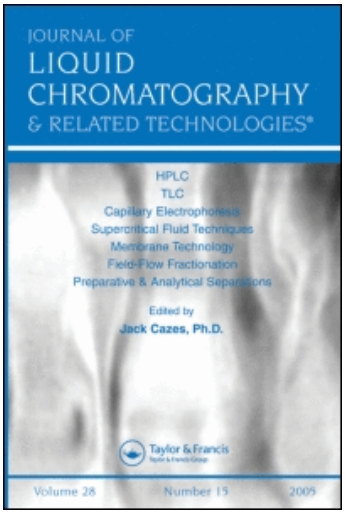

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OPTIMIZATION OF PEPTIDE SEPARATIONS IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

Optimization of peptide separations in HPLC involves more than simply optimizing the mobile phase in terms of flow-rate and gradient-rate. The first step in optimizing the separation of a complex peptide mixture obtained from chemical and/or proteolytic digestion is the selection of the mode or combination of modes required for the separation. For example, should a single or multimodal approach be used, and which mode(s) (SEC, IEC, RPC) should be selected? The second step is to make an assessment of the performance characteristics of the HPLC columns (selectivity, efficiency and deviations from ideal behaviour) to be used. The most logical approach to assessing column performance is to employ HPLC peptide standards designed specifically for this purpose under a set of standard chromatographic run conditions. The third step involves utilizing the information obtained with the standards and any knowledge concerning the expected size, charge and polarity of the peptide fragments to select the initial mobile phase conditions for each mode of chromatography. In SEC, we are concerned with the pH, ionic strength and whether or not denaturing or benign conditions should be used in the mobile phase. In IEC, we must consider pH which can drastically affect the net charge on the peptide fragments and, thus, the overall separation. Denaturing conditions may be required to eliminate conformational effects of large peptides. In RPC, decisions such as the ion-pairing reagent to be used and the pH of the mobile phase are important issues. The fourth step involves the optimization of the gradient-rate and flow-rate to provide maximum resolution in the minimum time period. A detailed explanation of these steps are included.

INTRODUCTION

High-performance liquid chromatography has proved very versatile in the isolation of peptides from a wide variety of sources. Investigation of the properties of peptides is vital in structure/function studies of proteins and it is often necessary to isolate a few peptides from a complex proteolytic and/or chemical cleavage mixture. The complexity of peptide mixtures may vary considerably depending on the source. The number of peptide fragments obtained from a protein cleavage, for instance, will depend on the nature and efficiency of the enzymatic or chemical agent used. Peptides obtained from biological tissues are often found in only very small quantities and may require extensive purification. The wide use of automated solid-phase peptide synthesis in recent years has also necessitated efficient isolation of peptides from various impurities. An efficient peptide synthesis should result in only a small number of synthetic impurities. However, these impurities are usually closely related to the peptide of interest (deletion, terminated or chemically modified peptides), perhaps missing only one amino acid residue, and may be difficult to separate.

Peptides derived from various sources differ widely in size, net charge and polarity, and the approach to their separation must be tailored to the separation goals. Thus, purification of a single peptide from a complex mixture will require a different approach to that necessary for separating all components of a mixture. The former approach may only require the application of a single HPLC mode. In contrast, the latter will require a combination of separation modes for efficient resolution of all desired peptides (1,2). The three main modes of HPLC used for peptide separations utilize differences in peptide size (size-exclusion HPLC or SEC), net charge (ion-exchange HPLC or IEC) or hydrophobicity (reversed-phase HPLC or RPC). Within these modes, mobile phase conditions may be manipulated to maximize the separation potential of a particular HPLC column. The eventual success of a particular peptide separation is inextricably bound up with the correct choice of column(s) and chromatographic conditions. The proper selection of the column will simplify optimization of the chromatographic conditions.

SELECTION OF MODE OF SEPARATION

Despite the widespread application of SEC to the separation of proteins and polypeptides in recent years, relatively little attention has been paid to its potential

for resolving peptides in the 200-5000 dalton range (2-50 residues). However, size-exclusion columns may still have great potential value in the early stages of a peptide purification protocol (2,3).

IEC is being increasingly used for peptide separations as commercially-available HPLC packings capable of retaining both highly charged and weakly basic or acidic peptides are being introduced. Both anion-exchange (AEX) (2,4-7) and cation-exchange (CEX) (1,2,6,8-10) columns have proved useful in peptide separations. The retention behaviour of a peptide in either ion-exchange mode will depend on a number of factors, including buffer pH and ionic strength of the anion or cation employed for displacement of acidic or basic peptides, respectively. The use of anion-exchange columns is limited to pH values (generally neutral pH) well above the pKa's of the acidic (aspartic and glutamic acid) side-chain carboxyl groups (~4.0) and C-terminal α-carboxyl group to ensure the full expression of their negative charges. In addition, the acidic nature of any histidine-containing peptides is increased above pH values of 6.0-6.5 by the loss of this residue's positive charge. In contrast, cation-exchange chromatography allows a wider choice of separation conditions. The basic residues, arginine and lysine, and the N-terminal α-amino group retain a positive charge over the acidic to neutral pH range. However, varying the pH from neutral to acidic conditions can significantly affect the net charge on the peptides and thus the elution profile. At pH levels below 3.5-4.0 (strong CEX) has the advantage of ensuring the protonation of side-chain carboxyl groups of acidic residues, thus emphasizing any basic, positively charged character of the peptides.

RPC is, by far, the most widely-used mode of HPLC at present (2). The ability of this technique to separate peptides of closely related structures has made it an extremely powerful, high-speed, analytical and preparative tool. Whatever the source of a particular peptide sample, the flexibility of RPC (parameters such as pH, mobile phase, counterion, temperature, etc. can be varied for optimum resolution) makes it the obvious choice for the initial HPLC run to gauge the complexity of the peptide mixture and help design the best approach for its resolution.

MULTIMODE HPLC

It is expecting a lot of a single HPLC technique to resolve very complex mixtures of peptides. A more efficient separation of all components of a mixture

will be obtained by the combined use of separation modes which utilize different selectivities (size, charge, hydrophobicity) (1,2). However, a multimodal approach should be considered even in the case of less complex peptide mixtures, since, even for a fairly simple peptide mixture, a combination of two or more HPLC modes is often much easier than attempting to optimize a single mode. By applying this approach, the separation demands on any one HPLC mode are lessened. In addition, a multimodal approaches allows a small, but efficient, preparative scale-up of peptide purification on analytical columns. It may be possible to achieve satisfactory peptide separation by a single HPLC technique, but a subsequent scale-up to higher peptide loads generally leads to a rapid loss in peptide resolution.

Having decided that a multimodal approach to the separation of a peptide mixture is worth pursuing, the next step is to consider the number of HPLC modes required and their order of use. Because a portion of the chromatographic sample is usually unavoidably lost at each separation step, it is important to minimize the number of steps required for satisfactory resolution. For peptides of known sequence, the columns required and the chromatographic conditions (e.g., pH in IEC) can be chosen accordingly. For unknown peptide sequences, the correct column order provides maximum information about the properties of the peptides as the purification proceeds in the minimum number of runs (1,2).

A. IEC → RPC

Although IEC has become increasingly popular for the analysis of proteins in recent years, less attention has been paid to its application to the separation of peptides, due mainly to the availability of reversed-phase supports for such purposes and the necessity of sample desalting prior to further analysis. However, RPC and IEC are often complementary, i.e., their combined use can provide optimal separation of a peptide mixture or assess the purity of a peptide preparation (2,8,11). Strong CEX and RPC are probably the most useful modes of HPLC for multistep peptides separations(1,2,6). Most peptides are soluble at low pH, where any basic character they possess is maximized due to the elimination of any negative charges. Apart from the powerful resolving capability of RPC, the use of volatile mobile phases at low pH enables it to act as a final desalting step after the initial ion-exchange separation. This is particularly important when preparing peptides for amino acid analysis and microsequencing, or during large-scale purification of peptides.

B. SEC → IEC→RPC

In some circumstances, even the combined resolving power of IEC and RPC may not be sufficient to entirely resolve a peptide mixture without extensive, and time-consuming, mobile-phase manipulations. A chemical or proteolytic cleavage mixture, for instance, may contain partial cleavage products and, perhaps, uncleaved protein, complicating the ion-exchange and reversed-phase chromatographic profiles. An initial separation, based on peptide size, would simplify subsequent ion-exchange and reversed-phase steps. This approach was applied by Mant and Hodges (1) to the resolution of the major cyanogen bromide fragments of rabbit skeletal troponin I. The initial SEC step was carried out with 0.1% aqueous TFA as eluting solvent, the volatile nature of which enabled direct application of the peptide fractions to a strong cation-exchange column. Fractions from this column were subsequently applied to a reversed-phase column for desalting and final purification. The use of a volatile eluting solvent for the SEC step precluded the necessity for sample desalting prior to its application to the ion-exchange column and kept the number of sample manipulations to a minimum. If it is not possible to use a volatile mobile phase for the SEC step, sample dilution prior to its application to an ion-exchange column may be an option.

C. SEC →RPC

For a relatively simple peptide separation, it may be possible to apply RPC as a desalting and final purification step directly after a size-exclusion separation. This would be especially useful if the SEC mobile-phase was, necessarily, non-volatile and sample dilution prior to an ion-exchange step was not a viable alternative.

D. SEC→RPC→IEC→RPC

On occasion, where all three major HPLC modes are required for efficient peptide resolution, a desalting and partial purification step may be required following an initial SEC separation with a non-volatile mobile phase and prior to the IEC step. Under these circumstances, only complex RPC fractions need be applied to the ion-exchange column, simplifying the remainder of the purification protocol. Following IEC in non-volatile buffers, the final RPC separation is usually only required as a desalting step.

COLUMN SELECTION

Columns of all three major HPLC modes come in a variety of sizes, containing a variety of packings and from a variety of manufacturers. Thus, once the general approach to a particular peptide separation has been decided, it is important to give careful consideration to the selection of column(s).

The major limitations to mobile phase conditions for peptide separations depend mainly on the nature and stability of the column support. Silica-based supports are still the most widely used (2). The rigidity of micro-particulate silica enables the use of high linear flow velocities of mobile phases. In addition, favourable mass-transfer characteristics allow rapid analyses to be performed. However, most silica columns are limited to a pH range of 2.0-8.0, since the silica matrix is rapidly dissolved in the presence of basic eluents. Thus, the use of acidic eluents for these silica-based packings helps to extend column lifetime. Column packings based on organic polymers are becoming increasingly used in all modes of HPLC (12). These materials, the most common of which are formed from crosslinked polystyrene divinyl benzene, have a broad pH tolerance (often pH 0-14). Though these non silica-based supports have been successfully used in SEC and IEC, little application has been made to the separation of peptides in RPC and their value remains largely untested.

A. SEC

The practical value of size-exclusion columns is, at present, rather limited for resolution of peptides (up to 50 residues) (2,3). A size-exclusion column designed specifically for peptides has yet to be produced and will probably require pore-size diameters less than any currently available. In addition, the high cost and rapid deterioration of peptide separation on currently available columns, designed mainly for protein separations, makes their purchase prohibitive. Thus, it is best to avoid the use of a size-exclusion step in a peptide purification protocol if the separation may be achieved without too much difficulty in its absence. If a size-exclusion step is necessary, packings with pore diameters of 60-100 Å offer the best peptide resolving capability at present.

B. IEC

Strong anion-exchange columns, consisting of quaternized supports, are the most useful mode of AEX for peptides. These columns yield essentially unchanged

peptide elution times over the acidic to neutral pH range (2,13). The advantage of strong cation-exchange columns, generally containing supports modified with sulphonate functionalities, for peptide separations lies in the ability of the sulphonate groups to retain their negative character in the acidic to neutral pH range (1,2,6).

The majority of silica-based supports for IEC in analytical and semipreparative applications are comprised of 5 μm-10μm spherical particles for optimum resolution. In general, 300Å pore size matrices give better resolution and recovery for peptides and proteins (2,14). Since pore diameter has little effect on peptide separations (15), and most researchers who separate peptides also separate proteins, the 300Å support is the most suitable for general use. Standard analytical columns are 5-30 cm in length with an internal diameter of 3-5 mm.

C. RPC

The most favoured reversed-phase columns for separation of most peptides and smaller proteins are silica-based supports containing octyl (C_8) or octadecyl (C_{18}) functionalities. Supports containing C_3 and C_4 functionalities have been used for more hydrophobic peptides and proteins (15,16). Standard analytical RPC columns generally have the same dimensions as IEC columns. Similarly, 5 μm-10 μm spherical particle size and 300Å pore size are suitable for most purposes.

ASSESSMENT OF COLUMN PERFORMANCE

Prior to the use of an HPLC column, it is important to assess its performance capabilities (selectivity, efficiency), since the peptide resolving power of column packings of all three major HPLC modes may vary from manufacturer to manufacturer or from batch to batch of support from the same manufacturer. In addition, any deviations from ideal column behaviour, i.e., non-specific interactions between the column and solute, must be identified and suppressed or eliminated.

The most logical approach to assessing column performance is to employ HPLC standards, preferably under a set of standard chromatographic run conditions, for a proper comparison of different HPLC packings (2). The need for standards to monitor column performance in chromatography is well established. Organic molecules such as the alkylphenone series, for instance, have seen much

use in monitoring reversed-phase columns (17). However, it is often preferable to use compounds that are structurally similar to the sample of interest and that presumably interact with the column packing in a similar manner to achieve the most precise and accurate analysis. Proteins are often used to demonstrate the utility of ion-exchange and size-exclusion columns, in the latter case often to calibrate the column for molecular weight determinations. However, the use of peptide standards designed specifically to monitor the peptide resolving capability of SEC, IEC and RPC has only recently been seriously addressed.

A. SEC

Separation of peptides by a mechanism based solely on peptide size (ideal SEC) occurs only when there is no interaction between the solutes and the column matrix. Although SEC columns are designed to minimize non-specific interactions, most modern SEC columns are weakly anionic (negatively charged) and slightly hydrophobic, resulting in deviations from ideal size-exclusion behaviour, i.e., non-ideal SEC (3,14,18-21). At the lower end of the fractionating range of a column, all small molecules should be eluted together under pure size-exclusion conditions. If they do not, the column is behaving in a non-ideal fashion. The volume of solvent required to elute small molecules in ideal SEC is the total permeation volume of the column. This column parameter is a combination of the void volume of the column (elution volume of a totally excluded species) and the pore volume of the size-exclusion matrix. By definition, under ideal size-exclusion conditions, no molecule will be retained beyond the total permeation volume of the column.

A series of synthetic peptide standards (Ac-[G-L-G-A-K-G-A-G-V-G]$_n$-amide, where n = 1-5), designed to monitor both non-ideal and ideal SEC behaviour, have proved extremely beneficial in enabling rapid development of the optional conditions for SEC of peptides (2,3). The increasing size of the peptide standards (800-4,000 daltons) enables an accurate molecular weight calibration of a column during ideal SEC; the increasingly basic character of the standards (+1 to +5) makes them sensitive to any anionic character of a size-exclusion support; the increasing hydrophobicity of the polymer series enables a determination of column hydrophobicity. The chromatographic profile of the five standards on a Spherogel TSK G2000SW silica-based column, coupled with the linear character of the $\log_{10}$ MW versus peptide retention time plot, clearly demonstrates the ability of the polymer series to monitor pure size-exclusion behaviour on SEC columns (Figure 1).

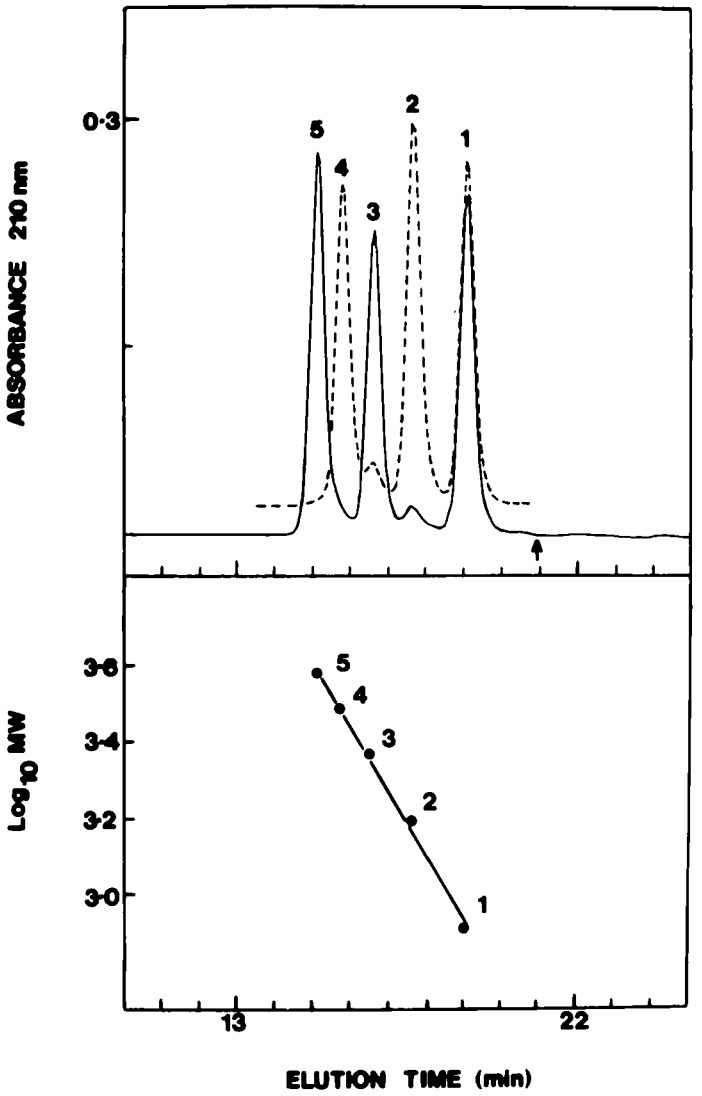

Figure 1. SEC of a mixture of synthetic peptide standards. Top: elution profile of peptide standards obtained with an Altex Spherogel TSK G2000SW column (300 x 7.5 mm I.D.; Beckman Instruments, Berkeley, CA, U.S.A.). Mobile phase: 0.1% aq. TFA (pH 2.0); flow-rate, 0.5 ml/min; temperature, 26°. Bottom: plot of log MW versus retention time of the peptide standards. Peptides 1-5 contain 10-50 amino acid residues, respectively. The arrow denotes the elution time for the total permeation volume of the column.

The chromatographic profile demonstrated in Figure 1 was achieved with a volatile 0.1% aqueous TFA eluent at a flow-rate of 0.5 ml/min. The choice of a volatile mobile phase is certainly advantageous in peptide separations, since it enables lyophilization of peptide fractions prior to immediate analysis or their direct application to ion-exchange or reversed-phase columns during multistep HPLC. However, it should be noted that hydrophobic and/or electrostatic interactions may be more pronounced with one size-exclusion column than with another (3,18), and the use of a simple, volatile mobile phase may be insufficient to suppress these interactions. Non-ideal SEC behaviour on a silica-based column is clearly illustrated in Figure 2. In the top panel, the separation of standards 1, 2 and 5 (+1,+2,+5 net charge, respectively) appears to be based on an ion-exchange rather than size-exclusion mechanism. The elution order is reversed from that expected of a size-exclusion mechanism, with the smallest peptide eluting first (n = 1; 10 residues) and the largest peptide eluting last (n = 5; 50 residues). In addition, all three peptides were retained longer than the total permeation volume of the column. Electrostatic effects between solutes and the column matrix may be minimized by the addition of salts to the eluent (2,3). The eluting solvent which produced the chromatographic profiles illustrated in Figure 2 was 0.1% aqueous TFA containing 5 mM (top), 10 mM (middle) or 200 mM KCL at a flow-rate of 1.0 ml/min. As the ionic strength of the mobile phase increased, electrostatic effects were gradually overcome until an essentially ideal size-exclusion mechanism was apparent at a salt concentration of 200 mM. Different peptide mixtures may require eluents of markedly different pH values for optimal SEC separation. The effect of pH variations may not only affect the net charge of a particular peptide, but may also influence any non-specific interactions between the solutes and the size-exclusion matrix. For instance, the non-specific interactions noted on the column which produced the chromatographic profiles shown in Figure 2 were more pronounced at pH 6.5 (5 mM KH_2PO_4 buffer) (3). However, the presence of 200 mM KCl was again sufficient to overcome these interactions. Aqueous solvents and buffers containing 100 mM to 400 mM salt are commonly employed as the mobile phase for SEC. In fact, efficient separation of peptide mixtures in their absence is generally the exception rather than the rule. Thus, although testing a size-exclusion column with a volatile mobile phase is worthwhile, particularly if a multistep HPLC approach to peptide separation is being considered, a non-volatile buffer (50 mM KH_2PO_4, pH 3.0-6.5, containing 200 mM KCl or NaCl is recommended) will

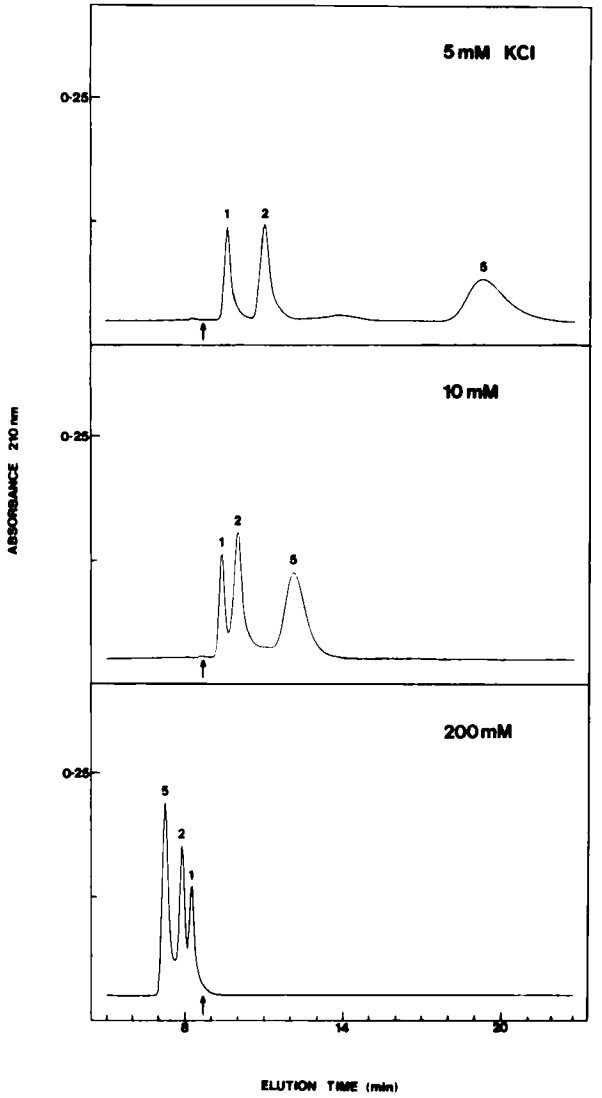

Figure 2. Effect of salt on non-specific interactions in SEC. Column: SynChropak GPC60 (300 x 7.8 mm I.D.; SynChrom, Linden, IN, U.S.A. Mobile phase: 0.1% aq. TFA (pH 2.0) containing 5 mM, 10 mM, or 200 mM KCl; flow-rate, 1 ml/min; temperature, 26°C. Synthetic peptide standards 1,2 and 5 contain 10, 20 and 50 residues, respectively. The arrows denote the elution time for the total permeation volume of the column. Non-specific interactions are observed on all SEC columns presently available.

probably be necessary to ensure non-specific solute-column interactions are eliminated.

B. IEC

Peptides may be removed from an ion-exchange column either by gradient or isocratic elution. However, linear gradient elution is generally the elution mode of choice when attempting to separate mixtures of peptides with a wide range of net charges (2,6). Gradient elution of peptides is usually performed with salt gradients of either sodium or potassium chloride in phosphate, tris or citrate mobile phase buffers. A linear sodium chloride gradient (20 mM salt/min) in 5 mM KH_2PO_4 buffer, pH 3.0 (strong CEX) or pH 6.5 (AEX or CEX), at a flow-rate of 1 ml/min are suitable standard conditions to evaluate analytical ion-exchange columns (2). Care should always be taken over the choice of ionic strength of the starting buffer. If it is too high, weakly acidic or basic peptides which may otherwise be retained by anion-exchange or cation-exchange columns, respectively, may elute with unretained compounds.

The value of standards in monitoring the retention characteristics of ion-exchange columns is twofold: firstly, to confirm that the column can, indeed, retain charged species (the weaker the charge that can be retained, the better the column); secondly, to assess the effect of pH variations on the resolving capability and loading capacity of an ion-exchange column. The latter is particularly important for strong CEX, where the manipulation of mobile phases over the acidic to neutral pH range is frequently employed for peptide separations (1,6). Figure 3 demonstrates the elution profiles at pH 6.5 (top) and pH 3.0 (bottom) of four undecapeptide cation-exchange standards (1-4 denote +1 to +4 net charge, respectively) on a silica-based strong cation-exchange column. The standards were subjected to gradient elution (buffer A = 5 mM KH_2PO_4, buffer B = buffer A + 1 M NaCl) at 20 mM salt/min and a flow-rate of 1 ml/min, following 5 min isocratic elution with buffer A. At pH 6.5, standards 2,3 and 4 (+2,+3,+4 net charge, respectively) were removed by the gradient, while peptide 1 (+1 net charge) was eluted during the initial isocratic elution. In contrast, at pH 3.0, only peptides 3 and 4 were removed by the gradient, while both peptides 1 and 2 were eluted during the initial isocratic step. In addition, the retention time of peptides 3 and 4 were reduced considerably. Ideally, there should have been no variation of elution time of the peptides with buffer pH. The observed effects apparently resulted from a reduction in column

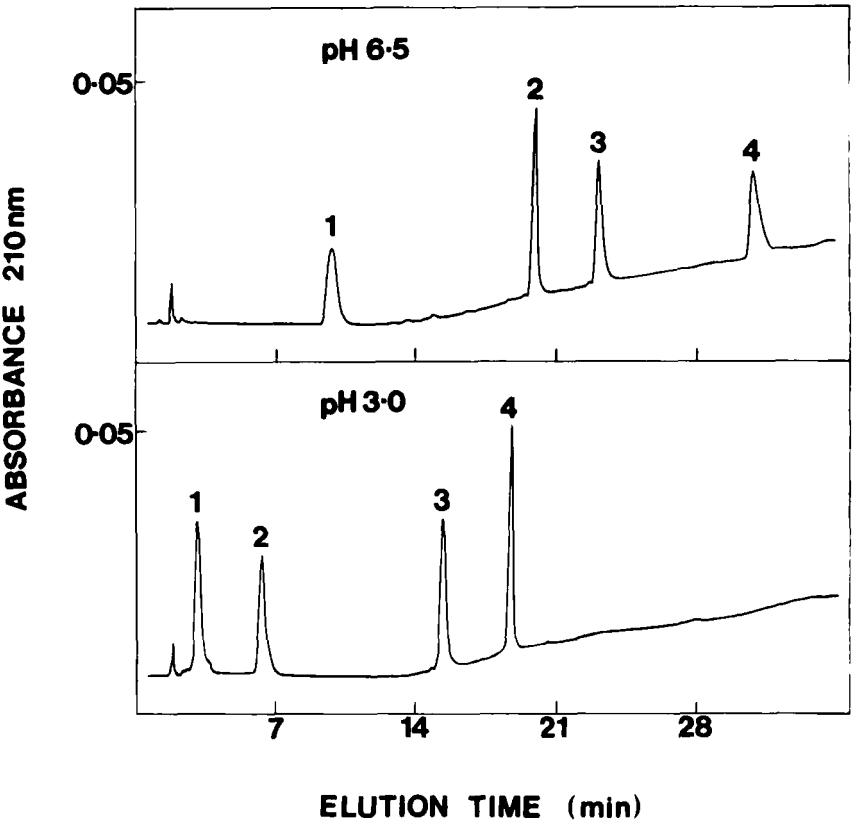

Figure 3. Strong CEX of a mixture of synthetic peptide standards at pH 6.5 (top) and pH 3.0 (bottom). Column: SynChropak S300 (250 x 4.1 mm I.D.; SynChrom, Linden, IN, U.S.A.). Mobile phase: linear AB gradient, following 5 min isocratic elution with buffer A, where buffer A is 5 mM KH_2PO_4, pH 3.0 or 6.5, and buffer B is 5 mM KH_2PO_4 + 1 M NaCl, pH 3.0 or 6.5; gradient-rate, 20 mM NaCl/min; flow-rate, 1 ml/min; temperature, 26°C. Peptides 1-4 are four synthetic undecapeptide standards containing 1-4 positively charged groups, respectively.

capacity to retain charged species as the pH became more acidic (6). A series of acidic peptide standards, with no basic residues present, would accurately monitor such pH-influenced properties of AEX columns.

The optimum separation of two peptides by gradient elution on anion- or cation-exchange columns will be obtained when there is a net charge difference of at least one unit between the two peptides. If an IEC column is performing correctly, and the overall net charge on peptides is being expressed, the retention times of peptides should show a linear relationship with net charge (2,6). This was well demonstrated by Mant and Hodges (6) (Figure 4) who showed that, under benign conditions (linear KCl gradient [5 mM/min] in 5 mM KH_2PO_4 buffer), the elution times of several basic peptides (9-21 residues) on the same strong CEX column described above (Figure 3) were linear with respect to their net charge at pH 3.0 (Figure 4, bottom left) and pH 6.5 (Figure 4, bottom right). Similar observations have been made for a series of acidic peptides (-2 to -5 net charge) chromatographed on a strong AEX column. These results may be useful in correlating the overall charge of a peptide at a given pH with its amino acid composition (2).

C. RPC

Though excellent resolution of peptide mixtures may be obtained at acidic or neutral pH, the majority of researchers have carried out RPC at pH values <3.0 (2). Reversed-phase silica-based columns may contain surface silanols which act as weak acids and are ionized above pH 3.5 - 4.0 (14). These weak acids may interact with the basic residues of peptides chromatographed on RPC columns and have an adverse effect on resolution, characteristically producing long retention times and peak broadening. Apart from the suppression of silanol ionization under acidic conditions, silica-based columns are more stable at low pH.

The ability to separate peptides closely related in hydrophobicity (e.g. differing by only one methyl group) should be a necessary requirement for a reversed-phase column. The best standard approach to assessing the peptide resolving power of a reversed-phase column is to employ a 0.1 % aqueous TFA to 0.1 % TFA-acetonitrile linear gradient (pH 2.0) of 1% acetonitrile/min at a flow-rate of 1 ml/min. Figure 5B demonstrates the results obtained when applying these conditions to the reversed-phase separation of a series of five synthetic decapeptide RPC standards (S1-S5) on an analytical C_{18} column (22). Each peptide contains two

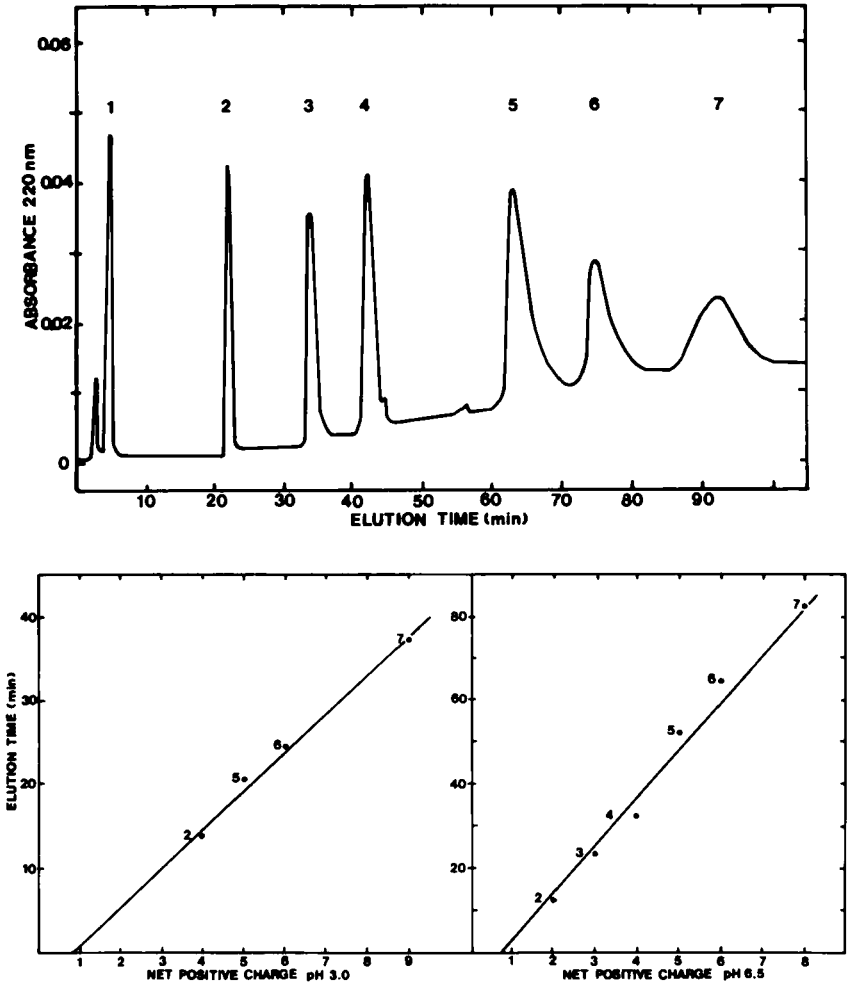

Figure 4. Relationship of peptide net charge to retention time in strong CEX. Column: SynChropak S300 (250 x 4.1 mm I.D.; SynChrom, Linden, IN, U.S.A.). Top: elution profile of basic peptides at pH 6.5. Bottom: plots of peptide elution time versus net positive charge at pH 3.0 (left) and pH 6.5 (right). Mobile phase: linear AB gradient, following 10 min isocratic with buffer A, where buffer A is 5 mM KH_2PO_4, pH 3.0 or 6.5, and buffer B is 5 mM KH_2PO_4 + 1 M KCl, pH 3.0 or 6.5; gradient-rate, 5 mM KCl/min; flow-rate, 1 ml/min; temperature, 26°C.

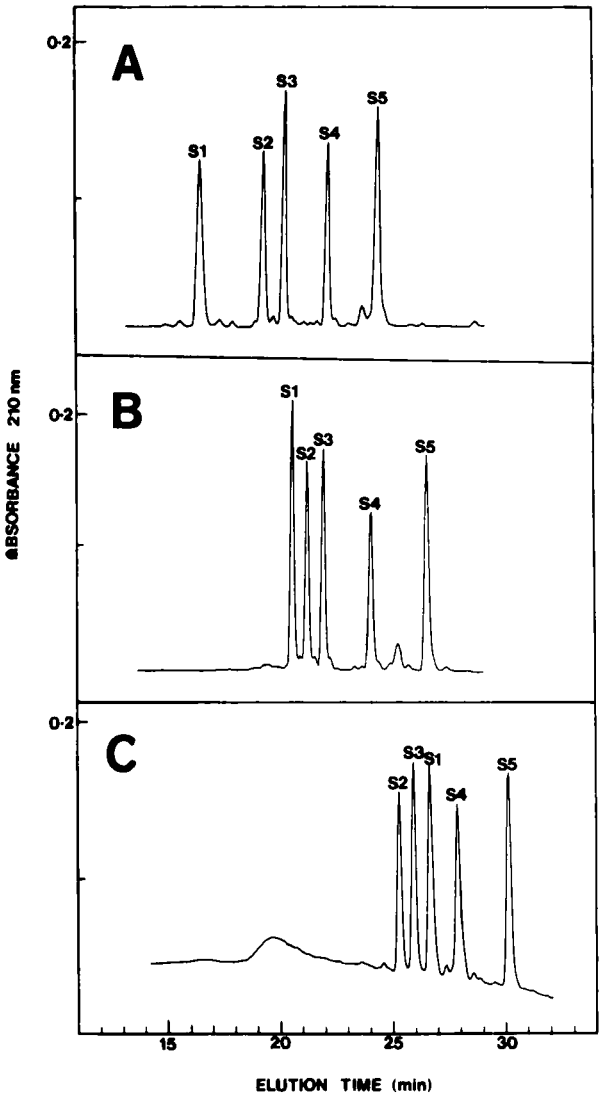

Figure 5. Effect of ion-pairing reagents on the RPC separation of a mixture of synthetic peptide standards. Column: SynChropak RP-8 C_8 (250 x 4.1 mm I.D.; SynChrom, Linden, IN. U.S.A.). Mobile phase: linear AB gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both containing 0.1% H_3PO_4 (panel A), 0.1% TFA (panel B) or 0.1% HFBA (panel C); flow-rate, 1 ml/min; temperature, 26°C. The decapeptide standards contained two basic residues with no acidic residues present. Each peptide also contained an Nα-acetylated amino terminal and a COOH-terminal amide, with the exception of S1, which resembled S3 in all respects save the presence of a free α-amino group.

basic residues, with no acidic residues present. The hydrophobicity of the standards increases only slightly between S2 and S5 — between S2 and S3 there is an increase of only one methyl group, between S3 and S4 there is an increase of two methyl groups, and between S4 and S5 there is an increase of an isopropyl group — enabling very precise determination of the resolving power of a reversed-phase column (23). The excellent separation obtained on the C_{18} column (Figure 5B) shows it to be eminently suitable for the resolution of peptide mixtures.

As well as differences in selectivity, performance characteristics of RPC columns may also vary dramatically due to non-specific interactions. Figure 6 demonstrates the elution profiles, over the acidic to neutral pH range, of four basic peptide standards (1-4) on two commercially-available RPC columns (24). Columns A and B were analytical C_{18} and C_8 columns, respectively. The standards (+1 to +4 net charge) are sensitive to any ionic, as opposed to hydrophobic, interactions with the hydrophobic stationary phase. The top panels of Figure 6 demonstrate the elution profiles obtained at pH 2.0 (linear AB gradient of 1% acetonitrile/min at a flow-rate of 1 ml/min, where solvent A was 0.05% aqueous TFA and solvent B was 0.05% TFA in acetonitrile). The middle panels demonstrate the profiles obtained at pH 4.5 (linear AB gradient of 1% acetonitrile/min and 1 mM TEAP/min [triethylammonium phosphate] at a flow-rate of 1 ml/min, where solvent A was aqueous 10 mM TEAP, pH 4.5, and solvent B was 50% aqueous acetonitrile containing 60 mM TEAP). The bottom panels demonstrate the profiles obtained at pH 7.0 (linear AB gradient of 1% acetonitrile/min and 1.67 mM $NaClO_4$/min at a flow-rate of 1 ml/min, where solvent A was aqueous 10 mM $(NH_4)_2HPO_4$, pH 7.0, and solvent B was 60% aqueous acetonitrile containing 100 mM sodium perchlorate). The contrast in the performances of the columns was striking. Column A exhibited significant ionic interactions with the basic peptide standards over the entire range available to researchers on silica-based reversed-phase packings. A satisfactory peptide elution profile was not even obtained at pH 2.0, where silanol ionization is usually effectively suppressed. In contrast, the elution profiles obtained on Column B were excellent at all three pH values, although some ionic interaction was apparent in the double gradient pH 7.0 system, specifically designed by Mant and Hodges (24) to monitor ionic interactions on RPC columns. The most basic peptide standards, 3 and 4 (+3 and +4 net charge, respectively) were particularly sensitive to ionic interactions with a hydrophobic stationary phase. Peptides with charges of +3 and

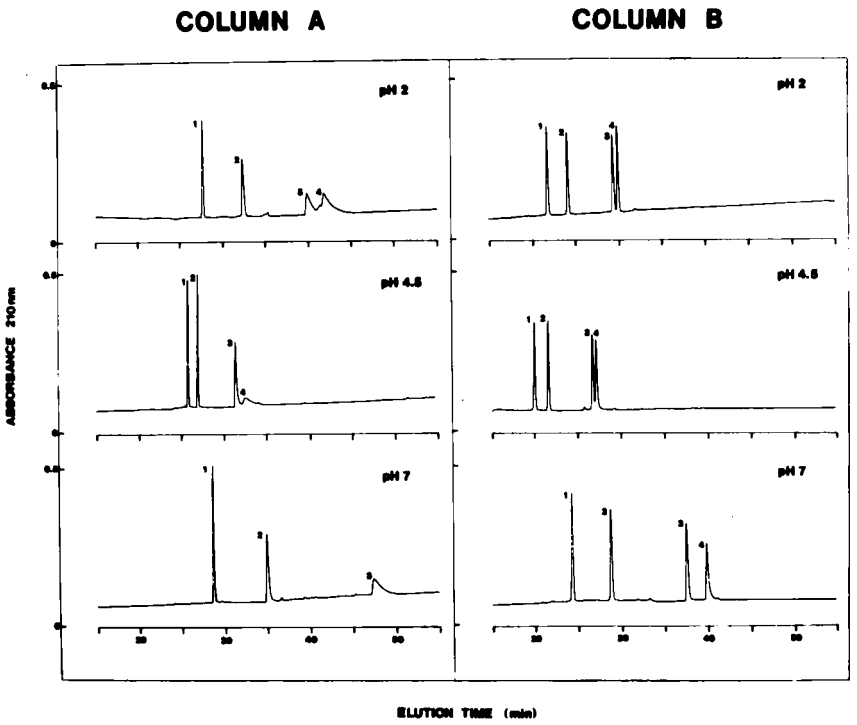

Figure 6. Monitoring of ionic interactions on commercial reversed-phase columns with peptide standards. Column A: C_{18} column (250 x 4.6 mm I.D.; 5 µm particle size; 300Å pore size). Column B: C_8 column (220 x 4.6 mm I.D.; 7 µm particle size; 300Å pore size). Mobile phase: pH 2.0, linear AB gradient (1% B/min), where solvent A is 0.05% aq. TFA and solvent B is 0.05% TFA in acetonitrile; pH 4.5, linear AB gradient (2% B/min, equivalent to 1% acetonitrile/min and 1 mM TEAP/min), where solvent A is aq. 10 mM TEAP, pH 4.5, and solvent B is 50% aq. acetonitrile containing 60 mM TEAP; pH 7.0, linear AB gradient (1.67% B/min, equivalent to 1% acetonitrile/min and 1.67 mM $NaClO_4$/min), where solvent A is aq. 10 mM $(NH_4)_2HPO_4$, pH 7.0, and solvent B is 60% aq. acetonitrile containing 100 mM $NaClO_4$ Flow-rate, 1 ml/min; 26°C. Undecapeptide standards 1-4 contain +1 to +4 net charge, respectively.

+4 are not uncommon, and researchers can quickly demonstrate with these standards whether a reversed-phase column is exhibiting undesirable ionic interactions.

OPTIMIZATION OF MOBILE PHASE

Having selected suitable HPLC columns, the researcher is now ready to subject the peptide sample of interest to the desired separation approach, i.e., single or multistep HPLC. Suitable initial chromatographic conditions will depend both on the mode of HPLC and the source of the peptide sample. As a general rule, the mobile phase should be optimized first, followed by any necessary adjustments to parameters such as flow-rate and gradient-rate.

A. SEC

(i) Mobile Phase

Since the use of SEC in a peptide purification protocol is generally limited to the preliminary separation of peptides of interest from a chemical or proteolytic protein digest, the ability to predict the position and/or elution order of peptides during SEC of a peptide mixture would be extremely useful. Under conditions of ideal SEC, large peptide fragments, resulting from incomplete protein digestion, can then be quickly identified and removed. Thus, it is important to achieve a linear relationship between logarithm of molecular weight and retention time over a wide molecular weight range (2,3).

The SEC mobile phases described previously (Figure 1: 0.1% aqueous TFA; Figure 2: 5 mM KH_2PO_4, pH 3.0 or 6.5, + 200 mM KCl) which produced ideal size-exclusion behaviour of a series of peptide standards were non-denaturing media. Under non-denaturing conditions, many proteins and large peptides may deviate from ideal size-exclusion behaviour due to their conformational characteristics. Thus, the tendency of peptides or protein fragments to maintain or reform a particular conformation, as opposed to a random coil configuration, in non-denaturing media will complicate retention time prediction (2,3). Mant *et al.*, (3) for instance, reported that a linear $\log_{10}$ MW versus retention time relationship for horse-heart myoglobin and its cyanogen bromide fragments (2500 - 17000 daltons) on silica-based TSK G2000SW and GPC 60 columns, and an agarose-based Superose 12 column, could only be obtained under highly denaturing

conditions [50 mM KH_2PO_4 (pH 6.5) - 0.5 M KCl - 8 M urea]. Figure 7 demonstrates the linear relationship and elution profiles of myoglobin, its cyanogen bromide fragments, and the five synthetic peptide standards for the TSK G2000SW column (3). The peptide fragments produced by cyanogen bromide cleavage of myoglobin constitute substantial portions of the whole protein and maintenance of a folded structure or reassociation of the large fragments in non-denaturing media is likely.

In situations where predictable size-exclusion behaviour is not necessarily required, non-denaturing media may be sufficient to produce an acceptable peptide separation. If this separation can be achieved in a volatile eluting solvent, then so much the better. Mant and Hodges (1), in fact, demonstrated ideal size-exclusion behaviour of a cyanogen bromide cleavage mixture of rabbit skeletal TnI on a TSK-250 SEC column with 0.1% aqueous TFA as eluting solvent. However, if the conformational character of a peptide protein mixture is uncertain, and predictable behaviour is desired, SEC should always be carried out under highly denaturing conditions (2,3).

(ii) Flow-rate

Generally, the slower the flow-rate, the better the separation of peptides in SEC. However, slow flow-rates can lead to excessively long run times, particularly for small peptides. Optimum flow-rates for analytical SEC columns are in the range of 0.2-1.0 ml/min for the best compromise between separation time and efficiency of resolution (2). In addition, sample volume must be kept as small as possible.

B. IEC

(i) Mobile phase

An advantage of peptide over protein separations in IEC lies in the fact that all charged groups in peptides are generally available to interact with the column even under benign conditions (2). Thus, the non-denaturing conditions described previously (linear NaCl gradient in 5 mM KH_2PO_4, pH 3.0 or 6.5) are adequate for most peptide separations. If the researcher already has information about the charge characteristics of the peptides in a peptide mixture, the use of a strong-cation exchange column enables manipulation of the pH of the mobile phase to optimize resolution and produce predictable elution profiles. Even if the peptide characteristics are unknown, varying the pH of the mobile phase during strong CEX (comparing

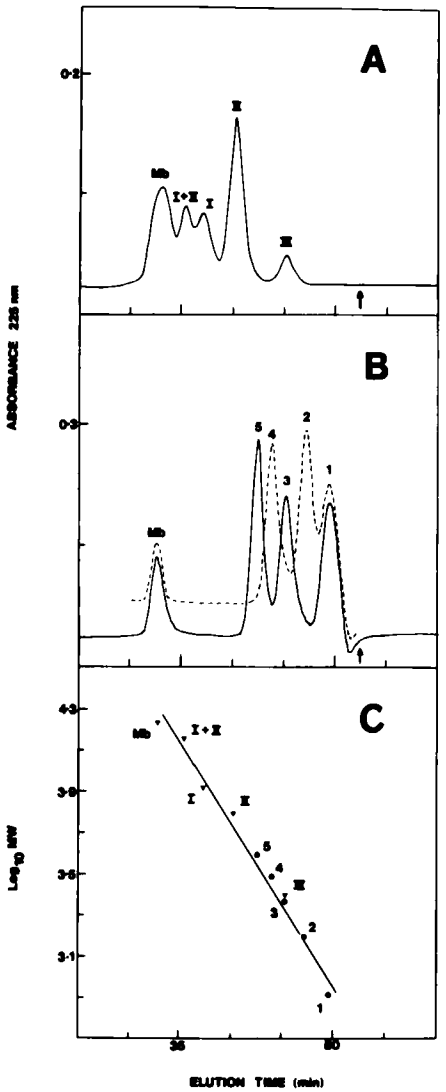

Figure 7. Ideal SEC of protein fragments and a mixture of synthetic peptide standards. Column: Altex Spherogel TSK G2000SW (300 x 7.5 mm I.D.; Beckman Instruments, Berkeley, CA, U.S.A.). Mobile phase: 50 mM KH_2PO_4 (pH 6.5) - 0.5 M KCl - 8 M urea; flow-rate, 0.2 ml/min, temperature, 26°C. (A) Elution profile of horse-heart myoglobin (Mb) and its cyanogen bromide cleavage fragments (I,II,I + II,III); (B) elution profile of horse-heart Mb and peptide standards 1-5 (10-50 residues, respectively); (C) plot of log MW versus retention time of Mb, cyanogen bromide fragments of MB, and the five peptide standards. The arrows denote the elution time for the total permeation volume of the column.

one run at acidic pH against one run at neutral pH) may be worthwhile to assess its effect on the separation of a peptide mixture.

Under non-denaturing conditions, there is a tendency for proteins or large protein fragments, following chemical or proteolytic cleavage, to assume a tertiary structure. In addition, large peptide fragments may reassociate. Under these circumstances, charged species may be shielded so that only a portion of the peptide or protein surface interacts with the IEC column (2). Hence, the overall net charge on the protein or peptide fragment will not be fully expressed. This is not necessarily an disadvantage during IEC of a peptide mixture, and non-denaturing conditions may still produce a perfectly adequate separation. However, in a similar manner to SEC, if predictable elution profiles are desired, and non-predictable behaviour due to conformational effects is possible,the presence of a denaturant (e.g., 8 M urea) may be necessary.

(ii) Flow-rate and gradient-rate

Flow-rates of 0.5 - 2.0 ml/min are favoured for analytical ion-exchange separations. The value of varying flow-rates is somewhat limited in IEC, despite a slight improvement in peptide resolution with increasing flow-rate.

In contrast to flow-rate, variations in gradient-rate (increasing counterion concentration per unit time) may have a potent effect on the efficiency of peptide separations. The choice of gradient-rate will be dictated by the complexity and charge distribution of the peptide mixture to be resolved, but an increase of 5 mM - 20 mM salt/minute is suitable for most purposes (2). Peptide resolution is improved with decreasing gradient rate.

C. RPC

(i) Mobile phase

Of the three major modes of HPLC, RPC offers the widest scope for manipulation of mobile phase characteristics to improve peptide separations. The best initial approach to most analytical peptide separations is to employ the standard chromatographic conditions described previously, i.e., linear AB gradient (1% B/min) at a flow-rate of 1 ml/min, where solvent A is 0.1% aqueous TFA and solvent B is 0.1% TFA in acetonitrile. The best resolution of a peptide mixture is usually obtained between 15% and 40% of the organic solvent in the gradient (25), and this is generally achieved with acetonitrile. TFA is completely volatile, eliminating the need for subsequent sample desalting, and enables detection at wavelengths below 210 nm due to its low UV transparency (2,26).

(a) Effect of organic solvent

In cases where a peptide mixture contains very hydrophobic or very hydrophilic peptides, a more non-polar solvent or a more polar solvent, respectively, may be advantageous (27). The order of effectiveness of the three organic solvents most commonly used in RPC has been shown to be isopropanol > acetonitrile > methanol (25,28,29). This order of effectiveness is reflected in Figure 8, which demonstrates the RPC elution profiles of a mixture of model synthetic peptide analogues with the sequence, Ac-Gly-X-X-$(Leu)_3$-$(Lys)_2$-amide, where position X is substituted by the 20 amino acids found in proteins. The peptides were chromatographed on an analytical C_8 column at a flow-rate of 1 ml/min, using a linear AB gradient (1% B/min), where A = 0.1% aqueous TFA and B = 0.1% TFA in isopropanol (Figure 8, top), acetonitrile (middle) or methanol (bottom). The much superior resolution and selectivity obtained with acetonitrile, compared to that with the alcohols (with the exception of the Pro/Ala peptides), confirmed its value as the best organic solvent for most practical purposes (27).

(b) Effect of ion-pairing reagent

Apart from the suppression of silanol ionization at low pH, TFA is also effective in separating complex peptide mixtures because of its ion-pairing properties. Since peptides are charged molecules at most pH values, the presence of different counterions will influence their chromatographic behaviour. Differences in the polarities of peptides in a peptide mixture can be maximized through careful choice of ion-pairing reagent (2,22). The resolving power of ion-pairing reagents is effected by its interaction with the ionized groups of a peptide. Anionic counterions, such as TFA, will interact with the protonated basic residues of a peptide. A hydrophobic anionic counterion (e.g., trifluoroacetate) is not only capable of ion-pairing with the basic solute, but, due to its hydrophobicity, can increase further the affinity of the peptides for the reversed-phase column. In contrast, a polar hydrophilic counterion (e.g., phosphate, chloride), following ion-pair formation with basic residues, would be unlikely to interact with the non-polar support. The increased peptide retention would only be due to reduction in hydrophilicity of positively charged residues by ion-pair formation (22).

An illustration of the effects of variations in anionic ion-pairing reagent hydrophobicity on the resolution of five basic peptide standards (S1-S5) is demonstrated in Figure 5. All five peptides contained two basic residues, while peptide S1 also contained a free α-amino group (23). The peptides were

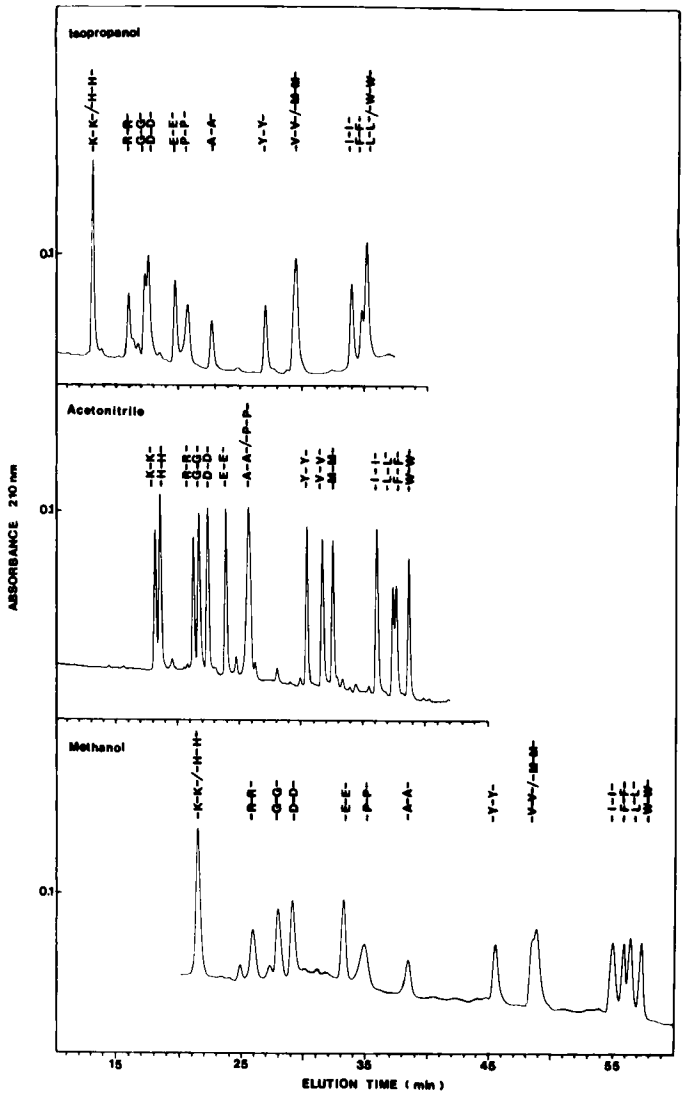

Figure 8. Effect of organic solvents on the reversed-phase separation of an identical mixture of synthetic octapeptides. Column: SynChropak RP-8 C_8 (250 x 4.1 mm I.D.; SynChrom, Linden, IN, U.S.A.). Mobile phase: linear AB gradient (1% B/min), where solvent A is 0.1% aq. TFA and solvent B is 0.1% TFA in isopropanol (top), acetonitrile (middle) or methanol (bottom); flow-rate, 1 ml/min; 26°C.

chromatographed on an analytical C_{18} column under linear AB gradient conditions (A = 0.1% aqueous orthophosphoric acid [Panel A], TFA [Panel B], or HFBA [Panel C] and B = 0.1% of the respective ion-pairing reagents in acetonitrile; 1% B/min, 1 ml/min). HFBA is used widely as the ion-pairing reagent of choice under circumstances where the resolving power of the TFA system has not been sufficient to separate satisfactorily a peptide mixture (22,26,30,31). Apart from its effectiveness as an ion-pairing reagent, it shares with TFA the advantage of volatility and, at low concentrations, UV transparency to permit monitoring of column effluent at 210 nm. Despite being non-volatile, phosphoric acid has proved useful as a hydrophilic ion-pairing agent for hydrophobic peptides and proteins (32-34). Its use, at 210 nm, permits a significant decrease in the concentration of organic solvent in the mobile phase, thus reducing the possibility of denaturation or precipitation. From Figure 5, it can be seen that although all five peptide standards demonstrated increasing retention times with increasing hydrophobicity of ion-pairing reagent (HFBA > TFA > H_3PO_4), the effect was most marked with the more highly charged S1 (+3 net charge), which changed its position in relation to the other four peptides (+2 net charge). A more dramatic demonstration of the value of manipulating counterion hydrophobicity during mobile phase optimization is illustrated in Figure 9. A mixture of seven basic peptides (10-14 residues) with varying numbers of positively charged residues (a peptide number also denotes its net positive charge) was subjected to RPC on an analytical C_{18} column using the same conditions described in Figure 5 (22). The peptides demonstrated increasing retention times with increasing hydrophobicity of the counterion: $HFBA^-$ (Figure 9C) > TFA^- (Figure 9B) >$H_2PO_4^-$ (Figure 9A). In addition, the greater the net charge on a peptide, the greater the effect on its retention time on increasing counterion hydrophobicity. Thus, the elution order of the peptides changed from one counterion to another. For instance, the elution order of peptides 1,3 and 6 (containing one, three and six positively charged residues, respectively) was reversed as the counterion changed from $H_2PO_4^-$ (Figure 9A) to $HFBA^-$ (Figure 9C). It should be noted that, although TFA is the counterion of choice for most purposes, it produced the least effective resolution of this particular peptide mixture, highlighting the value of counterion variations in optimizing peptide separations. In many cases in the purification of synthetic peptides, contaminating peptides and the desired peptide can be very similar in hydrophobicity under the conditions used. If the contaminants vary in the number of positively charged

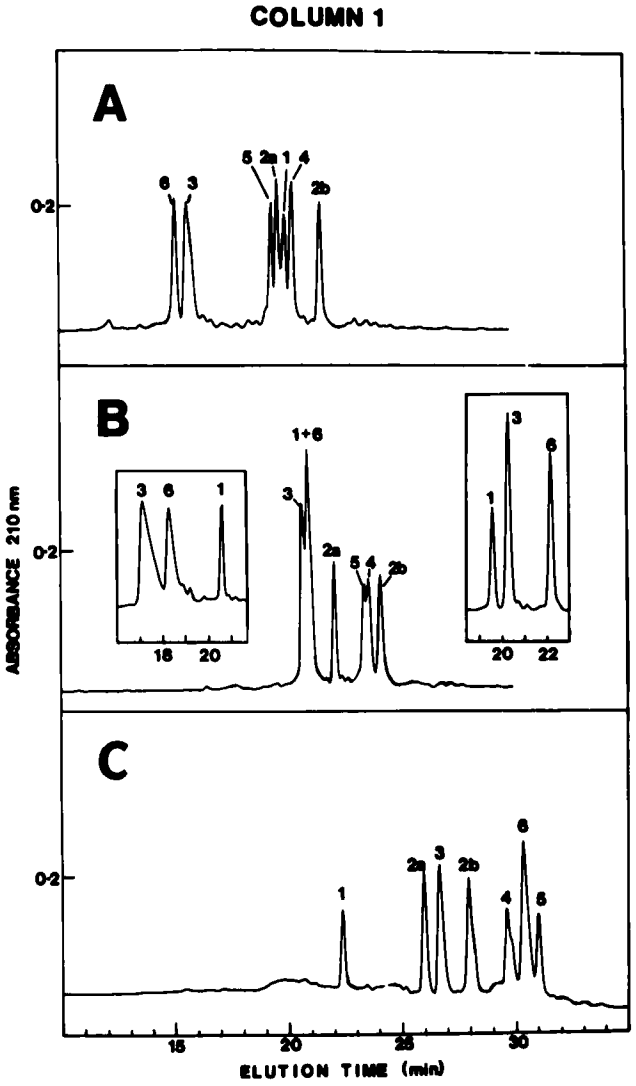

Figure 9. Effect of ion-pairing reagent on the separation of a mixture of basic peptides in RPC. Column: SynChropak RP-P C_{18} (250 x 4.1 mm I.D.; SynChrom, Linden, IN, U.S.A.). Mobile phase: linear gradient (1% B/min), where solvent A is water and solvent B is acetonitrile, both solvents containing H_3PO_4 (panel A), TFA (panel B) or HFBA (panel C); flow-rate, 1 ml/min; 26°C. Panel B, insets: left, 0.01% TFA in solvents A and B; right, 0.4% TFA in solvents A and B. Peptide numbers denote the number of positively charged residues the peptides contain.

residues they contain compared to the peptide of interest, changing the counterion hydrophobicity should resolve these contaminants from the desired peptide, presenting a useful test of peptide homogeneity (22).

Anionic ion-pairing reagents are generally used only at low concentrations (0.05 - 0.1% v/v) in the mobile phase. Counterion concentration may have a marked effect on the retention times of peptides (22,35-37), as demonstrated in Figure 9B (insets). As the concentration of TFA in the elution solvents was increased from 0.01% (left inset) to 0.1% (middle) to 0.4% (right inset), the retention times increased and elution order of peptides 1, 3 and 6 (+1, +3 and +6 net charge, respectively) changed accordingly. The greater the net charge of a peptide, the more its retention time increased with increasing TFA concentration. Thus, the elution order of these three peptides changed from 3, 6 and 1 in 0.01% TFA (left inset) to 1, 3 and 6 in 0.4% TFA. It is interesting to note that this change in peptide elution order is different to that observed when increasing the hydrophobicity of the counterion, i.e., 6, 3 and 1 with $H_2PO_4^-$ (Figure 9A) to 1,3 and 6 with $HFBA^-$ (Figure 9C). It is not a good idea to consistently use high concentrations of acidic ion-pairing reagents to separate peptide mixtures, since these can have a deleterious effect on silica-based RPC columns by gradually cleaving the *n*-alkyl functionalities from the reversed-phase support. However, Guo *et al.* (22) demonstrated that the greatest effect of variations in TFA (and HFBA) concentration on peptide retention was apparent over the 0.01% to 0.2% range of reagent concentration, with only a limited effect at higher acid levels. These results clearly demonstrate the importance of consistency in the concentration of ion-pairing reagent in the mobile phase for accurate run-to-run comparisons of peptide separations. In addition, and in a similar manner to counterion variations, the effect of counterion concentration on contaminating peptides in a synthetic peptide mixture can also be a test of homogeneity of the peptide of interest.

Manipulation of peptide resolution can also be effected through the ionized acidic residues in peptides by employing cationic ion-pairing reagents. Commonly used hydrophobic cationic reagents include tertiary alkylamines (38-40), particularly triethylammonium phosphate. Tetrabutylammonium phosphate has shown particular use as a strongly hydrophobic cationic counterion (2). The use of these reagents (generally in the 2-10 mM concentration range) is, of course, limited to pH values above the pKa's of acidic side-chain groups (~ pH 4.0), necessitating the employment of non-volatile mobile phases to maintain the required pH. Peptide

fractions will subsequently require a further RPC desalting step in a volatile mobile phase. Thus, although cationic ion-pairing can allow good control of the retention behaviour of peptides, it is best to avoid their use, if possible.

(c) Prediction of effect on peptide retention of varying ion-pair reagents

Prior knowledge of the effect of varying counterion hydrophobicity on a peptide elution profile obtained under the standard TFA system would greatly benefit the researcher. This would be particularly important where only limited amounts of sample were available.

Using a water-acetonitrile mobile phase containing H_3PO_4, TFA or HFBA, Guo *et al.* (22) clearly showed that these reagents effected changes in peptide retention solely through interaction with the basic residues in the peptide. In general, each positive charge, whether originating from a lysine, arginine or histidine side-chain, or from an N-terminal α-amino group, exerted an essentially equal effect on peptide retention. These results, demonstrating a simple relationship between peptide retention in different ion-pairing systems, enabled the determination of rules for prediction of peptide retention times in one ion-pairing system from observed retention times in another system.

The contribution of each positively charged residue to shifts in peptide retention is determined by chromatographing a basic peptide standard with both the desired counterion system and the counterion system employed initially. The average contribution of each basic residue to a change in retention time is denoted by Δ/N, where Δ is the shift (in min) in retention time of the standard between the two counterion systems, and N equals the number of positively charged residues in the standard. The column-dependent counterion correction factor (t_i) for a peptide of interest is then obtained by multiplying the number of positively charged residues of the peptide (n) by Δ/N for the standard,

$$t_i = n\,(\Delta/N).$$

This correction factor will have a negative value for a change from a more hydrophobic to a less hydrophobic counterion, while the reverse will require a positive correction factor.

When the retention time of a peptide of interest is known in the presence of one counterion, its predicted position in another counterion system is described by the expression,

$$\tau = t_R^{obs} + t_i,$$

where τ is the predicted peptide retention time in the desired counterion system, t_R^{obs} is the observed retention time in the initial counterion system, and t_i is the counterion correction factor.

This predictive method has proved extremely effective for gauging the possible effect on the resolution of peptide mixtures of varying the counterion hydrophobicity. The only initial requirement of this predictive approach to mobile phase optimization is that the composition of the components of a peptide mixture, or at least the numbers of positively charged groups they contain, must be known.

(ii) Flow-rate and gradient-rate

Although changes in ion-pairing reagent will often offer the more powerful peptide resolving capability, varying the flow-rate and gradient-rate can be very effective in optimizing peptide separations.

(a) Effect of flow-rate and gradient-rate

The resolution between two peaks is described quantitatively by the expression $2\Delta t/W_1 + W_2$, where Δt is the difference (min) between the retention times of the two retained components at their peak maxima, and W_1 and W_2 are the baseline peak widths (min). Generally, peptides demonstrate increasing resolution with increasing flow-rate and decreasing gradient-rate (41).

Under gradient elution conditions, flow-rate changes generally have little effect on peptide retention, provided the gradient-rate is kept low (29,32,41). Thus, the distances between peptide peaks (Δt) remain essentially constant regardless of the flow-rate. However, the tendency for peptides to diffuse decreases with increasing flow-rate, producing smaller peak widths (W_1,W_2) and, hence, improved resolution.

The retention times of peptides are inversely related to gradient-rate (28,41,42). Variations in gradient-rate affect different peptides to different extents, hence the significant effect gradient-rate may have on peptide separations. Mant *et al.* (43) examined the effect of gradient-rate on the reversed-phase separation of five synthetic peptide standards on a semipreparative C_{18} column with linear gradients (aqueous TFA to TFA-acetonitrile) of 0.5%-5.0% B/min at flow-rates of 1-5 ml/min. By plotting peptide retention times against the reciprocal of gradient rate,

these researchers demonstrated that, for most practical purposes, this inverse relationship may be considered linear for the usual working range of gradient-rates (1%-5% B/min). The resulting increase in Δt between two peptides as gradient-rate decreases more than compensates for any concomitant increase in peak widths as the gradient is shallowed, thus leading to improved resolution.

(b) Prediction of effect of flow-rate and gradient-rate

Manipulation of flow-rate and gradient-rate to optimize peptide resolution may be very time (and sample) consuming, particularly if it is necessary to test several permutations of these parameters to achieve the necessary separation. However, a predictive peptide standard approach developed by Mant *et al.* (43), enables rapid and accurate prediction of the effects of flow-rate and gradient-rate on peptide retention behaviour with maximum conservation of peptide sample.

The predicted retention time of a peptide at varying flow-rates and gradient-rates may be calculated by the expression:

$$\tau\,(y\%,y\text{ ml}) = t_R\,(x\%,x\text{ ml}) \times (x\%/y\%) + f,$$

where τ is the predicted retention time at the desired gradient-rate (y% B/min) and flow-rate (y ml/min), t_R is the observed retention time at an initial gradient-rate (x% B/min) and flow-rate (x ml/min), and f is a gradient-rate and/or flow-rate correction factor. This factor is determined from the expression,

$$f = t_R^{std(y\%,\,y\text{ ml})} - (t_R^{std(x\%,\,x\text{ ml})} \times [x\%/y\%]),$$

where t_R^{std} is the observed retention time of a peptide standard at the desired run conditions (y% B/min, y ml/min) and at the initial run conditions (x% B/min, x ml/min). Thus, accurate prediction of peptide retention times at various flow-rates, gradient-rates, or a combination of the two, simply requires chromatographing a peptide standard under two sets of conditions (initial and desired) in addition to our initial RPC separation of the peptide sample mixture. The number of reversed-phase runs may be reduced even further if, under the initial chromatographic conditions, the standard is included in the peptide sample mixture.

The excellent accuracy of this predictive approach is clearly demonstrated in Table 1. Five decapeptide standards, S1-S5, were subjected to reversed-phase gradient elution separation (0.05% aqueous TFA to 0.05% TFA in acetonitrile) under a wide range of flow-rate and gradient-rate conditions. With S4 as the

TABLE 1

Comparison of Predicted Versus Observed Peptide Retention Times [a]

Flow Rate (ml/min)	Gradient Rate (% B/min)	PEPTIDE S1 t_R^{obs} (min)	PEPTIDE S1 Δt[b]	PEPTIDE S1 Δ%B[c]	PEPTIDE S2 t_R^{obs} (min)	PEPTIDE S2 Δt	PEPTIDE S2 Δ%B	PEPTIDE S3 t_R^{obs} (min)	PEPTIDE S3 Δt	PEPTIDE S3 Δ%B	PEPTIDE S5 t_R^{obs} (min)	PEPTIDE S5 ΔT	PEPTIDE S5 Δ%B
1	0.5	36.8	0.8	0.4	40.7	0.2	0.1	42.9	0.2	0.1	54.1	0.2	0.1
	1.0	30.1	0.8	0.8	32.4	0.1	0.1	33.5	0.1	0.1	39.1	0.1	0.1
	2.0	25.8	0.5	1.0	27.0	0.1	0.2	27.6	0.1	0.2	30.3	0.1	0.2
	3.0	24.0	0.3	0.9	24.9	0.1	0.3	25.2	0	0	27.0	0.1	0.3
	5.0	22.6	0.1	0.5	23.3	0.3	1.5	23.3	0.1	0.5	24.3	0	0
3	0.5	19.0	0.2	0.1	22.3	0.4	0.2	24.4	0.4	0.2	35.1	0.3	0.2
	1.0	14.3	0.2	0.5	16.1	0.1	0.1	17.2	0.2	0.2	22.6	0.1	0.1
	2.0	11.2	0.2	0.4	12.3	0.1	0.2	12.7	0	0	15.5	0	0
	3.0	10.0	0.2	0.6	10.7	0	0	11.0	0.1	0.3	12.9	0	0
	5.0	8.9	0.1	0.5	9.3	0	0	9.5	0	0	10.6	0	0
5	0.5	13.6	0.5	0.3	16.7	0.5	0.3	18.8	0.5	0.3	29.2	0.3	0.2
	1.0	10.1	0.1	0.1	11.8	0.2	0.2	12.9	0.2	0.2	18.3	0.1	0.1
	2.0	7.8	0.1	0.2	8.8	0.1	0.2	9.3	0.1	0.2	12.0	0	0
	3.0	6.8	0.2	0.6	7.5	0.1	0.3	7.9	0.1	0.3	9.7	0	0
	5.0	5.9	0.1	0.5	6.4	0.1	0.5	7.5	0.1	0.5	7.7	0	0

(a) The observed retention times were obtained using a 250 x 10 mm I.D. semi-preparative reversed-phase column (Synchropak RP-P C-18, 300 Å, 6.5 μm particle size with approximately 10% carbon loading from SynChrom, Linden, IN, U.S.A). The predicted retention times were calculated from observed values on a 220 x 4.6 mm I.D. analytical reversed-phase column (Aquapore RP-300 C-8, 7μm particle size from Pierce Chemical Co., Rockford, IL, U.S.A.). The flow-rates on the analytical column were 0.5, 1.0, 1.5 and 2.0 ml/min. At each flow-rate, eight different gradient-rates were performed (0.5, 0.67, 1.0, 1.33, 2, 3, 4 and 5% B/min, where B = 0.05% TFA in acetonitrile).

(b) Each Δt value is the average error between the observed retention time and the 32 predicted retention times calculated from 8 gradient-rates at each of 4 different flow-rates.

(c) ΔB% is the average error in % organic modifier. For example, a Δt value of 0.1 min at a gradient-rate of 2% B/min is equivalent to an average error in % organic modifier of 0.2%.

internal standard, predicted retention values for peptides S1, S2, S3 and S5 were calculated for all combinations of five gradient-rates (0.5%, 1.0%, 2.0%, 3.0% and 5.0% acetonitrile/min) and three flow-rates (1,3 and 5 ml/min) on a semipreparative C18 column, from observed retention times of the peptides at all combinations of eight gradient-rates (0.5%, 0.67%, 1.0%, 1.33%, 2.0%, 3.0%, 4.0% and 5.0% acetonitrile/min) and four flow-rates (0.5, 1.0, 1.5 and 2.0 ml/min) on an analytical C8 column. The average error between all predicted and observed retention times of the peptides is only 0.3 min for peptide S1 and 0.1 min for each of peptides S2, S3 and S5. This represents the results obtained from a total of 480 separate predictions for each peptide. The accuracy of this approach is particularly impressive in light of the fact that variations in peptide retention for any one peptide was as much as ~46 min on either column.

In addition to allowing the researcher to predict rapidly the effect of flow-rate and gradient-rate variations on peptide retention, the results presented in Table 1 also demonstrates that this predictive approach allows for changes in column parameters (column dimensions, *n*-alkyl chain length and/or ligand density). This enables the researcher, if so desired, to scale up the final RPC step in a multimodal purification approach from an analytical to a semipreparative column.

CONCLUSION

It is our prediction that rapid growth in the computer simulation of HPLC elution profiles will be of immense aid to researchers' optimization of peptide separations in the future.

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REFERENCES

1. Mant, C.T. and Hodges, R.S., J. Chromatogr., 326, 349 (1985).
2. Mant, C.T. and Hodges, R.S., in "High-Performance Liquid Chromatography of Biological Macromolecules: Methods and Applications", (K. Gooding and F. Regnier, eds.), Marcel Decker Inc., 1988 (in press).

3. Mant, C.T., Parker, J.M.R. and Hodges, R.S., J. Chromatogr., 397, 99 (1987).

4. Gariépy, J., Sykes, B.D. and Hodges, R.S., Biochemistry, 22, 1765 (1983).

5. Takahashi, N., Ishioka, N., Takahashi, Y. and Putnam, F.W., J. Chromatogr., 326, 407 (1985).

6. Mant, C.T. and Hodges, R.S., J. Chromatogr., 327, 147 (1985).

7. Patience, R.L. and Rees, L.H., J. Chromatogr., 352, 241 (1986).

8. Takahashi, N., Takahashi, Y. and Putnam, F.W., J. Chromatogr., 266, 511 (1983).

9. Cachia, P.J., Van Eyk, J., Chong, P.C.S., Taneja, A.K. and Hodges, R.S., J. Chromatogr., 266, 651 (1983).

10. Kumagaye, K.Y., Takai, M., Chino, N., Kimura, T. and Sakakibara, S., J. Chromatogr., 327, 327 (1985).

11. Dizdaroglu, M. and Krutzsch, H.C., J. Chromatogr., 264, 223 (1983).

12. Benson, J.R. and Woo, D.J., J. Chromatogr. Sci., 22, 386 (1984).

13. Gooding, K.M. and Schmuck, M.N., J. Chromatogr., 327, 139 (1985).

14. Regnier, F.E., Methods Enzymol., 91, 137 (1983).

15. Lau, S.Y.M., Taneja, A.K. and Hodges, R.S., J. Chromatogr., 317, 129 (1984).

16. Taneja, A.K., Lau, S.Y.M. and Hodges, R.S., J. Chromatogr., 317, 1 (1984).

17. Kikta, E.J. and Stange, A.E., J. Chromatogr., 138, 41 (1977).

18. Pfannkoch, E., Lu, K.C. and Regnier, F.E., J. Chromatogr. Sci., 18, 430 (1980).

19. Engelhardt, H. and Mathes, D., Chromatographia, 14, 325 (1981).

20. Engelhardt, H., Ahr, G. and Hearn, M.T.W., J. Liq. Chromatogr., 4, 1361 (1981).

21. Kopaciewicz, W. and Regnier, F.E., Anal. Biochem., 126, 8 (1982).

22. Guo, D., Mant, C.T. and Hodges, R.S., J. Chromatogr., 386, 205 (1987).

23. Mant, C.T. and Hodges, R.S., LC, Liq. Chromatogr. HPLC Mag., 4, 250 (1986).

24. Mant, C.T. and Hodges, R.S., Chromatographia, 24, 805 (1987).

25. Hermodson, M. and Mahoney, W.C., Methods Enzymol., 91, 352 (1983).

26. Bennett, H.P.J., Browne, C.A. and Solomon, S., J. Liq. Chromatogr., 3, 1353 (1980).

27. Guo, D., Mant, C.T., Taneja, A.K., Parker, J.M.R. and Hodges, R.S., J. Chromatogr., 359, 499 (1986).

28. Wilson, K.J., Honegger, A., Stötzel, R.P. and Hughes, G.J., Biochem. J., 199, 31 (1981).

29. Mahoney, W.C. and Hermodson, M.A., J. Biol. Chem., 255, 11199 (1980).

30. Bennett, H.P.J., J. Chromatogr., 266, 501 (1983).

31. Browne, C.A., Bennett, H.P.J. and Solomon, S., Anal. Biochem., 124, 201 (1982).

32. O'Hare, M.J. and Nice, E.C., J. Chromatogr., 171, 209 (1979).

33. Grego, B., Lambrou, F. and Hearn, M.T.W., J. Chromatogr., 266, 89 (1983).

34. Gaertner, H. and Puigserver, A., J. Chromatogr., 350, 279 (1985).

35. Schaaper, W.M.M., Voskamp, D. and Olieman, C., J. Chromatogr., 195, 181 (1980).

36. Starratt, A.N. and Steven, M.E., J. Chromatogr., 194, 421 (1980).

37. Hearn, M.T.W. and Grego, B., J. Chromatogr., 203, 349 (1981).

38. Hearn, M.T.W. and Grego, B., J. Chromatogr., 255, 125 (1983).

39. Hearn, M.T.W. and Grego, B., J. Chromatogr., 266, 75 (1983).

40. Tarr, G.E. and Crabb, J.W., Anal. Biochem., 131, 99 (1983).

41. Guo, D., Mant, C.T., Taneja, A.K. and Hodges, R.S., J. Chromatogr., 359, 519 (1986).

42. Sasagawa, T., Okuyama, T. and Teller, D.C., J. Chromatogr., 240, 329 (1982).

43. Mant, C.T., Burke, T.W.L. and Hodges, R.S., J. Chromatogr. (submitted).