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Journal of Chromatography A, 816 (1998) 65–78

JOURNAL OF CHROMATOGRAPHY A

Hydrophilic interaction/cation-exchange chromatography for separation of amphipathic α-helical peptides

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Abstract

Mixed-mode hydrophilic interaction/cation-exchange chromatography (HILIC/CEX) is a novel high-performance technique which has excellent potential for peptide separations. Separations by HILIC/CEX are carried out by subjecting peptides to linear increasing salt gradients in the presence of high levels of acetonitrile, which promotes hydrophilic interactions overlaid on ionic interactions with the cation-exchange matrix. In the present study, HILIC/CEX has been applied to the separation of synthetic amphipathic α-helical peptides, varying in amphipathicity and the nature of side-chain substitutions in the centre of the hydrophobic or hydrophilic face. Observation of the retention behaviour of these amphipathic α-helical peptide analogues during HILIC/CEX and reversed-phase chromatography (RPLC) enabled the establishment of general rules concerning the applicability of these complementary HPLC techniques to peptides displaying a secondary structural motif of common occurrence.

Keywords: Hydrophilic interaction chromatography; Ion-exchange chromatography; Mixed-mode chromatography; Peptides

1. Introduction

Mixed-mode hydrophilic and ionic (in the present case, cationic) chromatography (HILIC/CEX) combines the most advantageous aspects of two widely different separation mechanisms: a separation based on hydrophilicity/hydrophobicity differences between peptides and the large selectivity advantages of ion-exchange chromatography for the separation of peptides of varying net charge [1–4]. Although reversed-phase liquid chromatography (RPLC) is the high-performance liquid chromatography (HPLC) method of choice for most peptide separations [5], including being commonly employed analytically to check the purity of a purified product, this laboratory has previously shown HILIC/CEX to be an excellent complementary method to RPLC [1–4]. Indeed, HILIC/CEX has rivalled or even exceeded RPLC for the resolution of specific peptide mixtures [2,4]. For instance, the efficacy of the HILIC/CEX approach has been shown to be particularly useful for the separation of deletion impurities differing only subtly from the desired synthetic peptide product [4] and unresolvable by the traditional RPLC approach. In addition, HILIC/CEX has also been successfully employed for protein separations where RPLC alone was unable to effect the required resolution [6,7].

Reasons for the widespread employment of RPLC for peptide separations are grounded in the capability of this high-performance mode to resolve efficiently peptide mixtures containing peptides of widely varying characteristics, e.g., peptide hydrophobicities and conformations. To date, the application of HILIC/CEX to peptide separations has only been reported

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0021-9673/98/$19.00
PII: S0021-9673(98)00507-X

for mixtures of linear peptides generally exhibiting little or no higher orders of structure. Thus, in order to explore fully the potential of this novel HPLC approach, it is important to extend its application to peptides displaying characteristics other than linear polypeptide chains. The present study describes the application of HILIC/CEX to the separation of series of synthetic amphipathic α-helical analogues. By comparing the retention behaviour of the peptides under RPLC and HILIC/CEX conditions, we set out to establish general rules concerning the applicability of these HPLC modes to the separation of model amphipathic peptides varying in amphipathicity and the nature of side-chain substitutions in the centre of the hydrophobic or hydrophilic face.

2. Experimental

2.1. Materials

HPLC-grade water and acetonitrile were obtained from BDH (Poole, UK). ACS-grade orthophosphoric acid and triethylamine (TEA, redistilled before use) were obtained from Anachemia (Toronto, Canada). Trifluoroacetic acid (TFA) was obtained from Aldrich (Milwaukee, WI, USA). Sodium perchlorate ($NaClO_4$) was obtained from BDH.

2.2. Instrumentation

Peptide synthesis was carried out on an Applied Biosystems Peptide Synthesizer Model 430A (Foster City, CA, USA). The HPLC system consisted of an HP1090 liquid chromatograph from Hewlett-Packard (Avondale, PA, USA), coupled to an HP1040A detection system, HP9000 Series 300 computer, HP9133 disc drive, HP2225A Thinkjet printer and HP7460A plotter.

2.3. Columns

Reversed-phase runs were carried out on a Zorbax SB300-C_8 reversed-phase column (150×4.6 mm I.D., 5-μm particle size, 300 Å pore size; void volume 1.7 ml) from Hewlett-Packard (Little Falls Site, DE, USA).

Hydrophilic interaction/cation-exchange runs were carried out on a poly(2-sulfoethyl aspartamide)-silica (PolySulfoethyl A) strong cation-exchange column (200×4.6 mm I.D., 5 μm, 300 Å; void volume, 1.9 ml) from PolyLC (Columbia, MD, USA).

2.4. Peptide synthesis

Amphipathic α-helical peptides were synthesized by the solid-phase technique (SPPS) on co-poly-(styrene–1% divinylbenzene)benzhydrylamine-hydrochloride resin (0.92 mmol/g resin) as previously described [8].

3. Results and discussion

3.1. RPLC vs. HILIC/CEX: general principles

The term hydrophilic interaction chromatography was originally coined to describe separations based on solute hydrophilicity [9]. Thus, separation by HILIC, in a manner similar to normal-phase chromatography (to which it is related), depends on hydrophilic interactions between the solutes and a hydrophilic stationary phase, i.e., solutes are eluted in order of increasing overall hydrophilicity (decreasing hydrophobicity). Characteristic of HILIC separations is the presence of a high initial organic modifier concentration to promote hydrophilic interactions between the solute and the hydrophilic stationary phase.

The major process governing peptide retention behaviour on ion-exchange columns involves ionic interactions between the column matrix and the peptide solutes; however, all such columns, in our hands have also exhibited some hydrophobic character, leading to long peptide retention times and peak broadening [10]. Although most researchers prefer to avoid separations based on such mixed-mode ionic-hydrophobic column behaviour by adding an organic solvent, such as acetonitrile, to the mobile phase buffer, this laboratory has demonstrated that manipulation of the acetonitrile concentration enables considerable flexibility in the separation of basic (potentially positively charged) peptides on a strong-cation-exchange column [1–4].

Concerning the aforementioned matrix hydropho-

bicity, it should be noted that different ion-exchange matrices exhibit differing degrees of hydrophobic characteristics [10]. In order to gain the full benefit of peptide separations by the HILIC mode, it is important to overcome unwanted hydrophobic properties of the matrix with as low a level of organic modifier (acetonitrile) as possible, i.e., the ion-exchange matrix should be as hydrophilic as possible. In this way, there is a greater organic modifier range open to the researcher to effect mixed-mode HILIC/CEX peptide separations. In our hands, the PolySulfoethyl A strong cation-exchange column [based on a polypeptide coating, poly(2-sulfoethyl aspartamide), covalently attached to silica] has proven to be very hydrophilic, particularly in comparison to other silica-based and non-silica-based matrices [10]; hence the use of this column in the present study.

Typical conditions for mixed-mode HILIC/CEX are a linear increasing $NaClO_4$ gradient (2 m*M*–20 m*M* $NaClO_4$/min) at pH 3, with both mobile phase buffers containing 15–80% acetonitrile. Thus, the cation-exchange column separates peptides based on net positive charge and this separation mode is overlaid by the presence of acetonitrile overcoming undesirable hydrophobic interactions with the column while promoting favorable hydrophilic interactions. $NaClO_4$ is suitable for this mixed-mode approach due to its excellent solubility characteristics in aqueous solution even in the presence of high concentrations of organic modifier [2,11]. The choice of a relatively low pH is governed by the desire to maximize the basic character of the peptide solutes to enhance ionic interactions with the negatively charged strong-cation-exchange matrix. Thus, at pH 3, any acidic (potentially negatively charged) residues (Asp, Glu) will be mainly protonated, i.e., uncharged. In addition, a full positive charge on the basic residue His (pK_a=6.5) is also assured at low pH. However, less obvious, perhaps, is the need to be cautious with the pH of the mobile phase when considering basic residues such as Lys (pK_a~10) and Arg (pK_a~12). Through the use of synthetic peptide models, Sereda et al. [8] demonstrated that the hydrophobic environment characteristic of RPLC (hydrophobic packings and organic modifiers) had a profound effect on the pK_a values of ionizable groups, these values being decreased to 5.8, 7.4 and 7.3 for His, Lys and Arg, respectively. Although an ion-exchange matrix is clearly far less hydrophobic than that of a reversed-phase packing, it was felt that the presence of high concentrations of acetonitrile (up to 90%) characteristic in HILIC/CEX mobile phases generally necessitates the use of relatively low pH conditions to ensure full protonation (i.e., a full positive charge) of basic side chains. As a side benefit, silica-based ion-exchange columns tend to be more stable over a period of time if utilized at pH 3 compared to pH values around neutrality.

Fig. 1 compares the separation of synthetic peptide RPLC standards, S2–S5, by RPLC (top), CEX (middle) and HILIC/CEX (bottom). Due to their lack of secondary structure, these peptides were useful to demonstrate the basic principles distinguishing RPLC from HILIC/CEX. This four-peptide mixture contains peptides with the same net positive charge (+2) and subtly increasing hydrophobicity (S2<S5). Note that the only difference between the CEX and HILIC/CEX runs was the presence of 10% (v/v) acetonitrile in the former compared to 80% (v/v) in the latter.

From Fig. 1, peptides were, of course, eluted from the RPLC column (top) in order of increasing hydrophobicity. Under characteristic cation-exchange conditions (middle) [the presence of 10% (v/v) acetonitrile helps to eliminate unwanted hydrophobic interactions between solutes and the column matrix [10]], the four peptides were very poorly resolved, as expected given the identical net charge on the peptides. Interestingly, the low concentration of acetonitrile (10%) has already induced hydrophilic interactions with the matrix in that the elution order is already opposite to that of RPLC (the most hydrophobic peptide was eluted first and the most hydrophilic last). In contrast, under HILIC/CEX conditions (bottom), the elution order remains the same but the peptides are now well resolved. Clearly, to effect a separation of these peptides on the cation-exchange column, an increased concentration (80%, v/v) of acetonitrile was required in the mobile phase in order to promote hydrophilic interactions with the column matrix to complement the ionic interactions.

3.2. Design of synthetic model amphipathic α-helical peptides

As noted above, the separations shown in Fig. 1

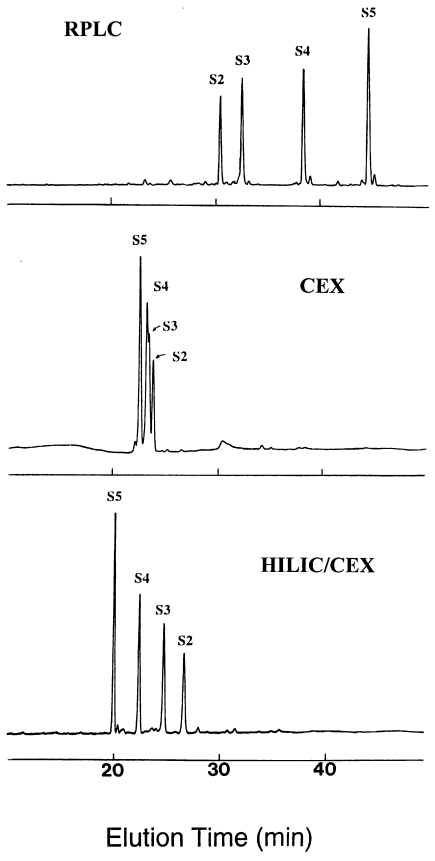


Fig. 1. General principles of RPLC vs. HILIC/CEX. Conditions: RPLC, linear A–B gradient (0.5% acetonitrile/min) at a flow-rate of 1 ml/min, where eluent A is 20 m*M* aq. TEAP, pH 3, and eluent B is eluent A containing 60% (v/v) acetonitrile, both eluents containing 100 m*M* $NaClO_4$; CEX, linear A–B gradient (5 m*M* $NaClO_4$/min, following 5-min isocratic elution with eluent A) at a flow-rate of 1 ml/min, where eluent A is 20 m*M* aq. TEAP, pH 3, containing 10% (v/v) acetonitrile and eluent B is eluent A containing 400 m*M* $NaClO_4$; HILIC/CEX, same conditions as for CEX, except for 80% (v/v) acetonitrile in eluents A and B; all runs carried out at 30°C and peaks detected by absorbance at 210 nm. The sequences of the synthetic peptide standards are Ac–Arg–Gly–X–X–Gly–Leu–Gly–Leu–Gly–Lys–amide, where X–X is substituted by Gly–Gly (S2), Ala–Gly (S3), Val–Gly (S4) or Val–Val (S5); Ac denotes N^{α}-acetyl and amide denotes C^{α}-amide.

were achieved using peptides of negligible secondary structure, i.e., random coils. However, it is well documented that conformational effects can have a dramatic effect on RPLC retention behaviour, particularly in the case of amphipathic α-helical peptides [12–16]. Thus, on binding to a reversed-phase column, the hydrophobicity of the stationary phase stabilizes secondary (α-helical) structure. Indeed, Zhou et al. [14] demonstrated that amphipathic peptides remain α-helical when bound to a reversed-phase column and, due to the preferred binding domain (pbd) created by the non-polar face of the α-helix, are considerably more retentive than peptides of the same composition but lacking this pbd. Similar observations have recently been reported for cyclic amphipathic β-sheet peptides [17,18].

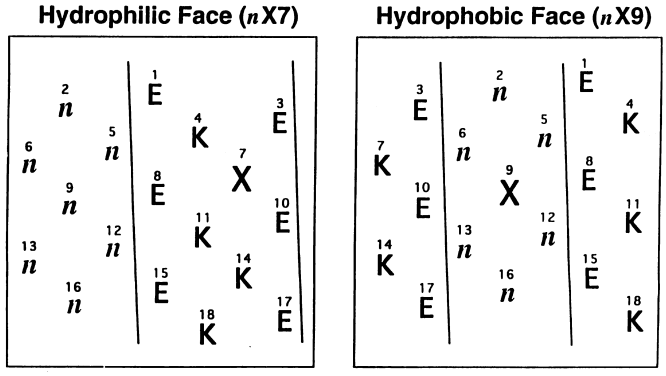
In a similar manner to the hydrophobic face of an amphipathic α-helical peptide binding preferentially to a reversed-phase matrix, it is not unreasonable to assume that the opposite face of the helix would represent a hydrophilic domain binding preferentially to a hydrophilic stationary phase such as an ion-exchange matrix. Indeed, preferential binding with ion-exchange columns of areas of a protein surface exhibiting high charge densities is a well-known phenomenon [19,20]. In addition, evidence for such hydrophilic preferred binding domains has recently been described for the cyclic amphipathic β-sheet peptides mentioned above [18].

In determining the design for model amphipathic α-helical peptides which would best compare the relative attributes of RPLC and HILIC/CEX for separation of such solutes, the following assumptions were made: (1) modifications or mutations in the hydrophilic face of amphipathic α-helical peptides should be best separated by HILIC/CEX; (2) modifications or mutations in the hydrophobic face of amphipathic α-helical peptides should be best separated by RPLC; and (3) the previous statements should hold true no matter whether the substitutions are made with hydrophobic (e.g., Leu, Val) or hydrophilic (e.g., Thr, Ser) amino acid side-chains.

Fig. 2 (top) shows the generic sequences of two series of model amphipathic α-helical peptides designed for the present study, based on the assumptions outlined above. The two series are denoted n X 9 and n X 7 depending on whether substitutions are made in the hydrophobic or hydrophilic face, respectively. The periodic distribution of non-polar residues (designated n) along the polypeptide chain ensures a wide hydrophobic face on the amphipathic helix, with non-polar residues 2, 5, 6, 12, 13 and 16 surrounding position 9, the substitution site, denoted X, on this hydrophobic face (see helical net presenta-

n X9 Ac-E-*n*-E-K-*n*-*n*-K-E-**X**-E-K-*n*-*n*-K-E-*n*-E-K-amide

n X7 Ac-E-*n*-E-K-*n*-*n*-**X**-E-*n*-E-K-*n*-*n*-K-E-*n*-E-K-amide



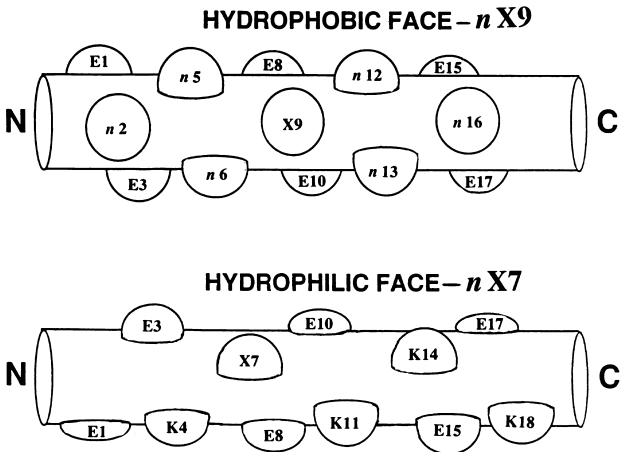


Fig. 2. Design of model synthetic amphipathic α-helical peptides. Top: sequence of model peptides, n X 7, where n (denoting non-polar residue) is substituted at each of the shown positions either by Ala or Leu; X denotes position substituted by Leu, Val, Thr or Ser at position 7 of the hydrophilic face (n X 7 peptides) or position 9 of the hydrophobic face (n X 9 peptides). Middle: n X 7 and n X 9 peptides represented as α-helical nets. The radius of the α-helix is taken as 2.5 Å with 3.6 residues per turn, a residue translation of 1.5 Å and thus a pitch of 5.4 Å. The area between the solid lines on the left hand net (n X 7) peptides represents the hydrophilic face (made up of Lys and Glu residues) of the peptides; the area between the solid lines on the right hand net (n X 9) represents the hydrophobic face (made up of Ala or Leu residues) of the peptides. Bottom: rod representation of the hydrophobic and hydrophilic faces of the peptides. N and C denote, respectively, N-terminal and C-terminal of peptides. Ac denotes N^{α}-acetyl and amide denotes C^{α}-amide.

tion of the hydrophobic face denoted n X 9 in Fig. 2). The hydrophilic face of the helix is made up of Lys and Glu residues surrounding position 7, the substitution site, denoted X, on this hydrophilic face (see helical net presentation of the hydrophilic face denoted n X 7 in Fig. 2). Fig. 2 also shows rod representations of the hydrophobic face of the n X 9 analogues and the hydrophilic face of the n X 7 analogues in order to illustrate schematically how these faces (and specifically the substituted position X) would be presented to, respectively, the reversed-phase and ion-exchange matrices.

Within the two series of peptides, there was also a division into two markedly different amphipathicities, where the non-polar positions (designated n) in the peptide sequences were either substituted by Ala (AX9, AX7 peptides) or the much more hydrophobic Leu (LX9, LX7 peptides). For the AX9/AX7 peptides, position X was substituted by Leu (denoted peptides AL9 and AL7), Val (AV9 and AV7), Thr (AT9 and AT7) or Ser (AS9 and AS7); similar denotions were applied for the LX9/LX7 peptides (LL9, LS7, etc.). The choice of which residues were substituted at position 7 (n X 7 peptides) or position 9 (n X 9 peptides) was predicated on the desire to have only a subtle change between related pairs (Leu/Val or Thr/Ser) of side-chains. Thus, for the hydrophobic residue pair Leu and Val, Leu has only one more carbon atom in its side-chain compared to Val; similarly, for the polar residue pair Thr and Ser, Thr has only one more carbon atom in its side-chain compared to Ser. It was felt that subtle changes such as these would be a stringent test of the assumptions outlined above.

The sequences of these model peptides was known to have a high potential to form α-helices [21,22], as determined by circular dichroism spectroscopy (in 50% trifluoroethanol, a helix-inducing solvent [23]). In addition, the considerable amphipathic character of such peptides has also been reported previously [21]; indeed, the latter study also confirmed that such peptides were eluted as single-stranded amphipathic α-helices during RPLC, interacting with the stationary phase through preferential binding with their hydrophobic faces.

It has also been previously shown that high concentrations of organic modifiers such as acetonitrile can induce helix formation in a potentially helical peptide [14,24]. Thus, under characteristic conditions of HILIC/CEX [high acetonitrile concentration in the mobile phase; 80% (v/v) in the present study], the model peptides would be expected to be α-helical, allowing interaction of the hydrophilic face with the ion-exchange matrix; indeed, the presence of 80% (v/v) acetonitrile serves to enhance such interactions.

Three additional peptides were also synthesized: a peptide designated AA9, with Ala at position 9 of the AX9 series; a non-amphipathic α-helical peptide, designated naA, with the same composition as AA9 but a different sequence (Ac–Glu–Glu–Ala–Lys–Ala–Lys – Ala – Glu – Ala – Glu–Ala–Lys–Ala–Lys–Ala–Glu–Glu–Lys–amide); and a non-amphipathic α-helical peptide, denoted naL, with the same composition as LL9 but a different sequence (same composition as naA but with Ala replacing Leu; Ac–Glu–Glu–Leu – Lys–Leu–Lys–Leu–Glu–Leu–Glu – Leu – Lys – Leu – Lys – Leu – Glu – Glu – Lys–amide). The term ''same composition, different sequence'' is frequently abbreviated as SCDS. The α-helical potential of AA9 and its non-amphipathic analogue have been demonstrated previously [16], as well as their single-stranded elution behaviour during RPLC. Note that AA9 and LL9 may be viewed as ''native'' peptide analogues.

3.3. RPLC and HILIC/CEX run conditions

Mixed-mode HILIC/CEX is best utilized at a pH which ensures hydrophilic interactions overlay ionic interactions between the peptide solutes and the negatively charged cation-exchange matrix. From Fig. 2, it can be seen that both the n X 7 and n X 9 peptide analogues contain six Glu (potentially negatively charged) residues but only four (n X 7 peptides) or five (n X 9) Lys (potentially positively charged) residues. Thus, at pH levels above the pK_a of Glu (pK_a~4), the peptides would have an overall negative net charge and would not be expected to be retained by the column through ionic interactions. A preliminary investigation of the effect of pH on the retention behaviour of AV9 and AV7 under CEX conditions (described in Fig. 1 for middle elution profile) demonstrated negligible retention of the peptides between pH 5.0 and pH 6.5; optimum retention of the peptide occurred only at pH levels

below pH 4. For the current study, pH 3 was chosen as an excellent compromise between satisfactory peptide retention and a desire not to approach too closely the pK_a value of the sulphonate cation-exchange functional group (pK_a~1–1.5). In addition, pH 3 is the best pH to choose if the net charges of peptides in a mixture are unknown, since the net positive charges on the peptides are then maximized.

RPLC conditions at pH 3 were designed to approximate the HILIC/CEX conditions as far as possible in terms of the environment to which the peptides were subjected; hence, the use of 20 m*M* aq. triethyl-ammonium phosphate (TEAP) in the presence of $NaClO_4$. In addition, the use of salt in the RPLC mobile phase at low pH had already been shown to confer advantages to peptide separations of specific peptide mixtures, compared to its absence, including superior peak shape [11]. By optimizing the peptide separation potential in this way and comparing the resultant RPLC separations with those achieved by HILIC/CEX, it was felt that the relative advantages of these two complementary HPLC modes for resolution of the amphipathic α-helical peptide analogues would be clearer.

RPLC using an aqueous TFA–CH_3CN mobile phase was also carried out since it was deemed important to include conditions most commonly employed by researchers in the peptide field [1,4,25,26].

3.4. RPLC and HILIC/CEX of amphipathic versus non-amphipathic α-helical peptides

Fig. 3 compares the RPLC and HILIC/CEX elution profiles of a mixture of amphipathic α-helical ''native'' peptide analogues, AA9 and LL9, and their non-amphipathic SCDS α-helical counterparts, naA and naL (the RPLC run had to be carried out at an elevated temperature due to the tendency of peptide naL to aggregate at room temperature). For both pairs of peptides (AA9/naA and LL9/naL), the amphipathic analogues (AA9 and LL9) were eluted later than their non-amphipathic analogues (naA and naL, respectively) during RPLC. This expected observation has been reported previously by Sereda et al. [16] for the naA and AA9 analogues and is due to the presence of a preferred hydrophobic binding domain in the amphipathic analogues compared to

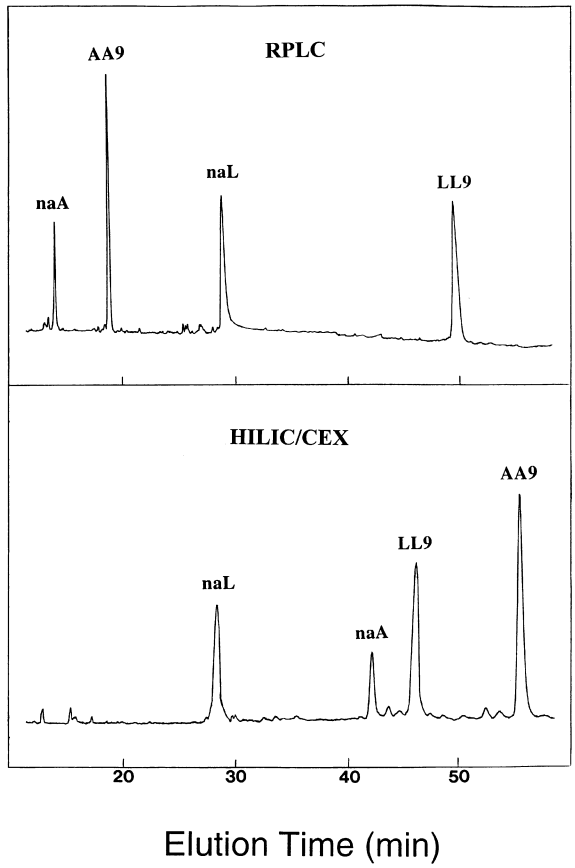


Fig. 3. RPLC and HILIC/CEX of amphipathic and non-amphipathic α-helical peptides. Conditions: same as Fig. 1 for RPLC and HILIC/CEX runs, except for a temperature of 60°C for RPLC. Peptides AA9 and LL9 denote amphipathic α-helical peptides of the n X 9 sequence shown in Fig. 2, where positions 2, 5, 6, 9, 12, 13 and 16 on the hydrophobic face were all substituted with Ala (AA9) or Leu (LL9). Peptides naA and naL denote the non-amphipathic helical analogues [same composition, different sequence (SCDS)] of AA9 and LL9, respectively; the sequences of these non-amphipathic analogues are Ac–Glu–Glu–X–Lys–X–Lys–X–Glu–X–Glu–X–Lys–X–Lys–X–Glu–Glu–Lys–amide, where each position denoted X is substituted by Ala (naA) or Leu (naL).

the non-amphipathic peptides. Note also the greater retention times of naL and LL9 compared to naA and AA9, respectively, due to the considerably greater hydrophobicity of Leu compared to Ala.

From Fig. 1, it was shown that, in the absence of any conformational effects on peptide retention behaviour, the elution order of a peptide mixture is reversed between RPLC and HILIC/CEX; thus, the

most hydrophobic (least hydrophilic) peptide is eluted last during RPLC and first during HILIC/CEX. From Fig. 3, this has already occurred for both pairs of α-helical peptides, where the order of elution has reversed between the two HPLC modes; thus, the more hydrophobic (less hydrophilic) naL was eluted after naA during RPLC but before naA during HILIC/CEX while LL9 was eluted after AA9 during RPLC but before AA9 during HILIC/CEX. In contrast, the elution orders of the SCDS peptide pairs were unchanged between the two HPLC modes, i.e., the non-amphipathic analogues, naA and naL, were eluted prior to their amphipathic versions, AA9 and LL9, respectively, in both RPLC and HILIC/CEX. This result can be explained by the presence of the hydrophilic preferred binding domains in the amphipathic peptides. With this domain binding preferentially to the cation-exchange matrix, the hydrophobes (Ala or Leu) on the hydrophobic face will generally be oriented away from the stationary phase during elution and hence have a lesser effect on HILIC/CEX retention time than they would if these residues were distributed evenly throughout the helix, as is the case with the non-amphipathic analogues. Thus, these results offered clear evidence that the Lys-containing hydrophilic faces of the amphipathic α-helical peptides do indeed bind preferentially to the negatively charged cation-exchange matrix, an important point when considering the relative efficiencies of RPLC and HILIC/CEX for separating such peptides. As an aside, such results also supported the view that the model amphipathic peptides would be helical in the presence of 80% (v/v) acetonitrile.

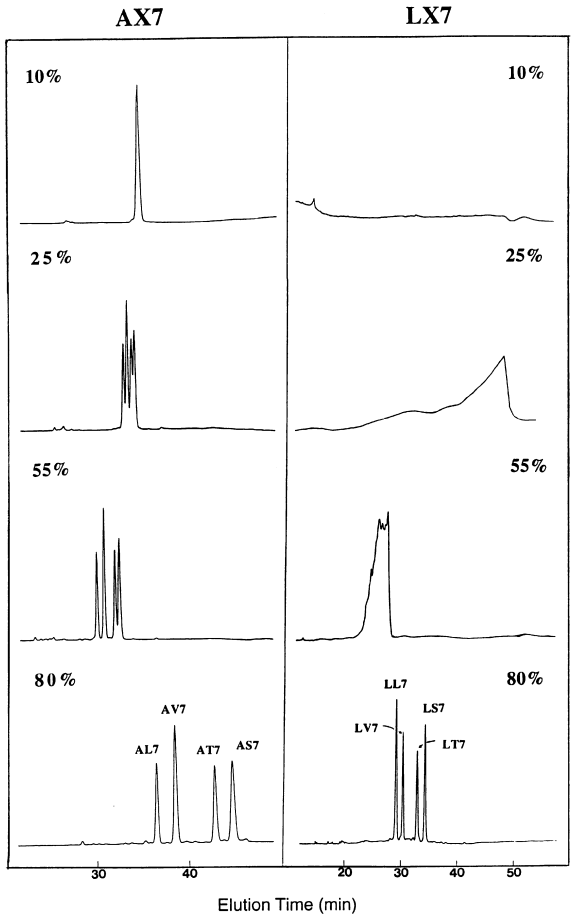


Fig. 4. Effect of acetonitrile concentration on HILIC/CEX separation of amphipathic α-helical peptides. Conditions: same as Fig. 1, except for 10%, 25%, 55% or 80% (v/v) acetonitrile in both eluents. Sequences of peptides AL7, AV7, AT7 and AS7 are shown in Fig. 2 for n X 7 peptides (all non-polar positions, denoted n, are substituted by Ala), where position X is substituted by Leu, Val, Thr or Ser, respectively; the sequences of LL7, LV7, LT7 and LS7 are similar save for the presence of Leu at all non-polar positions.

3.5. RPLC and HILIC/CEX of amphipathic α-helical peptides

3.5.1. Effect of acetonitrile concentration on HILIC/CEX separation of amphipathic α-helical peptides

Fig. 4 shows the effect of an increasing level of acetonitrile in the mobile phase on the HILIC/CEX elution profiles of the AX7 and LX7 series of peptides. These two series of peptides were chosen due to the assumption (see above) that such peptides with substitutions in the hydrophilic face of the helix would be best separated under HILIC/CEX conditions, i.e., it was important to demonstrate that the presence of elevated levels of acetonitrile to promote hydrophilic interactions between the hydrophilic face of the helices and the cation-exchange matrix was indeed necessary to separate peptides with the same overall net charge.

From Fig. 4, the efficacy of increasing the level of acetonitrile on the resolution of the AX7 analogues is quite clear. Thus, at a level of 10% acetonitrile in the

mobile phase to overcome any small hydrophobic character of the matrix (essentially representing a situation where only an ion-exchange mechanism is taking place), the four peptides are eluted as one peak, reflecting the identical net charge on the four peptides. As the acetonitrile concentration is raised further to 25% and 55%, hydrophilic interactions are now being increasingly promoted and overlaid on the ion-exchange mechanism, thus effecting a separation. At a level of 80% acetonitrile, the four peptides are now well resolved. At this high concentration of acetonitrile, hydrophilic interactions are likely dominating the mixed-mode separation process and strongly promoting such peptide–matrix interactions, hence the longer retention times of the peptides relative to those observed at lower acetonitrile levels. Note that the four peptides are eluted in order of increasing peptide hydrophilicity (AL7<AS7).

From Fig. 4, the response of the very hydrophobic LX7 peptides to variations in acetonitrile concentration clearly differed considerably compared to the only mildly hydrophobic AX7 analogues. Thus, at a level of 10% acetonitrile, no peaks were detected; a broad, severely tailing peak was only eluted when the acetonitrile level was raised to 25%. Even at an acetonitrile concentration of 55%, the four peptides were still completely unresolved, despite the further decrease in retention relative to the 25% acetonitrile run. Satisfactory resolution of the peptides was only achieved when the acetonitrile concentration was at a high 80%. Note again that the four analogues were once more eluted in order of increasing hydrophilicity (LL7<LS7). As noted above, the LX7 analogues are very hydrophobic, particularly compared to their AX7 counterparts, and the non-existent or poor elution profiles at acetonitrile levels of 10% and 25%, respectively, indicate considerable hydrophobic interactions of the peptides even with this hydrophilic matrix. The elution profile at an acetonitrile concentration of 55%, while satisfactory in terms of retention time, still shows poor resolution, indicative of peptide aggregation due to the extreme hydrophobicity of the non-polar face of the amphipathic α-helices. Thus, in addition to the necessity of the organic modifier to promote hydrophilic interactions to separate like-charged amphipathic α-helices (common to both the AX7 and LX7 series), high levels of organic modifier are also essential to suppress any hydrophobic/aggregation effects due to highly hydrophobic peptide solutes.

3.5.2. Comparison of the effect of substitutions in the hydrophilic face of an extremely hydrophobic amphipathic α-helix (peptides LL7, LV7, LT7, LS7)

From Fig. 5, in RPLC, as expected, the identical hydrophobic preferred binding domains of the peptides bind to the hydrophobic matrix, resulting in co-elution of all four peptides under both RPLC

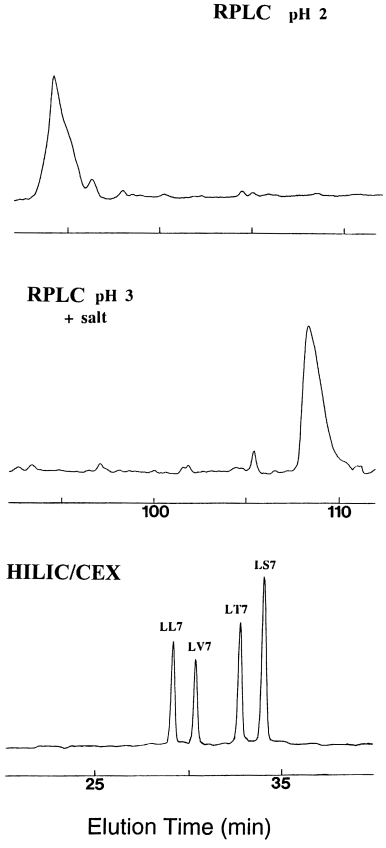


Fig. 5. RPLC and HILIC/CEX of highly amphipathic α-helical peptides where substitutions have been made in the hydrophilic face. Conditions: RPLC at pH 2, linear AB gradient (0.5% acetonitrile/min) at a flow-rate of 1 ml/min, where eluent A is 0.05% aq. TFA and eluent B is 0.05% TFA in acetonitrile, temperature, 30°C; absorbance, 210 nm; RPLC at pH 3 and HILIC/CEX, same as Fig. 1. Sequences of peptides LL7, LV7, LT7 and LS7 are shown in Fig. 2 for n X 7 peptides (all non-polar positions, denoted n, are substituted by Leu), where position X is substituted by Leu, Val, Thr or Ser, respectively.

conditions, i.e., substitutions in the hydrophilic face had little effect on the RPLC retention behaviour of the peptides. Note the longer elution times of the co-eluted peptides in the pH 3 system compared to pH 2, a consequence, possibly, of the ion-pairing properties of the anionic (negatively charged) perchlorate ion which interacts with the positive charges on the peptides. TFA is, of course, frequently used as an anionic ion-pairing reagent for peptide separations [5,25,26]; indeed, the trifluoroacetate ion is more hydrophobic than perchlorate, i.e., it might be expected that the peptides would be retained longer in the presence of TFA compared to sodium perchlorate. However, the concentration of sodium perchlorate (100 m*M*) in the mobile phase is considerably greater than that of TFA (0.05% or ca. 6.5 m*M*). Since an increase in concentration of an anionic ion-pairing reagent is known to lead to a concomitant increase in retention time of peptides containing positively charged residues [26], it seems likely that the much larger concentration of perchlorate ions compensates for the more hydrophobic nature of the trifluoroacetate ion. An alternative explanation for the observed increase in peptide retention times in the presence of $NaClO_4$ lies in the long accepted premise that salts can affect hydrophobic interactions in proteins. Specifically, salts such as KCl have been shown to promote a stronger hydrophobic effect between interacting hydrophobic protein domains [27,28]. In the present study, the reversed-phase packing would represent a hydrophobic domain with which the present single-stranded amphipathic α-helices are interacting via their hydrophobic faces. The presence of 100 m*M* $NaClO_4$ in the mobile phase may thus be enhancing the hydrophobic effect between the peptides and the stationary phase, resulting in increased peptide retention times.

In contrast to RPLC, all four peptides were well resolved by HILIC/CEX even though all peptides have the same net positive charge, with the substitution sites in the hydrophilic faces of the peptides able to interact intimately with the ion-exchange matrix and, hence, influence the retention behaviour of the four analogues. The elution order is in order of decreasing hydrophobicity with the Leu analogue being eluted first followed by the Val, Thr and Ser analogues.

3.5.3. Comparison of the effect of substitutions in the hydrophobic face of an extremely hydrophobic amphipathic α-helix (peptides LL9, LV9, LT9, LS9)

From Fig. 6, the effect of substitutions on the hydrophobic face of the helix is minimal during HILIC/CEX, i.e., all four peptides were essentially co-eluted, since the hydrophilic binding domain presented to the ion-exchange matrix is constant.

In contrast, the peptides were clearly better resolved in order of increasing hydrophobicity by RPLC, particularly LS9 and LT9, despite the co-

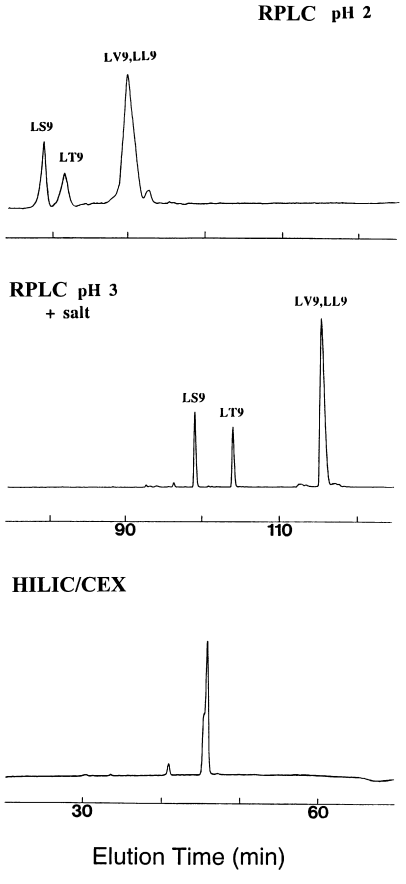


Fig. 6. RPLC and HILIC/CEX of highly amphipathic α-helical peptides where substitutions have been made in the hydrophobic face. Conditions: RPLC at pH 2, same as Fig. 5; RPLC at pH 3 and HILIC/CEX, same as Fig. 1. Sequences of peptides LL9, LV9, LT9 and LS9 are shown in Fig. 2 for n X 9 peptides (all non-polar positions, denoted n, are substituted by Leu), where position X is substituted by Leu, Val, Thr or Ser, respectively.

elution of LL9 and LV9 under both RPLC conditions employed, with the pH 3/perchlorate system effecting an improved separation over that of the pH 2/TFA mobile phase; also, note again the longer retention times in the former system compared to the latter. The excellent separation of LS9 and LT9 compared to the two more hydrophobic substitutions of LL9 and LV9 reflects previous work by Sereda et al. [21], who demonstrated that a very hydrophobic environment enhances the hydrophilicity of polar side-chains such as those of Thr and Ser.

3.5.4. Comparison of the effect of substitutions in the hydrophilic face of a moderately hydrophobic amphipathic α-helix (peptides AL7, AV7, AT7, AS7)

From Fig. 7, all four peptides were well separated by HILIC/CEX. Indeed, the separation of the peptide pairs (AL7/AV7, $\Delta t_R = 2$ min; AT7/AS7, $\Delta t_R = 2$ min) was actually greater than that observed for the more hydrophobic Leu analogues (LL7/LV7, $\Delta t_R =$ 1.2 min; LT7/LS7, $\Delta t_R = 1.3$ min; Fig. 5).

Unlike the co-elution of the LX7 peptides (Fig. 5), Fig. 7 illustrates that there was some separation of the corresponding AX7 peptides by RPLC under both run conditions, again with the pH 3 system showing an improvement over the TFA system, despite the co-elution of AT7 and AS7 in both mobile phases. Clearly, for these lesser amphipathic peptides (compared to the LX7 analogues), residues substituted into the hydrophilic face were still able to interact with the hydrophobic stationary phase. Peptides AL7 and AV7, in particular, were well resolved, perhaps reflecting the enhancement of hydrophobicity of non-polar side-chains in a minimally hydrophobic environment reported by Sereda et al. [21]. Such side-chains are then apparently able to exert an influence on HILIC/CEX retention behaviour for only moderately amphipathic peptides (Fig. 7) compared to strongly amphipathic analogues (Fig. 5).

Another point to note from Fig. 7 is the relative elution order of the peptides between the RPLC and HILIC/CEX runs, i.e., the order of elution was reversed between the two HPLC modes (as highlighted by the arrows), as was demonstrated in Fig. 1 for peptides of negligible secondary structure.

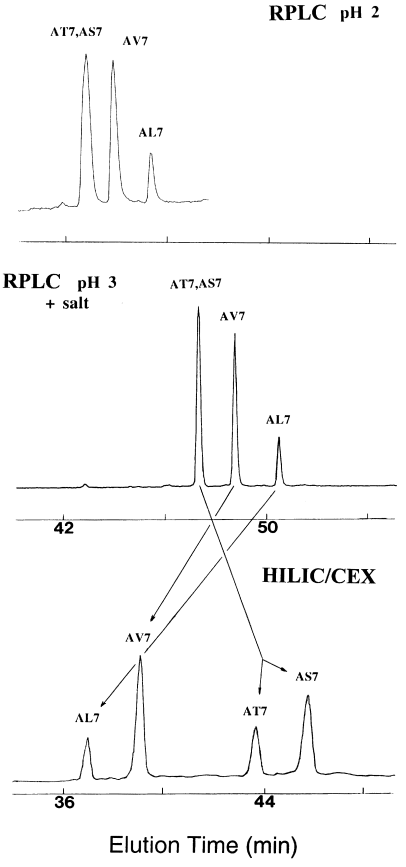


Fig. 7. RPLC and HILIC/CEX of moderately amphipathic α-helical peptides where substitutions have been made in the hydrophilic face. Conditions: RPLC at pH 2, same as Fig. 5; RPLC at pH 3 and HILIC/CEX; same as Fig. 1. Arrows denote reversal of peptide elution order between RPLC and HILIC/CEX. Sequences of peptides AL7, AV7, AT7 and AS7 are shown in Fig. 2 for n X 7 peptides (all non-polar positions, denoted n, are substituted by Ala), where position X is substituted by Leu, Val, Thr or Ser, respectively.

3.5.5. Comparison of the effect of substitutions in the hydrophobic face of a moderately hydrophobic amphipathic α-helix (peptides AL9, AV9, AT9, AS9)

From Fig. 8, all four peptides were well separated by RPLC, as expected, again showing the now typical longer retention times and generally superior elution profile of the pH 3 system. Also, in a similar manner to the better separation of the AS7 analogues by HILIC/CEX (Fig. 7) compared to the LX7

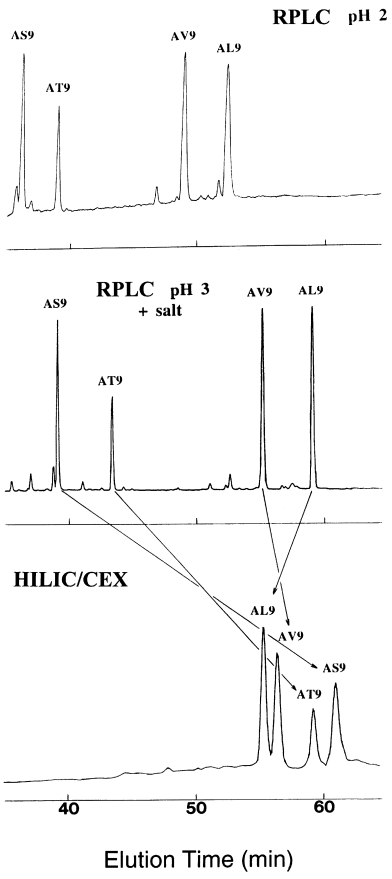


Fig. 8. RPLC and HILIC/CEX of moderately amphipathic α-helical peptides where substitutions have been made in the hydrophobic face. Conditions: RPLC at pH 2, same as Fig. 5; RPLC at pH 3 and HILIC/CEX, same as Fig. 1. Arrows denote reversal of peptide elution order between RPLC and HILIC/CEX. Sequences of peptides AL9, AV9, AT9 and AS9 are shown in Fig. 2 for n X 9 peptides (all non-polar positions, denoted n, are substituted by Ala), where position X is substituted by Leu, Val, Thr or Ser, respectively.

analogues (Fig. 5), the AX9 peptide pairs were generally better separated by RPLC (AL9/AV9, Δt_R=4 min at pH 3; AT9/AS9, Δt_R=4.3 min at pH 3) compared to the LX9 analogues (LL9/LV9, Δt_R= 0 min at pH 3; LT9/LS9, Δt_R=3.9 min at pH 3).

Some separation of the four peptides was achieved (compared to the co-elution of the LX9 peptides in Fig. 6) by HILIC/CEX, although clearly much inferior to that achieved by RPLC, as would be expected. Note again the reversal in peptide elution order (denoted by arrows) between the two HPLC modes. These results, together with the partial RPLC separation of the AX7 analogues shown in Fig. 7, as well as the clear HPLC mode preference seen for the highly amphipathic LX7 and LX9 peptides (Figs. 5 and 6, respectively), again suggests that peptide amphipathicity has a role in the relative separation power of RPLC and HILIC/CEX for such peptides.

One final point of interest concerning the longer peptide retention times for all four groups of peptides in the pH 3/perchlorate mobile phase compared to the pH 2 system lies in the relative effects on the very hydrophobic LX7/LX9 analogues compared to the moderately hydrophobic AX7/AX9 peptides. Thus, the effect of the mobile phase change is much more marked for the hydrophobic LX7 (Fig. 5) and LX9 (Fig. 6) analogues compared to the lesser hydrophobic AX7 (Fig. 7) and AX9 (Fig. 8) peptides. This observation again suggests that the presence of 100 m*M* $NaClO_4$ is indeed enhancing the hydrophobic interaction between the peptides and the stationary phase, the magnitude of this enhancement depending on the relative hydrophobicity of the hydrophobic preferred binding domain of the peptide.

3.5.6. Comparative efficiencies of RPLC and HILIC/CEX for separation of amphipathic α-helical peptides

Tables 1 and 2 summarize the results shown in

Table 1
Comparison of RPLC versus HILIC/CEX for separation of amphipathic α-helical peptide mixtures

Peptide type	RPLC Δt_R^a (min)	HILIC/CEX Δt_R^a (min)	$\Delta t_R/\Delta t_R^b$
AX7	3.2 (2.6)	8.6	2.7-fold (3.3-fold)
AX9	20.1 (16.1)	5.6	3.6-fold (2.9-fold)
LX7	Co-eluted[c]	4.9	–
LX9	16.3 (10.5)	Co-eluted	–

[a] Denotes difference in retention time between first and last eluted peptide.
[b] Denotes ratio of highest Δt_R to lowest Δt_R between the two HPLC modes, e.g., for AX7 peptides, $\Delta t_R/\Delta t_R$=8.6/3.2=2.7-fold greater Δt_R for HILIC/CEX mode compared to RPLC.
[c] Denotes that none of the four peptides showed any appreciable separation. Values in parentheses were calculated from aq. 0.05% TFA–acetonitrile, pH 2, RPLC run conditions; values without parentheses were calculated from aq. 20 m*M* TEAP–acetonitrile–100 m*M* $NaClO_4$, pH 3, RPLC run conditions.

Table 2
Comparison of resolution of individual peptide pairs by RPLC and HILIC/CEX

Peptide pairs	Resolution by RPLC[a]	Resolution by HILIC/CEX[a]
AS7/AT7	Co-eluted	3.1
AS9/AT9	13.4 (7.5)	1.9
AV7/AL7	6.5 (3.4)	4.1
AV9/AL9	9.2 (5.3)	1.3
LS7/LT7	Co-eluted	3.1
LS9/LT9	7.8 (1.7)	Co-eluted
LV7/LL7	Co-eluted	3.2
LV9/LL9	Co-eluted	Co-eluted

[a] Resolution was calculated according to the formula: resolution$=1.176\Delta t_R/W_1+W_2$, where Δt_R is the retention time difference between two peaks and W_1 and W_2 denote the peak widths (in min) at half peak height. Values in parentheses were calculated from aq. 0.05% TFA–acetonitrile, pH 2, RPLC run conditions; values without parentheses were calculated from aq. 20 m*M* TEAP–acetonitrile–100 m*M* $NaClO_4$, pH 3, RPLC run conditions.

Figs. 5–8 by quantifying the separations in terms of either the difference in retention time of first and last eluted peptides (Δt_R) (Table 1) or the resolution of specific peptide pairs (Table 2).

Original assumptions concerning the relative merits of RPLC and HILIC/CEX for separating amphipathic α-helical peptides included the premise that modifications in the hydrophilic (AX7, LX7 peptides) or hydrophobic (AX9, LX9 peptides) face of such peptides should be best separated by HILIC/CEX or RPLC, respectively. Table 1 demonstrates again that these assumptions hold up well. Thus, for the pH 3 RPLC run conditions, the RPLC Δt_R value for the AX9 peptides was 3.6-fold greater than that of the HILIC/CEX Δt_R value; conversely, for the AX7 peptides, the HILIC/CEX Δt_R value was 2.7-fold greater than that of the RPLC Δt_R value. Even more striking was the complete co-elution of the more amphipathic LX7 and LX9 peptides during RPLC and HILIC/CEX, respectively, compared to their good separation by HILIC/CEX and RPLC, respectively. Table 1 also confirms the overall superiority of the pH 3 RPLC run conditions compared to the more commonly employed aq. TFA–acetonitrile mobile phase, with the Δt_R values of this system consistently less than that of the aq. TEAP–acetonitrile–$NaClO_4$ mobile phase.

Concerning the above assumptions about which HPLC mode to employ for specific mixtures of amphipathic peptides, it was also stated previously that these assumptions should hold true no matter whether the substitutions in the hydrophobic or hydrophilic face of amphipathic α-helices are made with hydrophobic (e.g., Leu, Val) or hydrophilic (e.g., Thr, Ser) amino acid side-chains. Examination of Table 2, together with the observed elution profiles shown in Figs. 5–8, underlines the essential accuracy of this view. Thus, the resolution values of homologous peptide pairs where substitutions were made in the hydrophobic face of the helix (AS9/AT9, AV9/AL9, LS9/LT9) were generally significantly higher for the RPLC runs compared to the corresponding HILIC/CEX runs; only the LV9/LL9 peptide pair was co-eluted in both systems. Conversely, for homologous peptide pairs where substitutions were made in the hydrophilic face of the helix (AS7/AT7, LS7/LT7, LV7/LL7), the resolution values were generally better for the HILIC/CEX runs compared to RPLC. An interesting anomaly is the AV7/AL7 peptide pair where, in the salt-containing pH 3 mobile phase, the resolution value for RPLC (resolution=6.5) was a little greater than that of HILIC/CEX (4.1). However, in the aq. TFA–acetonitrile system, the resolution value for RPLC (3.4) had the expected lesser magnitude compared to HILIC/CEX. Indeed, save for the peptide pairs which were co-eluted in both RPLC mobile phases, the resolution of the peptides consistently deteriorated, at times considerably, on changing from the salt-containing pH 3 mobile phase to the aq. TFA–acetonitrile system.

4. Conclusions

This study has demonstrated the potential of

mixed-mode HILIC/CEX for the separation of amphipathic α-helical peptides. Such peptides were represented by model α-helical peptides varying in amphipathicity and the nature of the side-chain (Leu, Val, Thr or Ser) substituted in the centre of the hydrophilic or hydrophobic face of the helix. In addition to clarifying the relative values of HILIC/CEX and RPLC for specific model peptide separations, such results also have wider implications for resolving complex peptide mixtures such as those obtained from, for example, protein digests, where the occurrence of peptides with amphipathic α-helices is commonplace. Since the HILIC/CEX and RPLC modes of HPLC are inherently complementary, an initial purification of such peptides by HILIC/CEX may be followed by a final RPLC purification and de-salting step. In addition, since the need to identify mixture components as well as to separate and quantify them is great, e.g., HPLC in conjunction with electrospray mass spectrometry (MS), the utility of HILIC/CEX separations would be enhanced even further if they could be conducted with volatile mobile phase additives (as is the case with RPLC), an approach currently under investigation.

Acknowledgements

This work was supported by the Medical Research Council Group in Protein Structure and Function. We thank Paul Semchuk for synthesizing the peptides.

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