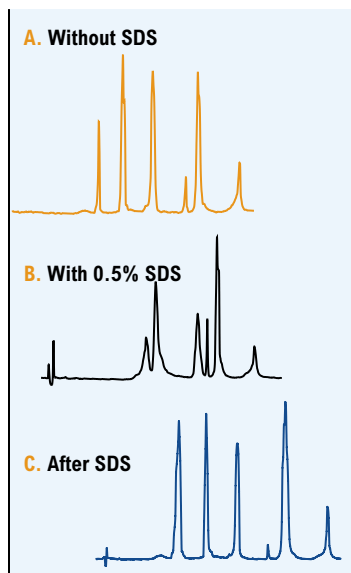
Figure C-2. Surfactant affects chromatography (B) but does not harm column or subsequent separations (C). **Column:** Vydac® 214TP54 column. **Eluent:** 24–95% ACN in 0.1% TFA over 30 min at 1.5mL/min **Sample:** ribonuclease, insulin, lysozyme, myoglobin and ovalbumin.

Results on a C4 column were slightly better than those obtained on the C18 column (**Figure C-2**). The presence of SDS in the protein sample affected the chromatography (**Figure C-2B**), however the effect was less than on the C18 column (compare with **Figure C-1B**). The SDS was removed in the gradient and did not affect the column or subsequent separations (**Figure C-2C**).

Peptide separations are seriously affected by the presence of surfactant. Even trace amounts of SDS in a peptide sample or protein digest can reduce separation efficiency significantly[12, 53]. Peptide maps of a protein digest containing small amounts of SDS showed that even small amounts of SDS affected the digest separation and higher amounts virtually destroyed resolution (**Figure C-3**).

Although surfactants usually degrade RP-HPLC peptide separations, the use of octylglucoside, urea and guanidine in the eluent have produced beneficial results in some cases[54, 55].

Surfactants usually degrade RP-HPLC polypeptide separations, however they do not harm the column. If surfactants are present in the sample, we recommend using a C4 reversed-phase column or removing the surfactant prior to chromatography.

Effect of Surfactants on Peptide Map
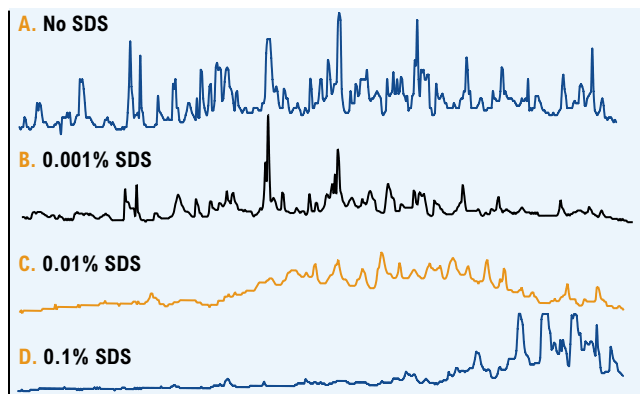
Figure C-3. The presence of even trace amounts of SDS causes a loss in resolution in a peptide map. **Column:** Vydac® 218TP52 column (Narrowbore). **Eluent:** 2–80% ACN with 0.06% TFA over 120 min at 0.25mL/min. **Sample:** tryptic digest of carboxymethylated transferrin *Data courtesy of K. Stone and K. Williams. Ref. 12.*

Appendix D: Optimizing Parameters for Narrow and Micro-Bore Analysis

Small bore columns can be beneficial in increasing sensitivity for LC-MS applications. Microbore HPLC columns can increase sensitivity over traditional analytical columns by up to 5000x (Figure D-1). In order to obtain the best results, the flow rate, injection volume, and tubing i.d. are important considerations (**Figure D-2**). Using small injection volumes and small i.d. tubing appropriate for the column i.d. will minimize band dispersion which ultimately maximizes column efficiency and sensitivity.

Peak Area as a Function of Column i.d.
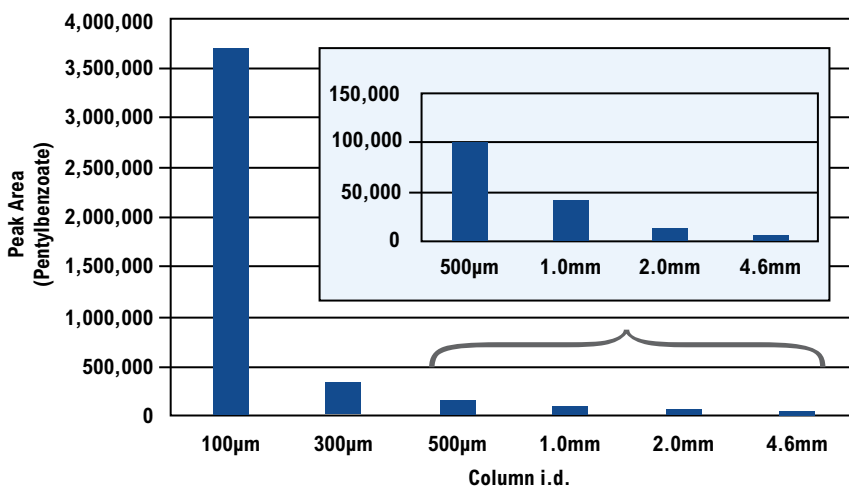
Figure D-1. 30 – 200nL injections of alkyl benzoates on columns of different i.d.

Parameters for Reduced Bore HPLC

Column i.d. (mm)	Optimum Flow (µL/min.)	Max. Injection Volume (µL)	Suggested Tubing i.d. (inch)	Suggested Tubing i.d. (µm)
4.6	1000	50	0.010	254
2.1	200	10	0.005	127
1.0	50	2	0.005	127
0.5	12	0.6	0.002	51
0.3	4	0.2	0.002	51
0.15	1	0.05	0.001	25
0.075	0.25	0.01	0.001	25

Figure D-2

■ When going from a 4.6 to a 2.1mm i.d. or smaller i.d. column, decrease the flow rate by the square of the ratios of column diameters:

$$[2.1/4.6]^2 \times 1000 = \text{new flow rate}$$

Column diameters   *Flow rate to reduce*

■ General rule of thumb for injection: < 5% of total column volume calculated by the equation for a cylinder, $\pi r^2 L$.

Appendix E: The Effect of System Hardware on Reversed-Phase Polypeptide Separations

Reversed-phase HPLC peptide separations are sensitive to the shape of the gradient and hence, to the characteristics of the system hardware being used. Pumps and gradient formers can affect peptide separations in subtle ways, especially at low flow rates.

## Gradient Systems and Response Delay Time

To experimentally examine the actual gradient produced by an HPLC system, replace the column with a short length of small diameter tubing and run a 30 minute gradient at 1.0mL/min from water to 0.3% acetone (for absorbance) in water and monitor at 254nm. The UV profile represents the gradient actually generated by the system hardware (**Figure E-1**). The gradient UV profile is used to:

- Check on system reproducibility;
- Determine system performance at the extremes of the gradient;
- Calculate the gradient response delay—the time from when the controller or computer signals a change in the gradient to when this change actually reaches the column.

In the example (**Figure E-1**) the gradient delay is about 3 minutes (3mL at 1mL/min) calculated from when the run begun to where the profile begins to rise. Hardware systems that differ in gradient response delay times will produce different gradient shapes, which may result in apparent differences in peptide selectivity.

Figure E-2 shows the effect that the gradient response delay has on narrowbore columns run at low flow rates. The peptide separation on a narrowbore HPLC column at 0.20mL/min (**Figure E-2B**) is compared with the separation on an analytical column at 1.0mL/min (**Figure E-2A**) using the same HPLC system and programmed gradient. The 10 minute gradient response delay distorts the peptide separation (**Figure E-2B**). Delaying sample injection and data collection ten minutes after starting the gradient cancels the effect of the gradient response delay and the resulting narrowbore separation (**Figure E-2C**) is similar to the analytical separation (**Figure E-2A**).

Gradient Hardware System Evaluation
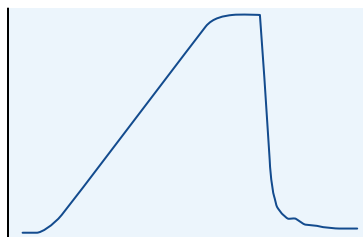
Figure E-1. The gradient generated by the system hardware is visualized by the profile of a gradient increasing in acetone. **Column:** Replaced by low-volume tubing. **Gradient:** 0–0.3% acetone in water over 30 min at 1.0mL/min. **Detection:** UV at 254nm.

Calculation of Desorbing Solvent Concentration

Because of internal volume in the flow system—tubing, mixing chamber, column void volume, etc— the solvent concentration given by the system when the polypeptide elutes is higher than the actual solvent concentration that desorbs and elutes the polypeptide.

To calculate the solvent concentration that desorbs the polypeptide (C_D):		
Enter the retention time of the peak	Example	33 min
Subtract the retention time of the injection peak	Example	2.5 min
Subtract the gradient response delay time	Example	2 min
And subtract any initial gradient hold time	Example	5 min
Equals corrected elution time (ET$_{corr}$)	*Example*	*23.5 min*

$$ET_{corr} = T_{retention} - T_{void} - T_{gradient\ Delay} - T_{hold}$$

The solvent concentration (C_D) at the corrected elution time is:
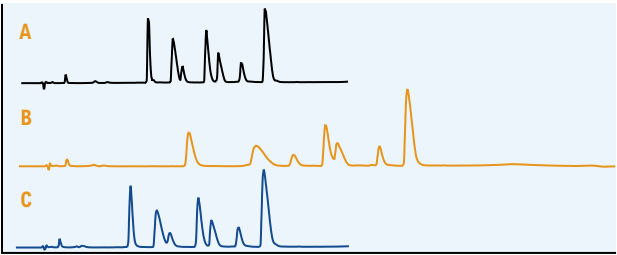
$C_D = C_S + (ET_{corr}/T_g)(C_E - C_S)$; where

    C_S = solvent concentration at start of gradient

    C_E = solvent concentration at end of gradient

    T_g = time duration of gradient

Effect of System Hardware on Gradient Shape in Narrowbore HPLC

Figure E-2. The system hardware gradient delay distorts the gradient shape at low flow rates and affects the peptide separation (B). Delaying sample injection to adjust for the gradient delay produces similar separation results (C) as obtained with an analytical column (A). **Column: A.** Vydac® 218TP54 (C18, 5µm, 4.6 x 250mm) column. **B and C.** Vydac® 218TP52 (C18, 5µm, 2.1 x 250mm) column. **Eluent:** 15–30% ACN in 30 min with 0.1% TFA. **Flow Rate:** A. 1.0mL/min B and C. 0.20mL/min **Peptides:** 1. bradykinin 2. oxytocin 3. angiotensin II 4. neurotensin 5. angiotensin I **Note:** In C, sample injection and data collection were delayed 10 min after initiating the gradient.

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About the Authors

David Carr, a graduate of U.C. Berkeley, first became involved in HPLC in 1971, when the technique was in its infancy. As the technical marketing manager of Vydac from 1984–1996, David was involved in the use of reversed-phase HPLC for protein and peptide separations for both analytical and preparative purposes. Working with companies such as Genentech, Amgen, and Immunex, David assisted in developing protein and peptide separation methods for quality control as well as consulting on large-scale preparative separations. Since 1996 David has developed and instructed courses in analytical biotechnology and HPLC. His short course, *Fundamentals in Analytical Biotechnology*, is very popular among biotechnology companies (details may be found at www.bioanalyticaltech.com). David is the author of the first three editions of *The Handbook of the Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC*, as well as an author of this Fifth Edition.

Reno Nguyen received his B.S. in Biochemistry in 1992 from the University of Maryland and his Ph.D. in 2000 where his research focus was in the area of microbial protein analysis and purification utilizing 2-D electrophoresis and HPLC coupled to fluorescence, ELSD, and mass spectrometry. Following his Ph.D., he attended The Ohio State University as a postdoctoral researcher responsible for studying nitrogen containing molecules by LC-MS. In 2002, he joined Grace as an applications chemist developing method applications for small molecules, peptides, and novel therapeutic proteins in analytical to preparative scale techniques. His current role is Media Technology Manager, Grace.

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