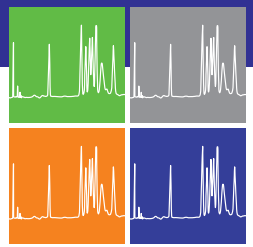
The Handbook of Analysis and Purification of Peptides and Proteins by Reversed-Phase HPLC



GRACE VYDAC
Enhancing the Science of Separations

Third Edition, 2002

This handbook presents the basic principles of reversed-phase HPLC for the analysis and purification of polypeptides. For further details regarding reversed-phase HPLC separations of polypeptides please refer to the technical references at the back of the Handbook or contact the Grace Vydac Technical Support Group.

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The Grace Vydac Technical Support Group is available for discussions regarding your bio-separation questions.

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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

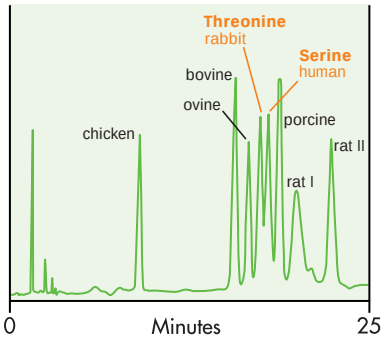

Figure 1. *RP-HPLC separates rabbit and human insulin that differ by only a single amino acid.* ***Column:*** *VYDAC® 214TP54* ***Eluent:*** *27–30% acetonitrile (ACN) in 0.1% TFA over 25 minutes at 1.5 mL/minute.*

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].

Reversed-Phase 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 patents referencing VYDAC® reversed-phase columns alone has grown exponentially over the past few years as illustrated in Figure 2 (Also see Reference 74).

Number of Patents Issued Using Grace Vydac Reversed-Phase HPLC Columns

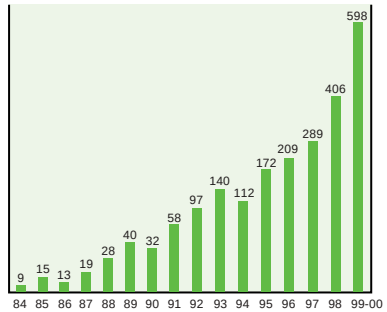

Figure 2. *The number of patents issued by the United States Patent Office in the years 1984–2000 in which VYDAC® Reversed-Phase HPLC columns are referenced in the patented process.*

Mechanism of Interaction Between Polypeptides and RP-HPLC 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

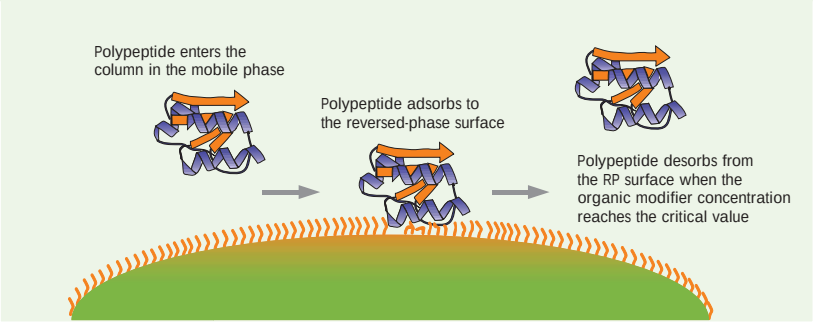

Figure 3. *Polypeptide enters the column in the mobile phase. The hydrophobic "foot" of the polypeptide adsorbs to the hydrophobic surface of the reversed-phase material where it remains until the organic modifier concentration rises to the critical concentration and desorbs the polypeptide.*

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

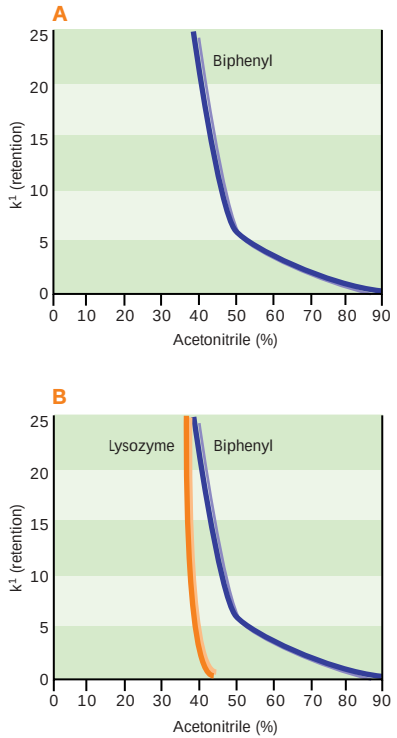

Figure 4. ***A:*** *The retention of small molecules such as biphenyl decreases gradually as the organic modifier concentration increases because they are retained by partitioning.* ***B:*** *The retention of polypeptides such as lysozyme changes suddenly and drastically as the organic modifier reaches 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

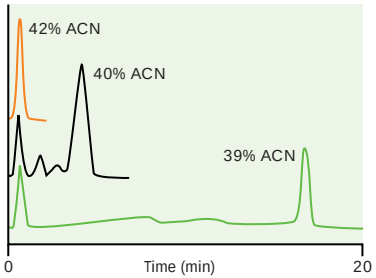

Figure 5. *At 39% ACN, the retention time of lysozyme is nearly 18 minutes. Increasing the ACN concentration to 40% reduces the retention time by more than half, to 7.6 minutes. Increasing the ACN concentration to 42% reduces the retention time of lysozyme again by more than half, to 3.1 mintues.* ***Column:*** *VYDAC® 214TP54* ***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 chromatograph 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

Retention Behavior of Peptides

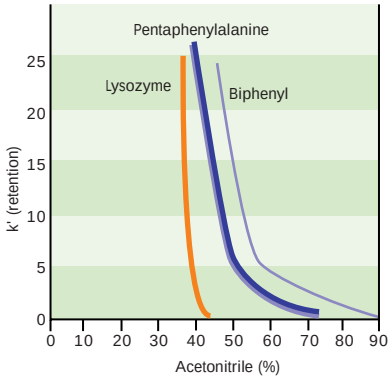

Figure 6. *The retention behavior on RP-HPLC of many peptides is midway between that of proteins and of small molecules. Pentaphenylalanine, a small peptide, desorbs more quickly than biphenyl, a small molecule, but more gradually than lysozyme. Small peptides appear to chromatograph by a mixed mechanism.*

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.

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

The HPLC column provides the hydrophobic surface onto which the polypeptides adsorb. Columns consist of stainless steel tubes filled with small diameter, spherical adsorbent particles, generally composed of silica whose surface has been reacted with silane reagents to make them hydrophobic. Spherical particles of synthetic polymers, such as polystyrene-divinylbenzene can also serve as HPLC adsorbents for polypeptides.

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 100 Å diameter. Polypeptides chromatographed poorly, in part because many polypeptides are too large to enter pores of this diameter. The development by Grace Vydac of large pore (~300 Å) 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 300 Å, although some peptides (<~2,000 MW) may also be separated on particles of 100 Å 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.

Five micrometer materials are recommended for capillary analytical and small scale preparative separations (columns up to 10 mm i.d.). Larger diameter laboratory columns are usually packed with 10 µm materials. Process chromatography columns of greater than 22 mm i.d. are normally packed with particles of 15 µm or greater and have wider particle size distributions than the particles used in analytical columns (see Pages 40–42).

Column Selection and Characteristics of Sample Molecule

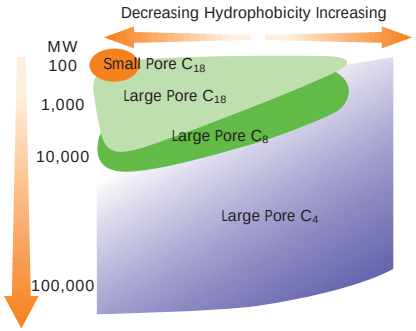

Figure 7. *This chart indicates the pore size and bonding recommended for various molecular weights and hydrophobicities.*

Adsorbent Phase Type

Reversed-phase HPLC adsorbents are formed by bonding a hydrophobic phase to the silica matrix by means of chlorosilanes, silicon-based molecules with chlorine as the reactive group and to which a hydrocarbon group is attached.

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. C_{18} 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 C_{18} columns. Proteins larger than 5,000 daltons or small polypeptides that are particularly hydrophobic are best chromatographed on C_4 columns. C_8 columns are similar to C_{18} columns in their application but sometimes offer a different selectivity or ability to separate particular peptides. Phenyl columns are slightly less hydrophobic than C_4 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 that can be used to optimize specific peptide 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 C_{18} and C_4 columns, although the C_4 column has slightly shorter retention. The phenyl column exhibits shorter retention times and a different selectivity than the C_{18} column. Bradykinin, with two phenylalanines, is retained somewhat longer, relative to the other peptides, on the phenyl column than on the C_{18} column.

Peptide Separation on Different Reversed-Phase Columns

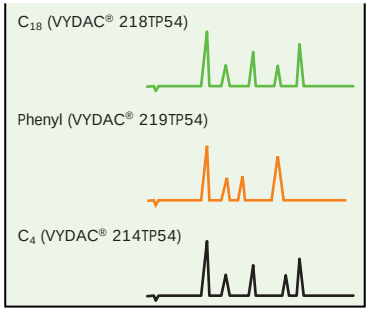

Figure 8. *Peptide separation on different reversed-phase columns* ***Columns:*** *VYDAC® 218TP54 (C_{18}); 214TP54 (C_4); 219TP54 (phenyl);* ***Eluent:*** *15–30 % ACN in 0.1% aqueous TFA over 30 minutes at 1.0 mL/min.* ***Sample:*** *1. oxytocin, 2. bradykinin, 3. angiotensin II, 4. neurotensin, 5. 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 small, but important ways. Some peptide separations are very sensitive to the density and uniformity of the hydrophobic phase bonded to the silica matrix (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 C_4 column has slightly less retention and a somewhat different peptide fragment elution pattern than the more commonly used C_{18} 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. 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.

Resolution Improvement with Low Carbon Load Column

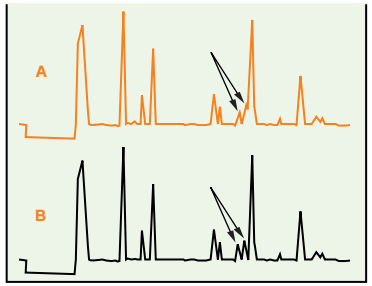

Figure 9. *Low carbon load C_{18} RP-HPLC column (B) separated two peptides that were only partially resolved on a standard carbon load column (A).* ***Columns: A.*** *VYDAC® 218TP52-standard C_{18}, 5 µm, 2.1 x 250 mm* ***B.*** *VYDAC® 218LTP52– low carbon load–C_{18}, 5 µm, 2.1 x 250 mm* ***Eluent:*** *6 mM TFA/4 mM HFBA, 11–95% ACN in 75 min at 0.25 mL/min* ***Sample:*** *Asp–N protein digest. Data courtesy of H. Catipon and T. Salati, Genetics Institute, Andover, MA.*

Separation of a Tryptic Digest on Different Reversed-Phase Columns

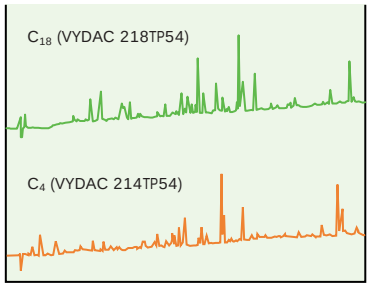

Figure 10. ***Columns:*** *VYDAC® 218TP54 (C_{18}); 214TP54 (C_4);* ***Eluent:*** *0–30 % ACN in 0.1% aqueous TFA over 60 minutes at 1.0 mL/min.* ***Sample:*** *tryptic digest of β-lactoglobulin A.*

The Separation of Synthetic Peptides on Monomerically Bonded and Polymerically Bonded C_{18} Reversed-Phase Columns

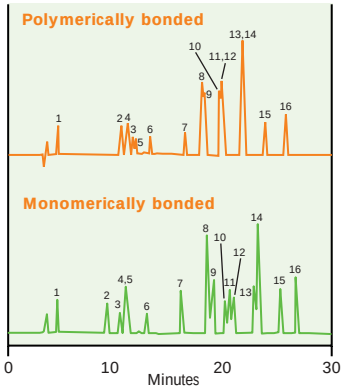

Figure 11. ***Columns:*** *VYDAC® 218TP54 polymeric and 238TP54 monomeric (C_{18}, 5 µm, 4.6 x 250 mm)* ***Eluent:*** *10–40% ACN with 0.1% TFA over 30 min.* ***Flowrate:*** *1.0 mL/min.*

Use of Synthetic Polymer Adsorbents

Although silica-based HPLC columns perform well under mild conditions of acidic pH and ambient temperatures, extreme conditions (high pH, high temperature) will degrade silica columns. Synthetic polymers such as polystyrene-divinylbenzene provide a very robust matrix for polypeptide separations.

Silica-based columns perform very well under moderate operating conditions of pH and temperature, but there is sometimes a need to operate at higher than normal pH or temperature or in the presence of high concentrations of chaotropic agents such as guanidine-HCl. Robust synthetic polymer matrices such as polystyrene-divinylbenzene are stable under harsh conditions and thus offer practical alternatives to silica. Figure 12 shows the separation of several peptides on a column based on synthetic polystyrene-divinylbenzene. Performance is similar to a silica based column, thus opening up the possibility of performing polypeptide separations under relatively harsh conditions on synthetic polymer matrices.

An advantage of separation materials made from synthetic polymers is that they are not degraded at extremes of pH. This allows the use of very acidic or basic solutions as cleaning reagents to remove proteins or other materials from columns after chromatography as illustrated in Figure 13. In this example, a column based on a polystyrene-divinylbenzene polymer was washed with strong base (1 N sodium hydroxide) and with strong acid (1 N sulfuric acid). Peptides chromatographed before washing, after washing with strong base and after washing with strong acid had similar peak shape, retention and resolution confirming that washing the column with strong reagents did not adversely affect column performance.

Separation of Several Peptides on a PS-DV3 Column

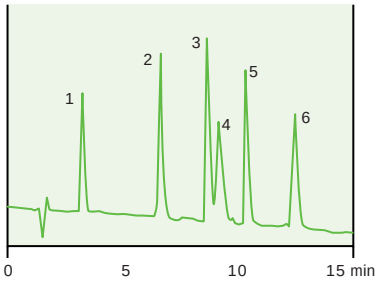

Figure 12. *Separation of peptides on synthetic polymer column (polystyrene-divinylbenzene).* ***Column:*** *VYDAC® 259VHP5415 (PS-DVB, 5 µm, 4.6 x 150 mm)* ***Eluent:*** *15–30% ACN over 15 min. with 0.1% TFA.* ***Flowrate:*** *1.0 mL/min. Peptides.1. oxytocin. 2. bradykinin. 3. neurotensin. 4. neurotensin 1–8. 5. angiotensin III. 6. val-4 angiotensin III.*

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 5–15 cm length are often used for protein separations. Small peptides, such as those from protease digests, are better separated on longer columns and columns of 15–25 cm 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 250 mm length–104 peaks–than on a column of 150 mm–80 peaks–or a column of 50 mm–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.

Peptide Separation Before and After Extreme pH Washes

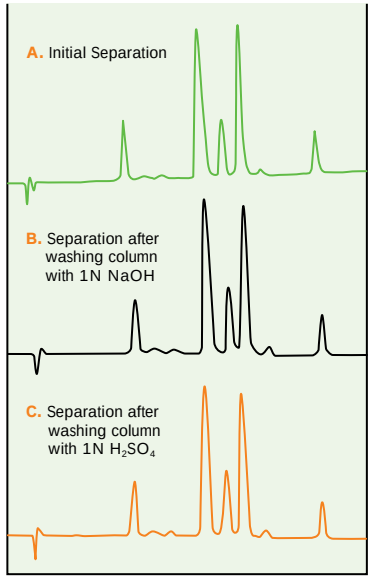

Figure 13. *Separation of peptides on a synthetic polymer (polystyrene-divinylbenzene) column before washing with strong reagents (A), after washing with 1N NaOH (B), and after washing with 1N sulfuric acid (C).* ***Column:*** *VYDAC® 259VHP5415 (PS-DVB, 5 µm, 4.6 x 150 mm)* ***Eluent:*** *15–30% ACN over 15 min. with 0.1% TFA.* ***Flowrate:*** *1.0 mL/min. Peptides. 1. oxytocin. 2. bradykinin. 3. angiotensin II. 4. eledoisin. 5. neurotensin*

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 4.6 mm. Larger diameter columns are used for purification of large amounts of polypeptide (see Pages 40–48 on preparative separations). The use of small diameter columns (0.075 mm to 2.1 mm) 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 50). Low flow rates can significantly reduce the amount of solvent needed for polypeptide separations.

Separation of the Tryptic Digest of Hemoglobin on a Microbore (1 mm Diameter) Column

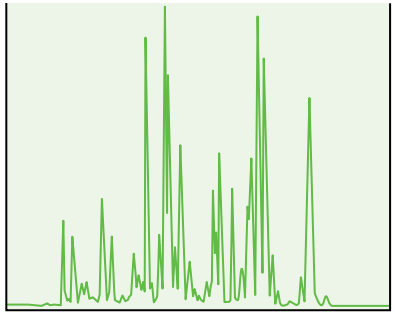

Figure 14. *Separation of the tryptic digest of hemoglobin on a microbore RP-HPLC column (Reference 26).* ***Column:*** *C_{18}, 1.0 x 250 mm (VYDAC® 218TP51).* ***Flow rate:*** *50 µL/min.* ***Eluent:*** *Gradient from 0 to 40% B over 50 minutes, where Solvent A is 0.1% TFA in water and Solvent B is 0.1% TFA in acetonitrile.*

Separation of the Tryptic Digest of Myoglobin on a Capillary (75 µm Diameter) Column

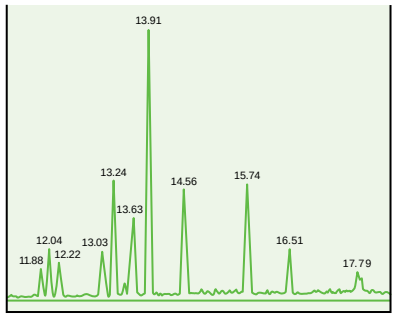

Figure 15. *Separation of the tryptic digest of myoglobin on a capillary RP-HPLC column.* ***Column:*** *C_{18} (VYDAC® 218MS), 75 µm i.d. capillary.****Flow rate:*** *0.5 µL/min.* ***Eluent:*** *water/TFA/acetonitrile gradient.*

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 1.0 mL/min.

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

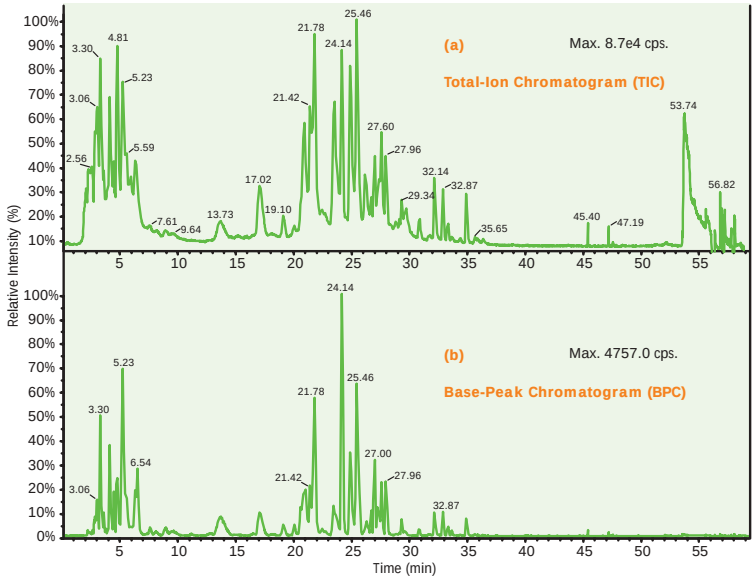

Figure 16. *Separation of the tryptic digest of bovine serum albumin (BSA) on a 300 µm i.d. capillary RP-HPLC* ***Sample:*** *3 pmole.* ***Column:*** *VYDAC® 218MS5.305 5 µm, 300 Å, polymeric-C_{18} reversed-phase (300 µm i.d. x 50 mm L).* ***Flow:*** *5 µL/min.* ***Mobile phase:*** *A = 0.1% formic 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 28–31).

Current Trends in Small Diameter Columns

Narrowbore columns

Narrowbore columns of 2.1 mm 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 lectrospray mass spectrometer interfaces.

Microbore and capillary columns

Columns of 1.0 mm 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 26–29).

Examples

Microbore. Figure 14 illustrates the separation of a tryptic digest of hemoglobin on a microbore (1.0 mm i.d.) column.

Capillary. Figure 15 is an example of the separation of a tryptic digest of myoglobin on a 75 µm i.d. capillary column.

Capillary sample load. Figure 16 illustrates that three picomoles of a tryptic digest of BSA can be separated on a 300 µm 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

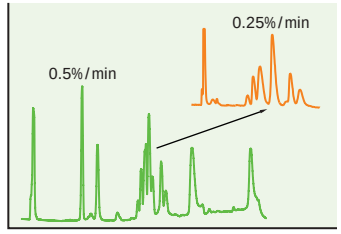

Figure 17. ***Column:*** *C_{18} (VYDAC® 218TP104)* ***Flow rate:*** *1 mL/min.* ***Eluent:*** *Gradient slope as shown. Gradient from 25–50% ACN in aqueous TFA.* ***Sample:*** *Subunits of cytochrome c oxidase. Data from reference 21.*

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 .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 17 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

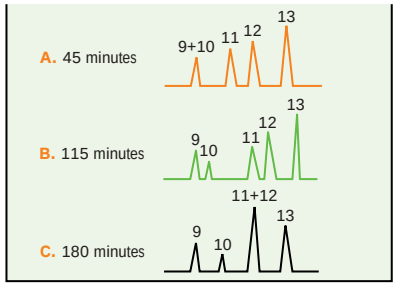

Figure 18. *The effect of gradient time (slope) on peptide selectivity.* ***Column:*** *C_{18}, 150 x 4.6 mm.* ***Flow rate:*** *1 mL/min.* ***Eluent:*** *Gradient from 0–60% ACN in aqueous 0.1% TFA in A. 45 min.; B. 115 min.; C. 180 min.* ***Sample:*** *tryptic digest of human growth hormone. Fragments 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 18 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 28).

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

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 250 nm 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 215 nm 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 19. 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

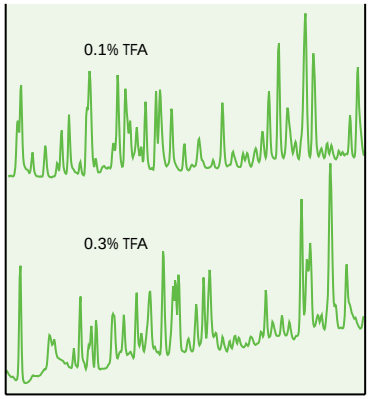

Figure 19. *Significant differences in the peptide separation pattern due to differences in TFA concentration are evident.* ***Column:*** *C_{18} (VYDAC® 218TP54).* ***Flow rate:*** *1 mL/min.* ***Eluent:*** *Gradient from 0–50% ACN in aqueous TFA, concentration as indicated.* ***Sample:*** *Tryptic 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 20) 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 (Fig. 20B). Hydrochloric acid also reverses the elution order of oxytocin and bradykinin and separates impurities not seen in TFA (Figure 20C).

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 26–29 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

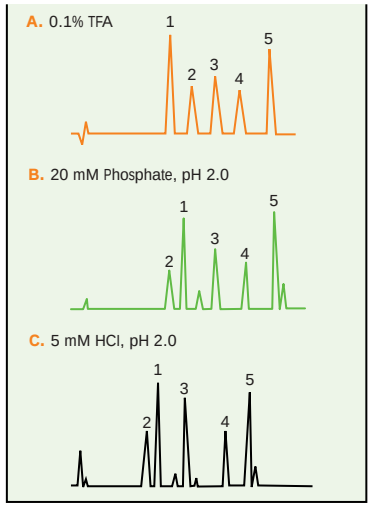

Figure 20. *Elution of five peptides using TFA (A), Phosphate (B) or HCl (C) as the buffer/ion-pairing agent.* ***Column:*** *VYDAC® 218TP54 (C_{18}, 5 µm, 4.6 x 250 mm).* ***Eluent:*** *15–30% ACN in 30 min at 1.0 mL/min; plus A. 0.1% TFA B. 20 mM phosphate, pH 2.0 C. 5 mM HCl, pH 2.0* ***Peptides:*** *1. oxytocin 2. bradykinin 3. angiotensin II 4. neurotensin 5. angiotensin I.*

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 21. All five peptides elute earlier at pH 4.4 (Figure 21B) than at pH 2.0 (Figure 21A) 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 21C) 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.

Synthetic polymer-based reversed-phase materials expand the practical pH range to nearly pH 14 (See Figure 13, Page 13). Peptides elute very differently at high pH than they do at low pH as illustrated in Figure 22. In going from pH 2 to pH 9 the peptides in the example change relative elution orders significantly.

The Effect of pH on Peptide Separations

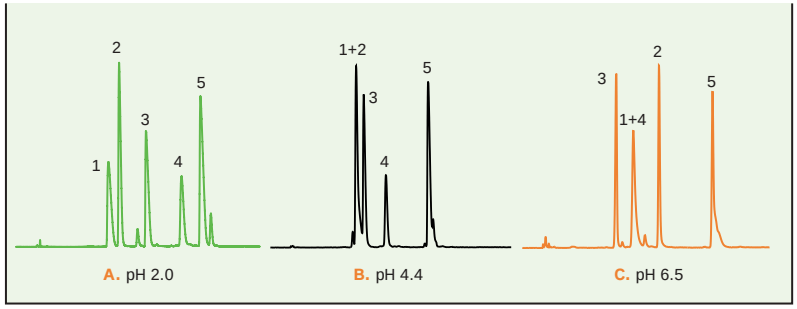

Figure 21. *Elution of five peptides at pH 2.0, 4.4 and 6.5 with phosphate as the buffer.* ***Column:*** *VYDAC® 218TP54 (C_{18}, 5 µm, 4.6 x 250 mm).* ***Eluent:*** *15–30% ACN in 30 min at 1.0 mL/min; plus A. 20 mM phosphate, pH 2.0 B. 20 mM phosphate, pH 4.4 C. 20 mM phosphate, pH 6.5* ***Peptides:*** *1. bradykinin 2. oxytocin 3. angiotensin II 4. neurotensin 5. angiotensin I.*

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 100 mM 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 10–20 mM 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.

Separation of Peptides on Synthetic Polymer (Polystyrene-Divinylbenzene) Column at Low and High pH

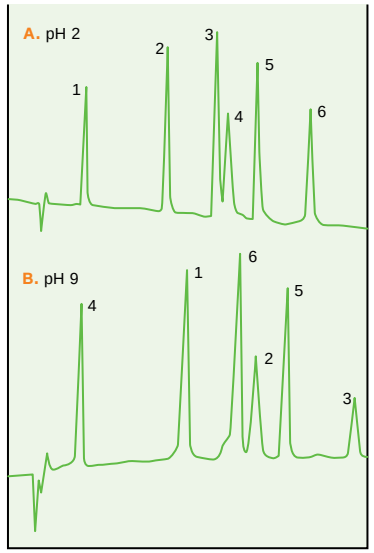

Figure 22. ***Column:*** *VYDAC® 259VHP5415 (PS-DVB, 5 µm, 4.6 x 150 mm)* ***Eluent:*** *15–30% ACN over 15 min. with A. 0.1% TFA, pH 2. B. 15 mM NaOH, pH 9.* ***Flowrate:*** *1.0 mL/min.* ***Peptides:*** *1. oxytocin. 2. bradykinin. 3. neurotensin. 4. neurotensin 1-8. 5. angiotensin III. 6. val-4 angiotensin III.*

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). 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 0.2 mL/min, compared to 116 fragments being resolved at 0.8 mL/min. From flow rates of 0.5 mL/min to 1.0 mL/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.

The Effect of Temperature on the Separation of Peptide Fragments

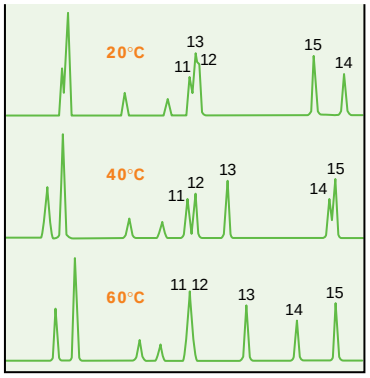

Figure 23. ***Column:*** *C_{18}, 4.6 x 150 mm.* ***Flow rate:*** *1 mL/min.* ***Eluent:*** *Gradient from 0–60% ACN in aqueous .1% TFA in 60 min.* ***Temperature:*** *As indicated.* ***Sample:*** *Tryptic digest of human growth hormone. Data from Reference 39.*

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 23 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.

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.

Rapid Separation of Proteins Using Short (50 mm) Column

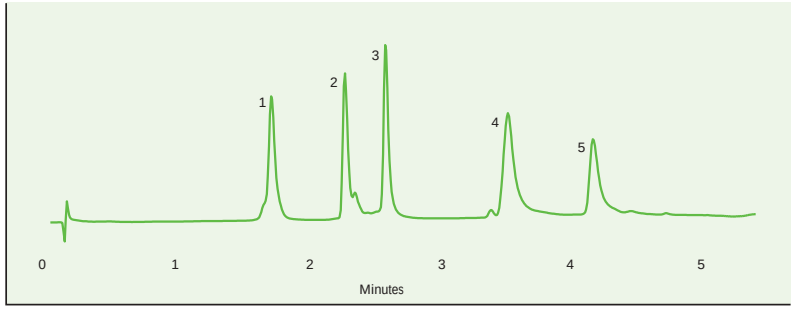

Figure 24. ***Column:*** *C_{18}, 3 µm 4.6 x 50 mm (VYDAC® 238TP3405).* ***Flow rate:*** *4.0 ml/min.* ***Eluent:*** *Gradient from 20–45% ACN in aqueous 0.1% TFA in 4 min.* ***Sample:*** *protein standards. (1) ribonuclease, (2) insulin, (3) cytochrome c, (4) BSA and (5) myoglobin.*

LC/MS Uses Short Columns for Rapid Analysis

The trend in LC/MS toward faster analyses with reduced resolution has led to the use of relatively short columns with very fast gradients.

The trend toward reduced resolution and faster separations has led to the use of short columns packed with smaller particle adsorbents than normal. The most commonly used particle size in short columns is three micrometers. Using three micrometer columns of five to ten centimeter length with fast gradients enables polypeptide separations to be completed in just a few minutes. Figure 24 shows the separation of five proteins in less than five minutes using a 50 mm long column packed with 3 µm particles using a fast gradient.

The Use of Low Concentrations of TFA for Peptide Separations

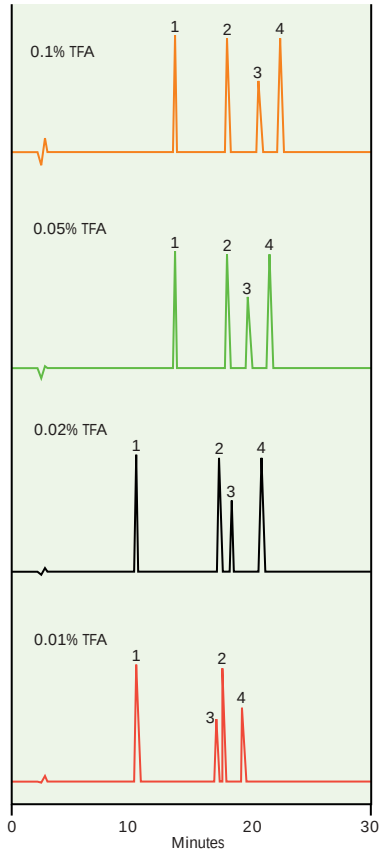

Figure 25. ***Column:*** *C_{18}, 5 µm, 4.6 x 250 mm (VYDAC® 218MS54).* ***Flow rate:*** *1.5 mL/min.* ***Eluent:*** *Gradient from 5–19% ACN in aqueous 0.1% TFA.* ***Sample:*** *1. neurotensin (1–8 frag) 2. oxytocin 3. angiotensin II 4. neurotensin.*

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.

Tryptic Map Replacing TFA with Acetic Acid (No TFA)

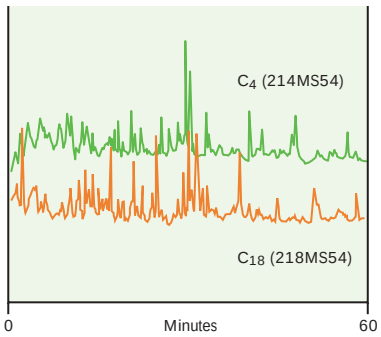

Figure 26. ***Columns:*** *Columns developed for peptide separations in the absence of TFA.* ***Top:*** *C_4 (VYDAC® 214MS54);* ***Bottom:*** *C_{18} (VYDAC® 218MS54;).* ***Flow rate:*** *1 ml/min. Eluent. Gradient from 0–30% ACN in 5 mM HOAc.* ***Sample:*** *Tryptic digest of apotransferrin.*

In some cases the TFA may be completely replaced with formic or acetic acid while retaining good resolution. Figure 25 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 26 demonstrates 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.

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.

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 27 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 27, 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 27, bottom). The peptide fragments can also be matched with a protein or DNA database to identify the protein.

Identification of Peptide Fragment of Proteins from Enzymatic Digest

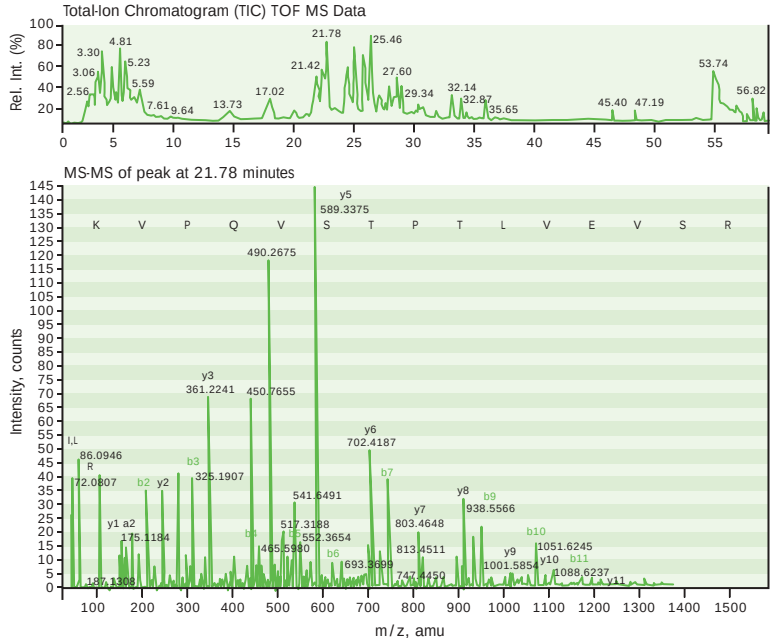

Figure 27. *Reversed-phase separation of tryptic digest peptides of bovine serum albumin (BSA) followed by MS determination of molecular weights of each peptide followed in turn by MS fragmentation of each peptide providing data to enable sequencing of the separated peptide.* ***Sample:*** *3 pmole of a tryptic digest of bovine serum albumin.* ***Column:*** *VYDAC® 218MS5.305, 5 µm, 300 Å, polymeric-C_{18} reversed-phase (300 µm i.d. x 50 mm).* ***Flow rate:*** *5 µL/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:*** *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 28).

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

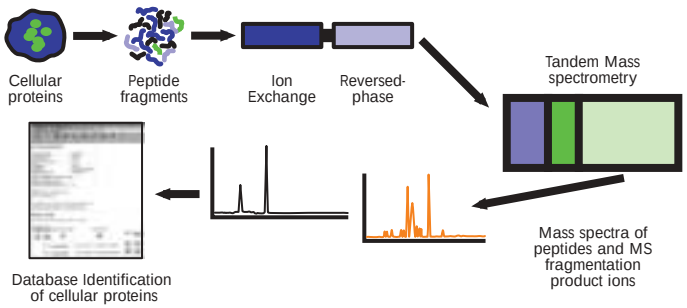

Figure 28.

Scientists from the Protein Characterization and Proteomics Laboratory at the University of Cincinnati College of Medicine reported using a capillary (300 µm i.d. x 100 mm) 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 29). 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*

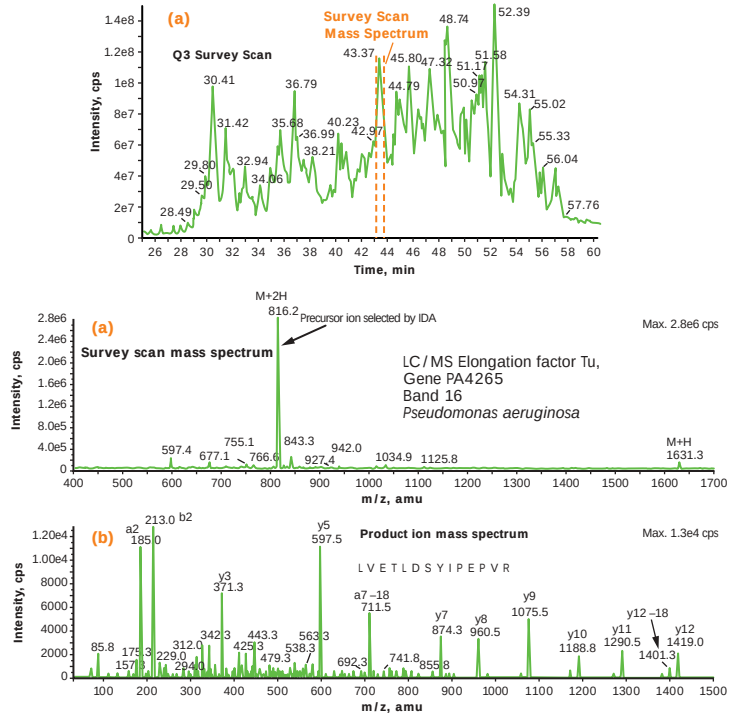

Figure 29.

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. C_{18} columns are most commonly used in the isolation of peptides as illustrated in Figure 30 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 49).

RP-HPLC Separation of Natural Peptides

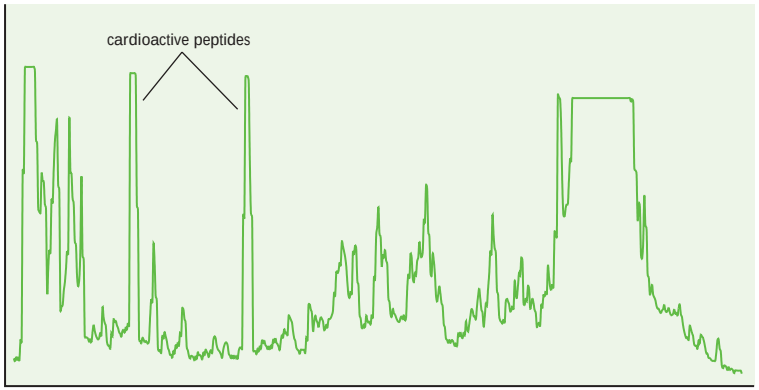

Figure 30. *RP-HPLC was used to separate two octapeptides with cardioacceleratory activity from an extract of Periplaneta Americana—american cockroach.* ***Column:*** *VYDAC® 218TP54 (C_{18}, 5 µm, 4.6 x 250 mm)* ***Eluent:*** *Hold at 18% ACN for 10 min; 18–30% ACN from 10–70 min, 30–60% ACN from 70–100 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 31. In this instance a monoclonal antibody was digested and the resulting fragments chromatographed on a C_{18} 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 32 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

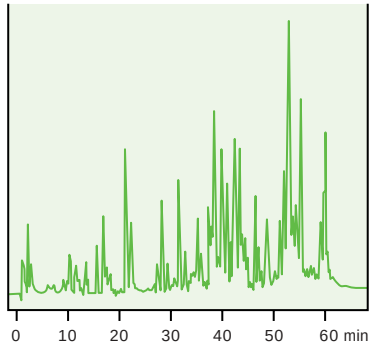

Figure 31. ***Column:*** *VYDAC® 218TP54 (C_{18}, 5 µm, 4.6 x 250 mm)* ***Eluent:*** *Gradient from 0–40% acetonitrile with 0.1% TFA over 65 minutes. Data from Reference 27.*

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.

Tryptic Maps of Normal Hemoglobin and Sickle Cell Hemoglobin

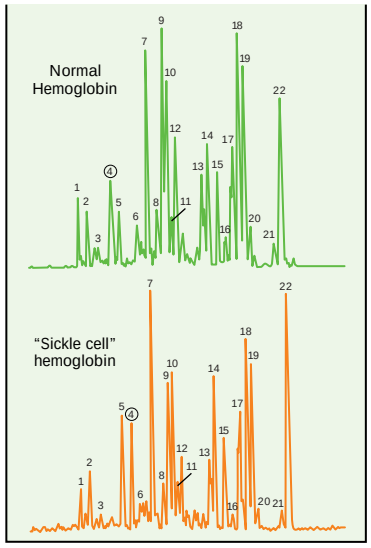

Figure 32. *Hemoglobin from normal and sickle cell subjects was tryptic digested and analyzed by RP-HPLC. Peptide 4 contains position six, which is mutated from glutamic acid to valine in sickle cell anemia subjects.* ***Column:*** *VYDAC® 218TP51 (C_{18}, 5 µm 1.0 x 250 mm)* ***Eluent:*** *0–40% ACN over 50 min, with 0.1% TFA, at 50 mL/min. Data from Reference 26.*

RP-HPLC Used in the Study of Protein Deamidation

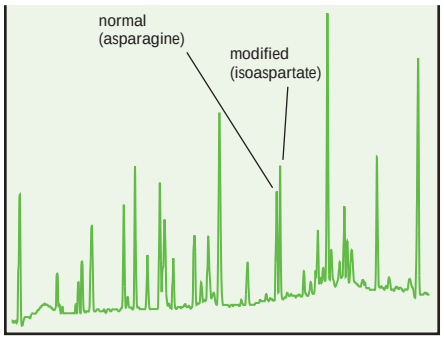

Figure 33. *RP-HPLC separation of peptide fragments from tryptic digests of normal bovine somatotropin (BST) with asparagine at position 99 and deamidated BST with the asparagine replaced by isoaspartate.* ***Column:*** *VYDAC® 218TP54 (C_{18}, 5 µm, 4.6 x 250 mm)* ***Eluent:*** *0–15% ACN over 20 min, 15–21% ACN over 12 min, 21–48% ACN over 27 min, 48–75% ACN over 4 min, all with 0.1% TFA, at 2.0 mL/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 34). 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

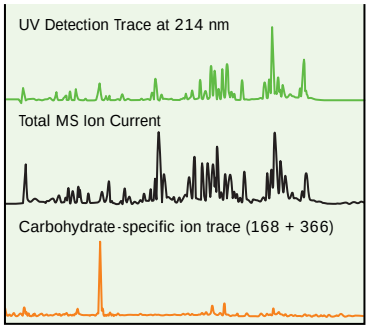

Figure 34. *Glycosylated peptides in a peptide map can be identified by the monitoring of "carbohydrate diagnostic ions" by on-line mass spectrometry.* ***Column:*** *VYDAC® 218TP54 (C_{18}, 5 µm 4.6 x 250 mm)* ***Eluent:*** *0–40% ACN over 65 min, with 0.1% TFA, at 1.0 mL/min. Data from Reference 40.*

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

In Figure 38, 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].

Detection of Deamidation by RP-HPLC

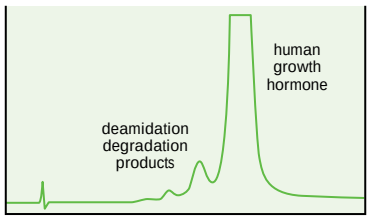

Figure 35. *The protein therapeutic recombinant human growth hormone deamidates during storage. Deamidation is detected by Reversed-Phase HPLC at slightly alkaline pH.* ***Column:*** *VYDAC® 214TP54 (C_4, 5 µm, 4.6 x 250 mm)* ***Eluent:*** *29% Isopropanol, 10 mM Tris-HCl, pH 7.5. Data from Reference 31.*

Separation of Oxidized Forms of Coagulent Factor from Native Protein

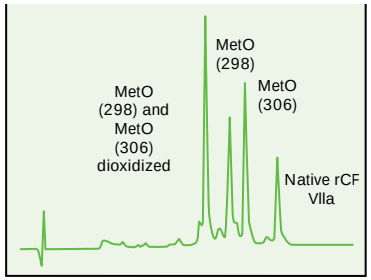

Figure 36. *Separation of oxidized forms of coagulant factor VIIa from the native protein.* ***Column:*** *VYDAC® 214TP54 (C_4, 5 µm 4.6 x 250 mm)* ***Eluent:*** *37–47% ACN over 30 min, with 0.1% TFA. Data from Reference 30.*

RP-HPLC of Insulin-Like Growth Factor

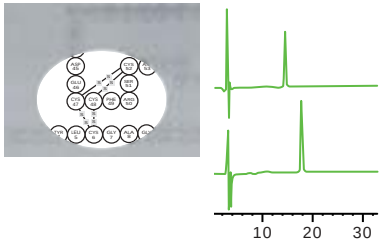

Figure 37. *Insulin-like growth factor has two adjacent disulfide bonds which can "switch". This changes the conformation of the protein, which, in turn, affects reversed-phase elution.* ***Column:*** *VYDAC® 214TP54 (C_{18}, 5 µm, 4.6 x 250 mm)* ***Eluent:*** *20–38% ACN:IPA (88:2) with 0.1% TFA. Data from Reference 37.*

RP-HPLC of Modified Insulin-Like Growth Factor

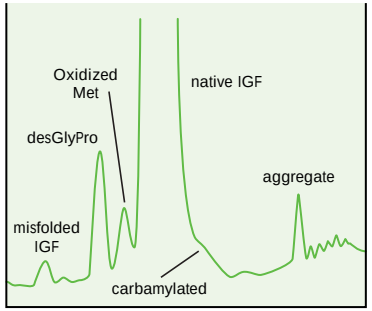

Figure 38. *Insulin-like growth factor modified during production was analyzed by RP-HPLC, revealing several modified forms.* ***Column:*** *VYDAC® 218TP54 (C_{18}, 5 µm, 4.6 x 250 mm)* ***Eluent:*** *A. 0.12% TFA in H_2O. B. 0.1% TFA in acetonitrile. Gradient 27.5–28.5% B over 9 minutes, followed by 28.5–40% B over 4 min., followed by 40–90% B over 90 minutes at 2 mL/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 39). The inset in Figure 39 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 C_4 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

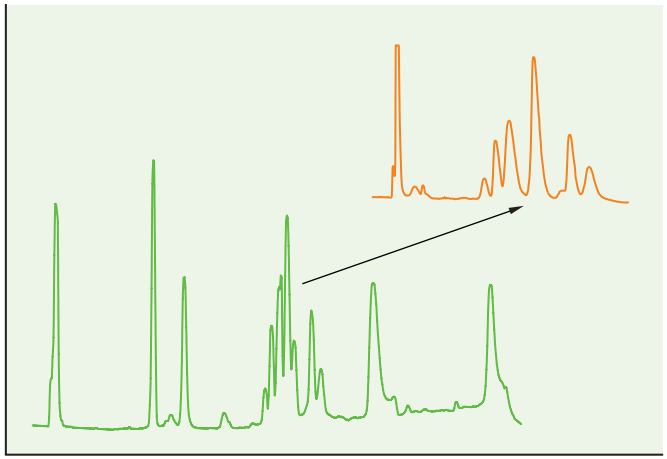

Figure 39. *Eleven subunits of bovine cytochrome c oxidase ranging in MW from 4962 to 56,993 are separated by RP-HPLC.* ***Column:*** *VYDAC® 214TP104 (C_4, 10 µm, 4.6 x 250 mm).* ***Eluent:*** *25–50% ACN over 50 min, then 50–85% ACN over 17.5 min; all with 0.1% TFA.* ***Flow rate:*** *1.0 mL/min.* ***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

A large, 105 kD, transmembrane protein from *Neurospora crassa* was dissolved in anhydrous TFA and purified by RP-HPLC using a C_4 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 C_4 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 (Reference 24). RP-HPLC profiles of alcohol-soluble endosperm proteins—glutelins—were obtained on C_4 columns and used to identify varieties of rice[32].

HPLC as a Tool to Purify and Isolate Polypeptides

RP-HPLC is routinely used in the laboratory to purify microgram to milligram quantities of polypeptides for research purposes. Columns of 50 mm 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® 300 Å silica is produced in particle sizes from less than five to nearly thirty micrometers (Figure 40). 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

Particle Size Distribution of VYDAC® TP-300 Å Pore Size Silica

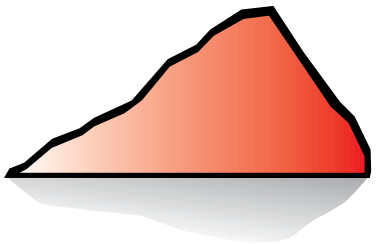

Figure 40. *Silica is produced in particle sizes from less than five to nearly thirty micrometers and particle size fractions are isolated for analytical, preparative and process applications.*

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 41). 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-30 µm—are normally used in 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 43). When columns are "overloaded", large particle materials perform nearly as well as small particle materials, as illustrated in Figure 42. 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

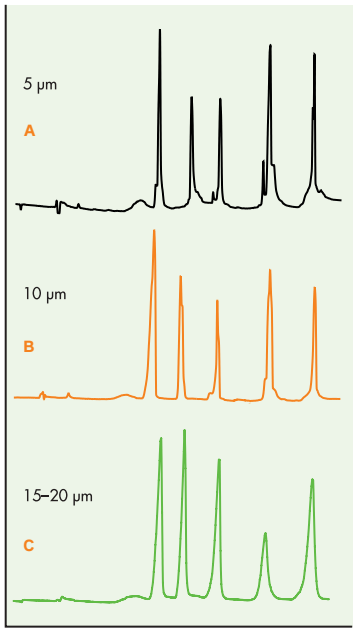

Figure 41. *Protein selectivity is the same on RP materials of different particle sizes. The only difference between materials of different particle sizes is that peak width increases and resolution decreases as particle size increases.* ***Column materials: A.*** *VYDAC® 214TP, 5 µm* ***B.*** *VYDAC® 214TP, 10 µm* ***C.*** *VYDAC® 214TP, 15–20 µm* ***Mobile phase:*** *24–95 % ACN with 0.1% TFA over 30 min at 1.5 mL/min.*

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 22 mm diameter column has about 23 times the volume of a 4.6 mm diameter column of the same length (22 divided by 4.6, squared). A 1.0 mL/min gradient over 30 minutes on an analytical column has a volume of 30 mL. To transfer the method to a 22 mm column, the gradient volume should be increased 23 times to 690 mL. 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 23 mL/min for 30 minutes would result in a gradient volume of 690 mL. However, a flow rate of 10 mL/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.

Protein Loading Capacity of RP-HPLC Materials of Different Particle Size

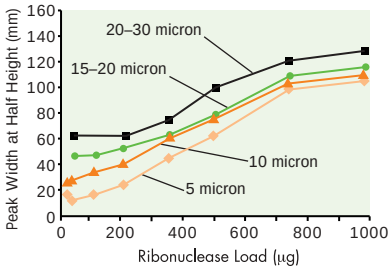

Figure 42. *Although peak widths are much narrower with small particle materials at low sample loads, there is little difference in peak widths at high loads, where the column is "overloaded". **Column materials:** VYDAC® 214TP, 5 µm; VYDAC® 214TP, 10 µm; VYDAC® 214TP, 15–20 µm; VYDAC® 214TP, 20–30 µm **Eluent:** 24–95 % ACN in 0.1% aqueous TFA over 30 min at 1.5 mL/min; **Protein:** ribonuclease.*

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 (4.6 mm diameter) columns, is about 100 to 200 µg for most polypeptides (Figure 43). 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 43 and 44), 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

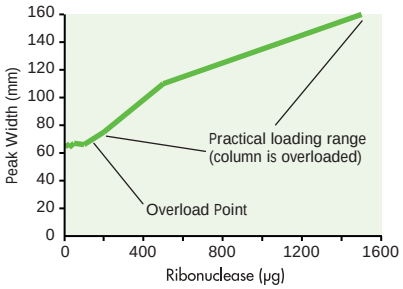

Figure 43. *Peak width is constant with sample loads up to 200 µg. Above 200 µg—the "overload" point—the peak width gradually increases. The practical loading region for ribonuclease is 200 to 5000 µg.* ***Column:*** *VYDAC® 214TP54 (C_4, 5 µm, 4.6 x 250 mm)* ***Eluent:*** *24–95% ACN with 0.1% TFA over 30 minutes* ***Sample:*** *ribonuclease.*

In Figure 44, 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 100 µg injections—in the region of optimum resolution—resolution between ribonuclease and the small impurity preceding it remains constant (Figure 44A, B). Resolution begins to decrease between ribonuclease and the impurity above 100 µg—the "overload" point. The 200 µg load shows a definite increase in peak width and consequent loss of resolution (Figure 44C). At 500 mg there is considerable loss in resolution (Figure 44D) and at 1,000 µg the impurity peak completely merges with the ribonuclease peak (Figure 44E).

Resolution between lysozyme and the preceding impurity peaks remains constant to about 200 µg, after which resolution is slowly lost. At 500 µg (Figure 44D) the impurity peaks appear only as shoulders on the lysozyme peak and by 1,000 µg (Figure 44E) 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 1,000 µg 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 30 cm column[46]. In a personal communication it was reported that 5 grams of synthetic peptide were purified on a 5 x 25 cm column in two steps.

Effect of Sample Load on Protein Peak Shape and Resolution

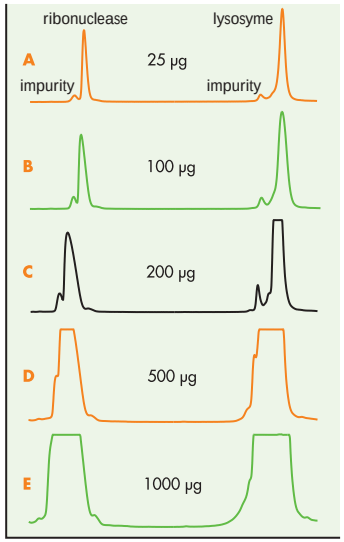

Figure 44. ***A.*** *25 µg each protein* ***B.*** *100 µg each protein* ***C.*** *200 µg each protein* ***D.*** *500 µg each protein* ***E.*** *1000 µg each protein* ***Column:*** *VYDAC® 214TP54 (C_4, 5 µm, 4.6 x 250 mm)* ***Eluent:*** *25–50% ACN in 0.1% TFA over 25 minutes at 1.5 mL/min.* ***Sample:*** *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 10 mg of peptide per gram of separation material—25 mg on a 4.6 x 250 mm column. Proteins have slightly higher binding capacities between 10 and 20 mg 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.

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 42). 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.

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.

HIV Protease Biological Activity

Dissolution

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

Refolding

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

Result

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

Figure 45. *Procedure used to regain biological activity of HIV protease after reversed-phase chromatography (Reference 56).*

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 45)[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 45)[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

Laboratory-scale purification

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

1. Establishing elution conditions with triethylammonium phosphate and acetonitrile on a five micrometer, 4.6 x 250 mm, column;
2. Loading the synthetic peptide onto a 5 x 30 cm column packed with 15–20 µm 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

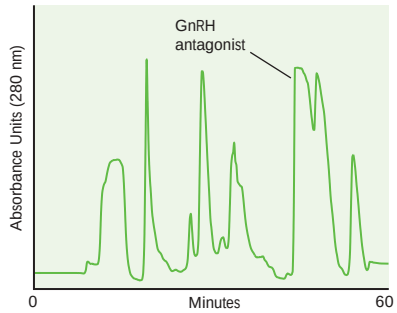

Figure 46. *Purification of 128 mg of a synthetic peptide, GnRH antagonist 1.2 grams of synthesis mixture were loaded onto a 5 x 30 cm column packed with VYDAC® 218TPB1520 (C_{18}, 15–20 µm) and eluted with a gradient of acetonitrile in water containing triethylammonium phosphate.*

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 47).

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

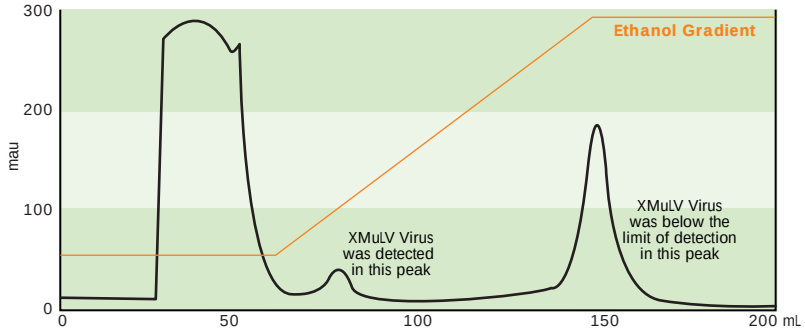

Figure 47. *Separation of Xenotropic Murine Leukemia Virus (XMuLV) from target protein during preparative HPLC.* ***Column:*** *VYDAC® C_4, 20-30 mm* ***Elution:*** *Ethanol gradient*
Data courtesy of Holly Harker and Marcus Luscher, Amgen, Boulder, Colorado

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 0.15 0.30 0.50	0.25 µL/min 1 µL/min 5 µL/min 10 µL/min	0.05 µg 0.2 µg 1 µg 2 µg	
Microbore	1.0	25–50 µL/min	0.05–10 µg	
Narrowbore	2.1	100–300 µL/min	0.2–50 µg	
Analytical	4.6	0.5–1.5 mL/min	1–200 µg	10 mg
Semi-preparative	10	2.5–7.5 mL/min	1,000 µg	50 mg
Preparative	22	10–30 mL/min	5 mg	200 mg
Process	50 100	50–100 mL/min 150–300 mL/min	25 mg 125 mg	1,000 mg 5,000 mg

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 100 µg of protein) or by injection of 100 µg 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—or 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)*
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
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 we recommend rinsing the column 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 C_{18} 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 C_{18} RP-HPLC of Polypeptides

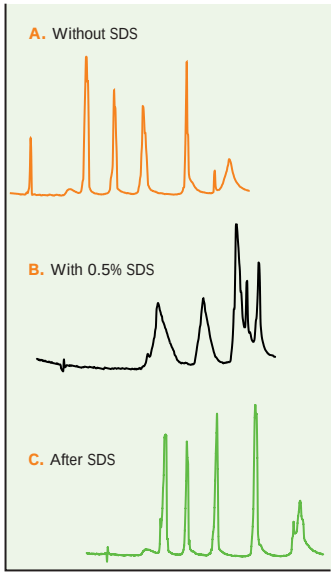

Figure C-1. *Surfactants affect chromatography (B) but do not harm column or subsequent separations (C).* ***Column:*** *VYDAC® 218TP54.* ***Eluent:*** *24–95% ACN in 0.1% TFA over 30 min at 1.5 mL/min* ***Sample:*** *ribonuclease, insulin, lysozyme, myoglobin and ovalbumin.*

Effect of Surfactants on C_4 RP-HPLC of Polypeptides

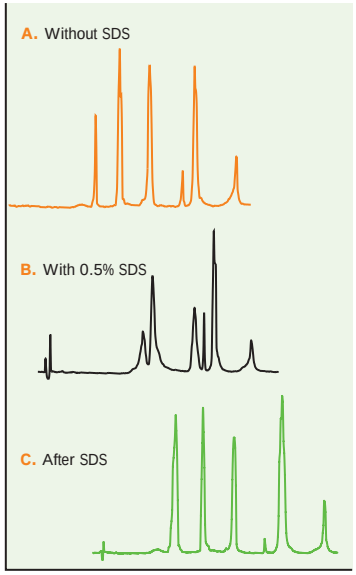

Figure C-2. *Surfactant affects chromatography (B) but does not harm column or subsequent separations (C).* ***Column:*** *VYDAC® 214TP54* ***Eluent:*** *24–95% ACN in 0.1% TFA over 30 min at 1.5 mL/min* ***Sample:*** *ribonuclease, insulin, lysozyme, myoglobin and ovalbumin.*

Results on a C_4 column were slightly better than those obtained on the C_{18} 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 C_{18} column (compare with Figure C-1). 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 C_4 reversed-phase column or removing the surfactant prior to chromatography.

Effect of Surfactants on Peptide Map

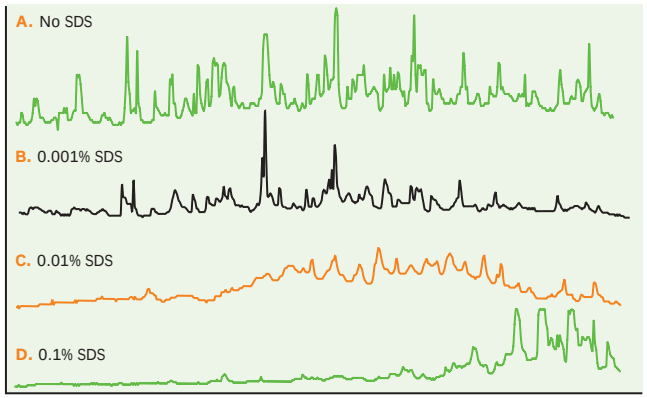

Figure C-3. *The presence of even trace amounts of SDS causes a loss in resolution in a peptide map.* ***Column:*** *VYDAC® 218TP52 (Narrowbore).* ***Eluent:*** *2–80% ACN with 0.06% TFA over 120 min at 0.25 mL/min.* ***Sample:*** *tryptic digest of carboxymethylated transferrin Data courtesy of K. Stone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

Appendix D: Ion Exchange Chromatography Orthogonal Analytical Techniques

Reversed-phase chromatography separates polypeptides on the basis of hydrophobicity; ion-exchange chromatography separates on the basis of charge. These complementary separation techniques offer synergistic capabilities in the analysis and purification of proteins and peptides and are often used together because of the different separation mechanisms. In series they offer better purification than can be achieved with either one alone; in parallel they offer mutual confirmation of analytical results. Comparison of the separation of several peptides by reversed-phase and cation exchange HPLC illustrates the complementary selectivity of the two techniques (Figure D-1). On the cation exchange column singly-charged oxytocin elutes early, followed by the three doubly-charged peptides—neurotensin, angiotensin II and bradykinin. Angiotensin I with four charges elutes last. On reversed-phase the peptides elute in the order of oxytocin, bradykinin, angiotensin I, neurotensin and angiotensin II. The complementary selectivities provide two dimensional resolving power.

Comparison of High Performance Reversed-Phase and High Performance Ion-Exchange Chromatography in the Separation of Peptides

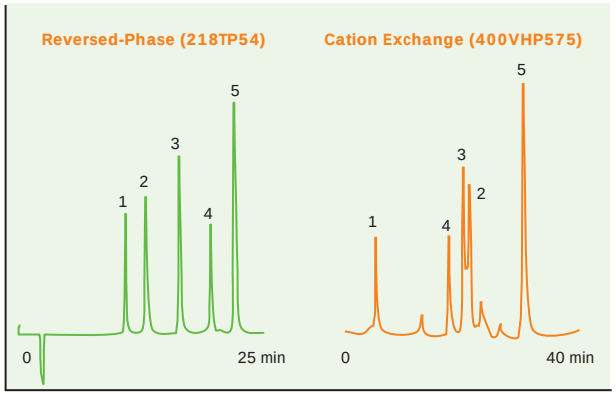

Figure D-1. ***Reversed-Phase Column:*** *VYDAC® 218TP54, C_{18}, 5 µm, 4.6 x 250 mm* ***Eluent:*** *15–30% ACN in 0.1% TFA over 30 minutes at 1.0 mL/min* ***Strong Cation Exchange Column:*** *VYDAC® 400VHP575, Cation exchange, 5 µm, 7.5 x 50 mm* ***Eluent:*** *10 mM phosphate, pH 2.7/25% ACN; gradient from 0-0.1 M NaCl in 20 min at 1.0 mL/min* ***Sample:*** *1. oxytocin 2. bradykinin 3. angiotensin II 4. neurotensin 5. angiotensin I.*

The Benefits of Ion-Exchange Chromatography

- Relatively high sample loading capacity compared to reversed-phase.
- Resistance to strong reagents such as 0.1 M NaOH, 0.1 M acid or 6 M guanidine because of the polymeric matrix. Relatively crude solutions can be loaded onto ion-exchange columns because adsorbed matrix components can be removed with strong reagents.
- Addition of urea, acetonitrile or non-ionic detergents to break-up complexes.
- Optimization of elution selectivity by adjustment of pH.

High Performance Reversed-Phase and Ion-Exchange Chromatography Used in Series to Remove Impurities in Lysozyme

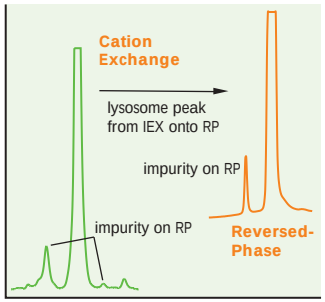

Figure D-2. ***Strong Cation Exchange Column:*** *VYDAC® 400VHP575, Cation exchange, 5 µm, 7.5 x 50 mm* ***Eluent:*** *10 mM phosphate, pH 6.5/25% ACN; gradient from 0–0.1 M NaCl in 25 min at 1.0 mL/min* ***Reversed-Phase Column:*** *VYDAC® 214TP54, C_4, 5 µm, 4.6 x 250 mm* ***Eluent:*** *10–35% ACN in 0.1% TFA, 50 minutes at 1.0 mL/min* ***Sample:*** *Lysozyme.*

The Benefits of Reversed-Phase Chromatography

- A high degree of selectivity based on differences in hydrophobicity or molecular conformation.
- Use of volatile buffers or ion-pairing agents.
- Freedom from interferences by salt or buffers from ion exchange.

Ion-exchange chromatography is normally used first, followed by reversed-phase chromatography (Figure D-2). Crude samples can be loaded onto a polymer-based ion exchange column without damaging the column; ion-exchange has a high loading capacity to accomodate complex samples; and chaotropes can be added to the sample to break up protein complexes. The partially purified polypeptide, containing salts and buffers from the ion exchange separation, can then be loaded onto a reversed-phase column. The salts are not retained and do not harm the reversed-phase column. Purification based on hydrophobicity or conformation then takes place and the collected sample elutes in a volatile solution, ready for final preparation.

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 1.0 mL/min from water to 0.3% acetone (for absorbance) in water and monitor at 254 nm. 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 (3 mL at 1 mL/min) calculated from when the run beguns 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 0.20 mL/min (Figure E-2B) is compared with the separation on an analytical column at 1.0 mL/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

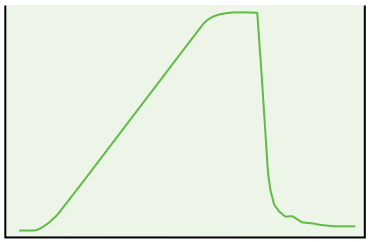

Figure E-1. *The gradient generated by the system hardware is visualized by the profile of a gradient increasing in acetone.* ***Column:*** *Replaced by low-volume tubing.* ***Gradient:*** *0–0.3% acetone in water over 30 min at 1.0 mL/min.* ***Detection:*** *UV at 254 nm.*

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

Tg = time duration of gradient

Effect of System Hardware on Gradient Shape in Narrowbore HPLC

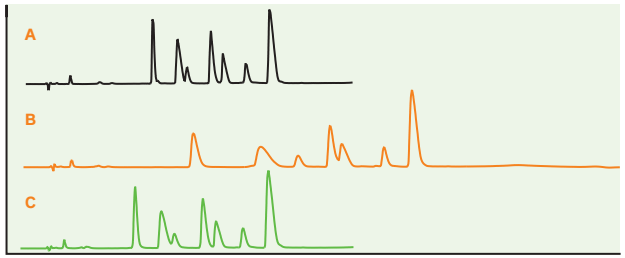

Figure E-2. *The system hardware gradient delay distorts the gradient shape at low flow rates and affects the peptide separation (B). Delaying sample injection to adjust for the gradient delay produces similar separation results (C) as obtained with an analytical column (A).* ***Column:*** *A. VYDAC® 218TP54 (C_{18}, 5 µm, 4.6 x 250 mm) B and C. VYDAC® 218TP52 (C_{18}, 5 µm, 2.1 x 250 mm)* ***Eluent:*** *15–30% ACN in 30 min with 0.1% TFA.* ***Flow rate:*** *A. 1.0 mL/min B and C. 0.20 mL/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.*

Technical References

Basic Principles and Analytical Conditions

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2 **Occurrence of Methionine Sulfoxide During Production of Recombinant Human Insulin-like Growth Factor (IGF-I)**, M. Hartmanis and A. Engstrom, *Second Symposium of the Protein Society, 1988, Abstract Number 502*

3 **Model of Protein Conformation in the Reversed-Phase Separation of Interleukin-2 Muteins**, M. Kunitani and D. Johnson, *J. Chrom. 371, 313–333 (1986)*

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