

## Reversed-Phase High-Performance Liquid Chromatography

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### 1. Introduction

Reversed-phase high-performance liquid chromatography (RP-HPLC) involves the separation of molecules on the basis of hydrophobicity. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase, i.e., the sorbent. A schematic diagram showing the binding of a peptide or a protein to a reversed-phase surface is shown in **Fig. 1**. The solute mixture is initially applied to the sorbent in the presence of aqueous buffers, and the solutes are eluted by the addition of organic solvent to the mobile phase. Elution can proceed either by isocratic conditions where the concentration of organic solvent is constant, or by gradient elution whereby the amount of organic solvent is increased over a period of time. The solutes are, therefore, eluted in order of increasing molecular hydrophobicity. RP-HPLC is a very powerful technique for the analysis of peptides and proteins because of a number of factors that include: (1) the excellent resolution that can be achieved under a wide range of chromatographic conditions for very closely related molecules as well as structurally quite distinct molecules; (2) the experimental ease with which chromatographic selectivity can be manipulated through changes in mobile phase characteristics; (3) the generally high recoveries and, hence, high productivity; and (4) the excellent reproducibility of repetitive separations carried out over a long period of time, which is caused partly by the stability of the sorbent materials under a wide range of mobile phase conditions (**1,2**). However, RP-HPLC can cause the irreversible denaturation of protein samples thereby reducing the potential recovery of material in a biologically active form.

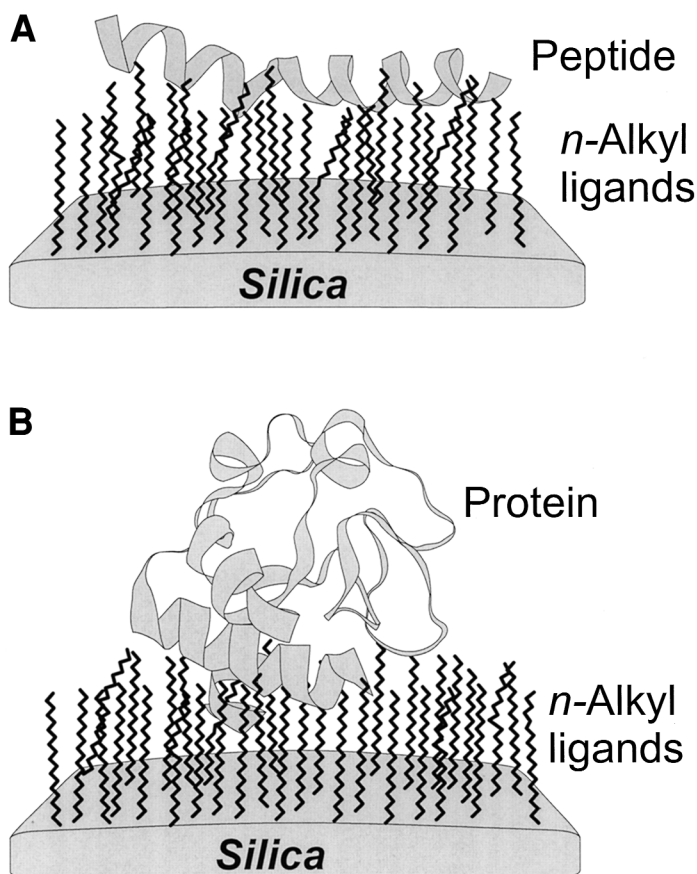


Fig. 1. Schematic representation of the binding of (A) a peptide and (B) a protein, to an RP-HPLC silica-based sorbent. The peptide or protein interacts with the immobilized hydrophobic ligands through the hydrophobic chromatographic contact region.

The RP-HPLC experimental system for the analysis of peptides and proteins usually consists of an *n*-alkylsilica-based sorbent from which the solutes are eluted with gradients of increasing concentrations of organic solvent such as acetonitrile containing an ionic modifier such as trifluoroacetic acid (TFA) (1,2). Complex mixtures of peptides and proteins can be routinely separated and low picomolar—femtomolar amounts of material can be collected for further characterization. Separations can be easily manipulated by changing the gradient slope, the operating temperature, the ionic modifier, or the organic solvent composition.

The extensive use of RP-HPLC for the purification of peptides, small polypeptides with molecular weights up to 10,000, and related compounds of pharmaceutical interest has not been replicated to the same extent for larger polypeptides

(molecular mass > 10 KDa) and globular proteins. The combination of the traditionally used acidic buffering systems and the hydrophobicity of the *n*-alkylsilica supports which can result in low mass yields or the loss of biological activity of larger polypeptides and proteins have often discouraged practitioners from using RP-HPLC methods for large-scale protein separations. The loss of enzymatic activity, the formation of multiple peaks for compositionally pure samples, and poor yields of protein can all be attributed to the denaturation of protein solutes during the separation process using RP-HPLC (3–6).

RP-HPLC is extremely versatile for the isolation of peptides and proteins from a wide variety of synthetic or biological sources and is used for both analytical and preparative applications (1–2, *see also* Chs. 10–21). Analytical applications range from the assessment of purity of peptides following solid-phase peptide synthesis (*see* Ch. 14), to the analysis of tryptic maps of proteins. Preparative RP-HPLC is also used for the micropurification of protein fragments for sequencing to large-scale purification of synthetic peptides and recombinant proteins. The complexity of the mixture to be chromatographed will depend on the nature of the source and the degree of preliminary clean-up that can be performed. In the case of synthetic peptides, RP-HPLC is generally employed both for the initial analysis and the final large-scale purification. The purification of synthetic peptides usually involves an initial separation on an analytical scale to assess the complexity of the mixture followed by large-scale purification and collection of the target product. A sample of the purified material can then be subjected to RP-HPLC analysis under the same or different elution conditions to check for purity. The isolation of proteins from a biological cocktail derived from a tissue extract or biological fluid for example, often requires a combination of techniques to produce a homogenous sample. HPLC techniques are then introduced at the later stages following initial precipitation, clarification, and preliminary separations using soft gels.

The challenge facing the scientist who wishes to analyze and/or purify their peptide or protein sample by RP-HPLC is the selection of the initial separation conditions and subsequent optimization of the appropriate experimental parameters. This chapter describes a standard method that can be used as an initial procedure for the RP-HPLC analysis of a peptide sample, and then different experimental options available to achieve a high-resolution separation of a peptide or protein mixture using RP-HPLC are outlined in **Subheading 4**.

## 2. Materials

### 2.1. Chemicals

1. Acetonitrile (CH<sub>3</sub>CN), HPLC grade.
2. Milli-Q water.
3. Trifluoroacetic acid (TFA).

## 2.2. Equipment and Supplies

1. HPLC solvent delivery system with binary gradient capability and a UV detector.
2. Reversed-phase octadecylsilica (C18) column (*see Note 1*) (4.6 mm id (internal diameter)  $\times$  250 mm length (*see Note 2*), 5  $\mu$ m particle size, 300 Å pore size (*see Note 3*).
3. C18 guard column.
4. Solvent filtration apparatus equipped with a 0.22- $\mu$ m Teflon filter.
5. Sample filters, 0.22  $\mu$ m porosity.
6. Buffer A: 0.1% (v/v) TFA in water (*see Note 4*).
7. Buffer B: 100% CH<sub>3</sub>CN containing 0.1% (v/v) TFA (*see Note 5*).

## 3. Methods

### 3.1. Sample Preparation

Dissolve 1 mg of sample in 1 mL of Buffer A. If there is some undissolved material, filter the sample through a 0.22- $\mu$ m filter.

### 3.2. Solvent Preparation

Filter all solvents through a 0.22- $\mu$ m filter before use. This removes particulates that could block solvent lines or the column and also serves to degass the solvent. If the HPLC instrument is not installed with on-line degassing capability, check with your instrument requirements to assess whether further degassing is required.

### 3.3. Column Equilibration and Blank Run

1. Connect the guard and the column to the solvent delivery system according to the HPLC system requirements and equilibrate under the following initial conditions.
  - a. Solvent: 100% Buffer A
  - b. Flow rate: 1 mL/min (*see Note 6*)
  - c. Detection wavelength: 215 nm (*see Note 7*)
  - D. Temperature: Ambient (*see Note 8*)
2. Once a stable baseline is obtained, inject 10  $\mu$ L of Milli-Q water (either manually or via an automatic injector). It is generally advisable to perform two blank runs to ensure proper equilibration of the column.

### 3.4. Sample Injection and Analysis

Once a stable baseline is obtained, inject 10  $\mu$ L of the sample (either manually or via an automatic injector) and use a linear gradient from 0 to 100% buffer B over 30 min to elute the sample (*see Note 9*).

**Figure 2** shows a typical chromatogram of a crude synthetic peptide. The large majority of components should normally elute within the gradient time. Thus, each individual method is relatively straightforward to perform. The

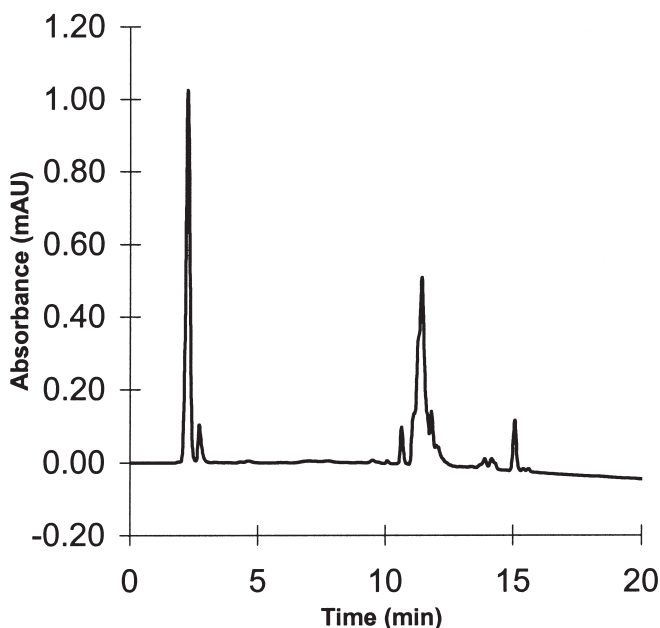


Fig. 2. RP-HPLC elution profile illustrating the purification of a synthetic peptide. Analytical profile (1 mg) of a crude peptide mixture from solid phase peptide synthesis. Column: Zorbax 300 RP-C18, 25 cm  $\times$  4.6 mm id, 5- $\mu$ m particle size, 30 nm pore size. Conditions, linear gradient from 0–60% acetonitrile with 0.1% TFA over 30 min, flow rate of 1 mL/min, 25°C.

scope lies in the wide range of operating parameters that can be changed in order to manipulate the resolution of peptide and protein mixtures in RP-HPLC. These parameters include the immobilized ligand (*see Note 1*), the column packing geometry (*see Note 3*), the column dimensions (*see Note 2*), the ionic additive (*see Note 4*), the organic solvent (*see Note 5*), the mobile phase flow rate (*see Note 6*), the gradient time and gradient shape (*see Note 9*), and the operating temperature (*see Note 8*).

#### 4. Notes

1. The most commonly employed experimental procedure for the RP-HPLC analysis of peptides and proteins generally involves the use of a C18-based sorbent and a mobile phase. The chromatographic packing materials that are generally used are based on microparticulate porous silica which allows the use of high linear flow velocities resulting in favorable mass transfer properties and rapid analysis times (7,8). The silica is chemically modified by a derivatized silane bearing an *n*-alkyl hydrophobic ligand. The most commonly used ligand is C18, whereas

*n*-butyl (C4) and *n*-octyl (C8) also find important application and phenyl and cyanopropyl ligands can provide different selectivity (9). The process of chemical immobilization of the silica surface results in approx half of the surface silanol group being modified. The sorbents are, therefore, generally subjected to further silanization with a small reactive silane to produce an end-capped packing material. The type of *n*-alkyl ligand significantly influences the retention of peptides and proteins and can therefore be used to manipulate the selectivity of peptide and protein separations. Although the detailed molecular basis of the effect of ligand structure is not fully understood, a number of factors including the relative hydrophobicity and ligand chain length, flexibility, and the degree of exposure of surface silanols all play a role in the retention process. An example of the effect of chain length on peptide separations can be seen in Fig. 3 (I). It can be seen that the peaks labeled T<sub>3</sub> and T<sub>13</sub> are fully resolved on the C4 packing but cannot be separated on the C18 material. In contrast, the peptides T<sub>5</sub> and T<sub>18</sub> are unresolved on the C4 column but fully resolved on the C18 material. In addition to effects on peptide selectivity, the choice of ligand type can also influence protein recovery and conformational integrity of protein samples. Generally higher protein recoveries are obtained with the shorter and less hydrophobic *n*-butyl ligands. However, proteins have also been obtained in high yield with *n*-octadecyl silica (10–12). Silica-based packings are also susceptible to cleavage at pH values greater than 7. This limitation can severely restrict the utility of these materials for separations which require basic pH conditions to effect resolution. In these cases, alternative stationary phases have been developed including cross-linked polystyrene divinylbenzene (13,14) and porous zirconia (15,16), which are all stable to hydrolysis at alkaline pHs.

2. The desired level of efficiency and sample loading size determines the dimension of the column to be used. For small peptides and proteins, increased resolution will be obtained with increases in column length. Thus, for applications such as tryptic mapping, column lengths between 15–25 cm and id of 4.6 mm are generally employed. However, for larger proteins, low mass recovery and loss of biological activity may result with these columns as a result of irreversible binding and/or denaturation. In these cases, shorter columns of between 2 and 20 cm in length can be used. For preparative applications in the 1–500 mg scale, such as the purification of synthetic peptides, so-called semipreparative columns of dimensions 30 cm × 1 cm id and preparative columns of 30 cm × 2 cm id can be used.

The selection of the internal diameter of the column is based on the sample capacity and detection sensitivity. Whereas most analytical applications are carried out with columns of internal diameter of 4.6 mm id, for samples derived from previously unknown proteins where there is a limited supply of material, the task is to maximize the detection sensitivity. In these cases, the use of narrow bore columns of 1 or 2 mm id can be used that allow the elution and recovery of samples in much smaller volumes of solvent (*see* Chapter 11). Capillary chromatography is also finding increasing application where capillary columns of internal

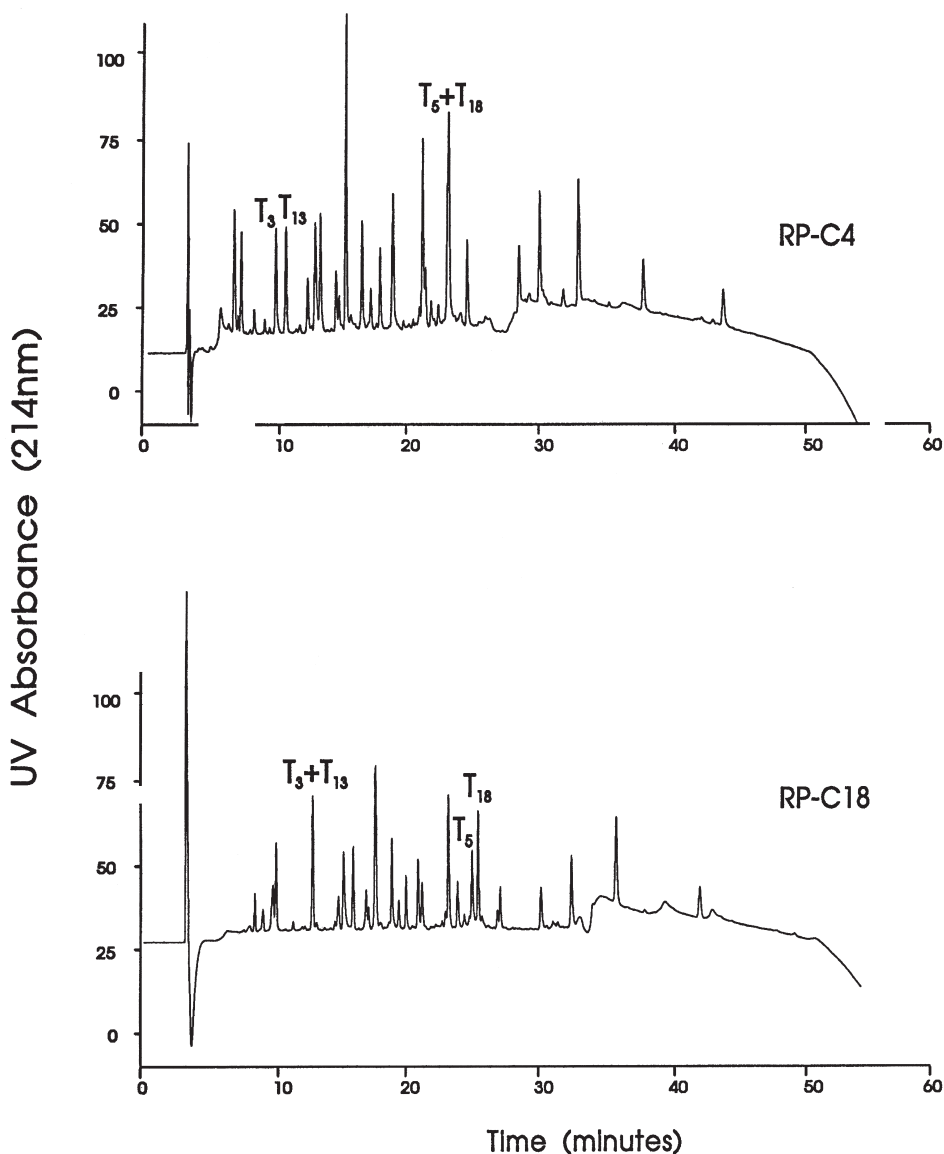


Fig. 3. The influence of *n*-alkyl chain length on the separation of tryptic peptides derived from porcine growth hormone. **Top:** Bakerbond (J. T. Baker, Phillipsburg, NJ) RP-C4, 25 cm  $\times$  4.6 mm id, 5  $\mu$ m particle size, 30 nm pore size. **Bottom:** Bakerbond (J. T. Baker) RP-C18, 25 cm  $\times$  4.6 mm id, 5  $\mu$ m particle size, 30 nm pore size. Conditions, linear gradient from 0–90% acetonitrile with 0.1% TFA over 60 min, flow rate of 1 mL/min, 25°C (Reproduced from **ref. 1** by permission of Academic).

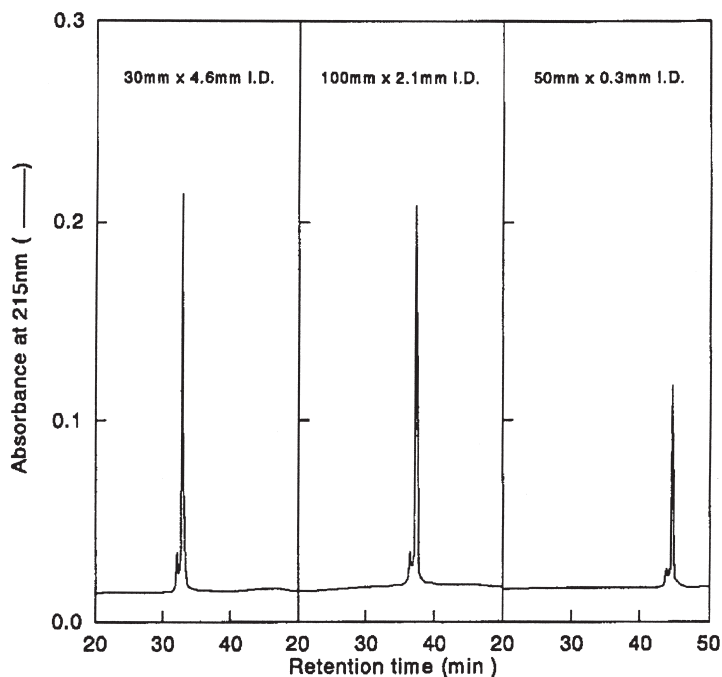


Fig. 4. Effect of column internal diameter on detector sensitivity. Column: Brownlee RP-300 C8 (7  $\mu\text{m}$  particle size, 30 nm pore size), 3 cm  $\times$  4.6 mm id and 10 cm  $\times$  2.1 mm id (Applied Biosystems) and 5 cm  $\times$  0.32 mm id. Conditions: linear gradient from 0–60% acetonitrile with 0.1% TFA over 60 min, 45°C. Flow rates, 1 mL/min, 200  $\mu\text{L}/\text{min}$ , and 4  $\mu\text{L}/\text{min}$  for the 4.6, 2.1, and 0.32 mm id columns, respectively. Sample loadings, lysozyme, 10  $\mu\text{g}$ , 4  $\mu\text{g}$ , and 0.04  $\mu\text{g}$  for the 4.6, 2.1, and 0.32 mm id columns, respectively. (Reproduced from **ref. 17** by permission of Elsevier Science, copyright 1992.)

diameter between 0.2–0.4 mm and column length of 15 cm result in the analysis of femtomole of sample (*see* Chapter 10). The effect of decreasing column internal diameter on detection sensitivity is shown in **Fig. 4** for the analysis of lysozyme on a C18 material packed into columns of 4.6, 2.1, and 0.3 mm id (**17**).

3. The geometry of the particle in terms of the particle diameter and pore size, is also an important feature of the packing material. Improved resolution can be achieved by decreasing the particle diameter and the most commonly used range of particle diameters for analytical scale RP-HPLC is 3–5  $\mu\text{m}$ . There are also examples of the use of nonporous particles of smaller diameter (**18**). For preparative scale separations, 10–20  $\mu\text{m}$  particles are utilized. The pore size of RP-HPLC sorbents is also an important factor that must be considered. For peptides, the pore size generally ranges between 100–300 Å depending on the size of the peptides. Porous materials of  $\geq 300$  Å pore size are necessary for the separation of proteins, as the

solute molecular diameter must be at least one-tenth the size of the pore diameter to avoid restricted diffusion of the solute and to allow the total surface area of the sorbent material to be accessible. The development of particles with 6000–8000 Å pores with a network of smaller pores of 500–1000 Å has also allowed very rapid peptide and protein separations to be achieved (19,20).

4. RP-HPLC is generally carried out with an acidic mobile phase, with TFA the most commonly used additive because of its volatility. However, for high sensitivity applications, the amount of TFA in buffer B can be adjusted downward because phosphoric acid, perchloric acid, formic acid, hydrochloric acid, acetic acid, and heptafluorobutyric acid have also been used (21–23). Alternative additives such as nonionic detergents can be used for the isolation of more hydrophobic proteins such as membrane proteins (24, *see* Chapter 22).
5. One of the most powerful characteristics of RP-HPLC is the ability to manipulate solute retention and resolution through changes in the composition of the mobile phase. In RP-HPLC, peptide and protein retention is a result of multisite interactions with the ligands. The practical consequence of this is that high resolution isocratic elution of peptides and proteins can rarely be achieved as the experimental window of solvent concentration required for their elution is very narrow. Mixtures of peptides and proteins are therefore routinely eluted by the application of a gradient of increasing organic solvent concentration. The three most commonly employed organic solvents in RP-HPLC are acetonitrile, methanol, and 2-propanol, which all exhibit high optical transparency in the detection wavelengths used for peptide and protein analysis. Acetonitrile provides the lowest viscosity solvent mixtures and 2-propanol is the strongest eluent. An example of the influence of organic solvent is shown in **Fig. 5** where changes in selectivity can be observed for a number of peptide peaks in the tryptic map. In addition to the elutotropic effects, the nature of the organic solvent can also influence the conformation of both peptides and proteins and will, therefore, have an additional effect on selectivity through changes in the structure of the hydrophobic contact region. In the case of proteins, this may also impact on the level of recovery of biologically active material.
6. The typical experiment with an analytical scale column would utilize flow rates ranging between 0.5–2.0 mL/min. With microbore columns (1–2 mm id) flow rates of 50–250  $\mu$ L/min are used, whereas for capillary columns of 0.2–0.4 mm id, flow rates of 1–4  $\mu$ L/min are applied. At the preparative end of the scale with columns of 10–20 mm id, flow rates ranging between 5–20 mL/min are required.
7. Detection of peptides and proteins in RP-HPLC, generally involves detection between 210 and 220 nm, which is specific for the peptide bond, or at 280 nm, which corresponds to the aromatic amino acids tryptophan and tyrosine. The use of photodiode array detectors can enhance the detection capabilities by the on-line accumulation of complete solute spectra. The spectra can then be used to identify peaks specifically on the basis of spectral characteristics and for the assessment of peak purity (24–26). In addition, second derivative spectroscopy can provide information on the conformational integrity of proteins following elution (27,28)

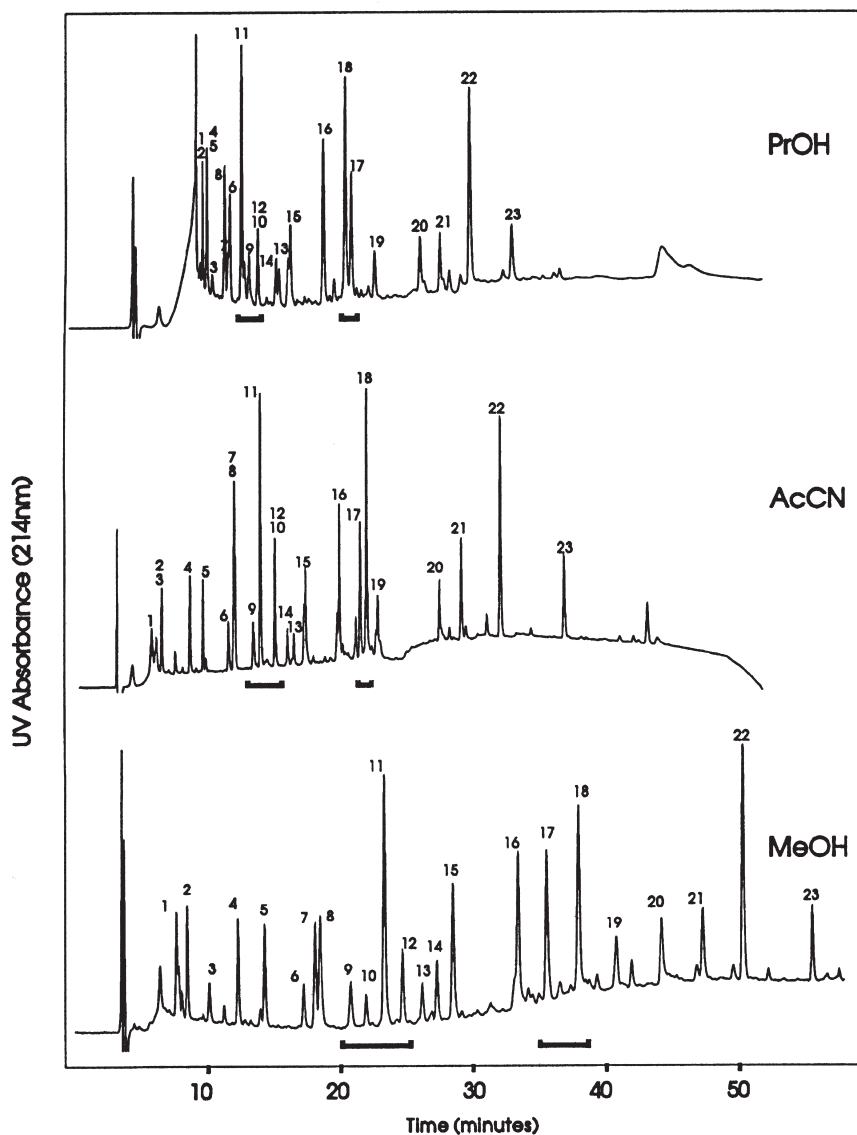


Fig. 5. The influence of organic solvent on the RPC of tryptic peptides derived from porcine growth hormone. Column: Bakerbond (J. T. Baker) RP-C4, 25 cm  $\times$  4.6 mm id, 5  $\mu$ m particle size, 30 nm pore size. Conditions, linear gradient from 0–90% 2-propanol (top), acetonitrile (middle) or methanol (bottom) with 0.1%TFA over 60 min, flow rate of 1 mL/min, 25°C. (Reproduced from *ref. 1* by permission of Academic.)

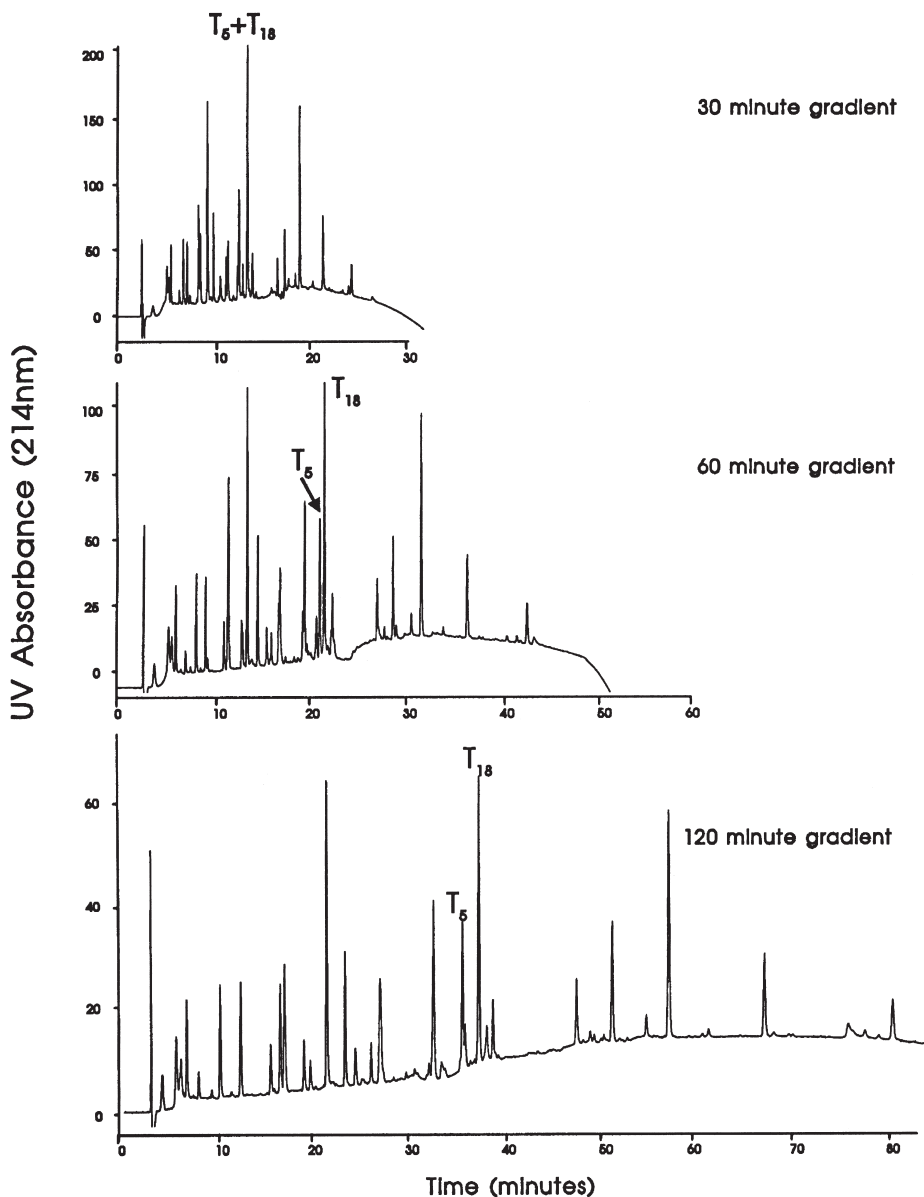


Fig. 6. Effect of gradient time on the reversed phase chromatography of tryptic peptides of porcine growth hormone. Column: Bakerbond (J. T. Baker) RP-C4, 25 cm  $\times$  4.6 mm id, 5  $\mu$ m particle size, 30 nm pore size. Conditions, linear gradient from 0–90% acetonitrile with 0.1% TFA over 30 min (top), 60 min (middle), or 120 min (bottom) at a flow rate of 1 mL/min, 25°C. (Reproduced from **ref. 1** by permission of Academic.)

8. The operating temperature can also be used to manipulate resolution. Although the separation of peptides and proteins is normally carried out at ambient temperature, solute retention in RP-HPLC is influenced by temperature through changes in solvent viscosity. In addition to this, peptide and protein conformation can also be manipulated by temperature. Changes in temperature can therefore also be used to manipulate the structure and retention of peptide mixtures. For peptides, it has been shown that secondary structure can actually be enhanced through binding to the hydrophobic sorbent (29). In the case of proteins that are to be subjected to further chemical analysis and thus where recovery of a biologically active protein is not essential, increasing temperature can be used to modulate retention via denaturation of the protein structure (3–6). However, if the efficient recovery of both mass and biological activity is of paramount importance, the use of elevated temperatures is not an option.
9. The choice of gradient conditions will depend on the nature of the molecules of interest. The influence of gradient time on the separation of a series of tryptic peptides proteins is shown in **Fig. 6 (I)**. Generally the use of longer gradient times provides improved separation. However, these conditions also increase the residence time of the peptide or protein solute at the sorbent surface, which may then result in an increase in the degree of denaturation.

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