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

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

Second Edition

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 Vydac Technical Support Group.

Table of Contents

Introduction ................................................................. Page 2
Mechanism of Interaction ............................................ Page 4
Chromatographic Conditions ........................................ Page 8
Column Selection Guide ............................................. Page 14
Applications of Reversed-Phase HPLC ......................... Page 20
Narrow-Bore, Micro-Bore and Capillary Columns ............ Page 27
Answers to Frequently Asked Questions
What effect does eluent flow rate have on RP-HPLC separations? Page 30
What effect does column length have on RP-HPLC separations? Page 33
How much polypeptide can be purified in one RP-HPLC run? Page 36
What effect do surfactants have on RP-HPLC separations? Page 40
Does RP-HPLC of polypeptides affect biological activity? Page 42
Scaling-Up Reversed-Phase HPLC Separations ................. Page 44
Synergistic Separation Techniques:
High Performance Ion Exchange Chromatography .......... Page 50
Appendices
A. The effect of column carbon load on RP-HPLC separations Page 52
B. The effect of system hardware on RP-HPLC polypeptide separations. Page 54
C. Guide to Column Maintenance and Troubleshooting .......... Page 56
Technical References .................................................... Page 59

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

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Biochemists and protein chemists have many separation and purification tools available to them. Why has Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) become such a widely used, well-established tool for the analysis and purification of biomolecules?

The answer 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. The separation of insulin variants shown in Figure 1 illustrates this point. Insulin variants have molecular weights of around 5300 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 reversed-phase HPLC was used to separate similar polypeptides. Insulin-like growth factor with an oxidized methionine has been separated from its non-oxidized analogue and interleukin muteins have been separated from each other. 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 muteins and were able to separate muteins that were nearly identical. In one instance an oxidized methionine derivative was separated from the native form and in other cases single amino acid substitutions were separated from native forms (Figure 2). They concluded that protein conformation was very important in reversed-phase separations and that RP-HPLC could be used to study protein conformation. In the process they demonstrated the resolving power of reversed-phase HPLC for similar polypeptides.

Reversed-phase HPLC is used for the separation of peptide fragments from enzymatic digests and for purification of natural and synthetic peptides. Preparative RP-HPLC is frequently used to purify synthetic peptides in milligram and gram quantities. RP-HPLC is used to separate hemoglobin variants, identify grain varieties, study enzyme subunits, and research cell functions. Reversed-phase HPLC is used to purify micro-quantities of peptides for sequencing and to purify milligram to gram quantities of biotechnology-derived polypeptides for therapeutic use.

In short, reversed-phase HPLC is widely used in the analysis and purification of proteins and peptides because it can perform polypeptide separations that cannot be obtained in any other way - and it does so in a practical, convenient manner.
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 by RP-HPLC 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.

Polypeptides may be thought of as "sitting" on the stationary phase, like an elephant sits on grass, 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 polypeptides being separated. Differences in the "hydrophobic foot" result from differences in amino acid sequences and in conformation.

**Figure 3**
The adsorption/desorption model of polypeptide/reversed-phase interaction

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.

Polypeptide adsorbs to the reversed-phase surface

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

Polypeptide enters the column in the mobile phase

**Figure 4**
The retention of small molecules such as biphenyl decreases gradually as the organic modifier concentration increases because they are retained by partitioning ....

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.

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 - 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 when the critical concentration is reached (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 and the sudden desorption on reaching the critical modifier concentration give RP-HPLC the ability to separate very closely related polypeptides (see Pages 2 - 3).
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 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 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 effect 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. 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.

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Because large polypeptides diffuse slowly, RP-HPLC results in broader peaks than obtained with small molecules. Peak widths of polypeptides eluted isocratically are a function of molecular weight, with large proteins such as myoglobin having column efficiencies only 5 - 10% of the efficiencies obtained with small molecules such as biphenyl. Gradient elution of polypeptides, even with shallow gradients, results in much sharper peaks than isocratic elution.
The desorption and elution of polypeptides from reversed-phase HPLC columns is accomplished with aqueous solvents which contain an organic modifier and either an ion-pairing agent or a 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.

**Ion-pairing agents and buffers.**

Ion-pairing agents interact with polypeptides in solution to enhance separation and to help solubilize polypeptides.

- The most common ion-pairing agent is trifluoroacetic acid (TFA), which is used in most RP-HPLC polypeptide separations. TFA is widely used because:
  - It is volatile and easily removed from collected fractions;
  - It has little UV adsorption at low wavelengths;
  - And, 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. The use of TFA concentrations below 0.1% may degrade peak shape and is not recommended for polypeptides larger than a few hundred molecular weight.

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**Figure 8**

UV spectra of TFA in solutions of various concentration ratios of acetonitrile:water

The adsorption spectrum of TFA shifts as the acetonitrile:water ratio changes causing a baseline shift in typical peptide gradient chromatograms.

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Elution gradients with a constant concentration of TFA - i.e., the same concentration of TFA in both Solvent A and Solvent B - 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, changes the adsorption spectrum in the 190 to 250 nm region (Figure 7).

A close look at the UV spectra of TFA at various organic modifier (acetonitrile) concentrations (Figure 8) shows that the wavelength must be adjusted to precisely 215-216 nm to avoid the shift in adsorption spectra which causes the baseline drift. In practice it is nearly impossible to adjust the detection wavelength that precisely.

**Recommendation:** adjust the wavelength as close to 215 nm as possible AND put 15% less TFA in Solvent B as in Solvent A to compensate for a slight adsorption 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 this 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 C). Water can also have hydrophobic impurities which chromatograph and cause spurious peaks.
The effect of pH on peptide separations

Peptide separations are often sensitive to the eluent pH, as illustrated in Figure 10. All five peptides elute earlier at pH 4.4 (Fig. 10B) than at pH 2.0 (Fig. 10A) and the relative retention of peptides changes. Bradykinin and oxytocin are well separated at pH 2.0 but co-elute at pH 4.4. Peptide retention at pH 6.5 (Fig. 10C) 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 affect on peptide selectivity and can be a useful tool in optimizing peptide separations.

**Suggestion:** TFA is widely used as an ion-pairing agent 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 develop a good peptide separation.

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**Figure 10**

The effect of pH on peptide separations

*Elution of five peptides at pH 2.0, 4.4 and 6.5 with phosphate as the buffer*

**Conditions:**

- **Column:** Vydac 218TP54 (C₁₈, 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. bradykinin
2. oxytocin
3. angiotensin II
4. neurotensin
5. angiotensin I

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**Figure 9**

Comparison of TFA and alternate ion-pairing agents/buffers for the separation of peptides

*Elution of five peptides using TFA (A), Phosphate (B) or HCl (C) as the buffer/ ion-pairing agent.*

**Conditions:**

- **Column:** Vydac 218TP54 (C₁₈, 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

Although TFA is widely used, other buffers or ion-pairing agents sometimes give better resolution or peak shape than TFA. In the separation of five small peptides (Figure 9) phosphate gave sharper peaks for some peptides than TFA and caused 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 much 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. 9B). Hydrochloric acid also reversed the elution order of oxytocin and bradykinin and separated impurities not seen in TFA (Fig. 9C).

Heptafluorobutyric acid (HFBA) is effective in separating basic proteins and triethylamine phosphate (TEAP) is widely used for preparative separations. One study found that sample capacity was greater using TEAP than with TFA. Formic acid, in concentrations of 10 to 60%, has been used for the chromatography of very hydrophobic polypeptides. 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.

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Organic modifiers

The organic modifier desorbs the polypeptide from the hydrophobic surface and solubilizes it.

- Acetonitrile (ACN) is the most commonly used 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;
  - And, it has a long history of proven reliability in RP-HPLC polypeptide separations.

- 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% isopropanol to acetonitrile has been shown to increase protein recovery in some cases.

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

- Methanol or exotic solvents such as tetrahydrofuran and DMSO have been suggested for polypeptide separations but have not found widespread use. They seem to offer little advantage over the more commonly used solvents.

Elution gradients

Solvent gradients are almost always used to elute polypeptides. While isocratic elution is possible, peaks tend to be broad in isocratic elution and it is difficult to control elution conditions using a single solvent (see Page 6). Slight evaporation of the organic modifier can result in significant changes in retention times. A typical solvent gradient has a slope of 0.5 to 1% per minute increase in organic modifier concentration. Very shallow gradients, as low as .05 to 0.1% per minute can be used to maximize resolution. Very shallow gradients are normally preferred to isocratic elution.

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. For the best reproducibility and equilibration, avoid extremes in organic modifier composition.

The effect of temperature on peptide separations

Temperature can have subtle effects on peptide separations. In the peptide map of a trypsin digest of β-lactoglobulin A, the peptide fragments elute a little earlier as the temperature increases from 30 to 50 degrees C (Figure 11). More importantly, a number of peptides move slightly in relation to one another giving rise to increased or decreased resolution between certain pairs. Although no one temperature is clearly the best, a peptide map already optimized with respect to column and eluent may be further optimized by adjusting the temperature. This also suggests the importance of temperature control for good reproducibility.

**Figure 11**
Peptide map of β-lactoglobulin A at various temperatures

**Conditions:**
- **Column:** Vydac 218TP54 (C18, 5 µm, 4.6 x 250 mm)
- **Eluent:** 0 - 30% ACN in 60 min. with 0.1% TFA
- **Flowrate:** 1.0 mL/min
- **Sample:** tryptic digest of β-lactoglobulin A
Selecting the optimum reversed-phase HPLC column for the analysis or purification of a polypeptide sample depends on the characteristics of the polypeptide and impurities to be chromatographed, the nature of the sample itself, and the purpose of the chromatography.

- **Silica pore size.** 300 angstrom pore silica dominates the reversed-phase separation of polypeptides because resolution is generally best with this pore diameter silica even for small peptides. Smaller pore silicas (60-120 angstrom) may sometimes separate small or hydrophilic peptides better than large pore silica.

- **Particle size.** Five micron materials give the best resolution and are recommended for analytical and small scale preparative separations with columns of 1.0 mm (microbore), 2.1 mm (narrowbore) and 4.6 mm (analytical) diameter. Larger 10 mm diameter (semi-preparative) columns may be packed with either five or ten micron materials and 22 mm diameter (preparative) columns are packed with ten micron materials. Process chromatography columns are generally packed with 10-15, 15-20 or 20-30 micron materials.

**Table 1**

Recommended HPLC columns for polypeptide separation applications

<table>
<thead>
<tr>
<th>Polypeptide Application</th>
<th>Recommended RP Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>✔ peptides &lt; 5,000 MW</td>
<td>C18 (218TP)</td>
</tr>
<tr>
<td>✔ enzymatic digest fragments</td>
<td></td>
</tr>
<tr>
<td>✔ natural and synthetic peptides</td>
<td></td>
</tr>
<tr>
<td>✔ polypeptides &gt; 5,000 MW</td>
<td>C4 (214TP)</td>
</tr>
<tr>
<td>✔ hydrophobic polypeptides</td>
<td></td>
</tr>
<tr>
<td>✔ peptides &lt; 20,000 MW</td>
<td>C8 (208TP)</td>
</tr>
<tr>
<td>✔ enzymatic digest fragments</td>
<td></td>
</tr>
<tr>
<td>✔ natural and synthetic peptides</td>
<td></td>
</tr>
<tr>
<td>✔ large, hydrophobic proteins</td>
<td>Phenyl (219TP)</td>
</tr>
<tr>
<td>✔ peptides with aromatic side-chains</td>
<td></td>
</tr>
<tr>
<td>✔ small peptides (2-10 aa)</td>
<td>Small pore C18 (201HS)</td>
</tr>
<tr>
<td>✔ basic or very hydrophilic peptides</td>
<td></td>
</tr>
</tbody>
</table>

- **Phase type.** The length of the hydrophobic ligand on the reversed-phase surface - whether it be C<sub>18</sub>, C<sub>8</sub>, C<sub>4</sub>, etc. - makes little difference in many polypeptide separations because of the mechanism of interaction with the reversed-phase surface (see Pgs. 4-7). Although many proteins and peptides can be separated equally well on any of several RP-HPLC columns, experience does suggest that certain reversed-phase columns will separate polypeptides with specific characteristics better than others. C<sub>18</sub> is normally used for small peptides and C<sub>4</sub> is used for larger peptides and proteins. C<sub>8</sub> offers an alternate reversed-phase with intermediate characteristics. Phenyl reversed-phase is slightly less hydrophobic than C<sub>4</sub> and may offer unique selectivity for some polypeptides. Table 1 and Figure 12 give specific recommendations.
The separation of tryptic digest fragments of β-lactoglobulin A on three different RP-HPLC columns illustrates the subtle effects that different phases sometimes have on reversed-phase separations of peptides (Figure 14). Although the separations are similar, there are distinct differences between them. The C₄ column has slightly less retention and a somewhat different peptide fragment elution pattern than the more commonly used C₁₈ column. The small pore C₁₈ column also has a different elution pattern than the large pore C₁₈ column, although fewer fragments appear suggesting that, for this digest, there is less resolution with the small pore C₁₈ column. For other digests or samples the small pore column may give better resolution. Testing the separation on 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 (Figure 17, Page 22).

Beyond the recommendations in Table 1, subtle differences in reversed-phase hydrophobic surfaces sometimes result in differences in RP-HPLC selectivity for peptides that can be used to optimize specific peptide separations. The separation of five peptides illustrates this (Figure 13). RP-HPLC selectivity for the five peptides is about the same on the C₁₈ and C₄ columns, although the C₄ column has slightly shorter retention (Figures 13A and 13B). The phenyl column has both shorter retention and different selectivity than the C₁₈ column. Bradykinin, with two phenylalanines, is retained somewhat longer, relative to the other peptides, on the phenyl column than on the C₁₈ column and angiotensin I - with one histidine - and angiotensin II - with two histidines - both elute earlier relative to the other peptides on the phenyl column than on the C₁₈ column (Figure 13C). Selectivity on the small pore C₁₈ column (Figure 13D) is markedly different from the large pore columns. All five peptides are more strongly retained, with the cyclic oxytocin being very strongly retained, switching places with bradykinin. Neurotensin, a larger peptide is also more strongly retained and an impurity appears after angiotensin I that does not appear on the large pore columns.

**Figure 13**
Peptide separation on different reversed-phase columns

**Conditions:**
Columns: Vydac 218TP54 (C18); 214TP54 (C4); 219TP54 (phenyl); 201HS54 (Small pore C18);
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.

**Figure 14**
Separation of a tryptic digest on different reversed-phase columns

**Conditions:**
Columns: Vydac 218TP54 (C18); 214TP54 (C4); 201HS54 (Small pore C18);
Eluent: 0 - 30 % ACN in 0.1% aqueous TFA over 60 minutes at 1.0 mL/min.
Sample: tryptic digest of β-lactoglobulin A
These data illustrate the empirical nature of reversed-phase column selection for peptide separations. Although certain recommendations can be made, each laboratory must determine experimentally which of the available reversed-phase columns will best separate their particular sample, always keeping in mind that subtle differences in reversed-phase surfaces may affect peptide selectivity (See Appendix A).

■ **Column diameter.** The choice of column diameter depends on the required sample load and flow rate. Analytical - 4.6 mm i.d. - columns are the standard for general HPLC separations and offer the best performance for samples of 1 - 100 µg. Small bore columns (1.0 and 2.1 mm i.d.) can improve sensitivity and reduce solvent usage (see Page 27). Larger diameter columns are used for purification of larger amounts of polypeptide with the optimum diameter a function of the required sample capacity and the flow rate capability of the HPLC system. Sample capacities and recommended flow rates for columns of various diameters are given in Table 2.

■ **Column length.** Column length does not significantly affect most polypeptide separations (see Page 33). For this reason columns of 5 - 15 cm length are recommended for proteins and larger peptides. Small peptides, such as those resulting from trypsin digests, are better separated on longer columns and columns of 15 - 25 cm length are recommended for the separation of synthetic and natural peptides and for enzymatic digest maps. Short columns can be used with fast gradients for rapid analysis at reduced resolution.

### Table 2

<table>
<thead>
<tr>
<th>Diameter (internal)</th>
<th>Recommended flow rate</th>
<th>Sample capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbore 1.0 mm</td>
<td>25 - 50 µL/min</td>
<td>Optimum: .05 to 10 µg</td>
</tr>
<tr>
<td>Narrowbore 2.1 mm</td>
<td>100 - 300 µL/min</td>
<td>Optimum: .2 to 50 µg</td>
</tr>
<tr>
<td>Analytical 4.6 mm</td>
<td>.5 - 1.5 mL/min</td>
<td>Optimum: 1 to 200 µg</td>
</tr>
<tr>
<td>Semi-preparative 10 mm</td>
<td>2.5 - 7.5 mL/min</td>
<td>Optimum: up to 1 mg Practical: up to 40 mg</td>
</tr>
<tr>
<td>Preparative 22 mm</td>
<td>10 - 30 mL/min</td>
<td>Optimum: up to 5 mg Practical: up to 200 mg</td>
</tr>
<tr>
<td>Process 50 mm</td>
<td>50 - 100 mL/min</td>
<td>Optimum: up to 25 mg Practical: up to 1-5 g</td>
</tr>
<tr>
<td>100 mm</td>
<td>150 - 300 mL/min</td>
<td>Optimum: up to 125 mg Practical: up to 5-25 g</td>
</tr>
</tbody>
</table>
Reversed-phase HPLC has been used extensively for the analysis and purification of proteins and peptides for more than a decade. Some common uses of RP-HPLC are discussed in these pages.

**Peptide Mapping of Enzymatic Digests**

Enzymatic digests of proteins yield peptide fragments that can be separated on reversed-phase HPLC for analysis or sequencing. RP-HPLC peptide maps are often used to characterize or sequence polypeptides and are used for quality control of biotechnology-derived proteins by comparison of test sample maps with maps of standard protein. Impurities down to a few percent can be detected. Peptide mapping was used to locate protein sequence errors in subtilisin and a tryptic map, coupling RP-HPLC with mass spectrometry, was used to characterize a growth hormone releasing hormone. Enzymatic digest separations are routinely performed on analytical columns (4.6 x 250 mm), however small bore columns are being used more often to work with small samples and microbore RP-HPLC coupled with mass-spectrometry is becoming more widely applied.

**Figure 15**
RP-HPLC separated 53 peptide fragments from the S. aureus V8 digestion of colicin Ia, a 626 amino acid transmembrane ion channel protein.

**Conditions:**

- **Column:** Vydac 218TP54 (C18, 5 μm, 4.6 x 250 mm)
- **Eluent:** 0 - 35% ACN with TFA (.1% in A, .08% in B) in 105 mins at 1.0 mL/min
- **Detection:** UV at 215 nm

The RP-HPLC map of a S. aureus V8 digest of Colicin 1a (Figure 15) is a good example of enzymatic digest mapping. Fifty-three peptide fragments were resolved using elution conditions typical of peptide fragment separations. The column was a C18, five micron, 4.6 x 250 mm - the column recommended for enzyme digests (see Pages 14 - 15). Acetonitrile was the organic modifier and the ion-pairing agent was TFA. To reduce baseline shift, 0.1% TFA was added to Solvent A and .08% TFA was added to Solvent B. The gradient was 0 to 35 % ACN in 105 minutes, a slope of .33% per minute. Peptide maps generally run 60 to 240 minutes depending on the size of the protein being digested and the number of fragments obtained.

Protein deamidation often results from sample degradation and requires monitoring. Violand and colleagues used RP-HPLC maps to study deamidation of bovine somatotropin. They were able to differentiate between normal bovine somatotropin with asparagine at position 99 and deamidated somatotropin with isoaspartate at this position by a slight movement of fragment 95-107 on a C18 reversed-phase column, demonstrating the resolving power of RP-HPLC for nearly identical peptides (Figure 16).

**Figure 16**
RP-HPLC used in the study of protein deamidation

**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.**

**Conditions:**

- **Column:** Vydac 218TP54 (C18, 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

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**Page 20**

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**Page 21**
Natural and Synthetic Peptides

RP-HPLC is often used for the isolation and characterization of natural peptides. Scarborough and colleagues used a C$_{18}$, five micron, 4.6 x 250 mm column - the recommended column for small peptides - to isolate two octapeptides with cardioacceleratory activity from a cockroach extract (Figure 18). The elution conditions are a good example for isolation of natural peptides. The organic modifier was acetonitrile and the ion-pairing agent was 0.1% TFA. Elution began with a ten minute hold at 18% ACN to quickly remove weakly bound components. The active peptides were then eluted with a gradual gradient from 18 to 30% ACN over 60 minutes, a gradient slope of 0.2% per minute. This is a good example of the use of a shallow gradient to maximize resolution for the peptides of interest. Finally, strongly bound components of the extract were removed with a more rapid gradient from 30 to 60% ACN over 30 minutes.

RP-HPLC was used to separate peptides related to Alzheimer's disease and is widely used to purify synthetic peptides (Pages 44-49).

Characterization of a high molecular weight, hydrophobic membrane glycoprotein illustrates the use of complementary RP-HPLC phases. The tryptic digest of the glycoprotein was initially separated on a C$_{4}$ RP column into more than 100 peaks (Fig. 17A). Several early eluting, relatively hydrophilic peaks were further separated into multiple fragments on a C$_{18}$ RP column (example: Peak 27, Fig. 17B) and several late eluting, more hydrophobic peaks, were separated into multiple peaks on a phenyl column (example: peak 82, Fig. 17C). The complementary selectivity of different RP-HPLC phases can be a powerful tool in two-dimensional separations.
Protein folding. The folding of insulin-like growth factor was studied using RP-HPLC. Oxidative refolding of reduced IGF-1 resulted in two major peaks on RP-HPLC which had identical linear sequences but different disulfide pairing.

Viral proteins. Water insoluble poliovirus proteins were chromatographed by RP-HPLC.

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

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

Proteins

Proteins as large as 105 kd and 210 kd have been chromatographed by RP-HPLC. Examples include:

- **Analysis of protein subunits.** Eleven subunits of bovine cytochrome c oxidase ranging from MW 4962 to 56,993 were separated and analyzed by RP-HPLC (Figure 19). The inset in Figure 19 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 C8 RP using heptafluorobutryric acid (HFBA) as the ion-pairing agent (Figure 20). This example also illustrates the use of a shallow gradient - 0.2% per minute - to separate similar proteins.

Figure 19
RP-HPLC separation of bovine cytochrome c oxidase subunits

*Eleven subunits of bovine cytochrome c oxidase ranging in MW from 4962 to 56,993 are separated by RP-HPLC.*

**Conditions:**
- **Column:** Vydac 214TP104 (C4, 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

Figure 20
Analysis of histones

*Basic, nuclear proteins have been analyzed using heptafluorobutyric acid as the ion-pairing agent.*

**Conditions:**
- **Column:** Vydac 214TP54 (C4, 5 µm, 4.6 x 250 mm)
- **Eluent:** 40 - 56% ACN over 80 min. with 0.13% HFBA
- **Flow rate:** 1.0 mL/min
Hemoglobin variants. A RP-HPLC method using a C₄ column has been developed for the separation of globin chains. 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.

Protein characterization. Proteins are routinely purified for sequencing and characterization by RP-HPLC, for example the purification of an acid soluble protein from Clostridium perfringens spores.

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 C₄ columns and used to identify varieties of rice.

Figure 21
Identification of rice varieties by RP-HPLC separation of extractable proteins

RP-HPLC profiles of ethanol-soluble endosperm proteins provide a method to identify rice varieties.

Conditions:
Column: Vydac 214TP54 (C₄, 5 µm, 4.6 x 250 mm)
Eluent: Solvent A: .11% TFA in H₂O Solvent B: .085% TFA in ACN
Gradient: 28 - 36% B in 5 min, 36 - 47% B in 45 min, with a final 4 min hold at 47%
Flow rate: 1.0 mL/min Temperature: 60°C Data from Reference 24

Protein profiles are unique to each variety

Variety IR-42
Variety Milyang 42
Variety H4

Small bore and capillary HPLC columns are often used to separate polypeptides. Benefits from using small bore columns include:

Reduction in solvent usage. Flow rates from 5 nanoliters per minute with 50 micron capillary columns to 200 microliters per minute with narrowbore columns drastically reduce the amount of solvent needed for polypeptide separations.

Increased detection sensitivity. Polypeptides elute in smaller volumes of solvent at the reduced flow rates of small bore columns and UV 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 5 nanomoles of protein have been separated and collected using narrowbore RP-HPLC columns.

Interface with mass spectrometry or other complementary techniques. Direct transfer of the HPLC eluent into instruments such as mass spectrometers is possible with small bore columns.

Figure 22
Separation of the tryptic digest of carboxymethylated transferrin on a narrowbore RP-HPLC column (Reference 12)

Conditions in text. Data courtesy of K. Stone and K. Williams, Yale University
An article by Davis and Lee provides valuable information for getting the best performance using microbore and capillary columns and is recommended reading for anyone embarking on the use of small-bore columns. Another recent article discussed the use of microbore columns with mass spectrometry.

The separation of a tryptic digest of myoglobin (Figure 23) was performed on a 1.0 mm C18 microbore column (218TP51) at a flow rate of 50 µL/min with a gradient from 5 to 70% acetonitrile with 0.1% TFA over 90 minutes.

Fragments from the Lys C digest of cytochrome c were separated (Figure 24) on capillary columns of 500 micron (i.d.) and 160 micron (i.d.) using a gradient from 2 to 62% acetonitrile with 0.1% TFA over 60 minutes. Flow rates were 20 µL/min on the 500 micron column and 2 µL/min on the 160 micron column. The capillary columns were packed with five micron, C18 material (Vydac 218TPB5).

Narrowbore columns

Narrowbore columns of 2.1 mm i.d. are run at flow rates of 100 - 300 microliters per minute. Narrowbore columns are a practical step for most laboratories to take in reducing solvent usage and improving detection sensitivity. Some standard HPLC systems can operate at these low flow rates with little or no modification. In the example shown (Figure 22), the tryptic digest of carboxymethylated transferrin was separated on a C18 narrowbore column (218TP52) at 200 microliters per minute using a complex gradient from 2 to 98% B, where Solvent A is .06% TFA in water and Solvent B is .056% TFA in 80:20 acetonitrile:water.

Microbore and Capillary columns

Microbore columns of 1.0 mm i.d. offer a five-fold increase in detection sensitivity and reduction in solvent usage over narrowbore columns but require specialized equipment. Capillary columns - less than 1.0 mm i.d. - offer even greater sensitivity and solvent reduction but also require specialized equipment and special care. Modifications to standard systems are possible but are expensive and require good HPLC experience. The use of microbore or capillary columns should be approached with great care.
The eluent flow rate does not significantly affect the resolution of polypeptides in reversed-phase HPLC because desorption from the hydrophobic surface is not affected by flow rate; desorption is the result of reaching the critical organic modifier concentration and the flow rate plays a minor role.

This is illustrated in Figure 25, which shows retention times and resolution as a function of flow rate for proteins separated on a RP-HPLC column. Retention times decrease as the flow rate increases because the eluent carries the proteins through the column faster after desorption. Resolution, however, is essentially the same at all flow rates, since the desorption process is largely independent of 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. 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.

**Figure 25**

Effect of flow rate on protein retention and resolution

Retention times decrease as the flow rate increases because the desorbed proteins elute more quickly at higher flow rates.

However, resolution is unaffected by flow rate because proteins elute at a specific organic modifier concentration, regardless of flow rate.

**Conditions:**
- **Column:** Vydac 214TP15204
- **Eluent:** 24 - 95% ACN with 0.1% TFA over 30 min at 1.5 mL/min

Figure 26 shows that, in the separation of fragments from the tryptic digest of β-lactoglobulin A with gradients run over similar volumes, there are some differences in the peptide maps run at 0.5, 1.0 and 2.0 mL/min. The 0.5 mL/min peptide map has better resolution.

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 affect other aspects of a separation such as:

- **Detection sensitivity.** High flow rates elute polypeptides in large volumes of solvent and, by Beer’s law, adsorption decreases. Low flow rates elute polypeptides in small volumes of solvent and adsorption and sensitivity increase. The major reason that narrowbore HPLC columns increase detection sensitivity is because they are run at low flow rates and polypeptides are eluted in small volumes of solvent.

- **Sample solubility.** High flow rates may improve the solubility of hydrophobic polypeptides but they also increase the amount of solvent to be removed from the purified sample.
What effect does column length have on polypeptide reversed-phase separations?

Column length only plays a small role in the separation of polypeptides because most of the interaction of polypeptides with the column takes place in a single adsorption/desorption step near the top of the column and what little interaction that takes place as the protein elutes down the column does not affect resolution.

Figure 27 shows the retention times and resolution for several proteins on columns of 10, 15 and 25 cm lengths. Retention times increase slightly with longer columns because the proteins must pass through more column as they elute (Figure 27, Top), however resolution between protein pairs is not increased with longer columns (Figure 27, Bottom). In fact, resolution decreases slightly, probably due to additional diffusion and peak broadening as the desorbed protein continues down the longer column. It was also found that increased retention times with longer columns were almost entirely due to increased void volume, indicating that little or no interaction was taking place between the protein and the column after desorption.

### Table 3

<table>
<thead>
<tr>
<th>Column Diameter (mm)</th>
<th>Recommend flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>.025 - .050</td>
</tr>
<tr>
<td>2.1</td>
<td>.1 - .3</td>
</tr>
<tr>
<td>4.6</td>
<td>.5 - 1.5</td>
</tr>
<tr>
<td>10</td>
<td>2.5 - 7.5</td>
</tr>
<tr>
<td>22</td>
<td>10 - 30</td>
</tr>
<tr>
<td>50</td>
<td>50 - 100</td>
</tr>
<tr>
<td>100</td>
<td>150 - 300</td>
</tr>
</tbody>
</table>

### Conclusion

The resolution of proteins is not affected by eluent flow rate because the adsorption/desorption process is independent of flow rate. The separation of enzymatic digests or small peptides may be affected by flow rate. Flow rate may also affect other aspects of separations such as detection sensitivity, column back pressure, the volume of collected fractions or gradient shape.
Column length plays a more important role in the separation of enzymatic digests and small peptides because small peptides are more like small molecules in their chromatographic behavior (see Page 4 - Mechanism of Reversed Phase Interaction). 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.

Tryptic maps of β-lactoglobulin A on C18 columns of 50, 150 and 250 mm lengths demonstrate that better resolution is achieved with the 250 mm column than with shorter columns (Figure 28).

**Conclusion**

Short, 50 to 150 mm, length columns are recommended for the separation of large peptides and proteins. Longer columns, 150 to 250 mm, are recommended for the separation of small peptides and enzymatic digests. Long columns (with the largest volumes) are recommended to achieve maximum sample capacity.
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 becomes important. 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**

When polypeptides elute close together, the sample load must be kept below the level at which peak widths begin to increase and resolution decreases, the so-called "overload" point. Peak width and resolution remain constant up to this point which, for analytical (4.6 mm diameter) columns, is about 100 to 200 µg for most polypeptides (Figure 29). Sample loads above the "overload" point result in broadened peaks and decreased resolution; the column is "over-loaded". For polypeptide separations requiring optimum resolution, the sample load must be kept below the overload point for the column being used (Table 4, page 39).

**Figure 29**

Sample loading curve for ribonuclease on analytical (4.6 mm i.d.) RP-HPLC column

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.

Conditions:
- **Column:** Vydc 214TP54 (C4, 5 µm, 4.6 x 250 mm)
- **Eluent:** 24 - 95% ACN with 0.1% TFA over 30 minutes
- **Sample:** ribonuclease

**Practical loading capacity: an "overloaded column"**

For preparative separations, maximizing throughput in conjunction with resolution, yield and purity, 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 29 and 30), 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.

In Figure 30, injections of 25, 100, 200, 500 and 1000 micrograms of ribonuclease and lysozyme illustrate the affect 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. 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. At 500 µg there is considerable loss in resolution and at 1000 µg the impurity peak completely merges with the ribonuclease peak.

**Figure 30**

Effect of sample load on protein peak shape and resolution

- 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

Conditions:
- **Column:** Vydc 214TP54 (C4, 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
Tips for optimizing throughput and resolution

**Tip # 1**
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 slightly better resolution.

_Suggestion: Use dilute samples to improve resolution and sample loading capacity._

**Tip # 2**
Resolution between closely eluting polypeptides may be improved by using a more shallow gradient slope. This is usually done by lengthening the gradient time. In Figure 31, the separation of 1000 µg each of ribonuclease and lysozyme shows better separation for closely eluting impurity peaks with the more shallow gradient. The improvement in resolution is particularly evident in the case of the lysozyme impurities which are merged with the lysozyme peak with the standard gradient slope but are nearly resolved with the more shallow gradient.

_Suggestion: Use longer elution times and shallow gradients to obtain maximum resolution for closely eluting peaks._

---

### Table 4
Sample capacity ranges for RP-HPLC columns of various diameters

<table>
<thead>
<tr>
<th>Column Diameter (mm)</th>
<th>Sample Capacity Optimum Resolution</th>
<th>Sample Capacity Practical Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>.05 - 10 µg</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>.2 - 50 µg</td>
<td></td>
</tr>
<tr>
<td>4.6</td>
<td>1 - 200 µg</td>
<td>2 - 10 mg</td>
</tr>
<tr>
<td>10</td>
<td>5 - 1000 µg</td>
<td>1 - 40 mg</td>
</tr>
<tr>
<td>22</td>
<td>25 - 5000 µg</td>
<td>5 - 200 mg</td>
</tr>
<tr>
<td>50</td>
<td>.125 - 25 mg</td>
<td>25 - 1000 mg</td>
</tr>
<tr>
<td>100</td>
<td>.5 - 125 mg</td>
<td>125 - 5000 mg</td>
</tr>
</tbody>
</table>

---

Resolution between lysozyme and preceding impurity peaks remains constant to about 200 µg, after which resolution is slowly lost. At 500 µg (Figure 30D) the impurity peaks appear only as shoulders on the lysozyme peak and by 1000 µg (Figure 30E) the impurity peaks have completely merged with the lysozyme peak. Some resolution can be restored by running a more shallow gradient (Figure 31).

Since resolution between the two, well separated, major peaks - ribonuclease and lysozyme - remains good even at the 1000 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. In one case 1.2 grams of a synthetic peptide mixture were purified on a 5 x 30 cm column. In another, 5 grams of synthetic peptide were purified on a 5 x 25 cm column in two steps (personal communication).

**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.
Polypeptide samples often 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 32 and 33).

The separation on a C18 column of the protein sample containing SDS was much worse (Fig. 32B) than the separation of the protein sample without SDS (Fig. 32A). Subsequent chromatography of the sample without SDS, however, showed no deterioration (Fig. 32C), confirming that the SDS was removed in the gradient and did not harm the column or affect subsequent separations.

Results on a C4 column showed parallel but slightly better results than those obtained on the C18 column (Figure 33). The presence of SDS in the protein sample affected the chromatography (Fig. 33B), however the effect was less than on the C18 column (compare with Fig. 33A). The SDS was removed in the gradient and did not affect the column or subsequent separations (Fig. 33C).

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. 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 34).

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.

**Question:** What effect do surfactants have on polypeptide reversed-phase separations?

**Figure 32**
Effect of surfactants on C18 RP-HPLC of polypeptides
Surfactants affect chromatography (B) but do not harm column or subsequent separations (C).

**Conditions:**
- **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

**Figure 33**
Effect of surfactants on C4 RP-HPLC of polypeptides
Surfactant affects chromatography (B) but does not harm column or subsequent separations (C).

**Conditions:**
- **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

**Figure 34**
Effect of surfactants on peptide map
The presence of even trace amounts of SDS causes a loss in resolution in a peptide map.

**Conditions:**
- **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
Biology activity of proteins depends on tertiary structure and permanent disruption of tertiary structure usually reduces biological activity. RP-HPLC may disrupt protein tertiary structure because of the hydrophobic solvents used for elution or because of the interaction of the protein with the hydrophobic surface of the material. The amount of biological activity lost depends on the stability of the protein and on the elution conditions used. The loss of biological activity can be minimized by proper post-chromatographic treatment. Small peptides and very stable proteins are less likely to lose biological activity than large enzymes. Some specific points to keep in mind are:

- 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.
- 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, such as enzyme cofactors, added to the chromatographic eluent, 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 (Table 5) 41.

Examples of biological activity after RP-HPLC

- Trypsin. Reversed phase chromatography has been used to purify trypsin for use in protein digestion 18,42.
- Poliovirus proteins. Poliovirus proteins purified by reversed phase chromatography were able to induce production of specific antibodies in rabbits, indicating a retention of biological activity 23.

Table 5

<table>
<thead>
<tr>
<th>Procedure used to regain biological activity of HIV protease after reversed-phase chromatography (Ref. 41)</th>
</tr>
</thead>
</table>

- 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 43.
- HIV protease. HIV protease regained most of its biological activity after reversed-phase chromatography and post chromatographic treatment to allow refolding (Table 5) 41.
  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 44.
- Leukine, a marketed polypeptide therapeutic, uses reversed-phase HPLC as an integral part of its purification procedure 45,46.
- Human recombinant insulin purification uses reversed-phase chromatography in its production 47.

Conclusion

*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.*
Laboratory scale purification of microgram to milligram quantities of polypeptides for research or small scale clinical trials can be accomplished on columns of 4.6 to 50 mm diameter. Scaling up separations from analytical columns usually involves the use of standard solvents and ion-pairing agents or buffers, choosing column dimensions with the necessary sample load characteristics (see Page 19), and optimization of the elution gradient.

Process separations of milligram to gram quantities of synthetic or recombinant polypeptides for clinical trials or for marketing is performed with columns of 50 to 200 mm diameter or greater. 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 both 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 angstrom silica is produced in particle sizes from less than five to nearly thirty microns (Figure 35). Physical sizing procedures are used to isolate fractions of five and ten micron particles for use in analytical and laboratory scale preparative separations.

Silica fractions with larger average particle size and broader ranges are separated for preparative and process scale applications. Process-scale reversed-phase materials based on silica from the same manufacturing process as analytical size silica and bonded by matched chemical procedures have nearly identical protein and peptide selectivity characteristics as analytical scale materials. The separation of several proteins on columns of five, ten and fifteen-to-twenty micron particle size materials illustrates this (Figure 36). 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 greater with the larger particle materials, causing some loss in resolution. Large particle materials - 10-to-15, 15-to-20 or 20-to-30 micron - 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 36). When columns are "overloaded", large particle materials perform nearly as well as small particle materials. Figure 37 shows that peak width and resolution are much higher for small particle materials.
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 peptide to be collected.

Process-scale purification: More than five grams of peptide

- Elution solvent: The organic solvents commonly used in laboratory scale chromatography often 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 and is available at low cost and known and understood by regulatory agencies such as the FDA. Ethanol is presently used in large scale process purifications.

- 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 bio-technology derived therapeutics.

- 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).
Process-scale purifications

Reversed-phase chromatography is currently used in the production of several biotechnology-derived therapeutics.

- **Erythropoetin.** Recombinant erythropoietin may be purified using RP-HPLC as an integral part of the purification process \(^{44}\).
- **Colony-stimulating factor.** Leukine (GM-CSF), a marketed polypeptide therapeutic, uses reversed-phase chromatography as an integral part of its purification procedure \(^{45,46}\). G-CSF may be purified using RP-HPLC as an integral part of the purification process (personal communication).
- **Human insulin.** A process using reversed-phase chromatography has been developed for the purification of biosynthetic human insulin \(^{47}\).

Tips for optimizing preparative separations

**Tip # 1**

Since sample capacity is a function of column volume, either column diameter or column length can be increased for increased sample load.

**Tip # 2**

Use an initial solvent concentration well below (10-20%) the elution organic modifier concentration of the polypeptide being purified.

**Tip # 3**

Load the sample in a solvent that will not interfere with adsorption of the polypeptide. This generally 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.

**Tip # 4**

Sample concentration does not affect column loading capacity, however loading dilute samples sometimes results in slightly better resolution than loading concentrated samples.

**Tip # 5**

When columns are "over-loaded", particle size becomes less significant in obtaining resolution. Small particle materials give only slightly better resolution than large particle materials under "over-load" 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.

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**Laboratory-scale purification**

Several examples of the purification of synthetic peptides by RP-HPLC have appeared in the literature \(^{32-35}\). In one case \(^{32}\) 128 mg of gonadotropin releasing hormone (GnRH) antagonist was purified from 1.2 grams of synthesis mixture in two RP-HPLC purification steps (Figure 38). The procedure involved (see Reference 32 for details):

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

**Figure 38**

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 micron) and eluted with a gradient of acetonitrile in water containing triethylammonium phosphate.

0 min 60 min
Reversed-phase chromatography separates 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 39). On the cation exchange column singly-charged oxytocin elutes early, followed by the three doubly-charged peptides - neuropeptide Y, 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, neuropeptide Y and angiotensin II. The complementary selectivities provide two dimensional resolving power.

**The benefits of ion-exchange chromatography include:**
1. A relatively high loading capacity compared to reversed-phase.
2. 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.
3. Addition of urea, acetonitrile or non-ionic detergents to break-up complexes.
4. Optimization of elution selectivity by adjustment of pH.

**The benefits of reversed-phase chromatography include:**
1. A high degree of selectivity based on differences in hydrophobicity or molecular conformation.
2. Use of volatile buffers or ion-pairing agents.
3. Freedom from interferences by salt or buffers from ion exchange.

When used in series, ion-exchange chromatography is normally used first, followed by reversed-phase chromatography (Figure 40). 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; 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.
Reversed-phase HPLC separation of peptides is often accomplished by subtle interactions of peptides with the reversed-phase surface. Various aspects of the reversed-phase surface can affect peptide separations in small, but important ways. In particular, some peptide separations are very sensitive to the density and uniformity of the hydrophobic phase bonded to the silica matrix.

Sensitive Test of Chromatographic Carbon Load

A very sensitive test for monitoring the carbon load on some types of C₁₈ reversed phase columns has evolved from a test developed at the U.S. NIST (National Institute for Standards and Technology) for evaluation of C₁₈ phase types. Based on studies of the influence of shape selectivity on the reversed-phase HPLC separation of polynuclear aromatic hydrocarbons, this test consists of the isocratic separation of three polyaromatic hydrocarbons - phenanthro(3,4c)phenanthrene, tetrabenzenophthalene and benzo(a)pyrene, and the calculation of a resolution factor - α - between tetrabenzenophthalene and benzo(a)pyrene (Figure 41). The original NIST studies relate the α factor to the phase type: α values greater than 1.7 result from monomeric bonding of the reversed-phase and α values less than 1.0 result from polymeric bonding.

Micro-combustion carbon analysis of C₁₈ reversed-phase is an imprecise measure of carbon load, whereas the NIST alpha value measured on polymerically bonded C₁₈ reversed-phase columns is very sensitive to minute variations in the carbon load. Use of the NIST-derived test has significantly improved the carbon load reproducibility of polymerically bonded C₁₈ columns (Figure 42).

The precise control of C₁₈ carbon load resulting from the NIST-derived carbon load test led to an observation by scientists at Genetics Institute that a pair of peptide fragments in an Asp-N protein digest were barely separated on standard C₁₈ columns but were well resolved on a C₁₈ column with a slightly lower carbon load (Figure 43). Nine of ten peptide fragments eluted at the same time on both columns, however one peptide fragment, with a pK of 7.3, eluted earlier on the lower carbon load column, increasing the resolution between it and an adjacent fragment with a pK of 4.3. This illustrates the subtle affect that C₁₈ reversed-phase carbon load can have on peptide separations.

Figure 41
NIST-Derived C₁₈ RP-HPLC Chromatographic Carbon Load Test

The resolution between polynuclear aromatic hydrocarbons constitute a sensitive test of chromatographic carbon load.

Conditions:
Column: Vydac 218TP54 (C₁₈, 5 µm, 4.6x150 mm)
Eluent: 85:15 acetonitrile:water
Sample: phenanthro(3,4c)phenanthrene, tetrabenzenophthalene and benzo(a)pyrene
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.

**Evaluation of 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 44). The gradient UV profile can be 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 44) the gradient delay is about 3 minutes (3 mL at 1 mL/min) calculated from when the run begins 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 45 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 (Fig. 45B) is compared with the separation on an analytical column at 1.0 mL/min (Fig. 45A) using the same HPLC system and programmed gradient. The 10 minute gradient response delay distorts the peptide separation (Fig. 45B). 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 (Fig. 45C) is similar to the analytical separation (Fig. 45A).

**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 ($C_D$):

1. Enter the retention time of the peak (Example 33 min)
2. Subtract the retention time of the injection peak (Example 3.5 min)
3. Subtract the gradient response delay time (Example 3 min)
4. And subtract any initial gradient hold time (Example 5 min)
5. Equals corrected elution time ($ET_{corr}$) (Example 21.5 min)

The solvent concentration ($C_D$) at the corrected elution time is:

$$C_D = C_S + \left(ET_{corr}/T_g\right)(C_{E} - C_S);$$

where $C_S$ = solvent concentration at start of gradient

$C_E$ = solvent concentration at end of gradient

$T_g$ = time duration of gradient
Appendix C: Guide to maintaining and restoring RP-HPLC column performance

Reversed-phase HPLC columns, if properly cared for, give good performance for hundreds to over a thousand injections. Although the following ideas are specifically applicable to Vydac RP-HPLC columns, they also apply to many other RP-HPLC 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 larger than 5-10,000 MW. 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).

Table 6

<table>
<thead>
<tr>
<th>Column Size (mm)</th>
<th>Flow rate (mL/min)</th>
<th>Typical Back-pressure (with 50:50 ACN:Water)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 x 250</td>
<td>0.20</td>
<td>1000 - 1800 psi</td>
</tr>
<tr>
<td>4.6 x 250</td>
<td>1.0</td>
<td>1000 - 1800 psi</td>
</tr>
<tr>
<td>4.6 x 150</td>
<td>1.0</td>
<td>600 - 1200 psi</td>
</tr>
<tr>
<td>10 x 250</td>
<td>5.0</td>
<td>1000 - 1800 psi</td>
</tr>
<tr>
<td>4.6 x 250</td>
<td>1.0</td>
<td>500 - 1000 psi</td>
</tr>
<tr>
<td>10 x 250</td>
<td>5.0</td>
<td>500 - 1000 psi</td>
</tr>
<tr>
<td>22 x 250</td>
<td>25</td>
<td>500 - 1000 psi</td>
</tr>
</tbody>
</table>

**Pressure and Temperature Limits**

RP-HPLC columns are generally stable to 60 degrees C. and up to 5000 psi (335 bar) back-pressure. Typical back-pressures for RP-HPLC columns are shown in Table 6.

**RP-HPLC column trouble-shooting**

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

- **High back-pressure.** Disconnect the column from the injector and run the pumps to ensure that the back-pressure is due to the column and not the HPLC system. If the column back-pressure is high, most HPLC columns can be reversed and rinsed to try to flush contaminants from the inlet frit. Begin the reverse rinse at a low flow rate - 10 to 20% of normal - for 10-15 minutes and then increase to the normal flow rate.

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

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 46). 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.

Figure 46
Evidence of solvent contaminants as source of ghost peaks

A. 10 min. equilibration - small "ghost peaks"
B. 30 min. equilibration - larger "ghost peaks"
- **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.

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**Figure 46**

Evidence of solvent contaminants as source of ghost peaks

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</tbody>
</table>

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**Technical References**

**Basic Principles and Conditions**


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Preparative Chromatography


Practical Aspects of Reversed-phase Chromatography


Digest of colicin Ia, a transmembrane protein (see Figure 15, Page 20)

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