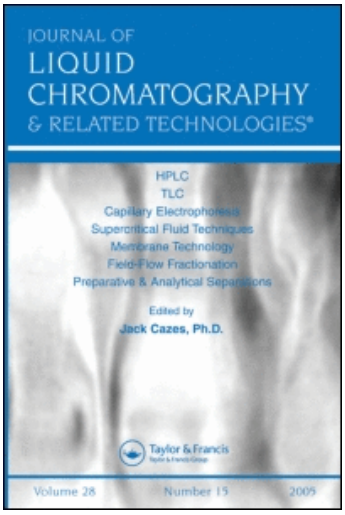

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Effect of Experimental Parameters on the HPLC Separation of Peptides and Proteins

Haleem J. Issaq[a]; Stephen D. Fox[a]; Megha Mahadevan[a]; Thomas P. Conrads[a]; Timothy D. Veenstra[a]
[a] Analytical Chemistry Laboratory, SAIC-Frederick, Inc., Frederick, Maryland, USA

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Effect of Experimental Parameters on the HPLC Separation of Peptides and Proteins

Haleem J. Issaq,* Stephen D. Fox, Megha Mahadevan, Thomas P. Conrads, and Timothy D. Veenstra

Analytical Chemistry Laboratory, SAIC-Frederick, Inc., NCI-Frederick, Frederick, Maryland, USA

ABSTRACT

This manuscript examines the effects of different experimental parameters on resolution, peak symmetry, peak width, and selectivity (peak elution order) of peptides by micro high performance liquid chromatography. The experimental parameters are: mobile phase composition, flow rate, organic modifiers, ion-pairing agents, column temperature, the effect of packing material properties: particle size, particle porosity and reversed-phase (RP) alkyl chain length, and column dimensions. When a mass spectrometer (MS) is used as the detector in micro-HPLC, certain experimental parameters such as mobile phase flow rate and buffer composition have to be adjusted in order to meet the requirements of the MS procedure employed. When electrospray ionization (ESI) is the selected MS mode of operation the mobile phase flow rate should be in

*Correspondence: Haleem J. Issaq, Analytical Chemistry Laboratory, SAIC-Frederick, Inc., NCI-Frederick, P.O. Box B, Frederick, MD 21702-1201, USA; E-mail: issaqh@ncifcrf.gov.

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nL min^{-1} and a volatile buffer should be used to achieve maximum sensitivity. Also, it was found that the elution order (selectivity) of peptides in RP-HPLC is affected by type and concentration of ion-pairing agent, organic modifier, column temperature, pH of the buffer, and the alkyl chain length of the derivatizing agent in RP. This manuscript includes work that has been done in our laboratory and is supplemented by data published by other researchers.

Key Words: Proteomics; Proteins; Peptides; μHPLC; ESI–MS.

INTRODUCTION

High performance liquid chromatography is a well established separation technique that has been used for the separation of small, as well as, large biomolecules such as proteins and peptides.[1–9] Recently, with the advent of proteomics and the use of mass spectrometry (MS) for peptide and protein identification, HPLC, a liquid separation technique that is easily coupled on-line to a MS, is playing a central role in the fractionation and separation of complex protein mixtures and digests. HPLC coupled to a MS allows, in addition to detection, accurate mass measurement that cannot be achieved by HPLC with UV detection. In order to successfully couple HPLC with electrospray ionization (ESI) MS, and to achieve maximum sensitivity it is necessary to lower the flow rate of the mobile phase and to use a volatile buffer. In analytical HPLC, with UV or fluorescence detection, buffer composition and flow rate are not as critical parameters as in the case of on-line HPLC-MS. In HPLC-UV and HPLC-fluorescence, the critical requirement is that the mobile phase should not absorb/fluoresce at the wavelength of the analyte. In HPLC-MS, the mobile phase composition and flow rate are two critical experimental parameters that should be adjusted to meet the requirements of ESI–MS. For example, although sodium phosphate is used to fractionate a peptide mixture by ion exchange chromatography with UV detection, such a buffer system cannot be used when a MS is the detector, because nonvolatile salt content of the buffering system, sodium phosphate, would interfere with the effective ionization of peptides and would affect the sensitivity, i.e., signal intensity, of the MS. The analyst then should use volatile buffers such as ammonium formate or ammonium acetate. Trifluoroacetic acid (TFA) is an excellent ion-pairing agent that is routinely used for the separation of proteins and peptides by HPLC-UV, however, TFA is not recommended for HPLC-MS because of competitive ionization that inhibits the MS signal. Also, compounds such as sodium dodecyl sulfate (SDS) or cyclodextrins that are used with HPLC-UV to

effect a separation, and protein solubilizing agents such as urea, cannot be used with ESI–MS. The MS sensitivity can be improved by lowering the mobile phase flow rates to below 1 μL min^{-1}. In a recent study, Smith and his coworkers[9] reported that MS sensitivity increases linearly with decreasing flow rate in the range of 20–400 nL min^{-1}.

This paper examines the effects of different experimental parameters on resolution, peak symmetry, peak width, and selectivity (peak elution order). The experimental parameters that will be examined are: mobile phase composition and pH, flow rate, organic modifiers, ion-pairing agents, and column temperature. Also, the effect of packing material properties; particle size, particle porosity and reversed-phase (RP) alkyl chain length, and column dimensions on the separation of peptides and proteins will be reviewed. This manuscript includes work that has been done in our laboratory and is supplemented by data published by other researchers.

EXPERIMENTAL

Materials

An HPLC peptide standard mixture and bovine serum albumin were purchased from Sigma (St. Louis, MO) and used without further purification. The peptide standard mixture contained 0.125 mg Gly-Tyr, and 0.5 mg each of Val-Tyr-Val, methionine enkephalin, leucine enkephalin, and angiotensin II. Acetonitrile and methanol were purchased from EM Science (Gibbstown, NJ). Triflouroacetic acid, acetic acid, formic acid (FA), and heptafluorobutyric acid (HFBA), were purchased from Fluka (Milwaukee, WI).

Instrumentation

The HPLC instrument used was from Agilent Technologies (Wilmington, Delaware), model 1100, equipped with a photodiode array UV detector (500 nL flow cell), and a Chem Station for data analysis. The columns used were, 0.5 mm i.d., 15 cm long, packed with Zorbax SB-C18, compliments of Agilent Technologies (Wilmington, Delaware) and a 75 μm × 10 cm fused silica capillary, packed in-house, with 5 μm C-18, 300 Å pore size, Jupiter, supplied free of charge by Phenomenex (Torrance, CA). Mass spectrometric results were generated using a LCQ Deca XP from ThermoFinnigan (San Jose, CA).

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Methods

Mobile phase gradients were prepared from methanol or acetonitrile in double distilled water using a NANOpure Diamond water system (Barnstead International, Dubuque, Iowa) and an ion-pairing agent. The solid peptide standard mixture was dissolved in 5 mL double distilled water. Detection was set at 214 nm. Bovine serum albumin was digested over night using trypsin and was analyzed by microHPLC-ESI/MS at a flow rate of 500 nL min^{-1}.

The Column

Column Selection

The column packing material in HPLC is determined based on the chemical (hydrophobic, hydrophilic, ionic) and physical (size) properties of the mixture to be resolved. After selection of the column packing material, the mobile phase is determined based on the column support material and the mixture's properties. Column dimensions are a function of different parameters that will be discussed in the following section.

Column Dimensions

The column dimensions in HPLC, in general, are a function of the packing materials' particle size; type, volume, concentration, and complexity of the sample to be analyzed; injection volume and detection mode. In analytical HPLC a 5 μm particle size is commonly used. The column dimensions are 1–4.6 mm i.d. and from 50 to 300 mm in length. Smaller particle sizes, 1–3 μm, are packed into shorter columns (30–100 mm) and not into long, 20–30 cm, columns due to (a) high back pressure and (b) the greater separation efficiency because of higher surface area. The column dimensions used in analytical HPLC requires flow rates of approximately 0.1–1.5 mL min^{-1}, that are not compatible with micro-HPLC/ESI–MS detection. Also, when analyzing proteins that are extracted from two-dimensional gel electrophoresis (2-DGE) spots the sample concentration is in the low nanogram range, which cannot be detected using an analytical HPLC column (4.6 × 50 mm) and UV/V is due to dilution effects. In micro- and nano-HPLC the column dimensions are 15 μm–1 mm in i.d. and 10–90 cm long. Although better separation is achieved using a longer column, the length of the column is limited by the degree of solute diffusion that, in turn, would affect peak height and sensitivity. Figure 1 shows the effect of the columns internal diameter on the detection of 2 pmol of a

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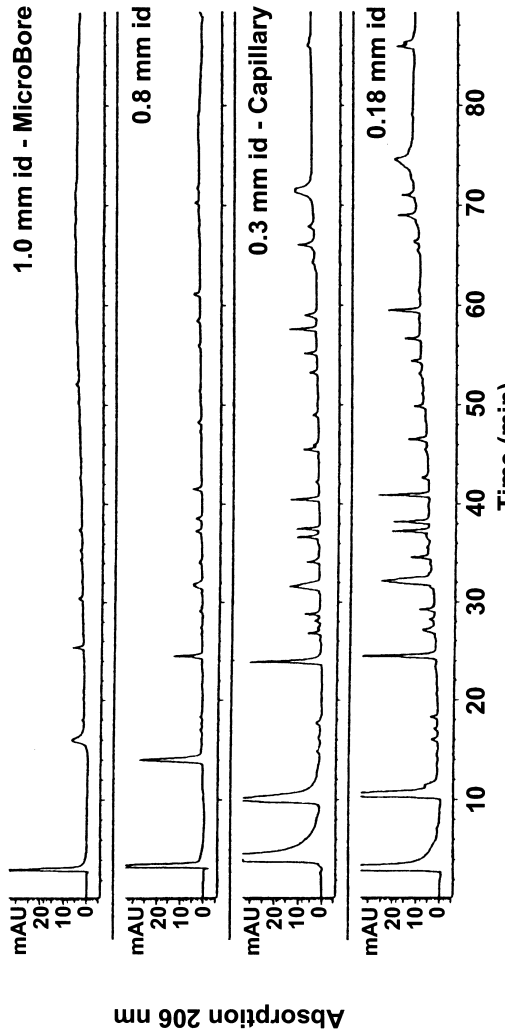

Figure 1. Comparative peptide map of a 2 pmol tryptic myoglobin digest on four columns of different inner diameter. Reprinted with permission from Agilent Technologies.

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myoglobin tryptic digest. Note that the sensitivity increased with decrease in column i.d. when the same sample load is used.

Column Packing Material—The Stationary Phase

Particle Size

Packing material particle size contributes to the efficiency of the column and resolution of the mixture. The smaller the particle size, the shorter the column length due to increased surface area, the higher the efficiency, and a better separation is achieved. The drawbacks in using very small particles, 1 μm, is: (a) an increase in backpressure; (b) may not be possible to find frits that will hold the particles in the column without them leaking out; and (c) clogging of such frits becomes a problem.

Particle Porosity

Porosity of the silica particles used for peptide analysis is a function of the size of the molecules to be resolved. When the column is used to resolve a small size peptide mixture, up to 30 residues, 80–90 Å pore size is acceptable. However, for resolving a polypeptide and a protein mixture, 300 Å pore size particles or larger are recommended. Also, proper pore size selection results in faster elution, sharper peaks, and better quantitation. Figure 2 demonstrates the effect of particle pore size, 90 Å vs. 300 Å, on the separation of a myoglobin tryptic digest.

Not all columns used for protein and peptide separation are packed with totally porous silica. Agilent Technologies, Inc., developed a solid core 5 μm silica particles with a 0.25 μm wide porous outer shell, 300 Å pore size for the fast separation of proteins and peptides. The porous outer shell has been derivatized with different alkyl chain lengths (C-3, C-8, C-18) and with a —CN group. The speed of analysis using the porous shell packed columns vs. conventional porous silica particles packed columns of the same size is due to the short diffusion distances. Four columns, each packed with different derivatized silica, were used for the separation of large polypeptides, Fig. 3, which resulted in different selectivities due to the different interactions between the polypeptides and the four different packing materials.

Nonporous Silica

Not all columns used for the separation of proteins/peptides are packed with porous silica. Wall et al.[10] used columns packed with 1.5 μm nonporous RP, C-18, silica particles for the separation of proteins having a molecular size of 12–75 kDa.

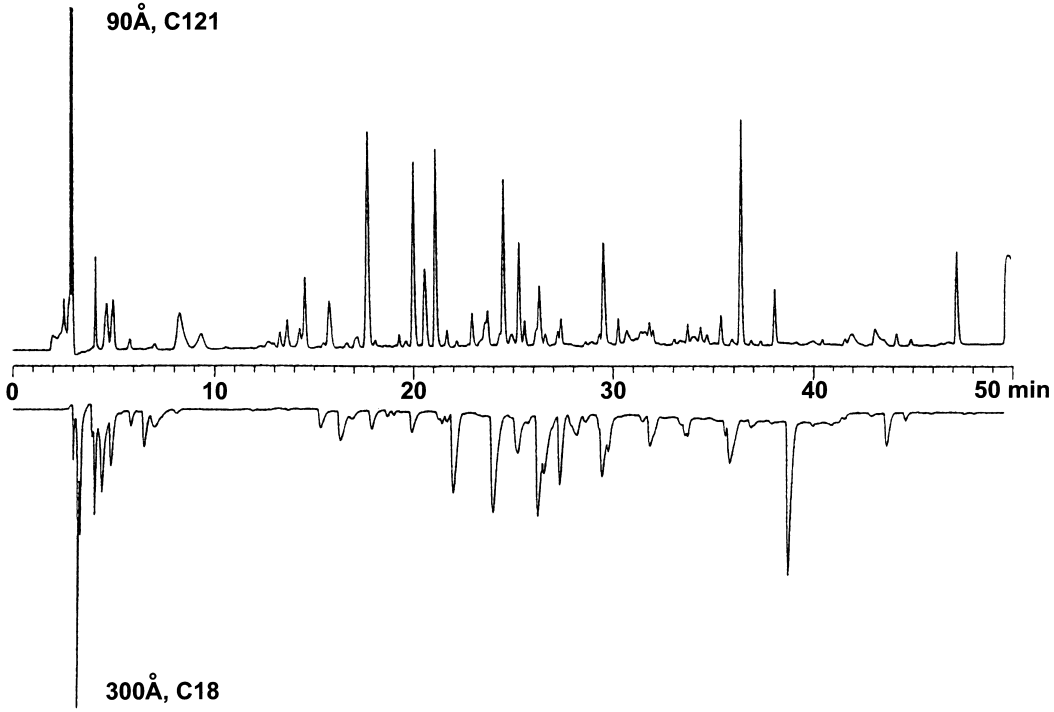

Figure 2. Effect of silica pore size, 90 Å vs. 300 Å, on the resolution of a myoglobin tryptic digest. Reprinted with permission from Phenomenex.

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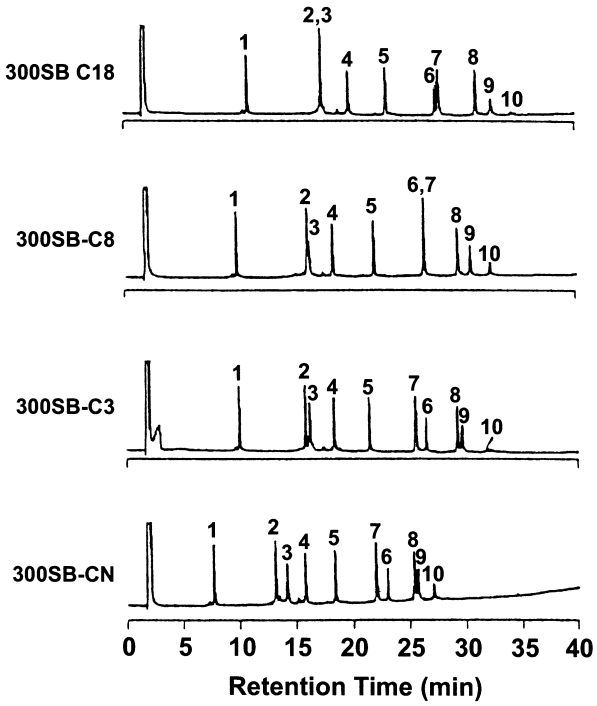

Figure 3. Comparison of the separation of polypeptides by HPLC using different bonded phases, namely C-18, C-8, C-3, and C—CN, using a linear gradient of 25–70% B in 40 min; A: 0.1% TFA in water and B: 0.09% TFA in 80% acetonitrile/20% water at 60°C. (1) Rnase, (2) insulin, (3) cytochrome C, (4) lysozyme, (5) pavalbumin, (6) CDR, (7) myoglobin, (8) carbonic anhydrase, (9) S-100b, and (10) S-100a. Reprinted with permission from Agilent Technologies.

Perfusion Chromatography

The columns in perfusion chromatography are made from a polymeric material, polystyrene/divinylbenzene (PS/DVB) particles, and have pores ranging between 800 and 8000 Å. Such columns give very fast separation of proteins due to increased flow rates. The polymeric particles are rugged and more stable than silica based particles. In contrast to conventional chromatography media, perfusion chromatography media particles are engineered to have two discrete classes of pores. Large "throughpores," 6000–8000 Å, allow convection flow to occur through the particles themselves, quickly carrying sample molecules to short "diffusive" pores, 800–1500 Å. By reducing

the distance over which diffusion needs to occur, the time required for sample molecules to interact with interior binding sites is also reduced. For more information consult Ref.[11]

Monolithic Columns

The most recent addition to column packing technology for the separation of proteins is the monolithic column.[12] Separation columns for micro-HPLC are usually prepared by packing fused-silica capillaries 50–320 μm i.d. with silica particles. However, the void volume between the packed particles, and the slow mass transfer of solutes are the limiting factors for the separation efficiency of porous packing materials, especially for proteins and peptides having low diffusivities. Monolithic stationary phases, in which the separation medium consists of a continuous rod of a rigid, porous polymer that has no interstitial volume but only internal porosity consisting of micro- and macro-pores, is a new stationary-phase with enhanced mass-transfer properties. The mobile phase flows through the channels of the porous separation medium, resulting in enhanced mass transport and improved chromatographic efficiency. Polymer-based monolithic chromatographic supports are usually prepared by the polymerization of a mixture of suitable monomers and porogens in the capillary forming one solid support. The permanent porosity in the monolith is created upon phase separation of the solid polymer from the liquid porogens during the course of polymerization. Monolithic capillary columns have been successfully applied to the separation of peptides[13,14] and proteins.[15] Monolithic PS/DVB has been used as a chromatographic support for on-line hyphenation of liquid chromatography and tandem mass spectrometry.[16]

Alkyl Chain Length

The alkyl chain length of the bonded aliphatic chain to the silica in RP-HPLC is an important parameter that deserves attention when resolving a mixture of proteins and peptides. The longer the alkyl chain, the more hydrophobic is the column's properties. Therefore, in resolving a protein/peptide mixture there exists the possibility of hydrophobic–hydrophobic interaction between the protein/peptide and the alkyl chain, which may lead to solute losses. It is hard to assess such losses when resolving a whole tissue or cell proteins or protein digest due to the large number, thousands, of proteins/peptides. Different selectivities are obtained when columns with different alkyl chain lengths are used, Fig. 3.

Other Packing Materials

In addition to RP chromatography, other chromatographic modes, which utilize packing materials other than alkyl derivatized silicas, have been used for protein and peptide fractionation and/or separation. These include ion exchange,[17] size exclusion,[17] affinity,[17] and hydrophobic interaction chromatography.[17] However, these separation techniques are used in proteomic research mostly as a first step in a two-dimensional separation scheme of complex protein/peptides mixtures.

The Mobile Phase

The mobile phase in HPLC is determined based on the sample properties and the type of packing material selected. A review of the scientific literature reveals that separation of peptides is mostly achieved using a RP column and a linear gradient of acetonitrile/water/TFA in varying percentages and gradient slopes. Gradient, not isocratic elution is required when analyzing a complex peptide mixture, such as cell protein digests, due to the complexity and the differing properties of the various peptides. Generally, gradient elution gives a higher degree of separation than isocratic elution.

Mobile Phase Composition

There are two main components to the RP-HPLC mobile phase used for the separation of peptides and proteins: (a) organic modifiers; and (b) ion-pairing agents. The discussion of the mobile phase will be limited to RP since it is the most popular mode of HPLC separation of proteins/peptides in proteomic research.

Organic Modifiers

Published studies indicate that acetonitrile is the preferred organic modifier for the separation of proteins and peptides. Methanol and tetrahydrofuran are rarely used. The gradients used are: solvent A is 0.1% TFA in water; and solvent B is 0.1 TFA in acetonitrile. Gradients start with 5% A to 30–70% B in 30 min and, in certain cases to hours, depending on the resolution required. Figure 4 shows the separation of a five peptides mixture using a 30 min gradient; 15–65% methanol/0.1% TFA (A) and 5–35% acetonitrile/0.1 TFA (B) as organic modifiers, both in 0.1% water. The five peptides were resolved in both systems with no change in selectivity. The organic modifier does, in certain cases, affect the selectivity. For example, the separation of the same five peptides mixture in different acetonitrile 0.1% TFA gradients, namely 5–35, 5–50, and 5–75%, Fig. 5, gave different

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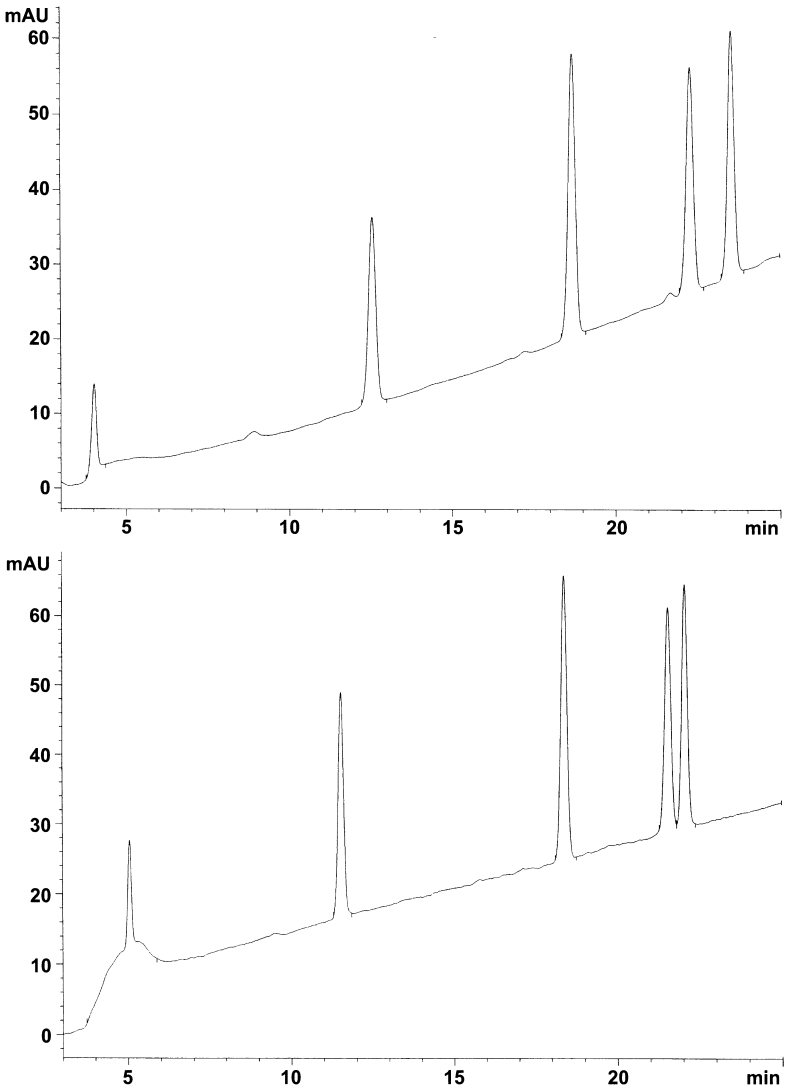

Figure 4. Effect of the organic modifiers methanol and acetonitrile on the separation of a test mixture of five peptides using a linear gradient in 30 min of 5–65% methanol (top) and 5–35% acetonitrile (bottom). The peptides, from left to right, are Gly-Tyr, Val-Tyr-Val, methionine enkephalin, leucine enkephalin, and angiotensin II.

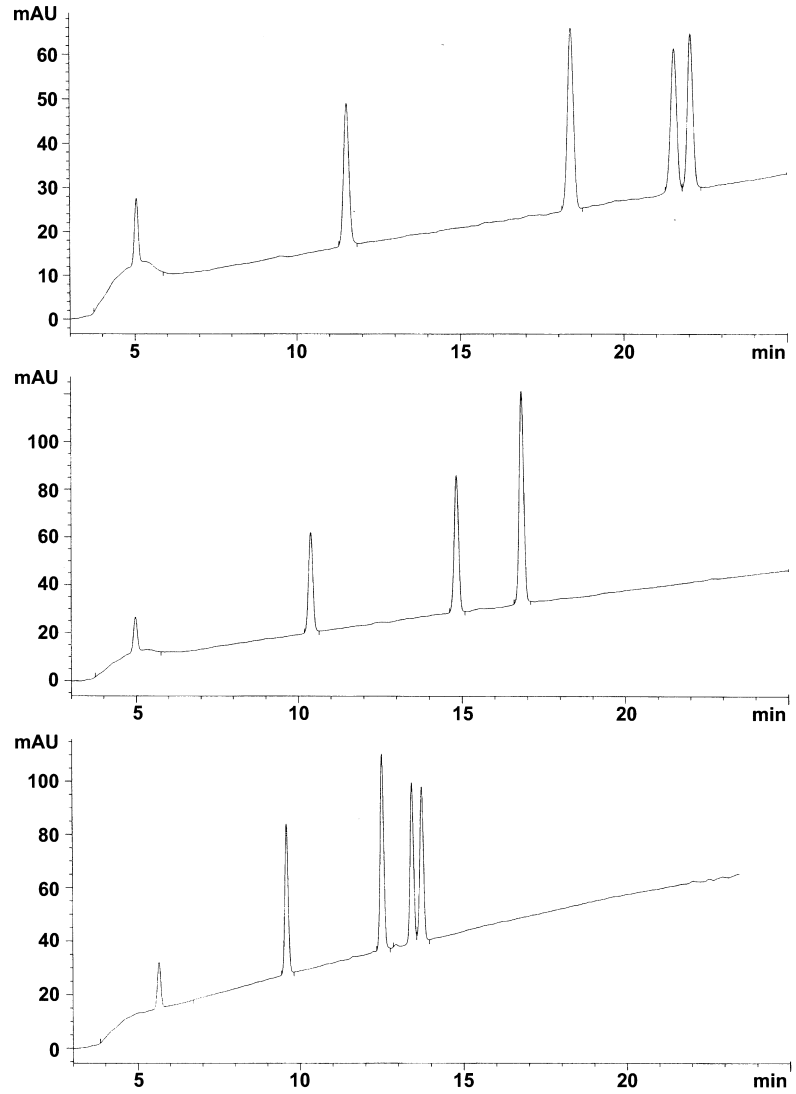

Figure 5. Effect of acetonitrile concentration in the linear gradient on the separation of the five peptide test mixture. Note that leucine enkephalin eluted before angiotensin II when 5–35% acetonitrile was used, coeluted in 5–50% and shifted positions in 5–75%. Gradient time 30 min. Other experimental conditions as in Fig. 4.

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selectivities of the last two peaks, which coeluted in 5–50% while they were resolved in 5–35 and 5–75% with different elution order. Table 1 shows the effect of the two organic modifiers, methanol and acetonitrile, when used for the separation of the test peptide mixture, on different experimental parameters.

The effect of ACN concentration in the mobile phase on the experimental parameters is given in Table 2.

Ion-Pairing Agents

Trifluoroacetic acid is the most commonly used mobile phase additive for RP-HPLC separation of protein/peptide mixtures. However, due to its interference in the ionization process in ESI/MS and loss of signal, FA is substituted for TFA. Different groups have studied the effect of different additives (ion-pairing agent's) type and concentration on the resolution of a mixture of proteins/peptides. Huber and Premstaller[18] studied the effect of volatile eluents on the separation of proteins by RP-HPLC-ESI-MS using microbore-columns packed with 2.3 μm highly crosslinked, octadecylated PS–DVB particles. Proteins were eluted at 80–90°C with gradients of acetonitrile in 0.10–0.50% aqueous solutions of TFA, FA or acetic acid (HOAc). Substitution of TFA by FA resulted in a 35–160-fold improvement in analyte detectability and 32–104% increase in peak width at half height.

In our laboratory, we studied the effect of different ion-pairing agents on the elution time, selectivity, resolution, and MS signal intensity by RP-C18 packed micro HPLC columns. Figure 6 shows the effect of different ion-pairing agents; TFA, HFBA, and FA on the resolution of a cytochrome C digest under the same experimental conditions. Table 3 gives the symmetry, peak width at half height, resolution, and selectivity of each of the tested

Table 1. Effect of organic modifiers on peak symmetry, peak width at half height, resolution, and selectivity of a test peptide mixture using gradient elution in 30 min.

Solvent (5–65%)	Symmetry		Peak width		Resolution		Selectivity	
	3[a]	5[a]	3[a]	5[a]	(2–3)[a]	(4–5)[a]	(2–3)[a]	(4–5)[a]
0.1% TFA in MeOH	1.13	1.02	0.20	0.19	14.23	2.60	1.30	1.03
0.1% TFA in ACN	1.06	1.01	0.12	0.12	16.44	3.85	1.34	0.99[b]

Note: Other details are in text.
[a]Peak(s).
[b]Peak 5 eluted before peak 4.

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Table 2. Effect of acetonitrile concentration in gradient HPLC on peak symmetry, peak width at half height, resolution, and selectivity of a test peptide mixture.

	0.1% TFA in ACN							
	Symmetry		Peak width		Resolution		Selectivity	
Gradient (%)	3[a]	5[a]	3[a]	5[a]	(2–3)[a]	(4–5)[a]	(2–3)[a]	(4–5)[a]
5–75	1.03	0.99	0.12	0.11	14.93	1.50	1.30	0.98[b]
5–70	1.03	1.01	0.12	0.12	15.34	1.69	1.32	0.98[b]
5–50	1.06	1.00	0.15	0.15	18.36	8.09	1.43	1.13
5–30	1.07	0.98	0.18	0.17	20.26	0.82	1.52	1.01

Note: Other details are in text.
[a]Peak(s).
[b]Peak 5 eluted before peak 4.

ion-pairing agents in a 5–35% acetonitrile gradient. The results indicate that TFA gives the best overall results.

Figure 7 shows the effect of ion-pairing agent (TFA) concentration on resolution and on selectivity of test peptides mixture. Note, that the elution order for peaks 4 and 5 at different concentrations of TFA, have changed, and the peak symmetry and other parameters have been affected as shown in Table 4.

We also tested the effect of HFBA concentration on peak symmetry, width at half height, resolution, and selectivity, Table 5. Changing the concentration of HFBA from 0.05% to 0.00625% affected the overall retention time and peak symmetry and resolution, but not the elution order of peaks 4 and 5 as in the case of TFA, Fig. 8.

Figure 9a–c, show the effect of TFA and FA on the separation and MS signal intensity of a peptide digest. Although TFA gives a better resolution, a stronger, 100 fold, signal was achieved by the ESI/MS when FA was used.

Mobile Phase Flow Rate and Detector Cell Volume

Normally a flow rate of $1\,\mathrm{mL\,min^{-1}}$ is used with analytical HPLC columns. In micro- and nano-HPLC the flow rate is adjusted according to the following equation:

$$F_2 = F_1\left(\frac{\mathrm{id}_2}{\mathrm{id}_1}\right)^2$$

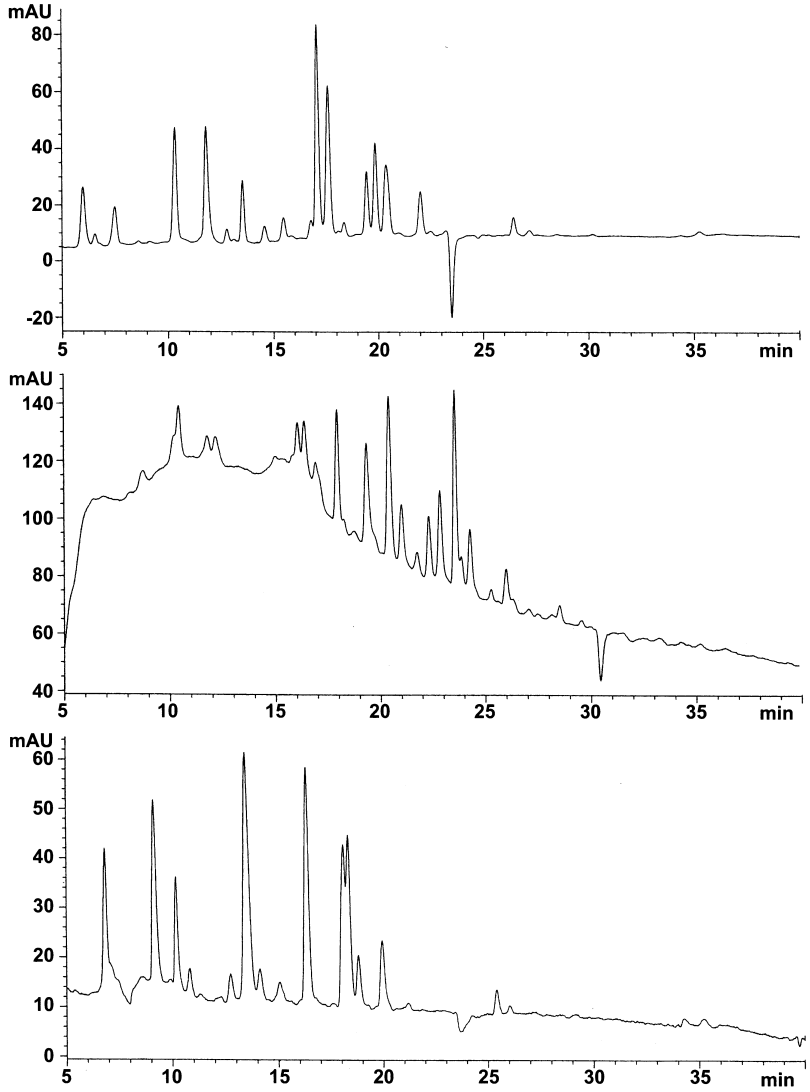

Figure 6. Effect of the ion pairing agents TFA, HFBA, and FA on the separation of a cytochrome C digest using a 5–50% acetonitrile linear gradient in 40 min. 0.1% TFA (top), 0.1% HFBA (middle), 1% FA (bottom). Other experimental conditions as in Fig. 4.

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Table 3. The effect of different ion-pairing agents on peak symmetry, peak width at half height, resolution, and selectivity of a test peptide mixture.

	ACN gradient 5–35%							
	Symmetry		Peak width		Resolution		Selectivity	
Ion pair	3[a]	5[a]	3[a]	5[a]	(2–3)[a]	(4–5)[a]	(2–3)[a]	(4–5)[a]
0.1% TFA	1.08	1.01	0.20	0.18	21.21	1.54	1.59	1.02
0.1% HFBA	0.97	0.84	0.22	0.20	5.51	—	1.09	1.01
0.1% HOAc	0.44	0.38	0.28	0.32	12.61	2.69	1.64	1.11
0.1% FA	0.71	0.66	0.22	0.24	13.90	6.36	1.64	1.16

Note: Gradient: 5–35% ACN in 30 min. Other details are in text.
[a]Peak(s).

where F_1 and F_2 are the mobile phase flow rates through columns 1 and 2, and id_1 and id_2 are the internal diameters of columns 1 and 2, respectively. Therefore, based on the above equation, if the flow rate is 1 mL min^{-1} for a 4.6 mm id column; then for 0.075 mm i.d. capillary column the flow rate should be 266 nL min^{-1}. Also, at 1 mL min^{-1} flow rate, the UV/Vis detector flow cell volume is normally 7–10 μL, which means that the volume in the cell is renewed 100–150 times min^{-1}. Therefore, at 266 nL min^{-1} the detector flow cell volume should be 2–3 nL in order to prevent peak broadening and loss of resolution. An increase of two orders of magnitude in peak height was achieved by decreasing the flow rate from 70 nL min^{-1} to 20 nL min^{-1}.[19]

Isocratic or Gradient Elution

Isocratic elution is not the right choice for resolving a complex mixture of proteins/peptides due to their different properties. Almost all separations recorded in the scientific literature for the separation of cell proteins or their digest used gradient elution or multidimensional separations.[17] Snyder et al.[20] derived the following equation for optimizing the resolution (R) in gradient elution:

$$R = \left(\frac{N}{4}\right)\alpha\left(\frac{t_g F}{S\Delta\phi V_m}\right)$$

where t_g = gradient ramp time, $\Delta\phi$ = volume fraction change of organic modifier, V_m = column dead volume, F = flow rate, α = separation factor, S = solvent strength parameter.

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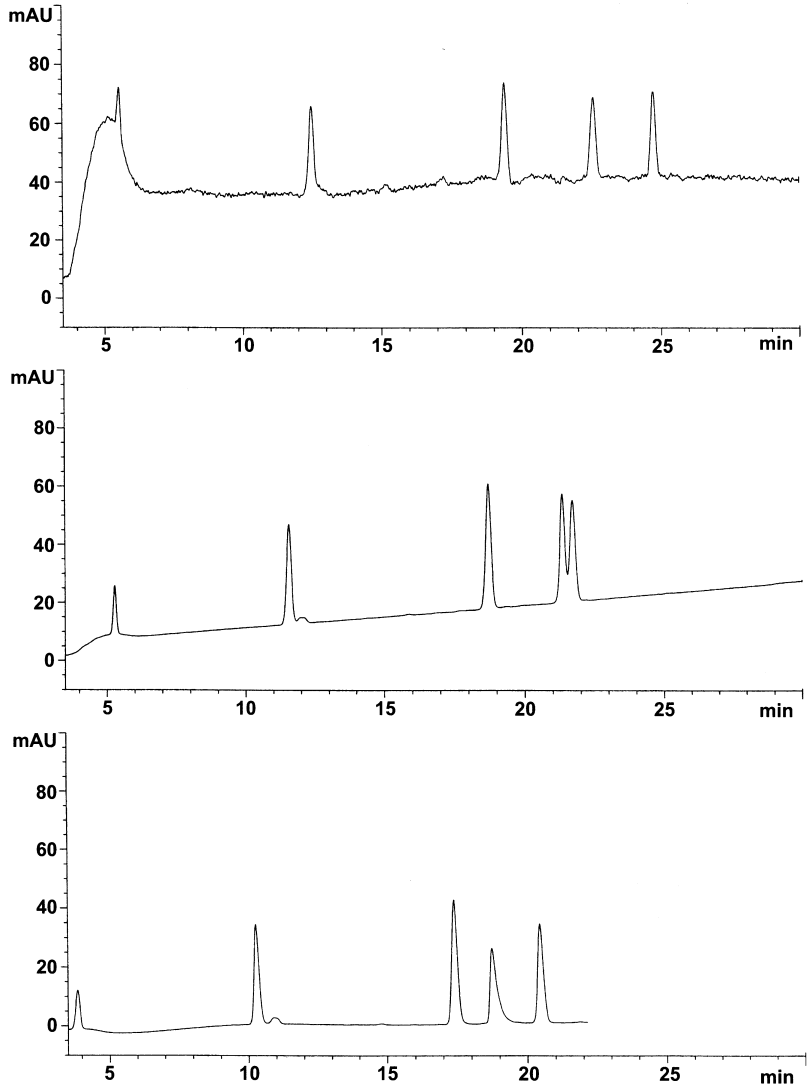

Figure 7. Effect of TFA concentration on the separation of (1) Val-Tyr-Val, (2) methionine enkephalin, (3) leucine enkephalin, and (4) angiotensin II in a 5–35% acetonitrile linear gradient in 30 min. Elution order: top, 1, 2, 3, 4; Middle, 1, 2, 4, 3; bottom, 1, 2, 4, 3. Other experimental conditions as in Fig. 4.

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Table 4. Effect of %TFA on peak symmetry, peak width at half height, resolution, and selectivity of a test peptide mixture.

	TFA ACN gradient 5–35%							
	Symmetry		Peak width		Resolution		Selectivity	
IP Conc. (%)	3[a]	5[a]	3[a]	5[a]	(2–3)[a]	(4–5)[a]	(2–3)[a]	(4–5)[a]
0.01	0.70	0.70	0.22	0.22	14.07	4.26	1.60	1.09
0.05	0.98	0.90	0.21	0.20	14.12	7.58	1.56	0.98[b]
0.50	1.06	0.94	0.33	0.35	16.40	—	1.02	1.01

Note: Gradient: 5–35% acetonitrile in 30 min. Other details are in text.
[a]Peak(s).
[b]Peak 5 eluted before peak 4.

It is clear, from the above equation, that resolution is directly proportional to t_g and F and indirectly proportional to $\Delta\phi$ and V_m.

The optimization of the organic modifier in gradient elution can be achieved by running a few experiments using either (a) constant time or (b) variable time. In constant time, the analyst determines the time required for a gradient. This time remains constant while the % organic modifier concentration is changed. For example, a series of experiments may be carried out as follows. Time of gradient 40 min, solvent A is 5% acetonitrile and solvent B 40% acetonitrile. In the next experiment solvent B is 50% acetonitrile, in the third experiment, solvent B is 60% and so on. The analyst then selects the condition that gave optimum separation. In the variable time, solvents A and B

Table 5. Effect of %HFBA on peak symmetry, peak width at half height, resolution, and selectivity of a test peptide mixture.

	HFBA in ACN gradient 5–35%							
	Symmetry		Peak width		Resolution		Selectivity	
IP Conc. (%)	3[a]	5[a]	3[a]	5[a]	(2–3)[a]	(4–5)[a]	(2–3)[a]	(4–5)[a]
0.05	0.93	0.79	0.21	0.20	1.61	11.05	1.04	1.15
0.025	0.87	0.85	0.21	0.21	17.10	8.05	1.38	1.11
0.0125	0.76	0.73	0.21	0.21	16.90	8.21	1.39	1.06
0.00625	0.65	0.60	0.22	0.23	16.40	8.39	1.42	1.03

Note: Gradient: ACN 5–35% in 30 min. Other details are in text.
[a]Peak(s).

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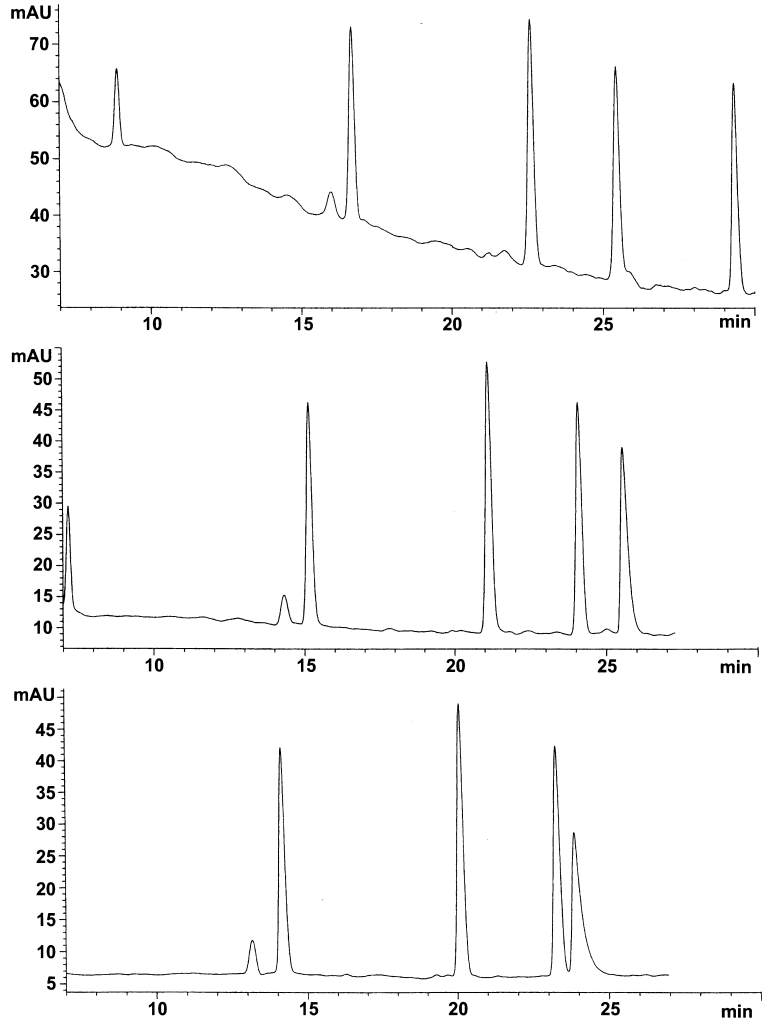

Figure 8. Effect of HFBA concentration on the separation of the peptide test mixture. Elution order as in Fig. 4. Other experimental conditions as in Fig. 4.

remain constant but the gradient time is variable. Such a process is preferable to constant time because solvents A and B remain constant and have to be prepared only once. Figure 10 shows the optimization of the separation of the cytochrome C digest using the variable time, gradient slope, approach. Solvent A is 5% acetonitrile in water and solvent B is 50% acetonitrile in water. Both

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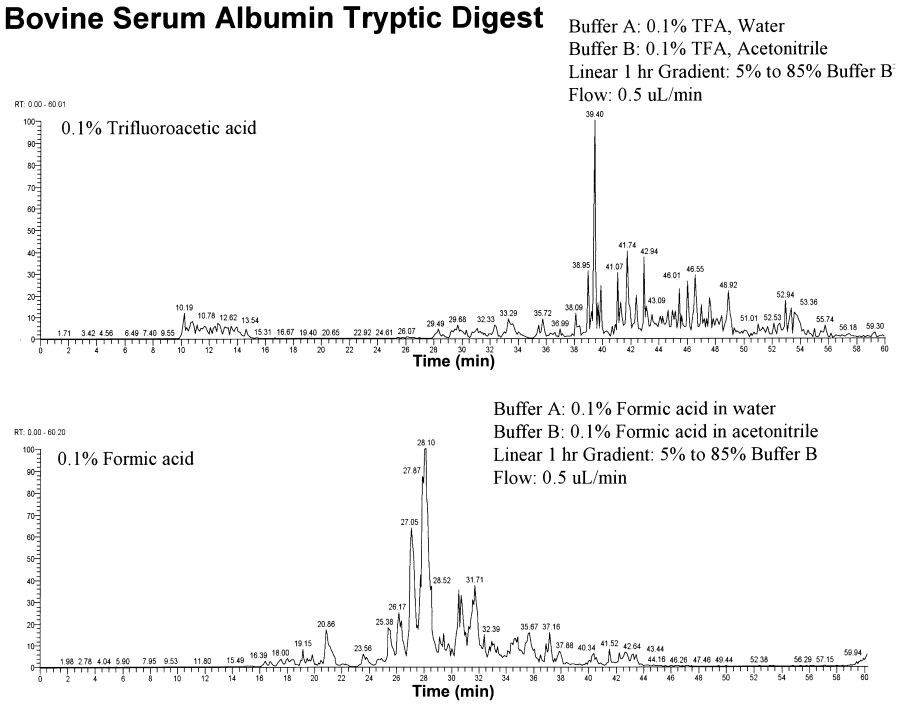

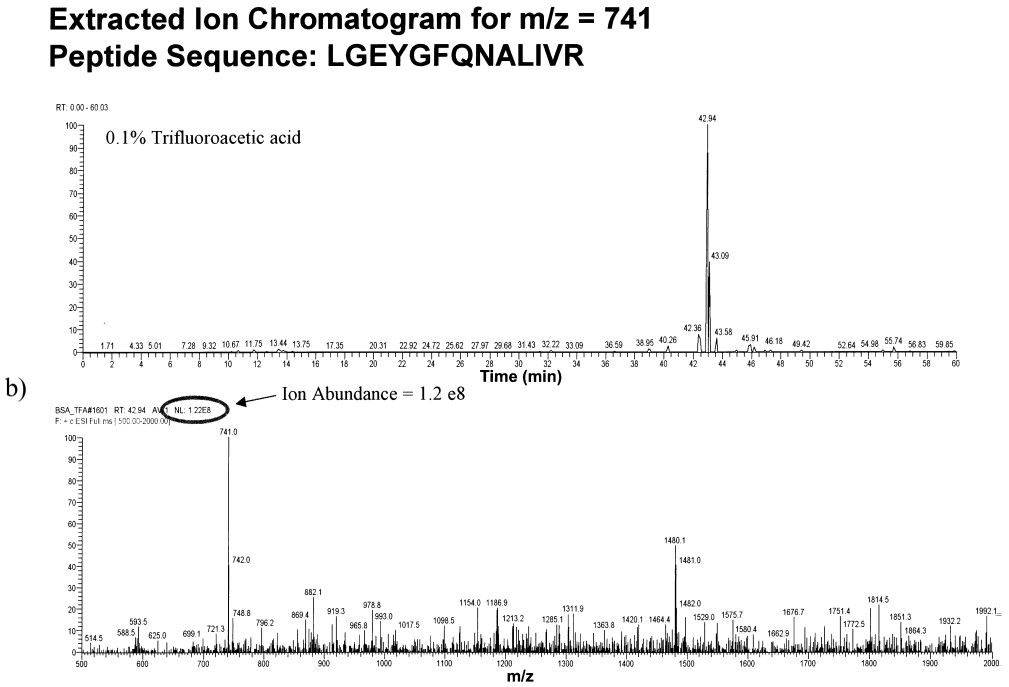

Figure 9. a. Chromatograms of the separation of bovine serum albumin tryptic digest using a 60 min linear gradient of 5–85% acetonitrile with 0.1 TFA (top) and 0.1 FA (bottom). 9b and 9c are extracted ion chromatograms (top) and ion intensity (bottom) for m/z of 741 in 0.1 TFA and 0.1 FA, respectively. Experimental parameters as in text.

(*continued*)

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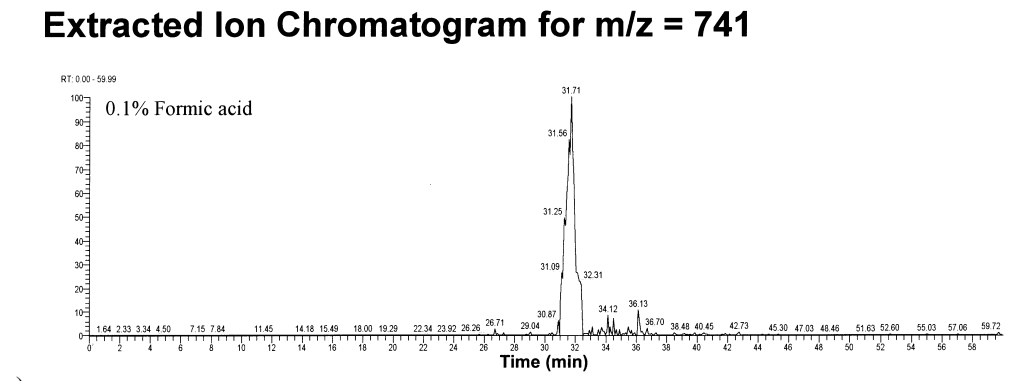

c)

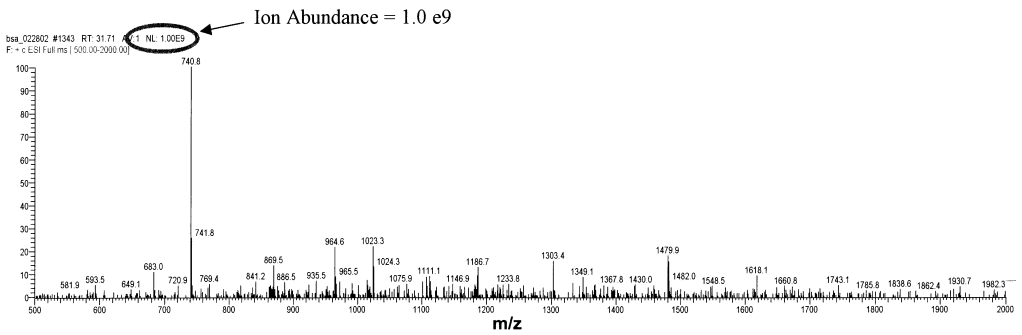

Figure 9. Continued.

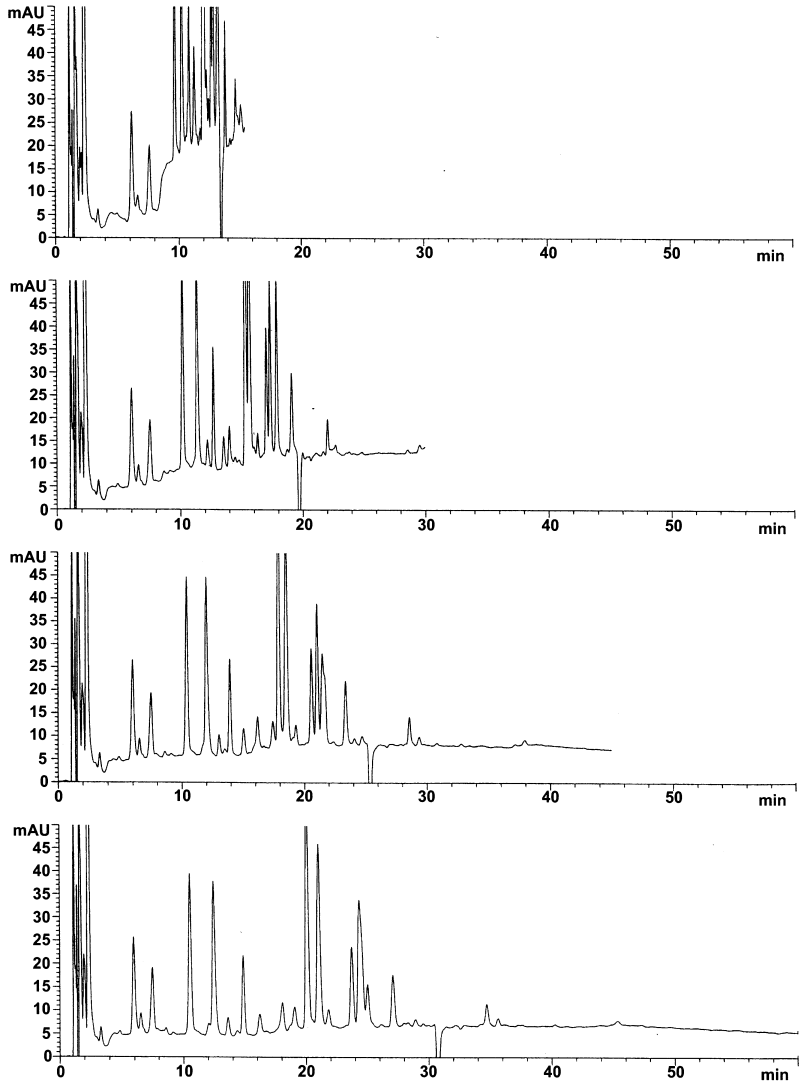

Figure 10. Effect of gradient time (slope) on the separation of bovine serum albumin tryptic digest using a gradient of 5–50% acetonitrile/0.1% TFA.

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solvents contain 0.1% TFA. Note that the separation of the peptides gets better as the gradient times get longer.

Effect of pH

The pH of the buffer plays an important role when resolving proteins/peptides. At an acidic pH proteins and peptides are positively charged while they are negatively charged at basic pH. Therefore, when ion exchange is the HPLC mode of separation the pH determines if anion or cation exchange should be used. In addition, it was observed that the selectivity in RP-HPLC is affected by the buffer pH. For example, the elution order of a mixture made of Bradykinin, Neurotensin, Bobesin, and Elodosin with an acidic pH buffer was different from that obtained with a basic pH buffer, Fig. 11. Other examples can be found in the literature.

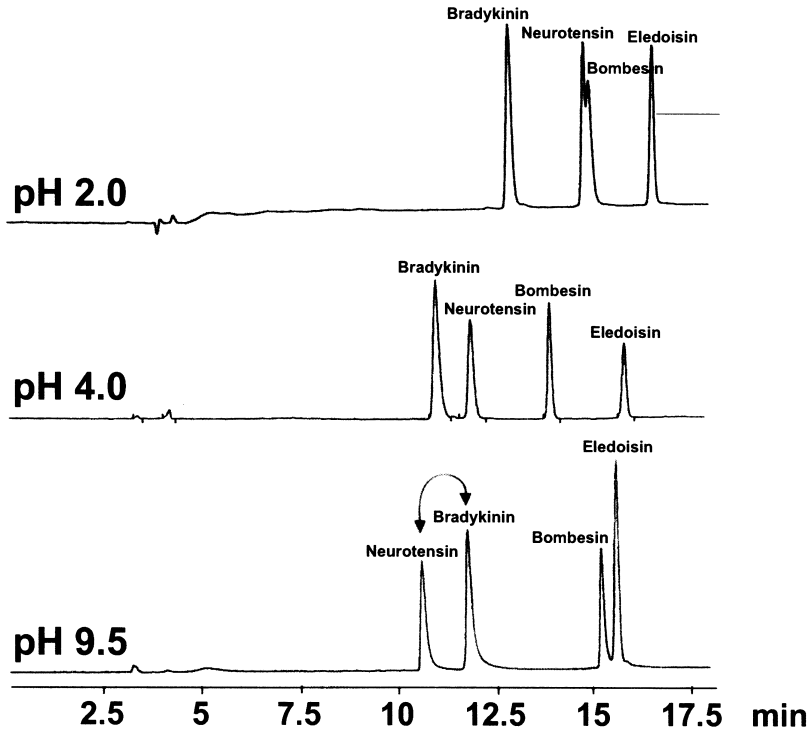

Figure 11. Effect of mobile phase pH on the separation of a mixture of Bradykinin, Neurotensin, Bobesin, and Elodosin. Reprinted with permission from Phenemenex.

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Experimental Temperature

The effect of experimental temperature on the separation of proteins/peptides in HPLC was studied by several investigators.[21–29] Temperature may affect the conformation of the protein and may lead to peak broadening. Karger and his coworkers[22] showed that changing the column temperature from 20 to 37, the peak shape of ribonuclease progressively improved to give a narrow symmetrical peak. Horvath and his coworkers[23,24] used elevated temperatures for the separation of proteins and peptides. Chen and Horvath[24] stated that in RP-HPLC with packed capillary columns, temperature programming offers an alternative to gradient elution in a relatively narrow range of the required elution strength. They also reported that a 5°C change in column temperature and a 1% change in acetonitrile concentration have almost the same effect on the separation of alkylbenzenes. They also reported the use of temperature programming, from 30°C at 30°C min^{-1} for 3 min, for the separation of β-lactoglobulins A and B. Temperature programming, from 30°C at 10°C min^{-1}, was used simultaneously with gradient elution to enhance the separation by RP chromatography of four different proteins; ribonuclease A, cytochrome C, lysozyme, and β-lactoglobulins B, in less than 2 min.[24] The four proteins were base line resolved in less than 3 min using gradient elution at two different temperatures; 30°C and 80°C. It is not clear what the contribution of programmed temperature was in this case?

Horvath and his coworkers[13,24] used temperature as means of increasing column efficiency and to speed the analysis time. Others[26–29] have shown that a change in temperature will also affect the selectivity of the separation by RP-HPLC. Hancock et al.[26,27] used a combination of mobile phase gradient steepness and temperature to resolve a tryptic digest of recombinant human growth hormone (rhGH) and several peptide and proteins. They reported that peak spacing changed significantly when column temperature was varied from 20°C to 60°C, and that the combined use of temperature and gradient steepness provided an efficient procedure for the control of peak spacing and separation optimization of rhGH digest.[26] We studied the effect of temperature variation in gradient RP-HPLC, from 20°C to 60°C, on the separation of a tryptic digest of cytochrome C. The mobile phase gradient was from 5% to 50% solvent B in 30 min using a 0.5×150 mm column packed with Zorbax SB-18 (Agilent Technologies). Solvent A is 0.05 TFA in water and solvent B is 0.05 TFA in acetonitrile. The chromatograms, Fig. 12, clearly show the elution changes with temperature. Our results agree with those of others that a change in temperature would affect the selectivity of the peptides separation. Other experimental parameters, in addition to temperature, can affect the elution order (selectivity) of peptides. These include

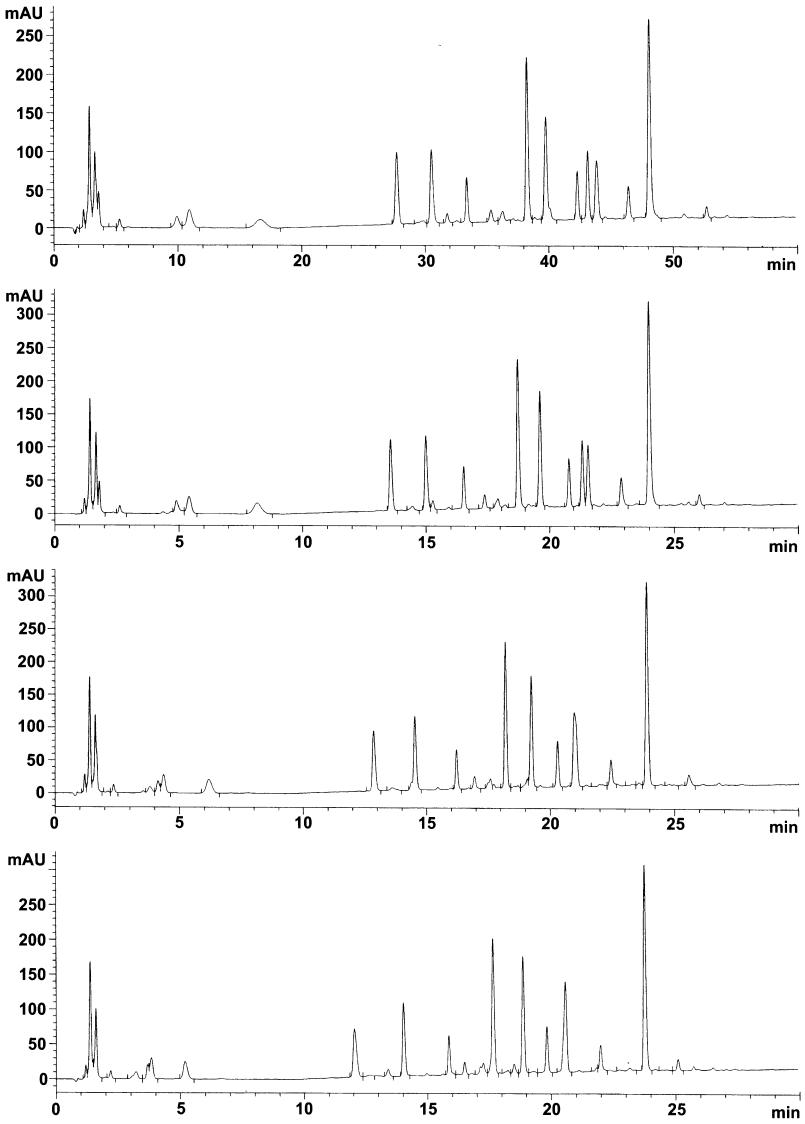

Figure 12. Effect of column temperature on the separation of bovine serum albumin tryptic digest.

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organic modifier's type and concentration, alkyl chain length, buffer pH, and ion-pairing agent concentration as was shown in previous figures.

CONCLUSION

A review of the scientific literature and our research indicate that in order to achieve a good separation of a mixture of proteins and peptides the analyst should first understand the influence of each parameter on the selectivity and resolution of the mixture. For example, it was found that many parameters affect the elution order of peptides by HPLC. These include pH of the mobile phase, experimental temperature, the packing material used in the column, the type of ion-pairing agent, and type of the organic modifier. Also, peak width and resolution are affected, among other things, by the mobile phase composition, pH, mobile phase flow rate, and gradient slope, in addition to column dimensions.

ABBREVIATIONS

ESI–MS	electrospray ionization–mass spectrometry
RP	reversed-phase
TFA	trifluoroacetic acid
HFBA	heptafluorobutyric acid
FA	formic acid
SDS	sodium dodecyl sulfate

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