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Use of mixed-mode sorbents for the electrochromatographic separation of thrombin receptor antagonistic peptides

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This manuscript is dedicated to the memory of Csaba Horváth, a friend, mentor, scientific innovator and a beacon of inspiration for this laboratory for the past 30 years.

Abstract

In this study, the thrombin receptor antagonistic peptide TRAP-1 and its alanine-scan analogues, TRAP 2–6, have been employed as probes to characterise the performance of C_{18}/SCX mixed-mode capillary electrochromatographic (CEC) columns. It was found that the resolution of this group of peptides could only be achieved in a narrow pH range with phosphate-based running electrolytes. The influence of the running electrolyte composition, e.g. the buffer choice, the ionic strength, the pH and the organic solvent content, on the electroosmotic flow (EOF) of these mixed-mode CEC columns was investigated. In addition, the retention mechanism for this group of peptide probes in the electrochromatographic process was studied by examining the effect of varying the running electrolyte composition. As a result, it can be concluded that the electrochromatographic separation of this set of peptides was mediated by a combination of electrophoretic migration and chromatographic retention involving both hydrophobic as well as ion exchange interactions. By modulating the running electrolyte composition, the hydrophobic or ion exchange components of the interaction process could be made to dominate the chromatographic retention of the peptides. Based on this strategy, a high-resolution separation of six closely related synthetic peptides was demonstrated with this mixed-mode CEC system.

Keywords: Capillary electrochromatography; Synthetic peptides; Separation; Hydrophobic interaction; Ion exchange; Retention mechanism

1. Introduction

Capillary electrochromatography (CEC) was introduced about two decades ago as an analytical technique that combines the features of high performance capillary electrophoresis (e.g. high efficiency) and high performance liquid chromatography (e.g. high selectivity and sample loading capacity) [1–4]. Initially, CEC was mainly used for the separation of neutral and non-polar compounds under conditions similar to those employed in reversed-phase HPLC [4–6]. With the development of CEC column technologies and concomitant improvement in instrumentation, the application of CEC has expanded in recent years to the separation of relatively complex polar biomolecules, such as nucleosides, synthetic peptide mixtures and protein digests [7–10].

To date, the majority of CEC applications described in the literature have utilized reversed-phase silica-based chromatographic sorbents as stationary phases [11]. The use of such non-polar phases with polar analytes, e.g. peptides, in CEC is associated with two constraints. Firstly, the surface charge of these sorbent materials varies with the pH of the running electrolyte. As the electroosmotic flow (EOF) is surface-charge dependent, capillaries packed with silica-based sorbents have an EOF dependence on pH. Secondly, silanophilic interactions between positively charged functional groups of the peptide and ionised silanol groups of the sorbent lead to severe peak tailing and can result in

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irreproducible separations [12]. End-capping the free silanol groups at the surface of the sorbents, in order to suppress these interactions, is not an acceptable solution in CEC due to the significantly decreased EOF. An alternative option is to suppress adverse silanophilic interactions through the use of low pH conditions. However, under these conditions the EOF progressively becomes reduced as well, as the silanol groups become increasingly protonated. The ideal sorbents for CEC applications, therefore, should be able to generate sufficiently high and stable electroosmotic flow over a wide pH range, whilst at the same time having an appropriate ligand density and composition that permit reproducible chromatographic retention.

Mixed-mode stationary phases containing hydrophobic and strong cation exchange ligands have been proposed by several research groups for the separation of simple peptides by chemically co-bonding the strong propylsulfonic acid and octadecyl groups onto the silica surface [13–15]. These sorbents exhibited a significant EOF over a wide range of pH values. So far, few investigations have been performed to systematically characterise these mixed-mode sorbents with sets of closely related peptides or other biomolecules [16–18]. In this paper, the separation of synthetic thrombin receptor antagonistic peptides (TRAPs) by HP-CEC with hydrophobic/strong cation exchange mixed-mode sorbents is presented. In particular, the influence of the pH value of the running electrolyte, the ionic strength of the background buffer and the organic solvent content on the retention behaviour of this peptide set has been studied. These investigations thus provide the basis to further understand the mechanism of differential migration and the retention processes underlying the separation of peptides by mixed-mode CEC sorbents.

2. Experimental

2.1. Chemicals and buffers

Acetonitrile (HPLC grade) was obtained from Biolab Scientific (Sydney, Australia). Water was distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, USA). Tris (hydroxymethylaminomethane) was purchased from Sigma (St. Louis, USA). Sodium dihydrogenphosphate, disodium hydrogenphosphate and ammonia acetate were purchased from BDH Chemicals Australia (Kilsyth, Australia); NaOH, hydrochloric acid 36% (v/v), orthophosphoric acid 85% (v/v) and acetic acid were obtained from Ajax Chemicals (Sydney, Australia). DMF (*N*,*N*-dimethylformamide) was purchased from Auspep (Melbourne, Australia). Unless otherwise stated, all the above reagents were of analytical grade.

The stock solutions of 100 mM phosphate buffers, pH 2.0–3.0, were prepared by titrating 100 mM sodium dihydrogenphosphate with phosphoric acid; whilst the 100 mM phosphate buffers, pH 6.0–7.5, were prepared by titrating 100 mM disodium hydrogenphosphate with 100 mM sodium dihydrogenphosphate. The stock solution of 100 mM ammonia acetate buffers, pH 4.0 and pH 5.0, were made by titrating 100 mM ammonia acetate with acetic acid; the 25 mM Tris-HCl buffer, pH 8.0, was made by titrating 25 mM Tris solution with hydrochloric acid. All of the CEC running electrolytes were prepared by mixing appropriate proportions of the stock solution, Milli-Q water and the organic solvent, and were degassed by ultra-sonication for 10 min before use.

2.2. Samples

All the peptides used in this study were synthesised and purified using procedures reported previously [19]. All peptides were dissolved in 10% (v/v) acetonitrile–water (1 mg/mL) since some were too hydrophobic to be dissolved in pure water. 5% of DMF in water was used as EOF marker. All samples and the EOF marker were injected electrokinetically at 5 kV for 4 s unless otherwise stated.

2.3. Instruments

Both the CEC and CE experiments were performed with a HP^{3D}CE capillary electrophoresis system from Agilent Technologies (Waldbronn, Germany) at ambient temperatures (the instrument was set up in an air-conditioned laboratory with room temperature maintained at $22 \pm 1\,^{\circ}C$). The temperature of the cassette containing the capillary was controlled by a circulating air fan, and registered by a temperature monitor (U-lab, Melbourne, Australia) and read 21–23 °C during the period over which the experiments were carried out. The inlet and outlet capillary ends were pressurised with 10 mbar during CEC analysis. The detection of peptides was performed using a diode-array detector at 214 nm.

2.4. CEC capillary columns

The CEC columns, 265 (350) mm × 100 μm, packed with 3 μm C_{18}/SCX sorbents were donated by Agilent Technologies. The total length of the columns was 350 mm and the packed length was 265 mm. All new CEC capillary columns were first conditioned before use for several hours in (25 mM Tris-HCl) pH 8.0-acetonitrile (1:4, v/v), according to the standard operating procedure recommended by the manufacturer.

2.5. Computational methods

The retention factor, κ_{CEC} was calculated as $\kappa_{CEC} = (t_M - t_{EOF})/t_{EOF}$, where t_M is the apparent migration time of the peptide and t_{EOF} is the elution time of an un-retained neutral molecule (EOF marker). Organic volume fractions were expressed as ψ, whereby 100% of organic modifier equals $\psi = 1$.

3. Results and discussion

3.1. Characteristics of the thrombin receptor antagonistic peptides

The thrombin receptor (PAR-1) is a transmembrane G-protein that is activated by serine protease cleavage of its extracellular N-terminus to expose an agonist peptide ligand that is tethered to the receptor itself [20]. Synthetic peptides that contain the agonist motif of human PAR-1, such as H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-OH (TRAP-1), are capable of receptor activation in the absence of thrombin [21]. Replacement of Phe^2 with Ala in TRAP-1, with elimination of the ß-phenyl side chain group, results in complete receptor inactivation [22]. Structure–function investigations with various bioactive thrombin receptor-activating peptide (TRAP) analogues have been reported [19,23], leading to the conclusion that an extended structure of the agonist peptide is responsible for receptor recognition, with a hydrophobic contact occurring between the side chains of Phe^2 and Leu^4.

The amino acid sequences, molecular weight, charge and relative hydrophobicity of six TRAP peptides synthesized in this laboratory are shown in Table 1. The peptides were derived by successively replacing the amino acids of the original sequence with alanine, except for the N- and C-terminal ends. These changes in the amino acid sequence are expected to result in differences in dipole characteristics and surface charge densities between the different members of this peptide. Thus, when a neutral amino acid within the sequence was replaced by alanine, no variation in molecular charge will be introduced, although other properties, for example, the intrinsic hydrophobicity will change. However, when a charged amino acid, such as arginine, as in the case of TRAP-5, was substituted by alanine, significant modification in the charge status of this peptide occurred. As shown in Table 1, TRAP-1, 2, 3, 4, 6 have the same positive charge at pH 6.5, while TRAP-5 is negatively charged.

As documented in our preliminary studies, the separation of TRAP-5 from other TRAP peptides can be easily achieved by a charge based separation technology, such as high performance capillary electrophoresis (HP-CZE). However, the separation of TRAP-1, 2, 3, 4, 6 from each other proved to be extremely difficult because of their close charge–mass ratios, with TRAP-3, 4, 6 not resolved by HP-CZE even under optimal conditions. These small peptides are not anticipated to adopt any secondary structure and are assumed to exhibit a random coil structure. In their solvated states they are expected to adopt globular shapes. By examining the peptides' structure as determined by molecular modelling experiments, the potential impact of changes in their amino acid sequence on their shape can thus be easily followed. The migrational similarity of TRAP-3 and TRAP-4 represents the major challenge in achieving a successful separation, as these peptides display identical amino acid composition but different sequences with respect to positions 3 and 4 (compositional isomers). Therefore, their difference at the molecular level was neither manifested in the available hydrophobicity coefficients nor in any charge calculations that may be used to determine their charge–size ratios.

3.2. Characteristics of mixed-mode CEC sorbents

CEC capillary columns employed in this investigation were packed with mixed-mode sorbents functionalised with immobilized octadecyl groups and propylsulfonic acid groups. The strong sulfonic acid groups can be readily ionised and therefore possess negative charges over a broad range of pH values. Apart from providing high electroosmotic flows, the sulfonic acid groups also induce strong cation exchange characteristics to the sorbent.

In order to assess the performance of the capillary columns packed with C_{18}/SCX sorbents at low pH, phosphate buffers from pH 2.0 to 3.0 were used. Under these conditions, the TRAP peptides could not be eluted from these capillary columns because of the strong electrostatic interaction between the negatively charged sorbents and highly positively charged peptides. When the pH of the running electrolyte was increased to pH 4.0 or 5.0 using an ammonium acetate buffer, only some of the peptides could be eluted, but with pronounced peak tailing. This outcome can be attributed to the positive charges on the peptides with buffers of this pH range. In contrast, when high pH buffers, for example, Tris-HCl buffers above pH 8.0, were employed, these peptides have a negative charge, and migrate towards the anode, which is opposite to the direction of the electroosmotic flow. If the migration times of the peptides are too long and their peaks too broad, they cannot be observed via UV detection, although their retention to the sorbents could be completely diminished because of electrostatic repulsion. It was found that the successful elution of all the TRAP peptides from these mixed-mode CEC columns could only be achieved in a neutral pH range with phosphate buffers. Under those conditions the peptides are weakly charged and their retention to the mixed-mode sorbent moderate.

3.3. The influence of the electrolyte composition on the EOF

To optimise the CEC separation of the TRAP peptides, besides the effects of the pH and type of background buffer used in the running electrolyte and buffer type, the influence of the ionic strength and the organic solvent content on the EOF must also be determined for the selected sorbent system. In these experiments, the electroosmotic velocity was measured with DMF as the unretained marker. The influence of the pH value of the running electrolyte on the electroosmotic flow of these mixed-mode CEC columns is illustrated in Fig. 1A. The running electrolyte was derived from 5 mM phosphate buffer containing 40% (v/v) acetonitrile. As can be seen from the plot, only a small change in the EOF occurred when the pH of the running electrolyte was increased from pH 6.0 to 7.5. This outcome can be mainly attributed to

Table 1
Amino acid sequences, molecular weights, calculated charges (q) at pH 6.5, charge to molecular mass ratios ($q/MW^{2/3}$) for globular peptides and relative hydrophobicities of TRAP peptides

Peptide	Sequence	q	MW	$q/MW^{2/3}$	Relative hydrophobicity
TRAP-1	S F L L R N P	0.86	846.00	0.0097	20.86
TRAP-2	S A L L R N P	0.86	769.90	0.0103	14.24
TRAP-3	S F A L R N P	0.86	803.92	0.0100	16.91
TRAP-4	S F L A R N P	0.86	803.92	0.0100	16.91
TRAP-5	S F L L A N P	−0.14	760.89	−0.0016	22.22
TRAP-6	S F L L R A P	0.86	802.97	0.0100	24.75

The pK_a values for the side chains and the C- and N-termini from references [28,29] were utilized to calculate peptide charges, and relative hydrophobicity was calculated with data from reference [30].

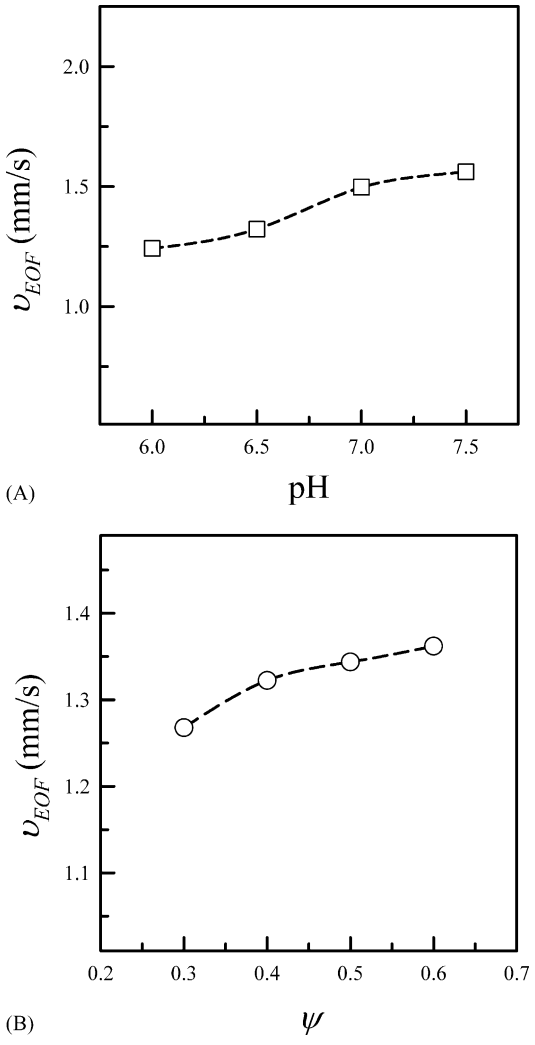

Fig. 1. (A) Plot demonstrating the relationship between the EOF, υ_{EOF}, and the pH values of the running electrolyte. The running electrolytes were composed of 4 mL acetonitrile, 0.5 mL 100 mM phosphate buffer, pH X, and 5.5 mL water, C_{18}/SCX CEC capillaries with a total length L_t = 35.0 cm, effective length L_e = 26.5 cm, 100 μm I.D. were used, and the applied voltage was 24.5 kV. Injections were made electrokinetically at 5 kV for 4 s. Detection was at 214 nm. (B) Plot demonstrating the relationship between the EOF, υ_{EOF}, and the volume fraction, ψ, of acetonitrile on a C_{18}/SCX CEC capillary. The running electrolytes were prepared from X mL of acetonitrile, 0.5 mL of 100 mM phosphate buffer, pH 6.5 with $(10 - X - 0.5)$ mL of water added to achieve a final volume of 10 mL. The applied voltage was 24.5 kV.

the enhanced ionisation of the residual silanol groups on the sorbent surface and the capillary wall.

The effect of the acetonitrile content in the running electrolyte was investigated by increasing the volume percentage from 30% (v/v) to 60% (v/v) whilst keeping the phosphate concentration of 5 mM and pH 6.5 (as measured with an Ag/AgCl combination pH electrode with the fully aqueous buffer electrolyte). As shown in Fig. 1B, the electroosmotic velocity slightly increased with increasing acetonitrile content. A decrease in the viscosity, η, of the running electrolyte, coupled with the effect of the organic solvent on the protic ionisation equilibrium as the acetonitrile content is increased, would account for the EOF increase, since firstly the EOF is inversely proportional to η and secondly the EOF is proportional to the zeta potential, ζ, of the sorbent. Since the zeta potential varies with the ionisation status of the sorbent surface, which in turn depends on the effective pH of the buffer electrolyte, with aqueous–organic solvent buffer electrolyte systems the effective pH can be as much as 0.5 pH units above the level measured with an Ag/AgCl combination pH electrode for the corresponding fully aqueous buffer electrolyte. These effects will result in a greater level of ionisation of the residual silanol groups present in the sorbent. The corresponding dependence of electroosmotic velocity on the ionic strength of the running electrolyte at a constant pH (pH 6.5), expressed in terms of the plots of υ_{EOF} versus $I^{-1/2}$ results in a linear relationship with a correlation coefficient of r^2 = 0.9906. The increased buffer concentration gives rise to double-layer compression, decreased zeta potential, and reduced EOF. As evident from these results, a small increase in the phosphate buffer concentration caused a pronounced decrease in υ_{EOF}. This outcome is consistent with the predicted linear dependence of υ_{EOF}, on $I^{-1/2}$ [4] with the correlation coefficients for the experimental data being r^2 = 0.9906.

3.4. The retention behaviour of TRAP peptides on mixed-mode sorbents

3.4.1. Influence of the acetonitrile content

In order to assess the influence of the acetonitrile content on the retention of the TRAP peptides on mixed-mode C_{18}/SCX CEC columns, its content in the running electrolyte was varied over the volume fraction range of ψ = 0.2–0.5, while the phosphate concentration was kept at 10 mM and

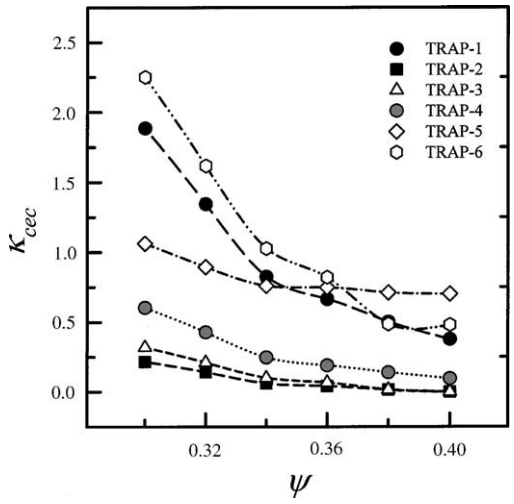

Fig. 2. Retention coefficients, κ_{CEC}, of TRAP peptides vs. the volume fraction, ψ, of acetonitrile. The running electrolytes were prepared from X mL of acetonitrile, 1 mL of 100 mM phosphate buffer, pH 6.5 with $(10 - X - 1)$ mL of water added to achieve a final volume of 10 mL. C_{18}/SCX CEC capillaries with a total length L_t = 35.0 cm, effective length L_e = 26.5 cm, 100 μm I.D. were used, and the applied voltage was 20.0 kV.

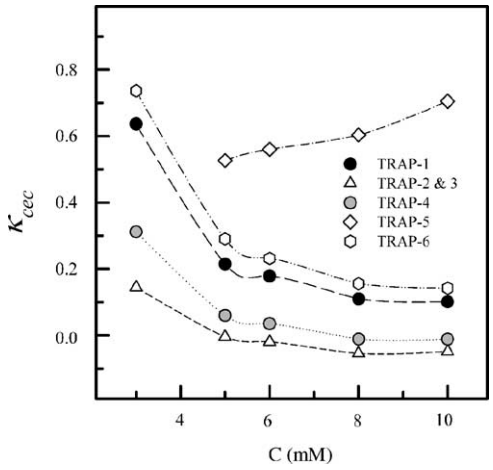

Fig. 3. The relationship between the retention coefficient, κ_{CEC}, of TRAP peptides and the concentration of the background buffer in the running electrolyte on a C_{18}//SCX CEC capillary. The running electrolytes were prepared from 5 mL of acetonitrile, X mL of 100 mM phosphate buffer, pH 6.5 with $(10 - X - 5)$ mL of water added to achieve a final volume of 10 mL. The applied voltage was 20.0 kV.

the pH at pH 6.5. When low volume fractions of acetonitrile were employed, e.g. $\psi < 0.3$, only the less hydrophobic peptides, for example, TRAP-2, 3, 4 were eluted. However, when higher elution strength running electrolytes were used, e.g. when the acetonitrile content was $\psi > 0.5$, some peptides co-eluted and were unresolved. It was found that the most effective separation of the six TRAP peptides could only be achieved in a narrow acetonitrile content range $0.3 < \psi < 0.4$. Fig. 2 shows the effect of acetonitrile content on the retention of TRAP peptides with the mixed-mode C_{18}/SCX CEC column. It can be seen that TRAP-6 has the highest relative hydrophobicity (Table 1) and shows the strongest retention. Moreover, the retention coefficients, κ_{CEC}, of these peptides decrease with an increase in the acetonitrile content. The more hydrophobic peptides, e.g. TRAP-5, 6, show more pronounced effects with very small changes in the acetonitrile content causing significant modifications to their retention with this mixed-mode CEC sorbent. The very hydrophobic peptide TRAP-5 is negatively charged at pH 6.5, and was repelled from the negatively charged surface of the sorbent. Although hydrophobic interactions may still play a role under these conditions, the influence of acetonitrile on the retention of TRAP-5, like the less hydrophobic peptides TRAP-2, 3, 4, was small. Therefore, due to differences in their mass to charge status, the relative changes in selectivity for TRAP-5, with respect to TRAP-1 and TRAP-6, were significant when even minor changes in the acetonitrile content in the running electrolyte was employed even though the hydrophobicity values of these three peptides were very similar.

Since both hydrophobic and the ion exchange groups are presented on the surface of the mixed-mode sorbent used in these investigations, the electrochromatographic retention behaviour of this set of TRAP peptides will reflect a combination of both interactions. As apparent from the results, changes in the buffer concentration and/or organic solvent content in the running electrolyte can be used to modulate the relative contribution of these processes. At relative low organic solvent conditions, increasing the buffer concentration in the running electrolyte reduces the contribution from electrostatic interactions. The relationship between the logarithmic values of the retention coefficient, $\log \kappa_{CEC}$, of TRAP peptides and the volume fraction, ψ, of acetonitrile in the running electrolyte is linear for all investigated peptides (data not shown). Under the regime of low organic solvent conditions, the electrostatic interaction will be greatly suppressed when a relatively high buffer concentration, i.e. >10 mM, was selected. Therefore, under these conditions the chromatographic retention behaviour becomes dominated [24] by hydrophobic interaction effects, as manifested by a linear relationship between the $\log \kappa_{CEC}$ and ψ over this limited range of $0.30 \leq \psi \leq 0.38$ values.

3.4.2. *Influence of the buffer concentration*

The retention mechanism of TRAP peptides on this mixed-mode sorbent was also studied by changing the buffer concentration in the running electrolyte whilst keeping the pH and organic solvent content of the running electrolyte constant. Fig. 3 illustrates the effect of buffer concentration, from 3 to 10 mM, on the retention of the TRAP peptides. The volume fraction, ψ of acetonitrile in the running electrolyte was fixed at $\psi = 0.5$, and was selected at this value to ensure that hydrophobic interaction of peptides with chromatographic sorbent was significantly suppressed. One direct consequence of a high organic solvent content in the running electrolyte is that the less hydrophobic peptides, TRAP-2 and TRAP-3, co-eluted and remained unresolved under this condition. As shown in Fig. 3, the retention coefficients, κ_{CEC}, of the pos-

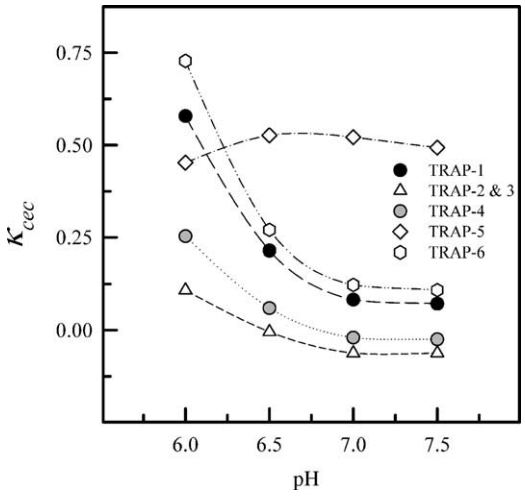

Fig. 4. The relationship between the retention coefficient, κ_{CEC}, of TRAP peptides and the pH of the running electrolyte on a C_{18}/SCX CEC capillary. The running electrolytes were prepared from 5 mL of acetonitrile, 0.5 mL of 100 mM phosphate buffer, pH X, with 4.5 mL of water added to achieve a final volume of 10 mL. The applied voltage was 24.5 kV.

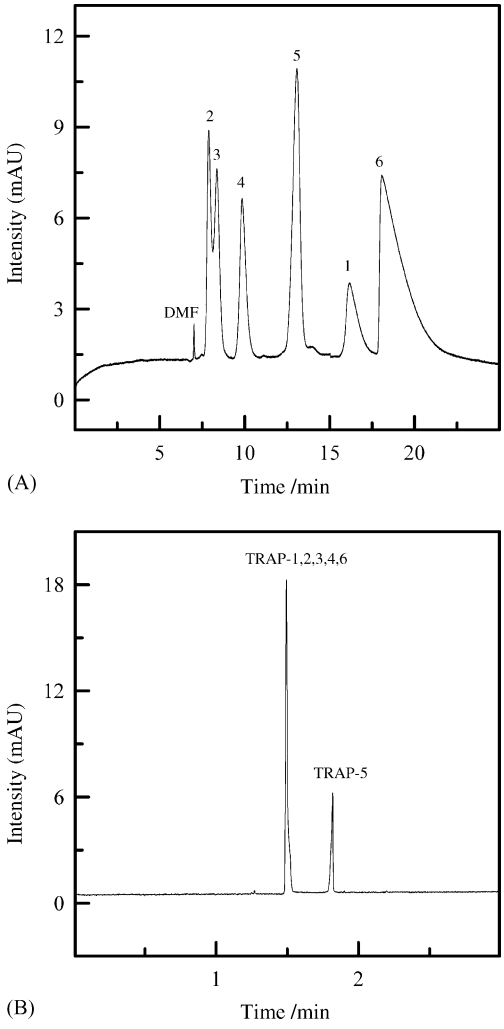

Fig. 5. (A) Separation of TRAP peptides on a C_{18}/SCX CEC capillary. Running electrolyte: 32/10/58 acetonitrile/100 mM phosphate buffer, pH 6.5/water (v/v). Other conditions: V = 20.0 kV, detection at 214 nm. (B) Separation of TRAP peptides with a bare-fused silica capillary with a total length of 34.0 cm, effective length of 25.5 cm, 100 μm I.D. Buffer: 32/10/58 acetonitrile/100 mM phosphate buffer, pH 6.5/water (v/v); applied voltage: 20.0 kV; detection at 214 nm.

itively charged peptides decreased with the increase in the buffer concentration in the running electrolyte, as the electrostatic interaction between the positively charged peptides and negatively charged surface of the sorbent is reduced. In contrast, the negatively charged peptide TRAP-5 increasingly underwent electrostatic repulsion from the surface of the sorbent, causing this peptide to move in the opposite direction of EOF.

As noted above, the running electrolyte contained 50% (v/v) acetonitrile to diminish the hydrophobic interaction of the peptides with the sorbent, and when low buffer concentrations were employed, electrostatic interactions became favourable for retention of these peptides [25,26]. Under these conditions, the dependency of the logarithmic retention coefficient, log κ_{CEC}, for the strongly retained peptides TRAP-1 and TRAP-6, versus the logarithmic buffer concentration in the running electrolyte followed linear relationships (with correlation coefficients, $r^2 = 0.9877$ and $r^2 = 0.9922$, respectively), indicating that electrostatic interactions dominated the chromatographic retention of the peptides under these conditions, with the migration behaviour reminiscent of that typical for the separation of similarly charged peptides in ion-exchange chromatography [27].

3.4.3. *Influence of the pH of the running electrolyte*

For sorbents containing strong ion exchange groups, such as the mixed mode sorbent used in the current studies, changing the pH value of the running electrolyte does not have a significant effect on the magnitude of EOF, as demonstrated above. However, it can have a profound effect on the retention of peptides, since the charge status of peptides is an important determinant of their electrostatic interaction with chromatographic sorbents. Changing the pH value of the running electrolyte can easily modulate this effect. As demonstrated in Fig. 4, over the range from pH 6.0 to 7.5, the retention coefficients, κ_{CEC}, of the positively charged peptides decreased, consistent with steadily weakened electrostatic interactions with the sorbent surface. On the other hand, since the negatively charged peptide TRAP-5 was repulsed from the surface of the sorbent, its retention coefficient stayed nearly unchanged over this pH range.

3.4.4. *Isocratic separation of TRAP peptides*

Although differences in hydrophobicity, as shown in Table 1, is a favourable feature for their separation of this set of peptides by reversed-phased HPLC, their resolution by CEC proved to be difficult, because of their very simi-

lar amino sequences and mass to charge status. In particular, the separation of the peptide compositional isomers, TRAP-3 and TRAP-4, initially proved to be challenging. Based on the understanding of the complex electrochromatographic process, including the interplay of electrophoretic migration and chromatographic retention, a successful separation of the six TRAP peptides could however be achieved, as illustrated in Fig. 5A. In this separation, a tailing peak shape was evident for TRAP-6 even when a high elution strength running electrolyte was utilized. This result can be attributed to the silanophilic interaction between the residual silanol groups on the surface of the mixed-mode sorbents and the positive moieties of the peptide [12]. The relative migration order of peptide isomers TRAP-3 and TRAP-4 is noteworthy. Molecular modelling investigations [19] have suggested that TRAP-3 adopts a more compact structure with a smaller hydrophobic patch accessible on its surface accessibility as compared to TRAP-4, with both features contributing to an earlier elution of TRAP-3. In comparison, Fig. 5B shows the corresponding results for the six TRAP peptides using the analogous high performance capillary electrophoretic techniques with the same running electrolyte. As can be seen from the Fig. 5B, application of HPCZE under these conditions results in the six TRAP peptides being separated into two peak zones, with all of the positively peptides unresolved because of their very close charge–mass ratios.

4. Conclusions

The results of this study demonstrate that the mixed-mode C_{18}/SCX sorbent described here can be used for the separation of closely related synthetic peptides under isocratic elution conditions by HPCEC. The results show that in order to achieve adequate resolution of peptides, advantage can be taken of the underlying complex electrochromatographic process, which can be influenced through a combination of both electrophoretic migration and chromatographic retention effect that involve hydrophobic as well as ion exchange interactions. Moreover, advantage can be taken of the charge status of a peptide, which has a more profound effect on its retention with mixed-mode sorbents compared to the typical reversed-phase sorbent material previously applied to the HPCEC of peptides. By modulating the running electrolyte composition, hydrophobic or ion exchange interaction can be tuned and used to dominate the overall chromatographic retention of the peptides, permitting the baseline resolution of synthetic peptides that have only very small physicochemical differences in their charge and hydrophobicity.

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