Use of sodium perchlorate at low pH for peptide separations by reversed-phase liquid chromatography
Influence of perchlorate ion on apparent hydrophilicity of positively charged amino acid side-chains

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Abstract

The reversed-phase liquid chromatography (RPLC) behavior of synthetic model peptides containing positively charged amino acid residues was studied in the presence or absence of 100 mM sodium perchlorate in order to determine the effect on apparent side-chain hydrophilicity of a charged residue at low pH. The peptides used in this study were either non-helical peptides or amphipathic α-helical peptides, where the effect of the negatively charged perchlorate ion on a charged residue in either the hydrophobic face or hydrophilic face of the helix was monitored. We have shown that the addition of 100 mM perchlorate to RPLC separations of positively charged peptides performed in a 20 mM aqueous phosphoric acid–acetonitrile system resulted in an increase in retention time of a peptide relative to the same peptide in the absence of perchlorate.

This effect occurred independent of conformation, i.e., whether comparing the effect of positively charged residue substitutions in the hydrophobic or hydrophilic face of an amphipathic α-helix or in a peptide with negligible secondary structure. From these results, suggesting that positively charged side-chain hydrophilicity is decreased by ion-pairing with the perchlorate ion, we have shown practical examples where mixtures of non-helical and amphipathic α-helical peptides showed enhanced resolution in the presence of perchlorate at pH 2, compared to in its absence. In addition, it was shown that an aqueous phosphoric acid–perchlorate–acetonitrile mobile phase may show markedly different selectivity for peptide separations at low pH compared to the more traditional aqueous trifluoroacetic acid–acetonitrile system. © 1997 Elsevier Science B.V.

Keywords: Ion-pairing reagents; Mobile phase composition; Peptides; Sodium perchlorate

1. Introduction

The current predominance of reversed-phase liquid chromatography (RPLC) as the high-performance liquid chromatography (HPLC) technique of choice for peptide separations [1] has resulted from its excellent resolving capability as will as the battery of stationary phase and mobile phase options open to the researcher for such applications [1–3]. Although satisfactory separation of peptides may be obtained at acidic or neutral pH, the majority of RPLC separations are carried out at pH values <3.0. Apart from the suppression of undesirable interactions of positively charged groups with weakly acidic silanols on the surface of a silica matrix [4,5], the siloxane bond linking the hydrophobic ligand to a silica-based support is more stable at low pH [6,7].

Peptides are charged molecules at most pH values,

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with basic (positively charged) character dominating at low pH, due to protonation of any potentially negatively charged acidic amino acid side-chains. Differences in the polarities of peptides at acidic pH can be maximized through careful choice of anionic ion-pairing reagent [e.g., phosphoric acid, trifluoroacetic acid (TFA), heptafluorobutyric acid (HFBA)], where the resolving power of the corresponding anionic counterions (phosphate, trifluoroacetate, heptafluorobutyrate, respectively) is effected through interaction with the positively charged (basic) residues of the peptides [8]. The actual effect on peptide retention time will depend strongly on the hydrophobicity of the anionic counterion as well as the number of positively charged groups in the peptide [8]. Thus, it is well known that the retention time of a peptide containing hydrophilic basic residues may be increased by the addition of hydrophobic counterions such as trifluoroacetate and heptaflourobutyrate to the mobile phase [8–12]; such counterions are not only capable of forming ion-pairs with positively charged peptide solutes but, due to their hydrophobic character, can further increase the affinity of the peptides for the hydrophobic stationary phase [8]. Hydrophilic counterions such as phosphate will also increase the retention times of basic peptides at low pH, albeit due only to a reduction of peptide hydrophilicity of positively charged residues by ion-pair formation [8]. Manipulation of the chromatographic profile of a peptide mixture may thus be attempted by varying the hydrophobicity of the anionic counterion at low pH. In a similar manner to the effect of increasing counterion hydrophobicity, increasing counterion concentration produces increasing retention times of basic peptides, the magnitude of this effect again being dependent on the number of positively charged groups a peptide contains [8]. Thus, manipulation of peptide elution profiles may also be effected by increasing the concentration of ion-pairing reagents such as phosphoric acid, TFA and HFBA from the usual working range of 0.05%–0.1% [1] to higher concentrations, e.g., up to 0.8% [8] or even 1% [13]. It should be noted, however, that the corresponding decrease in pH (~1.3 at 0.8% TFA [8]) with increasing reagent concentration may have a deleterious effect on silica-based column packings making this an undesirable option on a routine basis. In addition, Guo et al. [3]

reported that the magnitude of the concentration effect is small above counterion concentrations of about 0.2%.

One approach to the manipulation of RPLC profiles of peptides infrequently considered is the addition of salts to the mobile phase at low pH. Addition of salts (generally 50 mM–100 mM) to mobile phases over a pH range of ~4–7 is traditionally designed to suppress negatively charged silanol interactions with positively charged solutes [14–23]; any selectivity effects tend to be a secondary consideration. However, we believe that salt addition has the potential to offer useful gains in peptide separation selectivity at low pH through reduction of hydrophilicity of positively charged residues by ion-pairing with negatively charged ions. Thus, in the present study, we set out to examine the potential use of sodium perchlorate to modulate the hydrophilic character of model amphipathic α-helical peptides as well as peptides of negligible secondary structure. From these results, we will demonstrate how aqueous phosphate–perchlorate mobile phases may complement traditional aqueous TFA systems for specific peptide separations.

2. Experimental

2.1. Materials

HPLC-grade water and acetonitrile were obtained from BDH (Poole, UK). TFA was obtained from Aldrich (Milwaukee, WI, USA). ACS-grade orthophosphoric acid was obtained from Anachemia (Toronto, Canada). Sodium perchlorate was obtained from BDH.

2.2. Instrumentation

Peptide synthesis was carried out on an Applied Biosystems peptide synthesizer Model 430 (Foster City, CA, USA). Crude peptides were purified by RPLC using an Applied Biosystems 400 solvent delivery system connected to a 783A programmable absorbance detector.

The analytical HPLC system consisted of an HP1090 liquid chromatograph (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. Column and HPLC conditions

Unless stated otherwise, analytical runs were carried out on an Aquapore RP-300 C_8 reversed-phase column (220×4.6-mm I.D., 7-μm particle size, 300 Å pore size) purchased from Applied Biosystems.

Unless stated otherwise, separations were carried out by linear AB gradient elution (1% acetonitrile per min) at a flow-rate of 1 ml/min, where eluent A is 20 mM aqueous phosphoric acid, pH 2.0 and eluent B is 20 mM phosphoric acid in 50% aqueous acetonitrile; for separations in the presence of per-chlorate ion, both eluents contained 100 mM sodium perchlorate.

3. Results and discussion

The use of sodium perchlorate in these studies was prompted by a number of considerations: (1) it is a reagent employed in RPLC at both neutral [14,15,18,22] and acidic [14,15,24–27] pH values to improve peptide peak shape; (2) it is UV transparent, allowing peptide bond detection at 210 nm; (3) sodium perchlorate is highly soluble in aqueous acetonitrile eluents, even at relatively high concentrations of this organic modifier [28]; (4) sodium perchlorate is a strong chaotropic reagent (i.e., perchlorate is an inorganic ion that favours the transfer of nonpolar groups to water by altering water structure [14,29]), which may be useful in solubilizing hydrophobic peptides in a peptide mixture.

Prior to reporting the effect of perchlorate ion on peptide chromatographic profiles, it is useful to clarify the definition of hydrophilicity/hydrophobicity when applied to this parameter in the presence or absence of perchlorate or, indeed, any other negatively charged counterion. The assignment of hydrophilicity/hydrophobicity values to amino acid side-chains is very much a function of the technique and environment in which such an assignment has been made. In the case of hydrophilicity/hydrophobicity
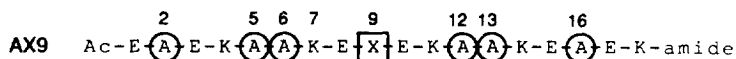
coefficients obtained through RPLC of peptides, the major determinant of the values obtained is the mobile phase, i.e., pH, presence of salts and/or ion-pairing reagent, organic modifier, etc. Values assigned to ionizable groups (N-terminal amino, C-terminal carboxyl, acidic and basic side-chains), in particular, are extremely sensitive to mobile phase variations; indeed, the influence of salts and/or counterions in the mobile phase is dependent on interactions with charged, rather than neutral, species [8]. Hence, in the present study, instead of assigning specific hydrophilicity values for basic side-chains, the influence of perchlorate at low pH on such side-chains is discussed in terms of changes in apparent hydrophilicity of side-chains.
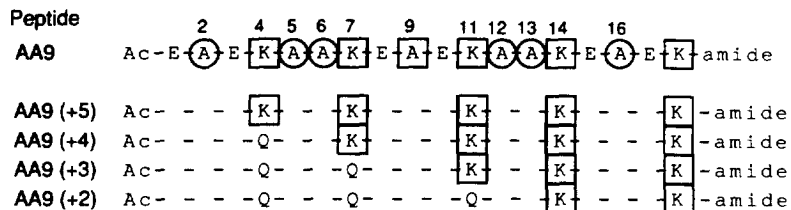
3.1. Model peptides

For a thorough understanding of the influence of perchlorate on a positively charged side-chain during RPLC, it is important to monitor the apparent hydrophilicity of the side-chain under different peptide environments, i.e., taking into account the influence, if any, of the position of the side-chain in the sequence on its apparent hydrophilicity under varying mobile phase conditions. Thus, our approach was to design and synthesize series of model peptide analogues with closely controlled environments influencing basic side-chains.

Fig. 1A shows the sequence of a series of 18-residue peptide analogues, denoted AX9, a sequence known to have a high potential to form an α-helix, specifically an amphipathic α-helix [30–33]. Position 9, denoted X, represents the position substituted by the 20 amino acids commonly found in proteins. The analogues of this series are identified by the substituted residue; thus, the designation AR9 refers to an arginine residue substitution at position 9 of the sequence, etc. From the helical net presentation in Fig. 1C (right), it can be seen that the substitution site at position 9 is in the centre of the hydrophobic face (between the solid lines) of the amphipathic helix surrounded by alanine residues. This design not only produces a consistent hydrophobic environment around the substituted residue, but also ensures intimate interaction of this residue with the hydrophobic stationary phase lying as it does in the centre of a preferred binding domain [32–35].
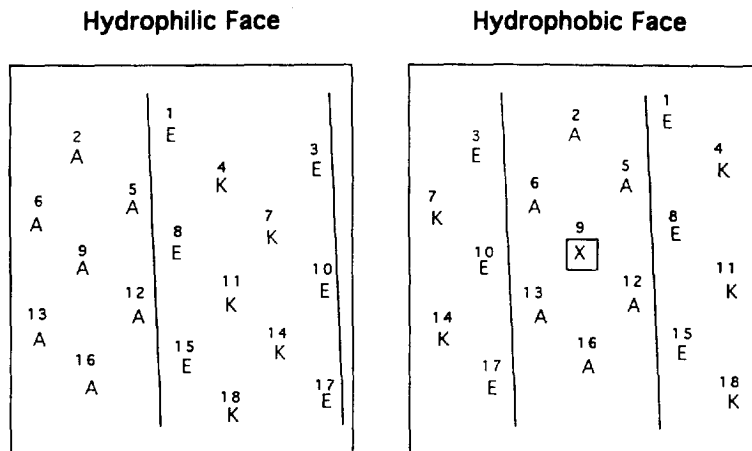
1A. Amphipathic α-helical peptides : Hydrophobic face substitution

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            2       5 6 7   9      12 13      16
AX9   Ac-E-(A)-E-K-(A)(A)-K-E-[X]-E-K-(A)(A)-K-E-(A)-E-K-amide
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1B. Amphipathic α-helical peptides : Hydrophilic face substitution

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Peptide      2     4 5 6 7    9     11 12 13 14    16
AA9     Ac-E-(A)-E-[K]-(A)(A)-[K]-E-[A]-E-[K]-(A)(A)-[K]-E-(A)-E-[K]-amide

AA9 (+5) Ac- - - -[K]- - -[K]- - - -[K]- -[K]- - - -[K]-amide
AA9 (+4) Ac- - - -Q- - -[K]- - - -[K]- -[K]- - - -[K]-amide
AA9 (+3) Ac- - - -Q- - -Q- - - -[K]- -[K]- - - -[K]-amide
AA9 (+2) Ac- - - -Q- - -Q- - - -Q- - -[K]- - - -[K]-amide
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1C. Helical net representation of hydrophobic and hydrophilic faces of model amphipathic α-helical peptides

Hydrophilic Face   **Hydrophobic Face**

1D. Non-helical peptides

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Peptide     1 2 3 4 5 6 7 8 9 10
X1      Ac-(X)-L-G-A-[K]-G-A-G-V-G-amide

K1(+4)  Ac-[K]- - - - - -[K]- - - -[K]- -[K]-amide
K1(+3)  Ac-[K]- - - - - -[K]- - - -[K]- -G-amide
K1(+2)  Ac-[K]- - - - - -[K]- - - -G- -G-amide
G1(+1)  Ac- G- - - - - -[K]- - - -G- -G-amide
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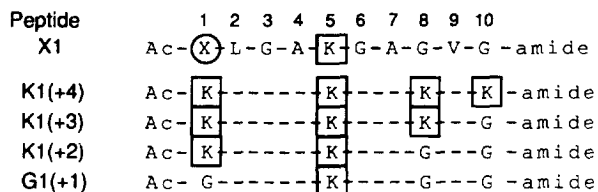
Table 1
Effect of sodium perchlorate on the apparent hydrophilicity of charged residues in non-helical peptides

Peptide[a]	t_R (min)[b] (−perchlorate)	t_R (min)[b] (+perchlorate)	Δt_R[c]	Δt_R/charge[d]
K1 (+4)	8.09	18.56	10.47	2.61
K1 (+3)	10.35	18.43	8.08	2.69
K1 (+2)	12.76	17.96	5.20	2.60
G1 (+1)	14.71	17.36	2.65	2.65
				2.64=Avg.

[a] Peptide sequences are shown in Fig. 1D.
[b] RPLC conditions [in the absence (−) or presence (+) of perchlorate] are shown in Section 2.
[c] Retention time difference of peptide in the absence and presence of 100 mM perchlorate (t_R in the presence minus t_R in the absence=Δt_R).
[d] Δt_R divided by the number of positively charged residues (Lys) in the peptide.

Fig. 1B shows the sequence of a series of peptide analogues, based on peptide AA9 of the amphipathic AX9 series, where substitutions have been made in the hydrophilic face of the helix. From Fig. 1B, substitutions of the neutral glutamine for positively charged lysine have been made at position 4 [peptide AA9 (+4)], positions 4 and 7 [AA9 (+3)], positions 4, 7 and 11 [AA9 (+2)] and positions 4, 7, 11 and 14 [AA9 (+1)], to give a range of net positive charges of +1 to +5 in the hydrophilic face: peptide AA9 is now referred to as AA9 (+5) to denote that this analogue contains 5 lysine residues (i.e., +5 net charge); AA9 (+4) contains 4 lysine residues (i.e., +4 net charge), etc. Fig. 1C (left) shows a helical net presentation of the hydrophilic face (between the solid lines) of peptide AA9 (or AA9 (+5)).

Fig. 1D shows the sequence of a peptide series, denoted X1, known to exhibit negligible α-helical structure [33], where X represents the amino acid substituted at position 1; thus, G1 represents a 10-residue peptide with a glycine residue substituted at position 1. A supplementary peptide series, shown in Fig. 1D, based on G1 [now denoted G1 (+1)] was

also prepared where substitutions of lysine for glycine have been made at position 1 [peptide K1 (+2)], positions 1 and 8 [K1 (+3)] and positions 1, 8 and 10 [K1 (+4)].

Thus, these peptide series allow an investigation of the effect of varying peptide environment and peptide conformation on the apparent hydrophilicity of basic side-chains.

3.2. Effect of sodium perchlorate on the apparent hydrophilicity of charged residues in non-helical peptides

Table 1 shows the effect of 100 mM sodium perchlorate on the retention time of four peptides of negligible secondary structure (X1 series in Fig. 1D) and varying numbers of positively charged lysine residues (+1, +2, +3 and +4) within the sequence. From Table 1, the presence of perchlorate increased the retention times of all four non-helical peptides, as would be expected with neutralization of hydrophilic positively charged groups through ion-pair formation with the negatively charged perchlorate ion. Thus, in

Fig. 1. Design of model synthetic peptides. A: sequence of model amphipathic α-helical peptides, denoted AX9 series, where substitutions have been made in the hydrophobic face of the helix; the X represents each of the 20 amino acids (boxed) (single letter code given in Table 5) found in proteins and substituted at position 9; the residues that are circled or boxed and labeled 2, 5, 6, 9, 12, 13 and 16 are in the hydrophobic face of the amphipathic α-helical peptides; lysine and glutamic acid make up the hydrophilic face of the amphipathic helix. B: sequence of model amphipathic α-helical peptides, based on peptide AA9 of the AX9 series shown in sequence 1A (with alanine substituted at position 9), where substitutions have been made in the hydrophilic face of the helix; lysine to glutamine substitutions have been made at the positions shown to give a range of net positive charges (+2 to +5) in the hydrophilic face. C: α-helical net representation of hydrophilic (1A) and hydrophilic (1B) faces of model amphipathic α-helical peptides; 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 α-helical nets represents the hydrophilic (left) or hydrophobic (right) faces of the peptides; D: sequence of model non-helical peptides, denoted X1 series, where X (circled) is substituted by each of the 20 amino acids found in proteins; the design of the four peptides of varying net positive charge [+1, +2, +3 and +4; G1(+1), K1(+2), K1(+3) and K1(+4), respectively] is based on peptide G1 (+1 net charge) of the X1 series.

the presence of perchlorate, a positively charged lysine residue appears less hydrophilic compared to the apparent hydrophilicity of this residue in the absence of perchlorate; for instance, for peptide G1 (+1), containing one lysine residue, the apparent hydrophilicity of this residue has been reduced by 2.65 min ($\Delta t_R = 17.36 - 14.71 = 2.65$ min in Table 1). Similar effects were observed for the other three peptides, the increase in retention time of these peptides in the presence of perchlorate clearly being related to the numbers of positive charges they contain; thus, in order of increasing effect (Δt_R) of perchlorate on peptide retention time, G1 (+1)<K1 (+2)<K1 (+3)<K1 (+4). In fact, the elution order of the four peptides is reversed in the presence of 100 mM perchlorate compared to its absence. Interestingly, the influence of perchlorate on the apparent hydrophilicity of lysine was consistently additive, i.e., each lysine residue contributed an equal reduction in hydrophilicity in the presence of perchlorate as expressed by Δt_R/charge (Table 1), representing the effect of perchlorate ion per charged residue in the peptide (an average decrease of 2.64 min of lysine side-chain hydrophilicity in the presence of perchlorate).

3.3. Effect of sodium perchlorate on the apparent hydrophilicity of positively charged side-chains in the hydrophobic face of an amphipathic α-helix

Table 2 shows the effect of 100 mM sodium perchlorate on the retention time of three am-

phipathic α-helical peptides (AK9, AR9 and AH9) containing positively charged side-chains in the hydrophobic face of the helix (Fig. 1A and C, right; AX9 series). In a similar manner to the non-helical peptides (Table 1), the retention times of all three peptides increased in the presence of perchlorate.

Since the AX9 series of peptides contains five positively charged lysine residues in the hydrophilic face of the amphipathic helix (Fig. 1B and C), the lysine-, arginine- and histidine-substituted analogues (AK9, AR9 and AH9, respectively) were referenced to the glutamine-substituted peptide (AQ9). In this way, the effect of perchlorate on the positively charged residue in the hydrophobic face of the helix only could be determined (expressed as Δt_R to AQ9 in Table 2). From Table 2, in the absence of perchlorate, AK9 is less retentive than AQ9 by 3.02 min (i.e., $\Delta t_R = -3.02$ min); however, in the presence of perchlorate, AK9 is less retentive by only 1.07 min. This suggests that, upon ion-pairing of the perchlorate ion with the charged lysine residue, the contribution of the lysine side-chain to peptide retention was increased by 1.95 min ($\Delta \Delta t_R$ in Table 2), i.e., the apparent hydrophilicity of lysine in the presence of 100 mM sodium perchlorate has decreased by 1.95 min [$\Delta \Delta t_R = -1.07 - (-3.02) = 1.95$]. Similar observations were made for the arginine (AR9) and histidine (AH9) residues, which became less hydrophilic by 2.68 min and 2.81 min, respectively, in the presence of 100 min perchlorate. Albeit with some variation for individual peptides, the magnitude of these hydrophilicity changes is similar

Table 2
Effect of sodium perchlorate on the apparent hydrophilicity of charged residues in the hydrophobic face of amphipathic α-helical peptides

Peptide[a]	−Perchlorate[b]		+Perchlorate[b]		$\Delta\Delta t_R$ [c]
	t_R (min)[c]	Δt_R AQ9[d]	t_R (min)[c]	Δt_R AQ9[d]	
AQ9	17.71	−	25.16	−	−
AK9	14.69	−3.02	24.09	−1.07	+1.95
AR9	15.50	−2.21	25.63	+0.47	+2.68
AH9	14.26	−3.45	24.52	−0.64	+2.81

[a] Peptide sequences are shown in Fig. 1A.
[b] Represents either the absence (−) or presence (+) of 100 mM sodium perchlorate in the mobile phase.
[c] RPLC conditions are shown in Section 2.
[d] Retention time difference between the basic residue-substituted peptide and the Gln-substituted analogue (t_R AX9 − t_R AQ9 = Δt_R).
[e] The change in retention time observed for a Gln to basic residue substitution in going from a mobile phase containing no perchlorate to that containing 100 mM sodium perchlorate. The value is obtained by subtracting the absolute value of the retention time of the basic residue substituted peptide relative to the Gln analogue (Δt_R; see above) in the absence of perchlorate from the Δt_R value in its presence.

to the average value of 2.64 min reported in Table 1 for non-helical peptide analogues.

3.4. Effect of sodium perchlorate on the apparent hydrophilicity of charged residues in the hydrophilic face of an amphipathic α-helix

Table 3 shows the effect of 100 mM sodium perchlorate on the retention time of amphipathic α-helical peptides containing varying numbers of positively charged residues in the hydrophilic face of the helix [Fig. 1B and C, left; AA9 (+) series]. In a similar manner to the results shown in Tables 1 and 2, the retention time of all four peptides increased in the presence of perchlorate. In addition, as seen previously for non-helical peptides (Table 1), the magnitude of this effect increases with increasing number of positive charges in the hydrophilic face; thus, in order of increasing effect (Δt_R) of perchlorate on peptide retention time, AA9 (+2)<AA9 (+3)<AA9 (+4)<AA9 (+5); in a similar manner to the peptides shown in Table 1, the elution order of the four peptides is reversed in the presence of 100 mM perchlorate compared to its absence. The average increase in retention time in the presence of 100 mM perchlorate per charged residue (Δt_R/charge), representing the average decrease in lysine hydrophilicity in the presence of the perchlorate ion, was 1.29 min. The significantly lesser magnitude of this figure compared to those obtained from substitutions in the hydrophobic face of the helix (1.95 min to 2.81 min; Table 2) or from lysine substitutions in non-helical peptides (average 2.64 min; Table 1) is not surprising considering that residues in the hydro-

philic face of the amphipathic helix would be expected to interact with the hydrophobic stationary phase to a considerably lesser extent than residues in the centre of the preferred binding domain (i.e., the hydrophobic face) of the helix or, indeed, residues substituted in a random coil.

Unlike the effect of perchlorate on apparent hydrophilicity of lysine in non-helical peptides (Table 1), this effect was not quite additive in the case of this amphipathic helix, i.e., Δt_R/charge decreased progressively from 1.47 min for AA9 (+5) to 1.14 min for AA9 (+2), suggesting a lesser effect of perchlorate on the hydrophilicity of individual lysine residues with each substitution of lysine by glutamine (Fig. 1B). However, compared to the overall retention times of the peptides in the presence of perchlorate (ranging from 28.37 min to 30.09 min), these sequence-dependent variations are not large.

The observation that the magnitude of the perchlorate effect increases with increasing number of positive charges in the hydrophilic face of the helix also raised another point of fundamental importance. It is known that the presence of high salt concentrations can enhance hydrophobic interactions between a solute and a hydrophobic stationary phase; indeed, this 'salting out' effect is the basis for the hydrophobic interaction mode of chromatography [36,37]. From Table 3, if the effect of 100 mM sodium perchlorate was merely to enhance the binding of the hydrophobic face of the amphipathic helix to the stationary phase (or if such a potential effect greatly outweighed any ion-pairing effects reducing lysine hydrophilicity in the hydrophilic

Table 3
Effect of sodium perchlorate on the apparent hydrophilicity of charged residues in the hydrophilic face of amphipathic α-helical peptides

Peptide[a]	t_R (min)[b] (−perchlorate)	t_R (min)[b] (+perchlorate)	Δt_R^c	Δt_R/charge[d]
AA9 (+5)	22.75	30.09	7.37	1.47
AA9 (+4)	24.29	29.61	5.23	1.33
AA9 (+3)	25.32	28.96	3.64	1.21
AA9 (+2)	26.09	28.37	2.28	1.14
				1.29=Avg.

[a] Peptide sequences are shown in Fig. 1B.
[b] RPLC conditions [in the absence (−) or presence (+) of 100 mM perchlorate] are shown in Section 2.
[c] Retention time difference of peptide in the absence and presence of 100 mM perchlorate (t_R in the presence minus t_R in the absence$=\Delta t_R$).
[d] Δt_R divided by the number of positively charged residues (Lys) in the peptide.

face), it might be expected that such enhancement of binding of the four peptides would be similar, i.e., the presence of varying numbers of positively charged groups in the hydrophilic face would have little, if any, effect and the elution order of the peptides would remain the same in the presence or absence of 100 mM perchlorate. However, as noted above (Table 3), the elution order of the four peptides has reversed in the presence of perchlorate, with the most highly charged peptide [AA9 (+5)] now being eluted last (as opposed to first in the absence of perchlorate) and the least highly charged AA9 (+2) now being eluted first (as opposed to last in the absence of perchlorate). This is also reflected by the increasing Δt_R values (t_R in the presence of perchlorate minus t_R in the absence) with increasing numbers of lysine residues in the hydrophilic face, clearly confirming the changes in lysine hydrophilicity effected by the perchlorate ion.

3.5. Comparison of effect of sodium perchlorate on apparent hydrophilicity of positively charged residues between the two faces of an amphipathic α-helix

The results shown in Tables 2 and 3, detailing the effect of perchlorate ion on the apparent hydrophilicity of positively charged residues in the opposing faces of an amphipathic α-helical peptide, now prompted a comparison of the relative magnitude of this effect between the two faces. To determine this effect, the data presented in Table 4 represent a reanalysis of data from Tables 2 and 3.

From Table 4, in the absence of perchlorate, a glutamine to lysine substitution in the hydrophobic face of the amphipathic α-helix [Fig. 1A and C (right)] resulted in a retention time decrease (Δt_R for Gln→Lys) of 3.02 min; whereas, in the presence of perchlorate, the same substitution resulted in a retention time reduction of only 1.07 min, indicating that the presence of perchlorate resulted in an apparent lysine hydrophilicity reduction ($\Delta\Delta t_R$; see Table 2) of 1.95 min. A glutamine to lysine substitution in the hydrophilic face of the amphipathic α-helix [AA9 (+4) to AA9 (+5); Fig. 1B and C, left] in the absence of perchlorate resulted in a retention time reduction of 1.54 min; whereas, in the presence of perchlorate, the same substitution resulted in a retention time increase of 0.48 min, indicating that the presence of perchlorate resulted in an apparent lysine hydrophilicity reduction ($\Delta\Delta t_R$) of 2.02 min. The similarity of these two $\Delta\Delta t_R$ values suggests

Table 4

Comparison of the effect of sodium perchlorate on the apparent hydrophilicity of positively charged residues between the hydrophobic and hydrophilic faces of amphipathic α-helical peptides

Peptide[a]	−Perchlorate[b]		+Perchlorate[b]		$\Delta\Delta t_R$[c]
	t_R (min)[c]	Δt_R Gln→Lys[d]	t_R (min)[c]	Δt_R Gln→Lys[d]	
Hydrophobic face					
AQ9	17.71	–	25.16	–	–
AK9	14.69	−3.02	24.09	−1.07	+1.95
Hydrophilic face					
AA9 (+4)	24.29	–	29.61	–	–
AA9 (+5)	22.75	−1.54	30.09	+0.48	+2.02
		$\Delta\Delta t_R'$[f] = +1.48		$\Delta\Delta t_R'$[f] = +1.55	

[a] Peptide sequences are shown in Fig. 1A (AQ9, AK9) and Fig. 1B [AA9 (+4), AA9 (+5)].

[b] Represents either the absence (−) or presence (+) of 100 mM sodium perchlorate in the mobile phase.

[c] RPLC conditions are shown in Section 2.

[d] Retention time difference between the lysine-substituted peptide and the Gln-substituted analogue [t_RAK9−t_RAQ9 or t_RAA9 (+5)−t_RAA9 (+4)].

[e] The change in retention time observed for a Gln to Lys substitution in going from a mobile phase containing no perchlorate to that containing 100 mM sodium perchlorate. The value is obtained by subtracting the absolute value of the retention time of the lysine-substituted peptide relative to the Gln analogue (Δt_R Gln→Lys; see above) in the absence of perchlorate from the Δt_R value in its presence.

[f] The change in retention time observed for a Gln to Lys substitution in going from the hydrophobic face to the hydrophilic face of the α-helix. The value is obtained by subtracting the absolute value of the retention time of the lysine-substituted peptide relative to the Gln analogue (Δt_R Gln→Lys; see above) in the hydrophobic face from the Δt_R value in the hydrophilic face.

that, when perchlorate ion-pairs with the positively charged lysine side-chain, the reduction in apparent residue hydrophilicity is of the same magnitude independent of its substitution in the hydrophobic or hydrophilic face of the amphipathic α-helix. An interesting point to note here is that, in the presence of 100 mM perchlorate, the apparent hydrophobicity of positively charged lysine in the hydrophilic face of the helix is greater than that of neutral glutamine; in contrast, in the absence of perchlorate, glutamine exhibits a greater hydrophobicity compared to lysine. This situation, where apparent glutamine hydrophobicity is greater than that of lysine, also holds for substitutions made in the hydrophobic face of the helix (Table 4). This is an excellent example of the way environment may have a profound effect on the relative hydrophilicity/hydrophobicity of amino acid side-chains.

Another point of interest apparent from Table 4 is the relative hydrophilicity/hydrophobicity of lysine expressed in the hydrophilic face versus the hydrophobic face of the helix in the absence or presence of perchlorate. Thus, in the absence of perchlorate, an addition of lysine to the hydrophobic face results in a retention time reduction (Δt_R Gln→Lys) of -3.02 min; whereas, for an addition of lysine to the hydrophilic face, this reduction is only -1.54 min i.e., the apparent hydrophilicity of lysine is greater in the hydrophobic face compared to the hydrophilic face. A similar observation was made in the presence of 100 mM perchlorate. Thus in the presence of perchlorate, an addition of a lysine side-chain to the hydrophobic face results in a retention time reduction of -1.07 min; whereas, for an addition of lysine to the hydrophilic face, there is actually a retention time increase of $+0.48$ min. These results support recently reported observations by Sereda et al. [32], who showed through the use of model amphipathic α-helical peptides that the apparent hydrophilicity/hydrophobicity expressed by a residue is dependent on its environment. Thus, maximizing the hydrophobicity of the environment surrounding a particular residue (in the present case, lysine substituted in the hydrophobic face of AA9) maximizes its apparent hydrophilicity (or minimizes its hydrophobicity); in contrast maximizing the hydrophilicity of the environment surrounding a lysine substituted in the hydrophilic face of AA9 minimizes its apparent hydrophilicity (or maximizes its hydrophobicity).

The change in apparent lysine hydrophilicity between the two faces of the amphipathic helix ($\Delta\Delta t_R'$, Table 4) in the absence of perchlorate was 1.48 min; whilst, in its presence, this value was 1.55 min. Thus, in a manner analogous to that noted above ($\Delta\Delta t_R$ values in Table 4), the similarity of these two values suggests that the change in apparent lysine hydrophilicity in the hydrophilic and hydrophobic faces of the helix is independent of the absence or presence of perchlorate.

3.6. Manipulation of RPLC peptide elution profiles by addition of sodium perchlorate to the mobile phase

Table 5 reports the effect on RPLC retention times of all 20 of the AX9 amphipathic α-helical series (Fig. 1A) and X1 non-helical series (Fig. 1D) of peptide analogues. In a manner similar to that noted for Table 1–3, the retention time of all peptides increases in the presence of 100 mM sodium perchlorate compared to its absence. This retention time increase which depends on the number of positive charges a peptide contains, is very consistent throughout both sets of analogues. Thus, for the amphipathic α-helical series, AX9, the Δt_R values (t_R in presence of perchlorate minus t_R in absence) for the analogues with neutral residue substitutions in the centre of the hydrophobic face (i.e., only the five lysine residues in the hydrophilic face have to be considered) range from just 7.45 min to 8.64 min with an average retention time increase of 1.61 min per positive charge ($\Delta t_R/5$). The greater effect of perchlorate on the retention times of AK9, AH9 and AR9 ($\Delta t_R = 9.40$ min, 10.26 min and 10.13 min respectively) is due to the extra positive charge in the hydrophobic face of the helix. Similarly for the non-helical series, X1, the Δt_R values for the analogues with neutral residue substitutions at position 1 (i.e., only the lysine residue at position 5 needs to be considered) range from 2.44 min to 3.38 min, with an average of retention time increase of 2.88 min per positive charge. The three analogues with an extra positively charged residue (K1, H1 and R9 with Δt_R values of 5.35 min, 5.70 min and 6.20 min, respectively) also showed an average retention time increase of 2.88 min per positive charge.

The results reported in Table 5 suggested that separation selectivity differences between am-

Table 5
Effect of sodium perchlorate on retention times of amphipathic α-helical and non-helical peptides

Amino acid[a] substitution	t_R (min) helical peptides[b]			t_R (min) non-helical peptides[b]			
	−perchlorate	+ perchlorate	Δt_R^c	− perchlorate	+ perchlorate	Δt_R^c	
Ile	(I)	27.13	35.73	8.60	21.44	24.82	3.38
Leu	(L)	27.20	35.55	8.35	22.19	25.42	3.23
Phe	(F)	26.78	35.23	8.45	23.66	26.56	2.90
Trp	(W)	26.23	34.60	8.37	24.43	27.35	2.92
Val	(V)	25.40	34.04	8.64	18.91	22.10	3.19
Met	(M)	24.96	33.32	8.36	19.83	22.78	2.95
Cys	(C)	23.03	31.20	8.48	16.90	20.17	3.27
Tyr	(Y)	23.20	31.20	8.00	19.41	22.44	3.03
Ala	(A)	22.81	30.31	7.50	15.69	18.52	2.83
Thr	(T)	20.00	28.17	8.17	15.53	17.92	2.39
Glu	(E)	19.77	27.27	7.50	15.62	18.18	2.56
Gly	(G)	18.77	26.38	7.61	14.73	17.55	2.82
Ser	(S)	18.36	26.16	7.80	14.66	17.10	2.44
Arg	(R)	15.50	25.63	10.13	13.03	19.23	6.20
Asp	(D)	17.67	25.42	7.75	14.92	17.62	2.70
Gln	(Q)	17.71	25.16	7.45	14.81	17.31	2.50
His	(H)	14.26	24.52	10.26	12.66	18.43	5.70
Lys	(K)	14.69	24.09	9.40	12.82	18.17	5.35
Asn	(N)	15.66	23.42	7.76	14.49	17.13	2.64
Pro	(P)	15.10	22.98	7.88	18.18	21.32	3.14

[a] Three-letter code and single-letter code for the 20 amino acids commonly found in proteins. Amino acid substitutions in either position 9 of α-helical AX9 series (Fig. 1A) or position 1 of non-helical X1 series (Fig. 1D).
[b] RPLC conditions [in the absence (−) or presence (+) of 100 mM perchlorate] are shown in Section 2.
[c] Retention time difference of peptide in the absence and presence of 100 mM perchlorate (t_R in the presence minus t_R in the absence=Δt_R).

phipathic helical and non-helical peptides may be enhanced through the addition of 100 mM sodium perchlorate. Fig. 2 shows examples of this approach by illustrating the beneficial effect of perchlorate on the separation of selected mixtures of α-helical and non-helical peptides from Table 5. Such mixtures are not uncommon when resulting from chemical or proteolytic digests, due to the high occurrence of amphipathic α-helices in proteins [38]. Indeed, Segrest et al. [39] noted that 50% of all α-helices in soluble globular proteins are amphipathic. Thus, the peptide mixtures shown in Fig. 2 (and, indeed, Fig. 3) offer good practical examples of the advantageous properties of perchlorate ion during RPLC at pH 2.

From Fig. 2A. the α-helical AN9 and non-helical A1 are coeluted in the absence of perchlorate, whilst the α-helical AM9 and non-helical W1 are only poorly resolved; from Fig. 2B, the addition of 100 mM perchlorate has resulted in excellent separation of all four peptides. A similar situation is seen in Fig. 2C, where α-helical (AP9, AA9) and non-helical

(D1, L1) peptide pairs are poorly resolved in the absence of perchlorate; in contrast, from Fig. 2D, the addition of perchlorate again produced excellent resolution of all four peptides. From Fig. 2E, all four components of a mixture of two α-helical (AG9, AE9) and two non-helical (V1, M1) peptides were poorly resolved in the absence of perchlorate; addition of perchlorate (Fig. 2F) now produced baseline separation of the four peptides.

Aqueous mobile phases containing 0.05%–0.1% TFA (v/v) are very commonly used for peptide separations [1,3] and Fig. 3 compares separations of selected mixtures of α-helical and non-helical peptides (Table 5) carried out either in an aqueous 0.1% TFA–CH$_3$CN, pH 2, mobile phase (Fig. 3A and C) or the aqueous 10 mM H$_3$PO$_4$–100 mM NaClO$_4$/CH$_3$CN mobile phase used throughout this study. From Fig. 3A, non-helical peptides K1 and N1 are coeluted in the TFA system, whilst pairs of non-helical and α-helical peptides are also either coeluted (I1/AS9) or eluted as a doublet (M1/AN9); in
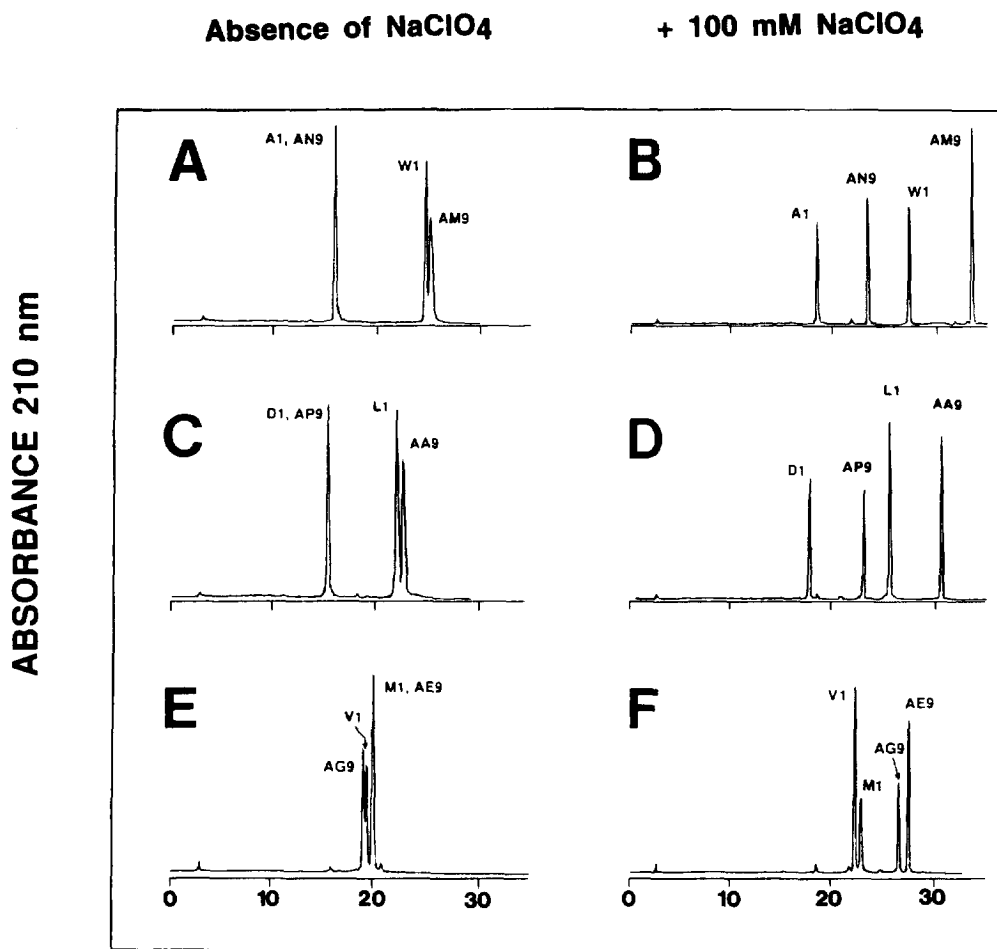
Fig. 2. RPLC of model synthetic peptides in the absence and presence of 100 m*M* sodium perchlorate at pH 2.0. HPLC column instrumentation and conditions: see Section 2. Peptide sequences and designations are described in Section 3.1 and Fig. 1.

contrast, all six peptides are well resolved to baseline in the H_3PO_4–perchlorate mobile phase system (Fig. 3B). In a similar manner, from Fig. 3C, non-helical peptide pair R1/G1 is unresolved in the TFA, system, whilst pairs of non-helical and helical peptides are also either coeluted (F1/AT9) or eluted as a doublet (Y1/AN9); from Fig. 3D, all six peptides are again baseline resolved by the H_3PO_4–perchlorate mobile phase. Note that the retention times of all of the peptides increased in the H_3PO_4–perchlorate system (Fig. 3B and D) compared to the TFA system (Fig. 3A and C), this increase being dependent on the number of positively charged groups a particular peptide contains. For instance, peptide K1 (con-

taining two lysine residues) showed an increase in retention time (Δt_R) of 2.91 min on changing from the TFA (Fig. 3A) to the H_3PO_4–perchlorate (Fig. 3B) mobile phase; in contrast, peptides N1, M1 and I1 (all containing just one lysine residue) showed an increase in retention times of 1.52 min, 2.23 min and 2.49 min, respectively. Similarly, an amphipathic α-helical peptide such as AN9, with five lysine resides in the hydrophilic face of the helix showed an increase of 3.6 min on changing from the TFA (Fig. 3A and C) to the H_3PO_4–perchlorate (Fig. 3B and D) system. Similar results were seen for the other non-helical and α-helical peptides. In a similar manner to that noted previously, the magnitude of
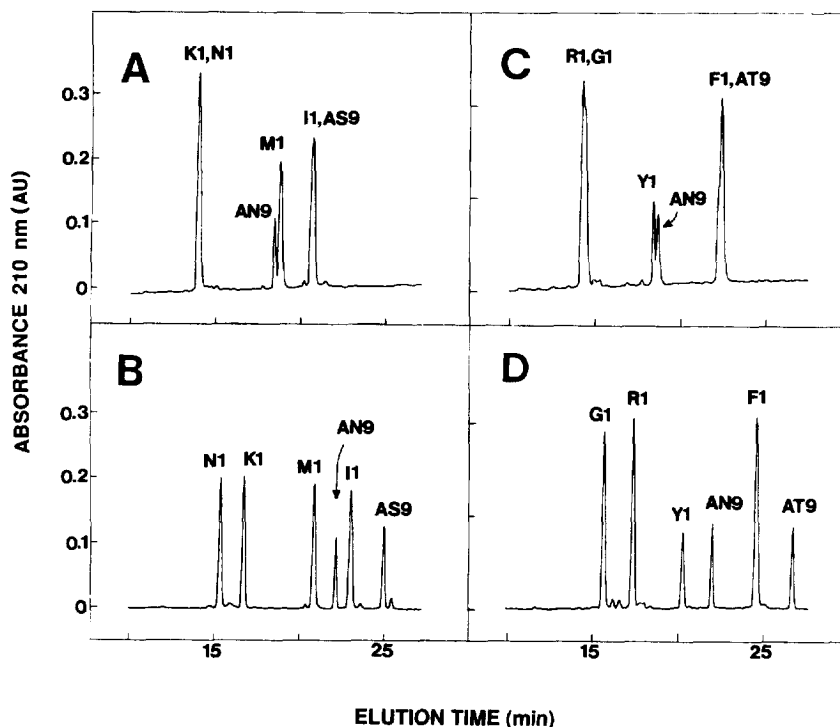
Fig. 3. RPLC of model synthetic peptides. Column: Zorbax 300-SB C$_8$ reversed-phase column (150×4.6-mm I.D., 5-μm particle size, 300 Å pore size; Rockland Technologies, Wilmington, DE, USA). Instrumentation: see Section 2. Conditions: panels A and C, linear AB gradient elution (1% acetonitrile per min) at a flow-rate of 1 ml/min, where eluent A is 0.1% aqueous TFA and eluent B is 0.1% TFA in acetonitrile; panels B and D, linear AB gradient elution (1% acetonitrile/min) at a flow-rate of 1 ml/min, where eluent A is 20 mM aqueous phosphoric acid and eluent B is 20 mM phosphoric acid in 50% aqueous acetonitrile both eluents containing 100 mM sodium perchlorate. Peptide sequences and designations are described in Section 3.1 and Fig. 1.

the increase in retention times of the amphipathic α-helical peptides is less than would have been expected if all five lysine residues had been fully exposed to the stationary phase (especially when compared to the Δt_R values of the non-helical peptides containing only one or two positively charged groups), due to the orientation of these residues away from the preferred binding domain (represented by the hydrophobic face of the helix) of the peptides.

Another point which can be gleaned from Fig. 2 and Table 5, as well as the selected peptides shown in Fig. 2, is that the retention times of peptides in both the α-helical and non-helical peptide series AX9 and X1 were increased in both the TFA and H$_3$PO$_4$–perchlorate systems relative to their observed times in an aqueous 0.1% H$_3$PO$_4$ mobile phase in the absence of perchlorate, i.e., a hydro-

philic counterion such as perchlorate may have a similar effect to a hydrophobic counterion such as trifluoroacetate if it is present in high enough concentration in the mobile phase [in the present study, 100 mM perchlorate versus 0.1% (13 mM) trifluoroacetate].

4. Conclusions

Through the use of model amphipathic α-helical and non-helical peptides, this study demonstrated how the apparent hydrophilicity of a positively charged residue may be reduced during RPLC by ion-pair formation with a hydrophilic anionic ion-pairing agent such as perchlorate ion. In addition, the results demonstrated that, even though the hydrophobic face of an amphipathic α-helix produces a

preferred binding domain in RPLC, the hydrophilic face of the helix still makes an important contribution to the retention behavior of the peptide. Indeed, when a charged residue is ion-paired with perchlorate, the decreased hydrophilicity of the side-chain was of similar magnitude whether the residue was in the hydrophobic face or the hydrophilic face of an amphipathic α-helical peptide. It was also shown that useful manipulation of selectivity may be effected through the use of 100 mM sodium perchlorate at pH 2 during separations of helical and non-helical peptides. In addition, aqueous phosphate/perchlorate mobile phases may complement traditional aqueous TFA systems for specific separations. This may prove especially useful practically for separating complex mixtures of α-helical (both amphipathic and non-amphipathic) and random coil peptides such as those frequently obtained from sources such as protein digests, or in situations where it is undesirable to isolate peptides as their trifluoroacetate salts.

Acknowledgments

References

[1] C.T. Mant and R.S. Hodges (Editors), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991.
[2] R.E. Majors, LC·GC 9 (1991) 686.
[3] C.T. Mant, N.E. Zhou and R.S. Hodges, in E. Heftmann (Editor), Chromatography, 5th Edition, Elsevier, Amsterdam, 1992, p. B75.
[4] K.E. Bij, Cs. Horváth, W.R. Melander, A. Nahum, J. Chromatogr. 203 (1981) 65.
[5] C.T. Mant, R.S. Hodges, Chromatographia 24 (1987) 805.
[6] J.J. Kirkland, J.L. Glajch, R.D. Farlee, Anal. Chem. 61 (1988) 2.
[7] J.J. Kirkland, C.H. Dilks Jr., J.E. Henderson, LC·GC 11 (1993) 290.
[8] D. Guo, C.T. Mant, R.S. Hodges, J. Chromatogr. 386 (1987) 205.
[9] W.M.M. Schaaper, D. Voskamp, C. Olieman, J. Chromatogr. 195 (1980) 181.
[10] H.P.J. Bennett, C.A. Brown, S. Solomon, J. Liq. Chromatogr. 3 (1980) 1353.
[11] D.R.K. Harding, C.A. Bishop, M.F. Tartellin, W.S. Hancock, Int. J. Peptide Protein Res. 18 (1981) 314.
[12] H.P.J. Bennett, J. Chromatogr. 266 (1983) 501.
[13] A.L. Hong, M.M. Brasseur and D. Kesuma, in C.T. Mant and R.S. Hodges (Editors), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991, p. 765.
[14] J.L. Meek, Proc. Natl. Acad. Sci. USA 77 (1980) 1632.
[15] J.L. Meek, Z.L. Rossetti, J. Chromatogr. 211 (1981) 15.
[16] A.I. Smith, J.R. McDermott, J. Chromatogr. 306 (1984) 99.
[17] J. Ishida, M. Kai, Y. Ohkura, J. Chromatogr. 356 (1986) 171.
[18] D. Guo, C.T. Mant, A.K. Taneja, J.M.R. Parker, R.S. Hodges, J. Chromatogr. 359 (1986) 499.
[19] B.S. Welinder, H.H. Sorensen, B. Hansen, J. Chromatogr. 398 (1987) 309.
[20] B.S. Welinder, H.H. Sorensen, B. Hansen, J. Chromatogr. 408 (1987) 191.
[21] R.E. Carraway, S.P. Mitra, Regul. Pept. 18 (1987) 139.
[22] C.T. Mant, R.S. Hodges, Chromatographia 24 (1987) 805.
[23] R.H. Snider, C.F. Moore, E.S. Nylen, K.L. Becker, Horm. Metab. Res. 20 (1988) 254.
[24] K.J. Wilson, A. Honegger, G.J. Hughes, Biochem. J. 199 (1981) 43.
[25] L.B. Klickstein, B.U. Wintroub, Anal. Biochem. 120 (1982) 146.
[26] R.L. Emanuel, G.H. Williams, R.W. Giese, J. Chromatogr. 312 (1984) 285.
[27] H. Gaertner, A. Puigserver, J. Chromatogr. 350 (1985) 279.
[28] B.-Y. Zhu, C.T. Mant, R.S. Hodges, J. Chromatogr. 594 (1992) 75.
[29] Y. Hatefi, W-G. Hanstein, Proc. Natl. Acad. Sci. USA 62 (1969) 1129.
[30] N.E. Zhou, C.M. Kay, B.D. Sykes, R.S. Hodges, Biochemistry 32 (1993) 6190.
[31] N.E. Zhou, O.D. Monera, C.M. Kay, R.S. Hodges, Protein Peptide Lett. 1 (1994) 114.
[32] T.J. Sereda, C.T. Mant, F.D. Sönnichsen, R.S. Hodges, J. Chromatogr. A 76 (1994) 139.
[33] T.J. Sereda, C.T. Mant, R.S. Hodges, J. Chromatogr. A 695 (1995) 205.
[34] N.E. Zhou, C.T. Mant, R.S. Hodges, Pept. Res. 3 (1990) 8.
[35] V. Steiner, M. Schär, K.O. Börnsen, M. Mutter, J. Chromatogr. 586 (1991) 43.
[36] J.L. Fausnaugh, E. Pfannkoch, S. Gupta, F.E. Regnier, Anal. Biochem. 137 (1984) 464.
[37] R.H. Ingraham, in C.T. Mant and R.S. Hodges (Editors), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991, p. 425.
[38] J.L. Cornette, K.B. Cease, H. Margalit, J.C. Spouge, J.A. Berzofsky, C.D. DeLisi, J. Mol. Biol. 195 (1987) 659.
[39] J.P. Segrest, H. DeLoof, J.G. Dohlman, C.G. Brouillette, G.M. Anantharamaiah, Proteins: Struct. Func. Gen. 8 (1990) 103.