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Mixed-mode hydrophilic and ionic interaction chromatography rivals reversed-phase liquid chromatography for the separation of peptides

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ABSTRACT

Peptide separations based upon mixed-mode hydrophilic and ionic interactions with a strong cation-exchange column have been investigated. The peptide separations were generally achieved by utilizing a linear increasing salt (sodium perchlorate) gradient in the presence of acetonitrile (20–90%, v/v) at pH 7. The presence of acetonitrile in the mobile phase promotes hydrophilic interactions with the hydrophilic stationary phase, these hydrophilic interactions becoming increasingly important to the separation process as the acetonitrile concentration is increased. At acetonitrile concentrations of 20–50% (v/v) in the mobile phase, the peptides utilized in this study were eluted in order of increasing net positive charge, indicating that ionic interactions were dominating the separation process. Peptides with the same net positive charge were also well resolved by an hydrophilic interaction mechanism, being eluted in order of increasing hydrophilicity (decreasing hydrophobicity). At higher acetonitrile concentrations (70–90%, v/v), column selectivity was changed dramatically, with hydrophilic interactions now dominating the separation process. Under these conditions, specific peptides may be eluted earlier or later than less highly charged peptides, depending upon their hydrophilic/hydrophobic character. This mixed-mode methodology was compared to reversed-phase liquid chromatography of the peptides at pH 2 and pH 7. The results of this comparison suggested that mixed-mode hydrophilic-ion-exchange chromatography on a strong cation-exchange column rivals reversed-phase liquid chromatography for peptide separations.

INTRODUCTION

The utility of ion-exchange chromatography (IEC) for peptide separations has been somewhat overshadowed in the past by the extensive employment of reversed-phase liquid chromatography (RPLC) for such applications. The major advantages of the latter technique, apart from its powerful resolving capability, include the availability of volatile mobile phases, aqueous trifluoroacetic acid (TFA)–acetonitrile systems being the most frequently employed [1]. Such volatile mobile phases avoid the need for sample desalting, frequently an important consideration for subsequent peptide characterization or use. Thus, although IEC has been successfully applied to peptide mixtures [1–8], occasionally as one part of a multi-step protocol (*e.g.*, IEC followed by RPLC for a final desalting/purification step) [1,2,7], its use to date has certainly not rivalled that of RPLC.

Although the major process governing peptide retention behaviour on ion-exchange columns involves ionic interactions between the column matrix and the peptide solutes, all ion-exchange packings have, in our hands, also exhibited some hydrophobic character leading to long peptide retention times and peak broadening [1,8]. Most researchers prefer to avoid separations based upon such mixed-mode ionic-hydrophobic column behaviour. Thus, an organic solvent, such as acetonitrile, is frequently added to the mobile phase buffers to suppress any such hydrophobic packing characteristics [1,8]. Recently, this laboratory demonstrated that manipulation of the acetonitrile concentration (20–50%) in the mobile phase buffers enabled considerable flexi-

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bility in the separation of basic peptides on a PolySulfoethyl A strong cation-exchange column [9]. Thus, at lower levels of acetonitrile, with hydrophobic interactions suppressed, the peptides were separated by an ionic mechanism only, *i.e.*, peptides were eluted in order of increasing net positive charge. However, as the level of acetonitrile was raised, while ionic interactions were still the chief separation mechanism (*i.e.*, peptides were still eluted in order of increasing net positive charge), hydrophilic interactions between the peptides and the column also came into play. Such a mixed-mode mechanism allowed the efficient resolution of peptides containing the same number of positively charged residues, with these identically charged peptides being eluted with excellent peak shape in order of increasing peptide hydrophilicity (or decreasing peptide hydrophobicity). The term hydrophilic interaction chromatography (HILIC) has been recently coined to describe separations based on solute hydrophilicity [10]. HILIC effects were more pronounced on the cation-exchange column in comparison to the HILIC column [9].

The present study extends our investigation of strong cation-exchange chromatography operated under controlled conditions favouring a mixed-mode hydrophilic-ionic interaction mechanism for peptide applications. By comparing the retention behaviour of a series of model peptides in this mixed-mode hydrophilic-ionic chromatography with that of their retention behaviour during RPLC, we have been able to draw some extremely positive conclusions concerning the potential of this novel cation-exchange approach to peptide separations.

EXPERIMENTAL

Materials

HPLC-grade water and acetonitrile and reagent-grade sodium perchlorate ($NaClO_4$) were obtained from J. T. Baker (Phillipsburg, NJ, USA). HPLC-grade trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, USA) and ACS-grade orthophosphoric acid (H_3PO_4) from Anachemia (Toronto, Canada). Synthetic model peptides were obtained from Synthetic Peptides (University of Alberta, Edmonton, Canada).

Instrumentation

The high-performance liquid chromatographic (HPLC) system consisted of a Spectra-Physics (San José, CA, USA) SP8700 solvent-delivery system, SP8750 organizer coupled to an Hewlett-Packard (Avondale, PA, USA) HP 1040A detection system, HP 3390A integrator, HP 85 computer, HP 9121 disc drive and HP 7470 plotter.

Columns

Peptides were separated on two columns: (1) a Zorbax SB-300 C_8 reversed-phase column, 150 × 4.6 mm I.D., 6 μm particle size, 250 Å pore size (Rockland Technologies, West Chester, PA, USA); and (2) a polysulfoethylaspartamide (PolySulfoethyl A) strong cation-exchange column, 200 × 4.6 mm I.D., 5 μm, 300 Å (PolyLC, Columbia, MD, USA).

RESULTS AND DISCUSSION

Synthetic model peptides

The relevant properties of the model peptides employed in this study are shown in Table I. Peptide pairs a–e, b–f, c–g and d–h possess identical amino acid sequences, the only difference being that peptides a, b, c and d contain a free N-terminal α-amino group, while peptides e, f, g and h are acetylated (*i.e.*, blocked) at their N-terminals.

Peptides i, j, k and l are commercially available as standards for monitoring cation-exchange column performance.

All of the peptides contain only basic (*i.e.*, potentially positively charged) residues (Lys, Arg, α-amino group), with no acidic residues (*i.e.*, potentially negatively charged) present, thus simplifying interpretation of results. In Figs. 2–5, the peptides are denoted by a number in addition to their letter. The number denotes the number of potentially positively charged groups a particular peptide contains, *e.g.*, k3 denotes that peptide k has three potentially positively charged groups. The presence of tyrosine in peptides d, h, j and l permits detection of these peptides at 280 nm in addition to peptide bond absorbance at 210 nm.

Reversed-phase liquid chromatography of model peptides

In order to determine the overall hydrophobicity/

TABLE I

PROPERTIES OF SYNTHETIC PEPTIDES USED IN THIS STUDY

Peptide	Peptide sequence[a]	No. of potentially positively charged residues[b]	Relative hydrophobicity[c]	
			pH 2	pH 7
a	*NH_2-*Arg-Gly-**Gly-Gly**-Gly-Leu-Gly-Leu-Gly-*Lys-amide	3	16.4	20.6
b	*NH_2-*Arg-Gly-**Ala-Gly**-Gly-Leu-Gly-Leu-Gly-*Lys-amide	3	17.1	21.4
c	*NH_2-*Arg-Gly-**Val-Gly**-Gly-Leu-Gly-Leu-Gly-*Lys-amide	3	19.6	24.2
d	*NH_2-*Arg-Gly-**Val-Tyr**-Gly-Leu-Gly-Leu-Gly-*Lys-amide	3	22.3	27.2
e	Ac-*Arg-Gly-**Gly-Gly**-Gly-Leu-Gly-Leu-Gly-*Lys-amide	2	17.5	20.9
f	Ac-*Arg-Gly-**Ala-Gly**-Gly-Leu-Gly-Leu-Gly-*Lys-amide	2	18.3	21.8
g	Ac-*Arg-Gly-**Val-Gly**-Gly-Leu-Gly-Leu-Gly-*Lys-amide	2	20.8	24.4
h	Ac-*Arg-Gly-**Val-Tyr**-Gly-Leu-Gly-Leu-Gly-*Lys-amide	2	23.7	27.5
i	Ac-Gly-Gly-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-*Lys-amide	1	17.7	19.2
j	Ac-*Lys-Tyr-Gly-Leu-Gly-Gly-Ala-Gly-Gly-Leu-*Lys-amide	2	20.4	23.2
k	Ac-Gly-Gly-Ala-Leu-*Lys-Ala-Leu-*Lys-Gly-Leu-*Lys-amide	3	26.0	31.7
l	Ac-*Lys-Tyr-Ala-Leu-*Lys-Ala-Leu-*Lys-Gly-Leu-*Lys-amide	4	27.1	35.0

[a] Ac = N^{α}-Acetyl; amide = C^{α}-amide. Variations in the composition of peptides a–h are shown in bold.
[b] Potentially positively charged residues (Lys, Arg, free α-NH_2 group) are denoted*.
[c] Relative peptide hydrophobicity is represented by their reversed-phase retention times, as described in Fig. 1.

hydrophilicity of the model peptides at pH 2 and pH 7, they were subjected to RPLC. Fig. 1A and D shows the elution profiles of peptides a to h and i to l, respectively, on a C_8 column following application of a linear gradient (1% B/min at a flow-rate of 1 ml/min), where eluent A was 0.1% aqueous TFA and eluent B was 0.1% TFA in acetonitrile. At the pH of this commonly employed mobile phase system (pH 2), all of the potentially positively charged groups in the peptides (Table I) possess a full positive charge. Thus, peptides a to d each exhibit a net charge of +3; peptides e to h each exhibit a net charge of +2; peptides i, j, k and l exhibit net charges of +1, +2, +3 and +4, respectively. The retention times of the peptides under these conditions represent their relative hydrophobicities/hydrophilicities at pH 2 (Table I). From Fig. 1A, it can be seen that for peptide pairs of identical sequence (a–e, b–f, c–g, d–h), the analogues containing a free α-amino group (peptides a, b, c and d) were always eluted prior to their acetylated versions (peptides e, f, g and h). This was not surprising, considering the hydrophilic nature of the full positive charge on the α-amino groups.

Fig. 1B and E shows the RPLC elution profiles of peptides a to h (Fig. 1B) and i to l (Fig. 1E) obtained under the same conditions as Fig. 1A and D, save for the substitution of 0.1% H_3PO_4 for 0.1% TFA. The pH of the two acidic mobile phases remains the same (pH 2). The elution profiles of the peptides under the H_3PO_4 system are markedly different from those obtained with the TFA system. Not only are all peptides eluted earlier on the H_3PO_4 system, but the relative elution order of peptides was occasionally changed. Thus, from Fig. 1B (compare to 1A), peptide c is now eluted prior to peptide f and peptide d is eluted prior to peptide g (the latter pair forming a doublet); from Fig. 1E (compare 1D), peptide l is now eluted with peptide k. The decrease in peptide elution times and the changes in peptide elution order in the H_3PO_4 system compared to the TFA system can be rationalized by considering the relative hydrophobicities/hydrophilicities of the anionic (*i.e.*, negatively charged) trifluoroacetate and phosphate counterions. Ion-pairing reagents such as H_3PO_4 and TFA effect changes in peptide retention time solely through interaction with positively charged groups on a peptide [1,11]. Since, the phosphate ion of H_3PO_4 is a significantly more hydrophilic counterion than the trifluoroacetate

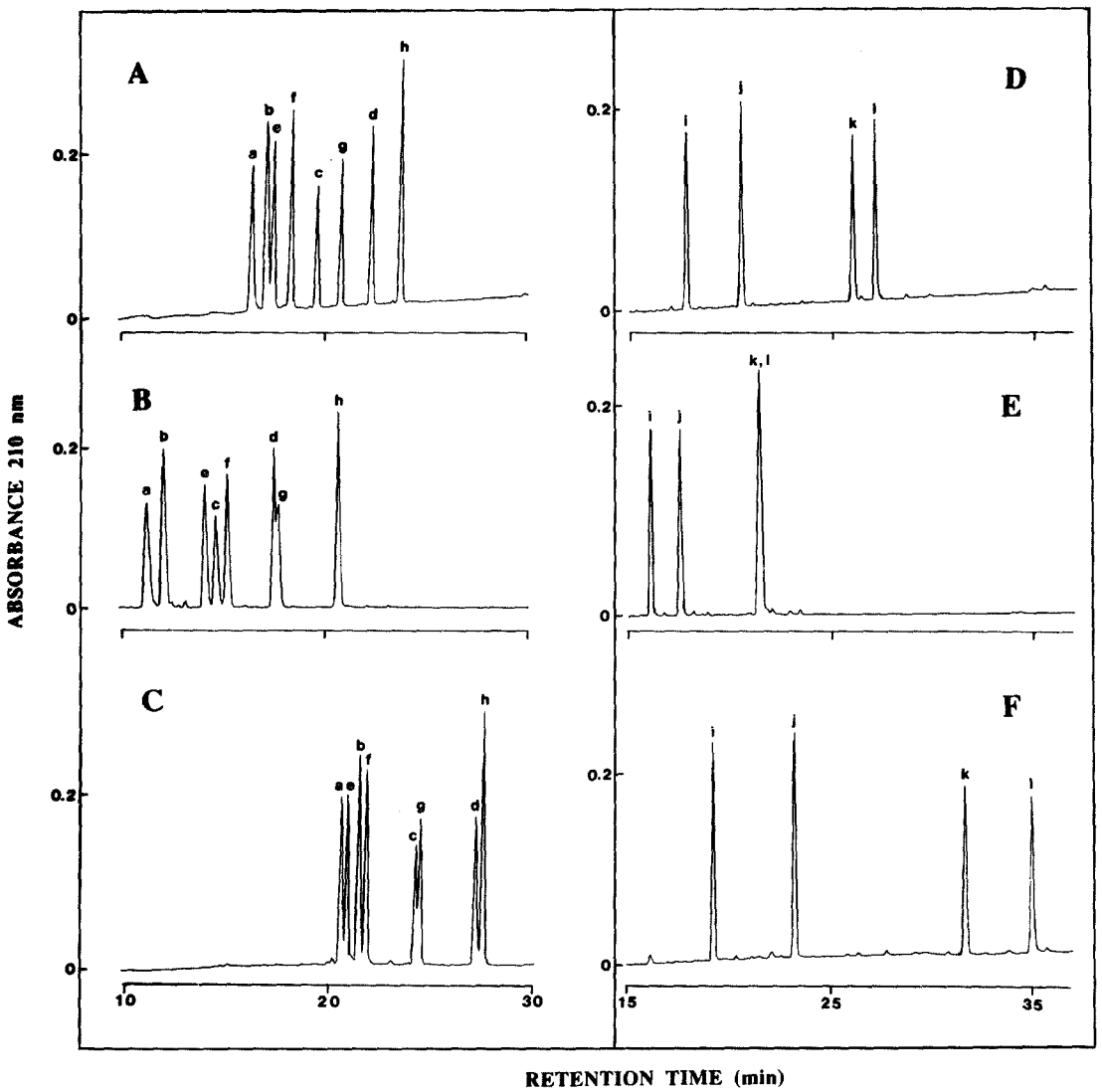

Fig. 1. Separation of mixtures of positively charged peptides by RPLC. Column, Zorbax SB-300 C_8 (150 × 4.6 mm I.D.). Mobile phase: panels A and D, linear A–B increasing acetonitrile gradient (1% B/min, starting with 100% A) at a flow-rate of 1 ml/min, where A is 0.1% aqueous TFA and B is 0.1% TFA in acetonitrile; panels B and E, linear A–B increasing acetonitrile gradient (1% B/min, starting with 100% A) at a flow-rate of 1 ml/min, where A is 0.1% aqueous H_3PO_4 and B is 0.1% H_3PO_4 in acetonitrile; panels C and F, linear A–B increasing acetonitrile gradient (2% B/min, equivalent to 1% acetonitrile/min,starting with 100% A) at a flow-rate of 1 ml/min, where A is 10 m*M* aqueous $(NH_4)_2HPO_4$, pH 7, and B is 50% aqueous acetonitrile containing 10 m*M* $(NH_4)_2HPO_4$, pH 7, both A and B also containing 200 m*M* $NaClO_4$. Temperature, 26°C.

ion, all 12 basic (*i.e.*, potentially positively charged) peptides shown in Table I would be expected to be eluted earlier in the presence of the phosphate ion compared to the trifluoroacetate ion, as was, indeed, the case. In addition, the magnitude of the effect on a particular peptide of changing from a more hydrophobic (less hydrophilic) ion-pairing reagent such as TFA to a less hydrophobic (more hydrophilic) ion-pairing reagent such as H_3PO_4 will depend on the number of positive charges the peptide contains —the greater the number of positive charges on a peptide, the greater the effect of increasing counter-

ion hydrophilicity, *i.e.*, the larger the decrease in retention time on substituting H_3PO_4 for TFA. Thus, comparing Fig. 1A and B, peptides with net charges of +3 (peptides a to d) exhibited an average decrease in retention time of *ca.* 5 min on substituting H_3PO_4 (Fig. 1B) for TFA (Fig. 1A); in contrast, peptides with net charges of +2 (peptides e to h) exhibited an average decrease in retention time of only *ca.* 3 min. Comparing Fig. 1D and E, the retention times of the four peptides decreased in the H_3PO_4 system (Fig. 1E) from that of the TFA system (Fig. 1D) by values of 1.8 min (peptide i; +1 net charge), 3 min (peptide j; +2), 4.5 min (peptide k; +3) and 5.6 min (peptide l; +4).

Fig. 1C and F shows the elution profiles of the peptides at pH 7. These elution profiles were obtained under linear gradient conditions (1% acetonitrile/min at a flow-rate of 1 ml/min), where the mobile phase pH 7 buffers also contained 0.2 *M* sodium perchlorate ($NaClO_4$). The addition of perchlorate was necessary for the suppression of ionic interactions between the positively charged peptides and negatively charged free silanols (pK_a *ca.* 4) present on the silica-based stationary phase. Such undesirable interactions, if unsuppressed, may lead to significant band broadening and peak tailing of basic solutes. The addition of perchlorate was not necessary for the TFA and H_3PO_4 systems, since silanol ionization is effectively suppressed at pH 2 on this column. From Fig. 1C and F, the retention times of all 12 peptides have increased in the pH 7 system compared to the TFA system (Fig. 1A and D). The dramatic change in elution orders and increase in retention times at pH 7 are probably due to a combination of effects. The high salt concentration in the mobile phase (200 m*M* sodium perchlorate) is possibly promoting hydrophobic interactions of the peptides with the stationary phase by increasing the hydrophilicity of the mobile phase, leading to a general increase in peptide retention times over those observed at pH 2. In addition, the high concentration of the negatively charged hydrophilic perchlorate counterion could decrease the hydrophilicity of the peptides by ion-pair formation with the positively charged groups in the peptides. This effect was previously demonstrated with the negatively charged trifluoroacetate counterion which, on increasing the counterion concentration, increased peptide hydrophobicity through ion-pair formation with positively charged residues [11]. Interestingly, the peptides having identical sequence are eluted in pairs (a–e, b–f, c–g and d–h) where peptides e, f, g and h are the peptides with their N-terminals acetylated while peptides a, b, c, and d have free α-amino groups. These results suggest that the α-amino groups at pH 7 are not fully charged and therefore the acetylated and non-acetylated peptides of the same sequence have similar overall hydrophobicities at pH 7 when compared to pH 2 (the differences in elution times of the peptide pairs in panel A varies from 1.1 to 1.4 min compared to 0.2 to 0.4 min in panel C). The pK_a of an α-amino group in free amino acids is generally in the range of 9 to 10, but may be lowered significantly in peptides and proteins, depending on the microenvironment created by the N-terminal residue side-chain. Hence, deprotonation of the α-amino group may occur at pH values around neutrality. This laboratory has demonstrated (unpublished results) that this effect is not limited to basic residues (such as the N-terminal arginine residues of peptides a–h), but occurs to a greater or lesser extent with all 20 amino acids. The different counterions and pH values were chosen for RPLC to allow for a better comparison of RPLC with the mixed-mode hydrophilic–cation-exchange chromatography.

Mixed-mode chromatography of peptides on a cation-exchange column

While the major separation mechanism of IEC is electrostatic in nature, ion-exchange packings may also exhibit significant hydrophobic characteristics, giving rise to mixed-mode contributions to solute separations. Fig. 2A and C shows elution profiles of peptides a3, d3, e2 and h2 (Fig. 2A) and peptides i1, j2, k3 and l4 (Fig. 2B) on the PolySulfoethyl A strong cation-exchange column. The peptides were eluted by a linear gradient (5 m*M* salt/min at a flow-rate of 1 ml/min) of $NaClO_4$ in triethylammonium phosphate (TEAP) buffer at pH 7. As would be expected, the major peptide separation mechanism was electrostatic in nature with the more highly charged a3 and d3 being eluted later than the lesser charged e2 and h2. Similarly, peptides i1, j2 and k3 (+1, +2 and +3 net charge, respectively) were eluted in order of increasing net positive charge. However, it is clear that a secondary hydrophobic separation mechanism is also present, with the more

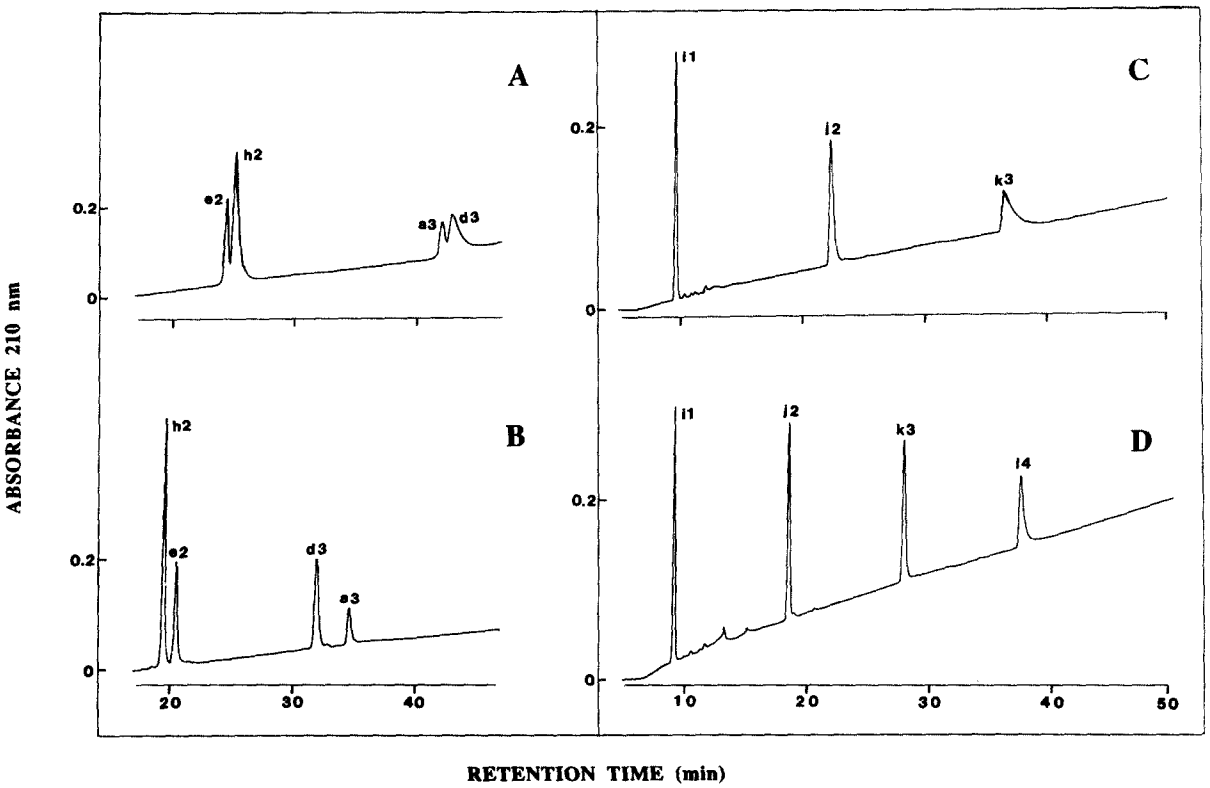

Fig. 2. Demonstration of hydrophobic and hydrophilic interactions between positively charged peptides and the stationary phase during strong cation-exchange chromatography. Column, PolySulfoethyl A strong cation-exchange column (200 × 4.6 mm I.D.). Mobile phase: panels A and C, linear A–B increasing salt gradient (2% B/min, equivalent to 5 m*M* $NaClO_4$/min, starting with 100% A) at a flow-rate of 1 ml/min, where A is 5 m*M* aqueous TEAP, pH 7, and B is A plus 0.25 *M* $NaClO_4$, pH 7; panels B and D, linear A–B increasing salt gradient (2% B/min, equivalent to 5 m*M* $NaClO_4$/min, starting with 100% A) at a flow-rate of 1 ml/min, where A is 5 m*M* aqueous TEAP, pH 7, and B is A plus 0.25 *M* $NaClO_4$, pH 7, both A and B containing 15% (v/v) acetonitrile. Temperature, 26°C. Peptides are denoted by both a letter and a number, with the number denoting the number of potentially positively charged groups a particular peptide contains, *e.g.*, d3 denotes that peptide d has three potentially positively charged groups. The sequences of the peptides are shown in Table I.

hydrophobic h2 and d3 being eluted after the less hydrophobic e2 and a3, respectively (Fig. 2A). Also, peptide l4 (+4 net charge and the most hydrophobic peptide employed in this study; Table I) was not even eluted from the column under these conditions.

Fig. 2B and D shows elution profiles of the same peptide mixtures under the same run conditions, save for the addition of 15% (v/v) acetonitrile to the mobile phase to overcome hydrophobic interactions. The excellent solubility of TEAP and $NaClO_4$ in such aqueous acetonitrile solutions recommended their employment in the present study. From Fig. 2B and D, the peptides (including l4) were eluted earlier, and with generally improved peak shape, compared to the elution profiles obtained in the absence of acetonitrile (Fig. 2A and C). In addition, when comparing Fig. 2B with A, it can also be seen that like-charged peptide pairs e2–h2 and a3–d3 have reversed their elution orders in the presence of 15% acetonitrile (Fig. 2B) compared to those observed in its absence (Fig. 2A). Thus, the peptides are now eluted in order of increasing hydrophilicity with e2 being eluted after h2, and a3 being eluted after d3. Hence, following suppression of hydrophobic interactions by the addition of acetonitrile, the peptide separation mechanism has become based upon

mixed-mode ionic and hydrophilic interactions (Fig. 2B), compared to the mixed-mode ionic and hydrophobic interactions apparent in the absence of acetonitrile (Fig. 2A). This mixed-mode hydrophilic-cation-exchange chromatography (HILIC-CEC) separation resulted in an excellent separation of identically charged peptides, with good peak shape and baseline resolution.

Effect of acetonitrile concentration on HILIC-CEC of peptides

The results shown in Fig. 2, concerning the induction of a mixed hydrophilic ionic mode of chromatography in the presence of acetonitrile, prompted a more thorough investigation of the effect of increasing acetonitrile concentration (up to 90%, v/v) on peptide elution profiles during HILIC-CEC. Fig. 3 shows the elution profiles of peptides a to h (Table I) obtained on the PolySulfoethyl A column under the same gradient elution conditions as Fig. 2, save for the presence of 80% (Fig. 3A), 70% (Fig. 3B), 30% (Fig. 3C) or 15% (Fig. 3D) (v/v) acetonitrile in the mobile phase.

From Fig. 3, hydrophilic interactions between the peptides and the stationary phase generally increased with increasing acetonitrile concentration. This is apparent from the progressively improved resolution of the identically charged peptides as the acetonitrile concentration was raised. Thus, at a level of 15% acetonitrile (Fig. 3D), each set of four peptides of like charge (a3 to d3; e2 to h2) were not completely resolved. In contrast, at acetonitrile concentrations of 70% (Fig. 3B) and 80% (Fig. 3A), all eight peptides are resolved to baseline with good peak shape and no tailing. At all levels of acetonitrile shown in Fig. 3, peptides of like charge were eluted in order of increasing hydrophilicity.

The increasing importance of an hydrophilic mechanism in the mixed-mode chromatography illustrated in Fig. 3 is also highlighted by considering the elution behaviour of peptides e2 and d3. Although more highly charged, d3 is considerably more hydrophobic (less hydrophilic) relative to e2 from Table I, d3 and e2 exhibited RPLC retention times of 27.2 min and 20.9 min, respectively, at pH 7. If the peptides were being separated solely by an hydrophilic mechanism, d3 would be eluted prior to e2, which is clearly not the case in Fig. 3. From Fig. 3, since the hydrophilic separation mechanism is

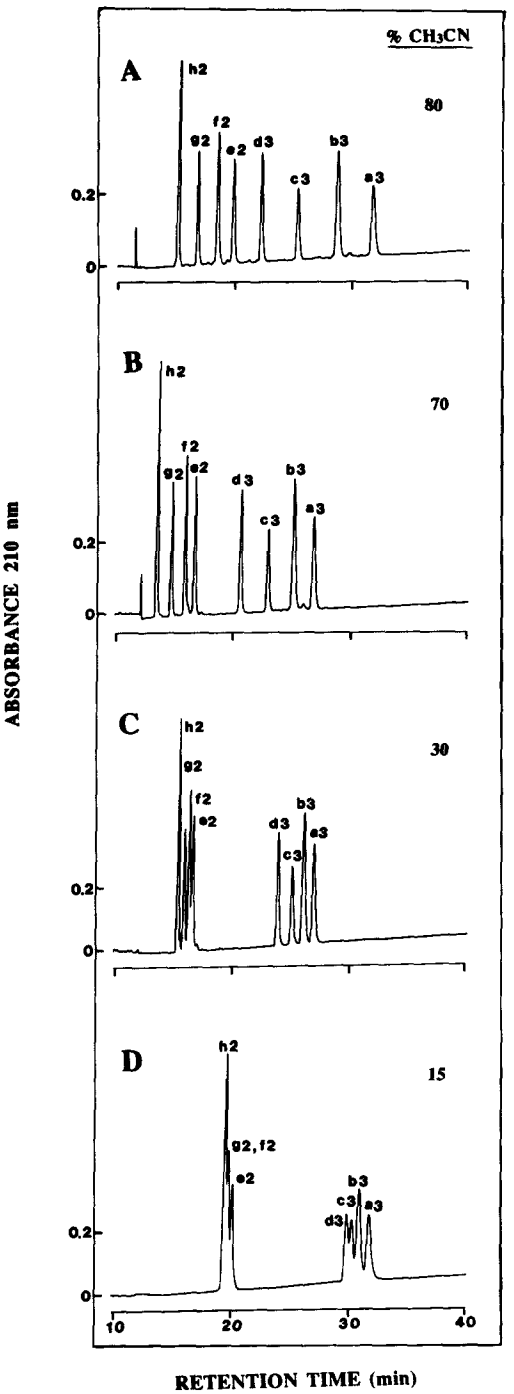

Fig. 3. Effect of acetonitrile concentration on peptide separations during strong cation-exchange chromatography. Column, same as Fig. 2. Mobile phase, linear A–B increasing salt gradient (2% B/min, equivalent to 5 m*M* $NaClO_4$/min, starting with 100% A) at a flow-rate of 1 ml/min, where A is 5 m*M* aqueous TEAP, pH 7, and B is A plus 0.25 *M* $NaClO_4$, pH 7, both A and B containing 80% (panel A), 70% (panel B), 30% (panel C) or 15% (panel D) (v/v) acetonitrile. Temperature, 26°C. The sequences of the peptides are shown in Table I. For peptide denotion, see Fig. 2.

secondary to that of the ionic interactions, particularly at the lower acetonitrile concentrations, d3 is eluted after e2. However, as the acetonitrile concentration is increased, the difference in retention times of these two peptides decreased. Thus, at an acetonitrile concentration of 20%, the retention time difference (Δt) between these two peptides was 10 min, decreasing to 6.5 min, 1.8 min and 0.6 min at acetonitrile concentrations of 50%, 80% and 90%, respectively, underscoring the increasing influence of hydrophilic interactions in the separation process.

Also from Fig. 3, acetonitrile concentration has little effect on the retention time difference between peptides of similar hydrophobicity/hydrophilicity. For example, Δt for h2 and d3 (RPLC retention times of 27.5 min and 27.2 min, respectively, at pH 7; Table I) was 12 min at acetonitrile concentrations of both 20% and 90%.

Fig. 4 summarizes the effect of acetonitrile concentration on the mixed-mode retention behaviour of peptides a3, d3, e2 and h2 on the cation-exchange column. From Fig. 4 (and Fig. 3), as the acetonitrile concentrtion in the mobile phase was raised from 0% to 20%, peptide retention times decreased as hydrophobic interactions were suppressed. Note the reversal in elution order of peptides a3 and d3, and e2 and h2 at 10% acetonitrile as hydrophilic interactions come into play. As the acetonitrile concentration was raised further to 70%, little effect on peptide retention times was observed, save for a slow decrease in retention time of d3 and h2. A further

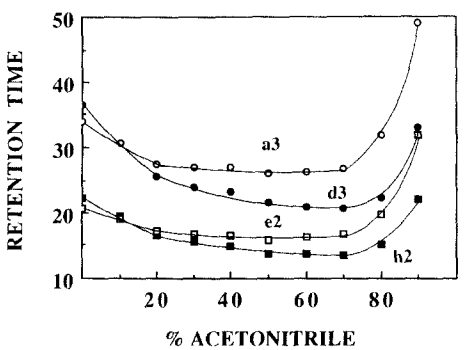

Fig. 4. Plot of peptide retention time *versus* acetonitrile concentration (v/v) in the mobile phase during strong cation-exchange chromatography. Column and mobile phase, same as Fig. 3. The sequences of peptides a3, d3, e2 and h2 are shown in Table I. For peptide denotion, see Fig. 2.

increase in the acetonitrile level up to 90% produced a marked increase in peptide retention times, probably due to an increase in hydrophilic interactions with increasing levels of acetonitrile, until such interactions may possibly be dominating the mixed-mode separation mechanism (note, as mentioned above, how peptides d3 and e2 are almost coeluted in the presence of 90% acetonitrile).

Effect of acetonitrile concentration on cation-exchange column selectivity

The possibility, suggested by the results of Figs. 3 and 4, that an hydrophilic interaction mechanism may be the dominant separation process during HILIC–CEC in the high acetonitrile concentrations was now further investigated. To this end, all 12 peptides in Table I were subjected to HILIC-CEC under the same gradient elution conditions, with varying acetonitrile levels in the mobile phase, as those employed for Figs. 2, 3 and 4. It was felt that, if hydrophilic interactions were to become dominant in the mixed-mode separation process (*i.e.*, no longer secondary to ionic interactions), then a peptide of higher net positive charge could be eluted earlier than a peptide of lower net positive charge if the overall hydrophilicity of the latter (expressed as RPLC retention time at pH 7; Table I) was greater than that of the former, *i.e.*, perhaps column selectivity could be dramatically changed for charged peptides depending upon the acetonitrile concentration [12]. From the results of Fig. 5, it can be seen that this was indeed achieved with certain peptides. Thus, at a level of 90% acetonitrile (Fig. 5A) peptide i1 (19.2 min RPLC retention time at pH 7) was eluted after the less hydrophilic peptide h2 (RPLC; 27.5 min). Similarly, e2 (RPLC; 20.9 min) was eluted after the much less hydrophilic k3 (RPLC; 31.7 min). Most dramatically, peptide l4 (RPLC; 35.0 min) was eluted before the more hydrophilic c3, b3 and a3 (RPLC; 24.2 min, 21.4 min, 20.6 min, respectively).

Fig. 5B demonstrates how dominant hydrophilic (over ionic) interactions may be maintained while reducing analysis time but retaining good column selectivity. This was achieved by employment of an increasing salt gradient combined with a decreasing acetonitrile gradient. The lower level of acetonitrile in buffer B (50% as against 90% in buffer A) led to a decrease in peptide retention relative to that effected by maintaining 90% acetonitrile in both mobile

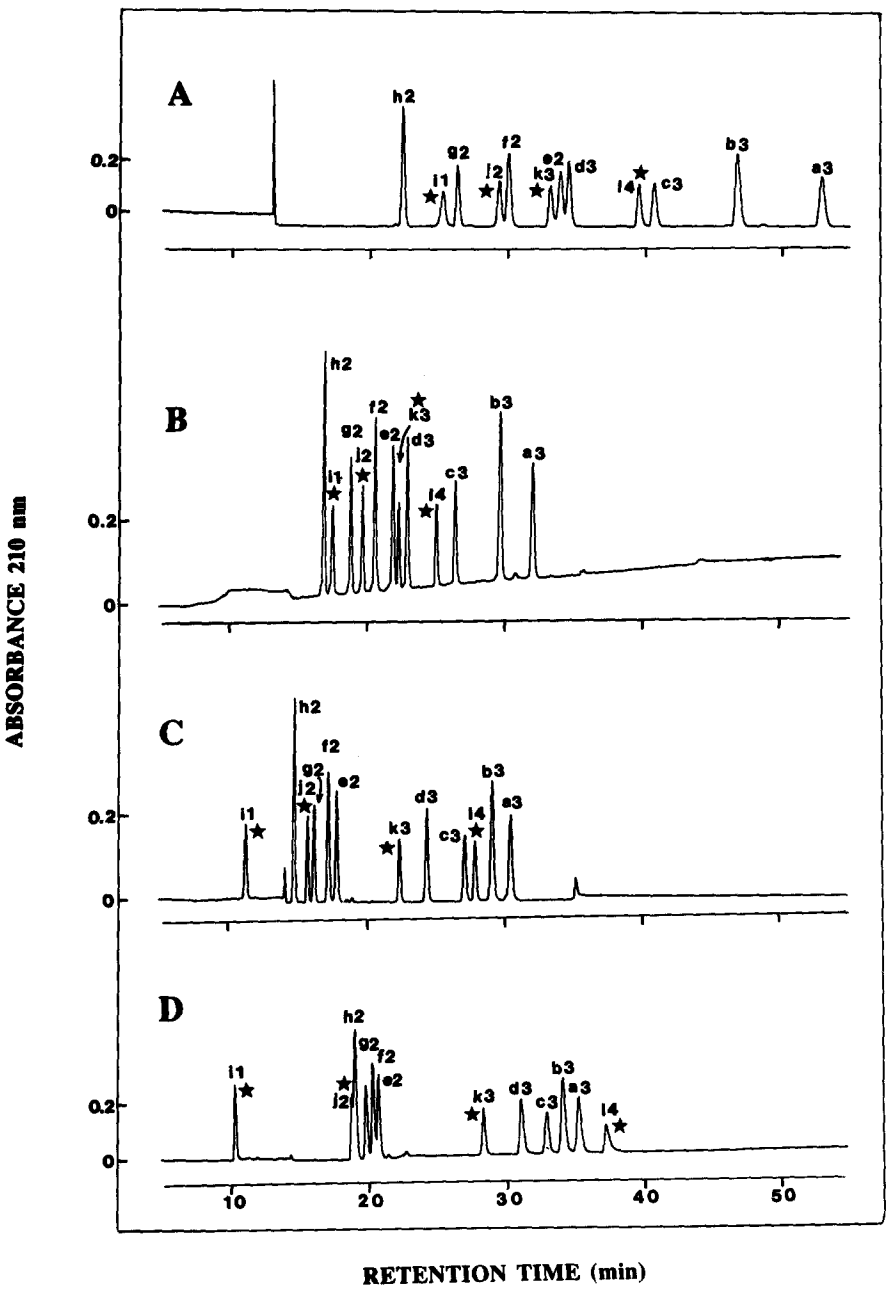

Fig. 5. Effect of acetonitrile concentration on column selectivity during HILIC-CEC of peptides. Column, same as Fig. 2. Mobile phase: panels A, C and D, linear A–B increasing salt gradient (2% B/min, equivalent to 5 m*M* $NaClO_4$/min, starting with 100% A) at a flow-rate of 1 ml/min, where A is 5 m*M* aqueous TEAP, pH 7, and B is A plus 0.25 *M* $NaClO_4$, pH 7, both A and B containing 90% (panel A), 50% (panel C) or 20% (panel D) (v/v) acetonitrile; panel B, linear A–B gradient (2% B/min, equivalent to a linear increasing salt gradient of 5 m*M* $NaClO_4$/min and a linear decreasing acetonitrile gradient of 0.8% acetonitrile/min, starting with 100% A) at a flow-rate of 1 ml/min, where A is 5 m*M* aqueous TEAP, pH 7, containing 90% (v/v) acetonitrile and B is 5 m*M* aqueous TEAP, pH 7, containing 0.25 M $NaClO_4$ and 50% (v/v) acetonitrile. Temperature, 26°C. The sequences of the peptides are shown in Table I. The stars denote the positions of peptides i1, j2, k3 and l4 (+1 to +4 net charge, respectively) relative to the non-acetylated (a to d) and acetylated (e to h) peptide analogues. For peptide denotion, see Fig. 2.

phase buffers (Fig. 5A). The peptide elution order obtained with this combined salt and acetonitrile gradient (Fig. 5B) was almost identical (save for a reversal of e2 and k3) to that shown in Fig. 5A, but was obtained in about 2/3 of the time and with sharper peptide peaks.

As the concentration of acetonitrile in the mobile phase was decreased to 50% (Fig. 5C), peptides were generally eluted in order of increasing net positive charge, except for l4 (RPLC retention time of 35.0 min at pH 7.0; Table I) which was still eluted earlier than the lesser charged a3 and b3 (RPLC retention times of 20.6 min and 21.4 min, respectively). Thus, even at relatively lower acetonitrile concentrations (*e.g.*, 50% as opposed to 90%), more highly charged peptides may still be eluted prior to less highly charged peptides if the latter are significantly more hydrophilic than the former.

At a concentration of 20% acetonitrile in the mobile phase (Fig. 5D), the overall elution order of all 12 peptides was based on increasing net positive charge, *i.e.*, ionic interactions are dominating the separation process.

An interesting anomaly of Fig. 5 is the retention behaviour of peptide j2 relative to peptides e2 to h2. All five of these peptides possess a net charge of +2. The elution order of e2 to h2 remains constant through all levels of acetonitrile concentrations employed in Fig. 5, *i.e.*, in order of increasing peptide hydrophilicity h2 < g2 < f2 < e2. However, j2, which may be expected to be eluted between f2 and g2 (see Table I for RPLC retention times at

TABLE II

COMPARISON OF RPLC AND MIXED-MODE CEC FOR PEPTIDE SEPARATIONS

Peptides[a]	$\Delta t(R_s)$[d]											
	RPLC[b], pH 2		RPLC, pH 7		CEC[c] 50/50		CEC 80/80		CEC 90/90		CEC 90/50	
A. Peptides with different net positive charges												
a–e	−1.1	(3.8)	−0.3	(1.3)	12.6	(33.7)	15.4	(31.3)	19.1	(26.7)	10.2	(29.2)
b–f	−1.2	(4.6)	−0.4	(1.5)	11.9	(33.3)	12.9	(28.1)	16.7	(25.8)	9.1	(28.9)
c–g	−1.2	(5.0)	−0.2	(0.9)	10.9	(32.9)	10.3	(30.9)	14.3	(26.8)	7.6	(26.3)
d–h	−1.4	(6.4)	−0.4	(1.4)	9.6	(31.4)	8.5	(24.8)	12.1	(24.5)	6.0	(22.9)
e–b	0.3	(1.3)	−0.6	(2.4)	−11.3	(30.8)	−11.2	(23.9)	−12.9	(19.0)	−7.8	(24.1)
c–f	1.3	(5.3)	2.4	(10.1)	9.9	(29.1)	7.9	(22.7)	10.6	(18.1)	5.8	(19.6)
g–d	−1.5	(6.7)	−2.7	(10.6)	−8.2	(25.3)	−5.8	(16.3)	−8.1	(15.9)	−4.1	(15.1)
B. Peptides with +3 net charge only												
a–b	−0.7	(2.3)	−0.9	(3.3)	1.4	(3.1)	4.2	(7.0)	6.2	(8.3)	2.4	(6.2)
b–c	−2.5	(8.8)	−2.8	(10.9)	2.0	(4.9)	5.0	(10.7)	6.1	(9.2)	3.3	(9.6)
c–d	−2.7	(11.2)	−2.9	(11.5)	2.8	(7.2)	4.4	(11.6)	6.2	(10.6)	3.5	(11.4)
k–d	3.7	(15.5)	4.6	(17.4)	−2.0	(5.7)	−0.3	(0.8)	−1.4	(2.4)	−0.6	(2.4)
C. Peptides with +2 net charge only												
e–f	−0.9	(3.8)	−0.9	(3.9)	0.6	(2.1)	1.8	(5.0)	3.8	(6.3)	1.3	(4.6)
f–g	−2.4	(11.0)	−2.6	(11.1)	1.0	(3.7)	2.4	(7.3)	3.7	(7.3)	1.8	(6.7)
g–h	−2.9	(13.1)	−3.1	(12.9)	1.4	(5.6)	2.6	(9.1)	4.0	(9.0)	1.9	(7.8)
e–j	−3.0	(12.9)	−2.4	(10.7)	2.1	(8.0)	3.4	(9.9)	4.5	(7.4)	2.2	(8.1)
g–j	0.3	(1.5)	1.2	(5.3)	0.5	(1.8)	−0.8	(2.4)	−3.0	(5.9)	−0.9	(3.2)

[a] The letters denote the peptides listed in Table I.

[b] The chromatographic conditions for RPLC at pH 2 (TFA system) and pH 7 are shown in Fig. 1.

[c] The chromatographic conditions for CEC are shown in Fig. 5. The values 50/50, 80/80, 90/90 and 90/50 denote the acetonitrile concentrations (%, v/v) in buffers A and B, respectively, used in linear AB gradients.

[d] Δt denotes the difference in retention time between two peptides. For example, "a–e" (RPLC, pH 2) denotes the retention time of peptide a minus the retention time of peptide e: 16.4 min–17.5 min = −1.1 min. Resolution (R_s) was calculated from the equation $R_s = 1.176\ \Delta t/(w_1 + w_2)$, where Δt is the retention time difference between two peptides and w_1 and w_2 are the peak widths at half height.

pH 7), exhibited a varying elution position relative to the other four peptides as the acetonitrile concentration is varied. Thus, from being eluted just prior to peptide f2, and well after peptides h2 and g2 at a concentration of 90% acetonitrile (Fig. 5A), peptide j2 gradually moved through the elution order as the acetonitrile concentration was decreased until, at a level of 20% acetonitrile (Fig. 5D), it was eluted prior to all four peptides possessing a net charge of +2. It is known that aqueous solutions of acetonitrile may affect the conformation of peptides, *e.g.*, the induction of α-helical structure in potentially helical peptides. The anomalous behaviour of peptide j2 relative to the other four like-charged peptides may be due to such conformational changes at different acetonitrile concentrations.

Comparison of RPLC and HILIC-CEC for peptide separations

Table II shows the resolution of selected peptide pairs following RPLC and HILIC-CEC of all 12 peptides in Table I. The aqueous TFA–acetonitrile, pH 2, RPLC mobile phase represents the run conditions employed by the great majority of researchers for peptide applications. The pH 7 run serves as a comparison. The HILIC-CEC results represent peptide separations obtained by a range of run conditions designed to manipulate the separation process. Thus, the peptide mixture was resolved by a separation process where ionic interactions were dominant (50/50, *i.e.*, 50% acetonitrile, v/v, in both buffers) or a separation process where hydrophilic interactions become increasingly more important as the acetonitrile concentration was raised.

For peptides with different net positive charges (and close relative hydrophobicities at pH 2), the selectivity advantage of HILIC-CEC over that of RPLC at either pH 2 or pH 7 is quite clear. During RPLC at pH 2, the resolution of peptide pairs ranged from 1.3 (e–b) to 6.7 (g–d); in contrast, HILIC-CEC (50/50) achieved resolution values ranging from 25.3 (g–d) to 33.7 (a–e).

From Table IIB and C, it can be seen that even instances where RPLC proved to be superior to that of HILIC-CEC for separating peptides of the same net charge, separations based on the latter approach were still excellent. In fact, depending on the conditions of HILIC-CEC, this approach frequently produced superior separation of such peptide pairs compared to that of RPLC at pH 2. For instance, for peptides of +3 net charge only, the resolution obtained by HILIC-CEC (80/80 and 90/50) was superior for three out of four peptide pairs, k–d being the exception. Similarly, for peptide pairs e–f and g–j (peptides with +2 net charge only), the resolution achieved by HILIC-CEC at the higher acetonitrile concentrations (80–90%) were superior to that obtained by RPLC at pH 2. Resolution of the other three peptide pairs in Table IIC by HILIC-CEC in the presence of the higher acetonitrile concentration also compared well with the RPLC results. It is difficult to make a meaningful comparison of peak capacity between the RPLC and HILIC-CEC approaches to peptide separations, since the two separation modes are so different mechanistically. Thus, we compared the effectiveness of the two HPLC approaches for resolving all 12 peptides in Table I by applying the equation, $\Delta t/W_{1/2}$, where Δt is the retention time difference between the first and last eluted peptide and $W_{1/2}$ is the average peak width of all 12 peptides. Representative values obtained were 75 (RPLC at pH 2), 88 (HILIC-CEC 90/90; Fig. 5A) and 98 (HILIC-CEC 50/50; Fig. 5C), indicating that the excellent selectivity of the HILIC-CEC approach is being achieved without any serious concomitant band broadening.

CONCLUSIONS

Mixed-mode hydrophilic and ionic interaction chromatography (HILIC-IEC) combines the most advantageous aspects of two widely different separation mechanisms: a separation based upon hydrophilicity/hydrophobicity differences between peptides and the large selectivity advantages of ion-exchange chromatography on the separation of peptides of varying net charge. Peptide separations were generally achieved by utilizing a linear increasing salt (sodium perchlorate) gradient in the presence of acetonitrile (20% to 90%, v/v) at pH 7. The presence of acetonitrile promotes hydrophilic interactions with the hydrophilic stationary phase, these hydrophilic interactions becoming increasingly important to the separation process as the acetonitrile concentration was increased. This mixed-mode methodology on a strong CEC column was compared to reversed-phase chromatography of

positively charged peptides at pH 2 and pH 7. The results of this comparison suggested that, although the mobile phases employed for HILIC-IEC in this study are somewhat less convenient than the volatile mobile phases characteristic of RPLC, HILIC-IEC may rival RPLC for peptide separations.

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