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Retention behaviour of peptides in capillary electrochromatography using an embedded ammonium in dodecacyl stationary phase

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Abstract

The potential of a silica stationary phase bearing an embedded cationic quaternary amine in dodecacyl chain, to separate peptides by capillary electrochromatography (CEC) has been evaluated. The ability of this stationary phase, to generate a consistent anodic electroosmotic flow was first evaluated. This flow was found to be independent of pH over a wide range (2–12), of the acetonitrile percentage in the electrolyte. The stability of the stationary phase evaluated through the electroosmotic flow variations was demonstrated at extreme pH values (2.5 and 9.1). A careful examination of the influence of mobile phase conditions (acetonitrile percentage, salt concentration and nature of buffer) on the electrochromatographic retention and electrophoretic migration behaviour of different standard peptides was carried out. In acidic conditions, the electrokinetic contribution appears to be predominant compared to the chromatographic one. Several types of chromatographic interactions, reversed-phase partitioning and anion exchange, were involved in the CEC of peptides, whereas repulsive electrostatic interaction could be considered as negligible. This stationary phase affords different selectivity compared to that observed on a C_{18} stationary phase. Finally, the method was applied to the peptide mapping of β-lactoglobulin and human growth hormone under unpressurized and isocratic elution.

Keywords: Capillary electrochromatography; Peptide mapping; Electroosmotic flow; Stationary phase; Human growth hormone; β-Lactoglobulin; Mixed-mode stationary phase

1. Introduction

During the last few years, interest in capillary electrochromatography (CEC) for analytical applications has grown. One of the most important advantages of the CEC over HPLC is the flat profile of the electroosmotic flow (EOF) generated, which reduces the band broadening caused by transchannel and eddy diffusion. Thus, higher column efficiencies can generally be attained in CEC. Moreover, this microseparative technique can also reach relatively high separation speeds with the potential to simultaneously separate neutral and charged, hydrophilic and hydrophobic compounds in the same system. Thus, CEC represents an ideal technique to analyse complex mixture of peptides such as protein digests.

Protein mapping is now widely employed in protein characterization for proteomic applications as well as for routine control of recombinant proteins in the pharmaceutical industry. New techniques are sought which permit faster analysis of samples with high efficiency. Although HPLC is traditionally employed for peptide mapping, several authors have shown the potential of HPLC assisted by electrodriven, in other words pressurized CEC (pCEC) for the separation of protein digests [1–7]. Wu et al. [1] reported for the first time the analysis of two protein digests (bovine cytochrome *c* and chicken ovalbumine) by pCEC. This technique provides a means of tuning selectivity for the separation of charged analytes by varying the electric field. However, in most cases, an elution gradient is necessary requiring an instrument especially dedicated to CEC. pCEC methods reported for peptide mapping utilize mostly C_{18} stationary phases [1–4,6], designed for HPLC, in combination with acidic mobile phases.

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However, under these conditions, EOF is often too low and an external pressure is necessary to ensure a consistent flow.

While the residual silanol functions of C_{18} stationary phases are not favourable for HPLC of peptides in acidic conditions due to the possible interaction between cationic peptides and anionic silanols, these functions generate the EOF in CEC. A major problem encountered in CEC with silica-based stationary phases is the inherent low EOF at low pH due to the neutralization of the silanol groups. The HPLC model quickly fails since the pH dependence of the flow rate, in capillaries packed with silica based particles, leads to separations of proteins and peptides at low pH, that are too slow.

The ideal columns for CEC have charge carrying groups that are compatible with a strong EOF at pH values over the range of ca. 2–9. One of the major obstacles to rapid growth of the CEC technique is the limited availability of specially designed stationary phases, which must exhibit strong EOF to speed up the analysis and must allow the separation of neutral and charged peptides.

Research have recently been directed towards the design of analytical capillary columns [8–14], with new chromatographic supports, e.g. polymeric monolith [11–14], as well as chromatographic functionalities, e.g. ions exchange packing or mixed mode packing. In this context, the use of silica-based mixed-mode stationary phases, i.e. phases combining both charge and hydrophobic groups, each fixed on the chromatographic support, is an attractive alternative. A specially designed anionic stationary phase for CEC has been developed by chemically bonding sulfonic acid and octadecyl groups onto silica [15–18]. Mixed-mode stationary phases with anion exchange groups have been also proposed for peptide mixture analysis, still via pressurized capillary electrochromatography [5,6].

Another type of mixed-mode stationary phase consists of charged and alkyl functionalities in the same coated-group. Within the context of monolithic column, fixed positive charges and alkyl moieties serving as hydrophobic binding sites were described but these new stationary phases were not employed for peptide separation [19,20]. Later Zhang et al. [21] reported the analysis of a protein digest (cytochrome *c*) on such a stationary phase by pure CEC on butyl-grafted monolithic columns.

Until now, no convincing results have been published on unpressurized CEC with a packed stationary phase for peptide mapping. In this paper, we have therefore tested the potential of a new stationary phase to perform peptide mapping without pressurisation or gradient elution. To generate a strong and stable EOF over a wide range of pH, a new stationary phase with a permanent positive charge Stability BS-C23 has been evaluated in this work. A quaternary ammonium group is covalently bonded via a spacer at the surface of silica support, forms a relatively hydrophilic and charged sublayer; while a non-polar upper layer of octadecyl functionalities plays the part of a hydrophobic binding layer (Fig. 1). In the present study, we have focused our investigation on acidic conditions, which are expected to reduce the severe peptide peak

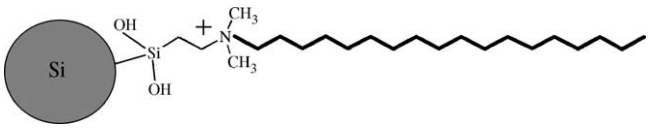

Fig. 1. Structure of the Stability BS-C23 stationary phase.

tailing due to silanophilic interaction. Moreover, the ammonium functionality was expected to shield the silanols while permitting a large EOF.

A particular attention has been devoted to EOF studies on this ammonium embedded stationary phase. The potential of this stationary phase for the separation of peptides by CEC has been then evaluated and the influence of several parameters of the mobile phase, such as acetonitrile (ACN) percentages, salt concentrations in the buffer have been studied for different standard peptides. A careful examination of the chromatographic retention and electrophoretic migration behaviour of different standard peptides was carried out. Thus, we have, attempted to determine and distinguish the influence of experimental conditions on the contribution of each mechanism chromatographic versus migration. Stability of this stationary phase under extreme pH was addressed by a monitoring of the EOF evolution over 1 week. Finally, a tentative peptide mapping of β-lactoglobulin and human growth hormone (hGH) with isocratic elution was performed.

2. Materials and methods

2.1. Reagents

Eledoisin, eledoisin RP, epidermial growth factor (EGF), thyrotropin releasing hormone (TRH) precursor peptide, Arg–Arg gastrine fragment 22–30, valosin porcine, renin substrate tetradécapeptide, angiotensin I and [Gln^{11}]-amyloid β protein fragments 1–16 were purchased from Sigma (St. Louis, MO, USA). Analytical concentrations were: Arg–Arg gastrin 0.24 mM, valosine porcine 0.01 mM, eledoisin 0.14 mM, renin substrate porcine 0.1 mM, EGF 0.07 mM, angiotensin I 0.25 mM, eledoisin RP 0.38 mM, protein amyloïde 0.17 mM, TRH precursor peptide 0,33 mM with EOF marker dimethyl sulfoxide (DMSO) 3.3 mM in water. HPLC-grade ACN was from Prolabo (Fontenay-sous-bois, France), DMSO and hydrochloric acid 1.0 M from Sigma and sodium hydroxyde 1.0 M was from VWR (Fontenay-sous-bois, France).

The hGH, somatotropin SCR was a standard obtained from the European Pharmacopoeia (Strasbourg, France). β-Lactoglobulin 18 400 subunits and trypsin treated with *N*-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK-trypsin) were from Sigma.

2.1.1. Apparatus

All CEC experiments were carried out using a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter,

Fullerton, CA, USA) equipped with an UV detector and a data handling system comprised of an IBM computer and Karat 32 software.

2.1.2. Protein digestion

The β-lactoglobulin was digested at 37 °C with TPCK-trypsin in 50 mM Tris–HCl buffer pH 8.0 containing 1 mM calcium chloride as described previously [22]. The enzyme was added to the incubation medium at an enzyme: substrate weight ratio of 1:100 at time 0, followed by a second addition after 1 h (ratio of 2:100 w:w). The digestion process was stopped after 3 h by addition of trifluoroacetic acid to a final concentration of 5% (v/v). The digest was desalted on octadecyl cartridge (J.T. Backer, Noisy le sec, France) with 0.1% TFA in ACN–water elution.

The hGH was digested at 37 °C according to the procedure described in European Pharmacopoeia, with a solution of 1 mg/ml of TPCK-trypsin, in 50 mM Tris–HCl buffer pH 7.5. The enzyme was added to the incubation medium: substrate weight ratio of 1:100. The digestion process was stopped after 4 h by addition of trifluoroacetic acid to a final concentration of 5% (v/v). The digest was then stored at −20 °C.

2.1.3. Stationary phases and columns

Stationary phases: Stability BS-C23, endcapped and non-endcapped, 5 μm, 300 and 100 Å, and Stability octadecyl were provided by C.I.L. (Sainte-Foy-La-Grande, France).

Fused-silica capillaries with an internal diameter of 75 μm and an outer diameter of 375 μm were purchased from Beckman Coulter.

All CEC columns used in this report were packed in-house using a slurry packing technique as reported in the literature [23,24]. Briefly, a stainless steel reservoir was constructed with an internal volume of approximately 70 μl. One port of the reservoir was connected to an HPLC pump (Shimadzu LC 9 AD-VP); the other port allowed for the insertion of the fused-silica capillary for packing. Capillary stock was cut to lengths of 50 cm and fitted with a stainless steel mesh frit (2 μm pores) in a removable inline mini micro filter (Upchurch, Oak Harbor, USA). Slurries were made at a concentration of 10 mg per 150 μl of acetonitrile. Columns were filled with acetonitrile and packed with water. After packing, sintered frits of packed columns were subsequently fabricated with an ACF electrical burner (Innovatech, Stevenage, UK) while the capillary was still under pressure. The total length of the columns was 312 mm and the packed length was 98 or 203 mm.

To estimate the stability of the stationary phase under extreme pH values, two independent capillary columns were exposed to either an acidic buffer (phosphate buffer, ionic strength 10 mM, pH 2.5–ACN, 40:60, v/v) or alkaline buffer (Tris–HCl, ionic strength 10 mM, pH 9.1–ACN, 40:60, v/v) under an applied voltage of −5 kV and during a period of 7 days. The EOF was measured everyday, six times by injecting a DMSO solution (4 mM in water, for 10 s at −5 kV).

2.1.4. CEC separation

Buffers used in this study, were prepared using Milli-Q water (Millipore, Molsheim, France) filtered through a 0.2 μm membrane (Millex, Millipore). Tris–HCl and phosphoric acid (reagent grade), used in the preparation of background electrolyte solutions, were purchased from Sigma. Adjustements of pH of the electrolyte solutions were achieved throught the addition of hydrochloric acid 1.0 M or sodium hydroxyde 1.0 M. pHs were measured in the aqueous phase before mixing with the specified ACN volume. The organic solvent and the background electrolyte were thoroughly degassed individually via ultrasonication for 15 min prior their use.

The samples were injected hydrodynamically under 1.7 × 10^5 Pa for 100 s. All samples were detected at 200 nm. All experiments were carried out at 25 °C and both column ends were pressurized at 6.9 × 10^5 Pa to prevent bubble formation. Except as otherwise specified, columns packed with end-packed Stability BS-C23, 300 Å, 5 μm, 31.2 cm (effective length 9.8 cm) × 75 μm were employed and analyses were made under −15 kV, with a voltage ramp of 0.5 min.

2.2. Capillary zone electrophoresis (CZE) separation

To study the different mechanisms (chromatographic versus electro-migration) involved in CEC, the electrophoretic mobilities of peptides were estimated by CZE using the same buffer conditions. The CZE capillary was a fused silica capillary, 32.1 cm (21 cm effective length) × 75 μm. In between runs, the capillary was rinsed step-wise by water (1 min), 0.1 M NaOH (3 min), water (1 min) and the running buffer (5 min). The samples were introduced into the capillary by applying a pressure of 6.9 × 10^2 Pa for 5 s. Other separation conditions were as in CEC analyses.

2.3. Retention factor

All CEC analyses included a ramp time in voltage. The elution times were therefore corrected, using the following equation [25] (Eq. (1)):

$$t_i = t_{i\,\mathrm{measured}} - \tfrac{1}{2} t_{\mathrm{ramp}} \quad (1)$$

where t_i is the corrected elution time, $t_{i\,measured}$ the elution time obtained experimentally and t_{ramp} is the duration of voltage ramp.

The CEC retention factor (k_{CEC}), calculated in this study is analogous to the retention factor employed in HPLC, excepted that it represents the contribution of both electrochromatographic retention and electrophoretic migration. The value of k_{CEC} can then be calculated according to Eq. (2):

$$k_{\mathrm{CEC}} = \frac{t_i - t_{\mathrm{eof}}}{t_{\mathrm{eof}}} \quad (2)$$

where t_i is the corrected elution time of the analyte and t_{eof} is the corrected elution time of the neutral and unretained component DMSO. However, the CEC system can be defined by a combination of a "retention factor" (k_{c}) that estimates

the electochromatographic retention in CEC and a velocity factor (κ_e) that characterizes the electrophoretic migration.

The parameter k_c can be estimated by Eq. (3), where the product $t_i(1 + \kappa_e)$ represents the retention time of the peptide in the absence of electrophoretic migration.

$$k_c = \frac{t_i(1 + \kappa_e) - t_{eof}}{t_{eof}} \quad (3)$$

According to Rathore et al. [26], κ_e can be estimated by the following ratio (Eq. (4)):

$$\kappa_e = \frac{\mu_{ep}}{\mu_{eo\ packed}} \quad (4)$$

where the electrophoretic mobility μ_{ep} is obtained from separated CZE measurements for each peptide, using the same buffer conditions. $\mu_{eo\ packed}$ represents the electroosmotic mobility in the packed portion of the capillary and is calculated from the migration time of an EOF marker (Eq. (5)).

$$\mu_{eo\ packed} = \frac{L_e^2}{t_{eof} V_{packed}} \quad (5)$$

where V_{packed} and L_e are an estimation of, respectively, the potential drop across the packed segment of the column and the length of the actual flow path followed by the marker traversing the packed segment. These parameters can be calculated from the values of current generated in fused silica capillary and packed columns [27]. To this purpose, capillaries having different portions of packed sections of BS-C23 (L_{packed} = 9.5 and 20 cm, for a total length of 31.2 cm) were employed.

3. Results and discussion

3.1. Electroosmotic flow

Following column fabrication, the dependence of the reduced plate height (h) on the migration velocity for naphthalene through the BS-C23 packed capillary was studied by varying the applied voltage to evaluate the packing quality and column performances. As shown in Fig. 2, the Van Deemter curves recorded for a retained but non-charged tracer (naphtalene) showed the expected shape generally observed in CEC, i.e. a low C-term and hence a plot largely dominated by the A- and B-terms. Mass transfer contribution to the plate height is small, even at higher velocity. Reduced plate height (h = 1.4, N = 160 000 plates/m) becomes almost independent of mobile phase velocity, for velocities higher than 0.05 cm/s indicating that high velocity and also high speed of analysis could be attained without a loss in efficiency. No significant differences were observed with the 300 and 100 Å pore size stationary phases except at low velocities where 300 Å pore size stationary phase affords a slightly higher efficiency.

On the other hand, several experiments were conducted to estimate the magnitude of the EOF in this capillary as a function of the electrolyte buffer pH, for columns packed with end-capped and non end-capped BS-C23. A similar study was conducted with a fused silica capillary and also with a capillary containing only two frits formed by sintering end-capped BS-C23 phase (without stationary phase in-between). Fig. 3 shows the electroosmotic flow velocity measured at different pH from 2 to 9, using a mobile phase containing 60% of ACN and 40% (v/v) of buffer. DMSO was used as

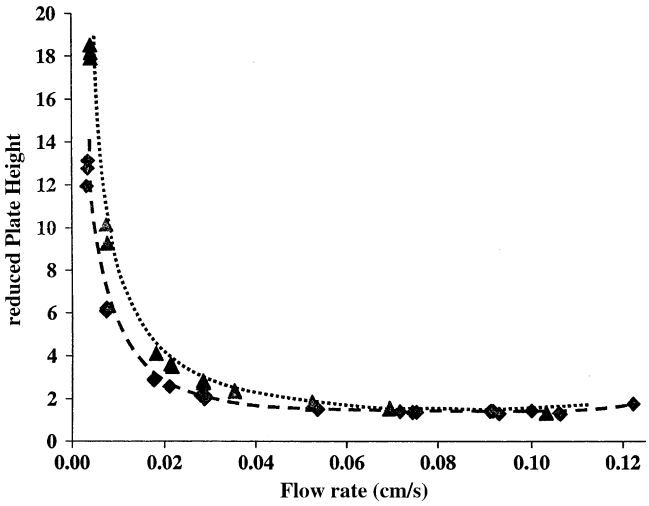

Fig. 2. Plot of the reduced plate heights calculated from naphthalene peek vs. flow rate. Plot of the reduced plate heights calculated from naphthalene peek vs. flow rate obtained for an end-capped BS-C23, 9.8 cm effective length, with a pore size of (▲) 100 Å or (♦) 300 Å. Conditions: injection 15 s under 1.75 bar of DMSO (100 mM) and naphthalene (6 mM), BGE: Tris–HCl pH 2.7, 150 mM–ACN (40:60, v/v), applied voltage from −0.5 to −20 kV.

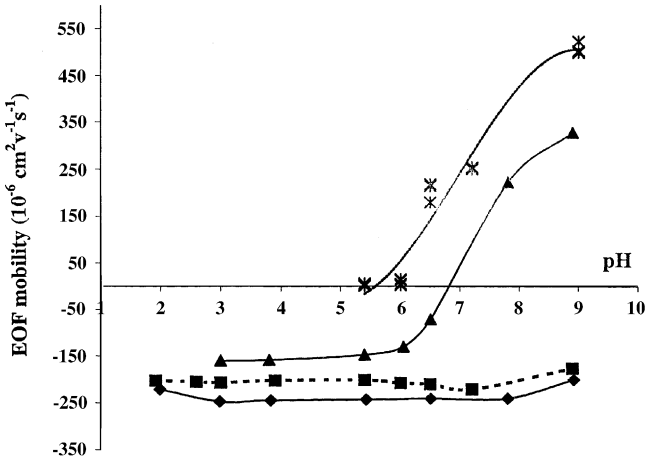

Fig. 3. Plot of the EOF mobility measured with DMSO as the unretained neutral marker vs. pH of BGE. BGE: Tris–HCl, 50 mM, pH from 2 to 9–ACN (40:60, v/v). Analyte: DMSO 100 mM, in H_2O/ACN, 40/60 (v/v). Injection for fused silica capillary: 5 s under 7 mbar, injection for other columns: 5 s under 0.7 bar. (✱) Fused silica capillary, 31.2 cm (effective length 21 cm) × 75 μm, (▲) capillary with only two frits, 9.4 cm in between the two frits, 31.2 cm (effective length 9.8 cm) × 75 μm, (■) column packed with non end-capped BS-C23 300 Å 5 μm, 31.2 cm (effective length 9.8 cm) × 75 μm, (♦) column packed with end-capped BS-C23 300 Å 5 μm, 31.2 cm (effective length 9.8 cm) × 75 μm. Other conditions of separation as described in Section 2.

the dead time marker. As expected in our conditions, the EOF decreases with the pH and was negligible below pH 5.5 in a bare fused silica capillary. This absence of EOF at pH below 5.5 probably reflects the effective silanol pK_a, which would be higher in hydro-organic buffer than in aqueous buffer.

In contrast, under the same experimental conditions, a strong EOF, with a magnitude of 2.4×10^{-4} cm^2 s^{-1} V^{-1}, was observed in the reversed direction (from cathode to anode) with our phase. Obviously, the opposite flow due to silanols from silica wall or to residual silanols from stationary phase particles is totally counteracted by the quaternary ammonium groups. A similar conclusion was drawn by Smith and Evans [15] who studied the contribution of charges of stationary phases to the EOF and found that in a CEC column, the EOF is predominantly generated by the charge of the silica particles rather than by those of the capillary wall. Similar flow magnitudes were previously observed with embedded ammonium groups either with monoliths, or with polymethacrylate microspheres or with open-coated capillaries [19,21,28]. In addition, no significant difference was obtained between the EOF values obtained with end-capped and non end-capped BS-C23 stationary phases. In both cases, the EOF was found to be independent of the pH of the mobile phase.

To evaluate the influence of the column frits on the EOF, a bare silica capillary containing only two sintered frits of 4 mm length was prepared. In this case, the sign of the EOF was pH-dependent. Below pH 6.5 an anodic flow was observed and was stable below pH 6. Its magnitude is however weaker than that of BS-C23 end-capped column (1.5×10^{-4} cm^2 s^{-1} V^{-1} against 2.4). Thus, we deduced that the ammonium groups were still present in formed frits and are responsible for weak anodic EOF in acidic conditions. Under alkaline conditions the cathodic flow, stemming from the silanols of capillary walls, largely compensated for the inversed EOF generated by the frits.

An evaluation of the effect of the ACN percentage in the mobile phase, was performed, keeping the same proportion of phosphate buffer (concentration 50 mM, pH 2.5) in the mobile phases. The electroosmotic mobility was measured according to the percentage of ACN. The EOF was found nearly constant over a wide range of organic modifier content (from 0 to 80%). The increase of ACN proportion in the electrolyte has two consequences: a decrease of the ionic strength and a variation of ε_r/η ratio (ε_r and η being the permittivity and the viscosity of the mobile phase, respectively). The ionic strength and the ε_r/η ratio of the mobile phase have an opposite influence on the magnitude of the EOF in the range of ACN percentages investigated (0–80%). This explains the apparent constancy of the EOF in the investigated conditions. Furthermore, any link between the buffer nature and the EOF magnitude has been underlined when phosphate, Tris–HCl, citrate or ammonium formate buffers have been employed.

Finally, the strong anodic EOF generated by the Stability BS-C23 was found to be independent on the pH, a feature that is explained by the presence of a strong cation exchange group

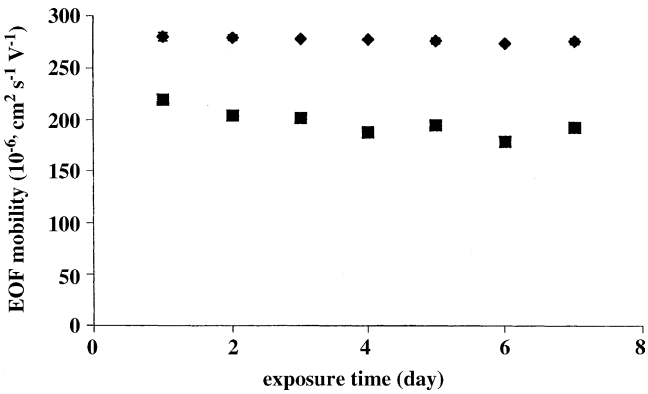

Fig. 4. Stability test of packed columns submitted to acidic or alkaline conditions over a period of 7 days, under voltage. BGE: (■) Tris–HCl ionic strength 10 mM, pH 9–ACN (40/60% (v/v)), (♦) phosphate, ionic strength 10 mM, pH 2.5–ACN (40:60, v/v), injection: DMSO 4 mM in water, 10 s, −5 kV, n = 6. Analytical voltage: −15 kV with a ramp of 0.5 min, both column ends were pressurized at 6.9×10^5 Pa. Temperature: 25 °C, detection: 200 nm. Column packed with end-capped BS-C23 300 Å 5 μm, 31.2 cm (effective length 9.8 cm) × 75 μm.

(ammonium) in the stationary phase. In addition, the EOF was stable with variation on the percentage of organic modifier in the mobile phase in our conditions. Thus, the selectivity can be tuned by varying different parameters without affecting the EOF velocity.

3.2. Column stability

The stability of the stationary phase under extreme pHs conditions (pH 2.5 and 9.1) was studied by monitoring the EOF over 1 week. To this purpose two capillaries were exposed to acidic and alkaline conditions respectively, under an applied voltage of −5 kV, as described in Section 2. As shown in Fig. 4, in both conditions the average intra-day EOF mobility was nearly constant with R.S.D. less than 1.4 and 2.7%, for acidic and alkaline conditions, respectively. In addition, the average day-to-day mobility for each condition did not vary by more than 1.1%, 3.4% for acidic and alkaline conditions, respectively. The stability of EOF observed in both conditions, demonstrate the good middle-term stability of the capillary packed with Stability BS-C23 under extreme pH values. The slight difference observed between the magnitude in the EOF mobility in the two conditions (acidic and basic) tested may be attributed to some contribution of the silanols for the silica capillary with the alkaline buffer and also to the fact that two different columns were employed.

3.3. Analyses of standard peptides

Because of the multiplicity of separation processes that can be involved in CEC separation of charged peptides, mobile phase composition is expected to play a major role in the separation of peptides by tuning the extent of each mechanism.

Table 1
Physico-chemical characteristics of investigated peptides

Peptides	Molecular mass ($g\,mol^{-1}$)	pI[a]	Frictional ratio[b] ζ_{fric} × 100 at pH 2.5	Hydrophobicity[c]
Eledoisin RP	707	10.1	1.8	1.45
TRH precursor peptide	1006	12.4	5.4	−3.19
Eledoisin	1207	4.1	1.2	0.10
Angiotensin I	1296	7.9	2.8	−0.20
Arg–Arg gastrin	1380	4.1	1.9	−2.10
Renin substrate	1759	7.8	2.3	0.28
[Gln^{11}] amyloid β protein	1954	6.3	3.4	−1.82
EGF	2319	7.2	1.9	−0.67
Valosin porcin	2918	9.4	2.1	−0.18

[a] The embl-heidelberg.de: EMBL WWW Gateway to Isoelectric Point Service was employed for calculating the isoelectric points (p*I* values) as well as the net-charge values of peptides at different pH values.
[b] $\zeta_{fric} = q/M^{2/3}$, with q net-charge value and M the molecular mass.
[c] Hydrophobicity was estimated on http://www.expasy.org with Protscale, using parameter of Kyte and Doolittle [31].

Initially, we have investigated the effect of the organic modifier and the buffer concentrations in the electrolyte on the migration behaviour of standard peptides. These peptides were chosen with different isoelectric points, molecular masses and hydrophobicities in order to reflect as much as possible the diversity of peptide structures that can be found in a protein digest (Table 1). The influence of ACN content in the mobile phase on the retention factor of peptide was evaluated. The CEC retention factors of these peptides were estimated as the apparent retention factors k_{CEC} at different percentages of ACN in the mobile phase, which consisted of 50 mM phosphate buffer, pH 2.5. With reversed stationary phases, a low content of organic solvent in the mobile phase is expected to favour hydrophobic interactions but also to decrease possible electrostatic or hydrogen-bond interactions. As shown in Fig. 5A, relatively low retention factors (less than 2), for percentages of ACN from 20 to 60% are observed for most of the investigated peptides, although they possess quite different hydrophobic properties. Under 10% ACN, all retention factors increase significantly. Interestingly, the eledoisin exhibits the highest retention factor at a low percentage of ACN, although it is quite hydrophilic. This indicates that reversed-phase retention is probably not the predominant mechanism in this separation. Generally, electrochromatographic behaviour of peptides based on the organic solvent composition of the mobile phase is quite a complex phenomenon. The concentration of the organic modifier may influence three main factors involved in retention/migration behaviour of analytes: (1) the magnitude of EOF in the capillary, (2) the electrophoretic mobility of the peptides and (3) the chromatographic retention based on solute-bonded phase interactions and/or ionic interaction with a charged stationary phase. However, as discussed previously, with the Stability BS-C23, EOF is not affected by variation in ACN concentrations.

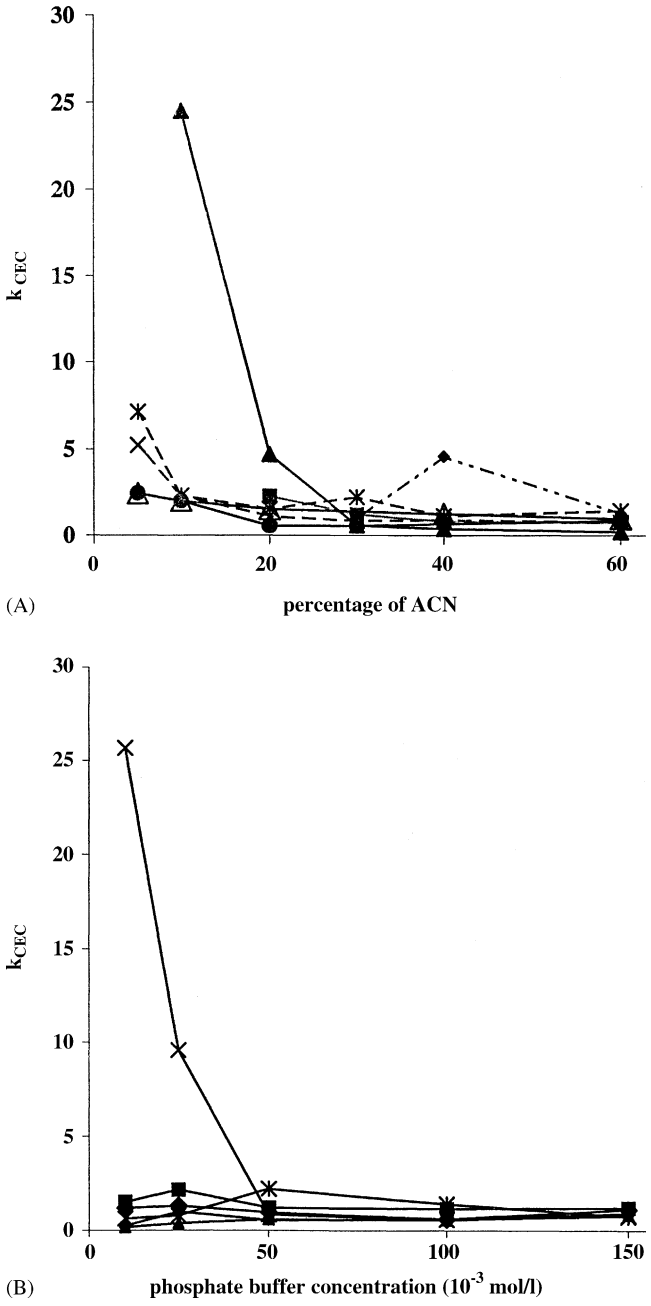

Fig. 5. Effect of (A) ACN percentages and (B) buffer concentrations in the mobile phase on peptide retention in CEC (k_{CEC}). (×) Arg–Arg gastrin, (♦) valosine porcine, (▲) eledoisin, (△) eledoisin RP, (■) renin substrate porcine, (●) EGF, (✳) angiotensin I, DMSO. (A) BGE: phosphate, 50 mM, pH 2.5–ACN, from 95:5 to 40:60 (v/v). (B) BGE: phosphate, concentration from 10 to 150 mM, pH 2.5–ACN (70:30, v/v), hydrodynamical injection: 100 s under 1.7×10^5 Pa; analytical voltage: −15 kV with a ramp of 0.5 min, both column ends were pressurized at 6.9×10^5 Pa. Temperature: 25 °C, detection: 200 nm. Column packed with end-capped BS-C23 300 Å 5 μm, 31.2 cm (effective length 9.8 cm) × 75 μm.

Rathore and Horváth [29] pointed out that k_{CEC}, unlike its equivalent in HPLC, does not yield any thermodynamic or mechanistic information. Thus, to draw conclusions about the degree of charged peptides-stationary phase interaction, a k_c, which is an indication of contribution of the chromatographic

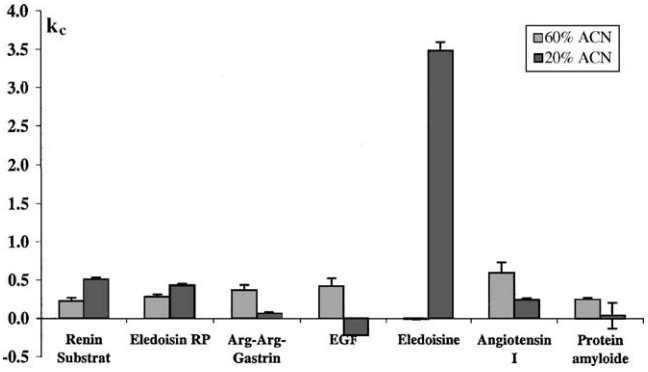

Fig. 6. Chromatographic retention factors (k_c) of different peptides at 20 and 60% of ACN in the BGE. BGE: phosphate, 50 mM, pH 2.5–ACN, 40:60 and 80:20 (v/v). Other conditions as described in Fig. 4. Bars indicate the standard deviation of each point.

retention in a CEC separation, was estimated for these peptides (under two conditions of low and high ACN percentages in the mobile phase). This estimation requires electrophoretic measurements of peptides by CZE and an estimation of the "actual" EOF in the packed section of the capillary. This value is obtained by measuring values of the current generated in capillaries having different packed lengths and in an empty fused silica capillary. Fig. 6 compares the electrochromatographic retention factor k_c of several peptides (Eq. (3)) at low and high ACN percentages in the mobile phase (20 and 60%).

Firstly, k_c values are very low and reflect peptide electrochromatographic behaviours dominated by their relatively strong electrophoretic migration with marginal electrochromatographic retention. Indeed, in acidic conditions, peptides are overall positively charged, and their electrophoretic mobility is in the opposite direction of the anodic EOF. The velocity factor κ_e (Eq. (2)) is therefore negative and the electrochromatographic factor k_c is always lower than k_{CEC}.

Secondly, we observed that k_c of the most hydrophobic peptides (eledoisin RP, eledoisin, renin substrate) increases with the percentage of aqueous portion in the mobile phase. That is the expected trend in RP chromatography partitioning. This increase is particularly important for eledoisin, which possesses the weakest frictional ratio and thereby the weakest electrophoretic mobility. This demonstrates that hydrophobic peptides can exhibit relatively high retention factors when a limited electrophoretic migration is involved.

On the other hand, the most hydrophilic peptides (Arg–Arg gastrin, [Gln^{11}] amyloïd β peptide and EGF) have a k_c at 60% of ACN lower than that at 20%. Modification of peptide configuration, with increasing percentages of ACN could explain this phenomenon. However, repulsive phenomena between the quaternary ammonium groups of the stationary phase and positively charged peptides are another possibility.

Finally, several hypotheses can be drawn up to explain the marginal electrochromatographic retention globally low and the unexpected electrochromatographic behaviour observed for several peptides (Arg–Arg gastrin, [Gln^{11}] amyloïd β peptide and EGF) at acidic pH: (1) the positive charges of the ammonium functionalities of the stationary phase could induce a repulsive electrostatic interaction with the overall positively charged peptides, (2) the EOF is too intense, and the electrokinetic contributions in the CEC separation is probably dominant with regard to the electrochromatographic retention. In addition, we cannot exclude that peptides, under an electric field or according to the percentage of ACN in the electrolyte, adopt conformations that preclude hydrophobic interaction with the chromatographic support.

To test the first hypothesis, the CEC apparent retentions of standard peptides were monitored using buffers composed from 5 to 150 mM phosphate pH 2.5 in water–ACN (70:30, v/v). Indeed, the potential repulsive electrostatic interaction should readily decrease by using a higher concentration of phosphate buffer pH 2.5 in the mobile phase. Fig. 5B shows the influence of phosphate concentration in the mobile phase on the k_{CEC} of several peptides. As a general trend, the phosphate concentration has low influence on k_{CEC}, except for Arg–Arg gastrin. The absence of migration time increase at high buffer concentrations indicates that repulsive electrostatic interactions are not responsible for the weak retention of peptides observed in our conditions. However, at low buffer concentrations, the less positively charged Arg–Arg gastrin within the hydrophilic peptide group, has a high retention factor (>20). This retention at weak ionic strength may be attributed only to electrostatic interaction with the stationary phase, which arises from an ion-exchange chromatographic mechanism between the fixed cationic charges of the stationary phase and some anionic charges of the glutamic acid-rich Arg–Arg gastrin. These results highlight that anion-exchange is a possible interaction mechanism of retention, whereas repulsive electrostatic interaction is probably not involved in CEC of peptides at acidic conditions.

An excessively strong EOF could also explain the low retention factor k_c observed. The impact of this electrokinetic phenomenon can be ascertained by a study of the influence of the applied voltage on the retention. Fig. 7 represents a plot

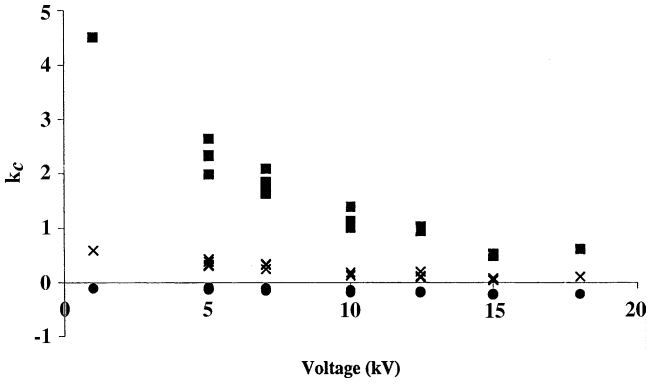

Fig. 7. Effect of the applied voltage on the retention factor k_c of three peptides. BGE: phosphate, 50 mM, pH 2.5–ACN (80:20, v/v). Analyte mixture of (●) EGF 0.3 mM, (■) renin substrate porcine 0.75 mM, (×) Arg–Arg gastrin 1 mM, DMSO 3.3 mM in water. Voltage from −1 to −18 kV. Other conditions as described in Fig. 4.

of k_c of three peptides versus the applied voltage under 20% of ACN in the BGE. Except for EGF, which exhibits no retention ($k_c \approx 0$), an increase in k_c is observed for the two other peptides with a decrease in the applied voltage. In fact, the possibility of chromatographic interactions is probably enhanced with longer analysis times. When ion-exchange CEC is performed, Ye et al. [30] have demonstrated that the retention of acidic components increased when the applied voltage increased. The opposite tendency observed here, supports the hypothesis that ion exchange mechanism is not the predominant retention mechanism in our conditions. It appears that the electrokinetic contribution is probably much more important than the electrochromatographic retention and may be reduced by a decrease in the applied voltage. An alternative, to exploit the favourable anodic EOF while permitting a larger extent role for the chromatographic process, would be to fine-tune the EOF through the choice of a suitable counter ion.

The overall results proved that the retention of peptides at acidic conditions could be tuned by changing either electric field strength and/or the BGE ionic strength.

A comparison was made between BS-C23 and octadecyl columns on the CEC of a synthetic peptide mixture (Fig. 8). The electropherograms showed that higher efficiencies and improved peak symmetries are attained with the Stability BS-C23 stationary phase for most of the investigated peptides, feature is also combined with a lower analysis time. The elution orders observed with the two stationary phases clearly demonstrate that BS-C23 affords a different selectivity compared to that obtained with a C_{18} stationary phase. Indeed, with the inverted EOF generated in a BS-C23 CEC column, the peptide eluting order was also expected to be also inverted in the case of a pure RP chromatography mechanism.

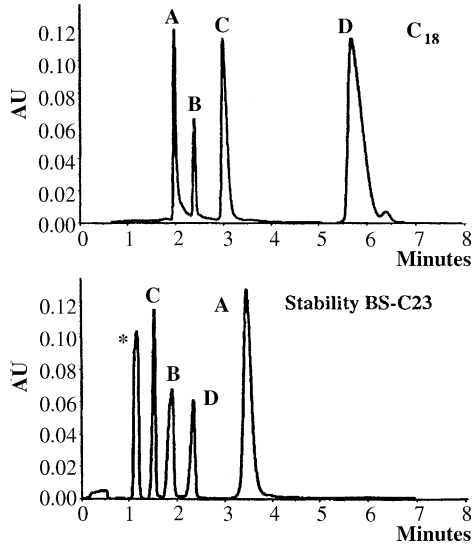

Fig. 8. Comparison of CEC profiles obtained with a peptide mixture on BS-C23 and C_{18} stationary phase. BGE: Tris–HCl 50 mM, pH 3–ACN (40:60, v/v), (*) DMSO 8 mM, (A) TRH precursor peptide, (B) EGF, (C) eledoisin, (D) eledoisin RP in H_2O/ACN: 20/80% (v/v). Other conditions as described in Fig. 4.

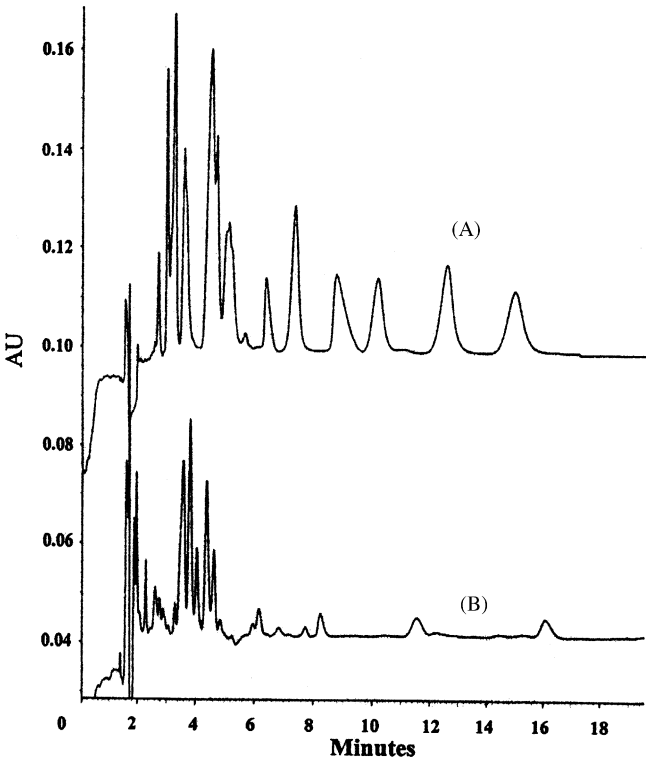

Fig. 9. Peptide mapping of (A) β-lactoglobulin and (B) hGH by CEC on a BS-C23 column. BGE: Tris–HCl 75 mM, pH 2.6–ACN (80:20, v/v). β-Lactoglobulin 10 μg/μl, hGH 2 μg/μl. Other conditions of separation as described in Fig. 4.

3.4. Applications

In view of the retention behaviour of peptides with this new stationary phase, we have performed the analysis of a β-lactoglobulin and hGH digest at acidic pH (2.6), with a relatively low level of acetonitrile (20%) in the buffer. Even if separations of the 20 and 18 tryptic peptides, expected for hGH and for β-lactoglobulin digest, respectively, are not complete (Fig. 9), the profile obtained under unpressurized CEC and without eluting gradient is quite encouraging. In addition we observed that the separation performances were clearly improved when a Tris–HCl buffer was employed instead of a standard phosphate buffer.

4. Conclusion

Among the different packing materials that are used in CEC, Stability BS-C23, which incorporates a permanent charge and RP properties, provides several advantages. First, an accelerated anodic EOF as compared to RP columns was evidenced. Second, a nearly independent EOF over a wide range of pH (2–9), acetonitrile percentages in BGE and buffer concentrations was observed. Moreover, slight differences in selectivity between BS-C23 and a common octadecyl stationary phase were demonstrated. Furthermore the stability

of this stationary phase under alkaline and acidic conditions was demonstrated.

The study performed on standard peptides underlined that the electrochromatographic behaviour is a complex phenomenon that superimposed a predominant electrophoretic migration process, an interplay of hydrophobic as well as electrostatic interactions (between the stationary phase and peptides). This extensive study pointed out the low electrochromatographic retention of peptides in CEC, in our conditions, which could not be explained by a charge repulsive phenomenon, but probably by a too intense EOF. Ion-exchange was possible in some seldom cases; however the benefit of several separation mechanisms in CEC cannot yet be used in a controlled manner in our conditions.

This stationary phase permitted the peptide mapping under unpressurized CEC and without eluting gradient. Further development will be devoted toward the investigation of other mobile phase conditions permitting a higher contribution of the chromatographic process in the CEC.

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