Journal of Chromatography A, 1217 (2010) 450–458

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

High performance liquid chromatography separation of structurally related enkephalins on quaternary ammonium-embedded stationary phase in isocratic mode

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ARTICLE INFO

Article history:
Received 27 May 2009
Received in revised form
13 November 2009
Accepted 17 November 2009
Available online 24 November 2009

Keywords:
Peptides
Enkephalins
Mixed-mode stationary phase
Eluent anions
Selectivity

ABSTRACT

Separation of twelve enkephalins was investigated on a quaternary ammonium-embedded stationary phase (Stability BS-C23). Variation of buffer pH of the mobile phase highlighted the complex relationship between repulsive/attractive electrostatic interactions and the reversed-phase partitioning mechanism. The effect of three different anions employed as additives (phosphate, chloride and perchlorate) was examined at various concentrations and two pH values (acidic and neutral). At pH 2.5, an increase in the anion eluent concentration resulted in a higher retention factors of positively charged enkephalins. This effect was more pronounced when perchlorate ions were added to the mobile phase rather than phosphate and chloride ions, due to chaotropic and ion-pairing effects. In contrast, at pH 7.5, retention factors of negatively charged enkephalins decreased when these salts were added, due to an anion-exchange mechanism. Perchlorate caused a sharper decrease than chloride and phosphate anions did. The results presented here provide insight into the possible adjustment of retention and separation of peptides on a mixed-mode stationary phase (BS-C23) by a careful control of the buffer pH, the nature and concentration of anions, added to the buffer, and organic modifier content.

1. Introduction

Peptides regulate most physiological processes, acting as endocrine or paracrine signals or as neurotransmitters. Leucine^5-enkephalin (LE), H-Tyr-Gly-Gly-Phe-Leu-OH, and Methionine^5-enkephalin (ME), H-Tyr-Gly-Gly-Phe-Met-OH are pentapeptides naturally synthesised in the human body and are termed as endogenous opiates. They originate from the large precursor protein, preproenkephalin [1] and are involved in the regulation of many physiological processes including temperature control, feeding behaviour, respiration, and pain [2]. A synthetic analogue of Leu-enkephalin [D-Ala^2, Arg^6] Leucine^5-enkephalin (Dalargin), H-Tyr-D-Ala-Gly-Phe-Leu-Arg-OH, has also shown analgesic properties [3].

Capillary zone electrophoresis (CZE) has been employed to check the purity of synthetic preparations of LE, ME and dalargin and also for their determination in biological fluids (serum, plasma, or cerebrospinal fluids) [4–7]. However, the efficiency and/or repeatability of CZE may not be reliable, because interactions between peptides and the silanol groups can introduce broadened and tailing peaks. Therefore, Catai et al. proposed to coat the capillary with a noncovalent bilayer to analyze enkephalin-related peptides [8]. However, even CZE can sometimes fail to fully separate peptides that only differ in the substitution of one or two hydrophobic residues. Thus, capillary micellar electrokinetic chromatography (MEKC) was used to analyze LE, ME and five analogues, which differed in their uncharged side chain [9,10]. Another approach was based on a multidimensional separation system consisting of the on-line coupling of size-exclusion chromatography (SEC), a reversed-phase C18 trapping column and CZE [11,12].

Several high performance liquid chromatography (HPLC) methods are available to analyze structurally related ENKs [13–20]. In metabolic studies of enkephalin peptides (ENK), various RP-HPLC methods, coupled with electrochemical, fluorescent or ultraviolet detections were developed to achieve simultaneous separation of parent endogenous opioid peptides and their metabolites in acidic conditions. Mixtures of the smallest (T, TG, and TGG) or largest metabolites and the pentapeptides were separated using C18 columns under isocratic conditions [13,14]. However, due to the large difference in polarities between the parent peptide and the shorter metabolites, a gradient elution method has

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doi:10.1016/j.chroma.2009.11.058

been often required to separate enkephalins and their metabolites simultaneously [15–18]. A new generation of stationary phases offers possibilities to improve separation selectivity of ENKs. For instance, a comparative study by Soukupová et al. analysed four analogues of ENKs using a conventional RP (reversed-phase) silica-based and a polybutadiene (PBD) or polystyrene (PS) coated zirconia stationary phase [19]. This study showed that differences in the interactions on both types of phases led to different selectivity. More recently, Lisi et al. developed an isocratic HPLC method to measure LE, ME and endomorphins (1, 2), in one sample using a polar end-capped reversed-phase column [20].

The mixed-mode reversed-phase/ion-exchange stationary phase chromatography can bring interesting selectivity to achieve the separation of complex mixtures [21–23]. This type of phase can be obtained by chemically introducing an ionisable or charged group, anionic [24,25] or cationic [21–23,26,27], into the alkyl chain grafted onto a silica-based support. These charged groups can establish different types of interactions with analytes, in addition to reversed-phase partitioning. Therefore, two or more separation mechanisms are used in conjunction. Furthermore, they offer several advantages over conventional RP, such as an increased stability in aqueous conditions, improved peak shape for basic compounds. These mixed-mode stationary phases have been investigated for the separation of biological molecules such as amino acids [28], nucleic acids [29], peptides and proteins [22,30–32].

Stability BS-C23 is one of these mixed-mode stationary phases which carries a permanent positively charged group, quaternary ammonium, embedded in a hydrophobic alkyl chain C21 on a silica support [22,32–34]. In 2006, we reported on this column for the first time a study exploiting its potential in electrochromatography (CEC) [22]. Recently, our group compared this column to a conventional C18 column (Kromasil C18) for analysing peptides differing in their physicochemical properties [32]. This study demonstrated that BS-C23 offers a unique selectivity as shown by the significant reversal elution of the peptides. This study highlighted the involvement of several retention mechanisms such as electrostatic interactions, and reversed-phase partitioning. These complex mechanisms suggested that the peptides studied interacted with the quaternary ammonium located deep inside the bonded layer.

In this study, our investigation was extended to twelve ENKs including LE, ME and ten of their structural analogues (see Table 1). The ten ENK analogues chosen differed from LE and ME by: (1) substitution at position 2 (Gly by D-Ala) ([D-Ala^2] Leu-enkephalin (ALE) and [D-Ala^2] Met-enkephalin (AME)), and/or (2) amidation of the terminal carboxylic group Leu-enkephalinamide (LEA), Met-enkephalinamide (MEA), [D-Ala^2] Met-enkephalinamide (AMEA) and [D-Ala^2] Leu-enkephalinamide (ALEA), and/or (3) addition of an amino acid (Arg) ([Arg^0] Met-enkephalin (Arg ME) and [D-Ala^2, Arg^6] Leu-enkephalin (Dalargin)), and/or (4) deletion of Tyr ([Des-Tyr^1] Met-enkephalin (Des Tyr ME)) and ([Des-Tyr^1] Leu-enkephalin (Des Tyr LE)). These changes in the structure of the parent peptides (LE and ME) lead to slight differences in their physicochemical properties (more hydrophobic, one added positive charge, or one suppressed negative charge) offering a set of compounds to achieve a better understanding of the mechanisms involved in the retention of peptides on the BS-C23. The aim of the study was to: (i) evaluate the benefits of the unique selectivity previously observed on BS-C23 [32] in the simultaneous analysis and separation of structurally related peptides such as ENKs, (ii) adjust the contribution of each mechanism to modify retention, and (iii) investigate the effect of different eluent anions on selectivity.

Table 1
Physicochemical characteristics of the investigated enkephalins.

Groups	Peptides	Abbreviation	pI^a	Molecular mass	Structure	Ionisable groups	
						Acidic	Basic
Group 1	Met-enkephalin	ME	5.92	573.8	YGGFM	1 carboxylic acid (-Met)1 phenol (Tyr-)	1 amino group (Tyr-)
	Leu-enkephalin	LE	5.92	555.8	YGGFL	1 carboxylic acid (-Leu)1 phenol (Tyr-)	1 amino group (Tyr-)
	[D-Ala^2] Met-enkephalin	AME	5.92	575.7	YaGFM	1 carboxylic acid (-Met)1 phenol (Tyr-)	1 amino group (Tyr-)
	[D-Ala^2] Leu-enkephalin	ALE	5.92	554.7	YaGFL	1 carboxylic acid (-Leu)1 phenol (Tyr-)	1 amino group (Tyr-)
	[Des-Tyr^1] Met-enkephalin	Des Tyr ME	5.92	410.5	GGFM	1 carboxylic acid (-Met)	1 amino group (Gly-)
	[Des-Tyr^1] Leu-enkephalin	Des Tyr LE	5.92	394.6	GGFL	1 carboxylic acid (-Leu)	1 amino group (Gly-)
Group 2	Met-enkephalinamide	MEA	9.9	587.6	YGGFM-NH_2	1 phenol (Tyr-)	1 amino group (Tyr-)
	Leu-enkephalinamide	LEA	9.9	569.6	YGGFL-NH_2	1 phenol (Tyr-)	1 amino group (Tyr-)
	[D-Ala^2] Met-enkephalinamide	AMEA	9.9	586.7	YaGFM-NH_2	1 phenol (Tyr-)	1 amino group (Tyr-)
	[D-Ala^2] Leu-enkephalinamide	ALEA	9.9	568.7	YaGFL-NH_2	1 phenol (Tyr-)	1 amino group (Tyr-)
Group 3	[Arg^0] Met-enkephalin	Arg ME	9.84	729.9	RYGGFM	1 carboxylic acid (-Met)1 phenol (Tyr-)	2 amino group (Arg-)
	[D-Ala^2, Arg^6] Leu-enkephalin	Dalargin	9.84	725.8	YaGFLR	1 carboxylic acid (-Arg)1 phenol (Tyr-)	2 amino group (Tyr-/-Arg)

[a] The embl-heidelberg.de: EMBL WWW Gateway to Isoelectric Point Service was employed for calculating the isoelectric points (p*I* values).

2. Materials and methods

2.1. Reagents and chemicals

HPLC grade acetonitrile (ACN), phosphoric acid (85%), sodium hydroxide 1.0 M, sodium chloride (NaCl) and hydrochloric acid 1.0 M were purchased from VWR (Fontenay-sous Bois, France). Thiourea, TRIS [hydroxymethyl] aminomethane and sodium perchlorate were purchased from Sigma (St. Louis, MO, USA).

The ENK peptides (ME, MEA, AME, AMEA, Arg ME, Des Tyr ME, LE, LEA, ALE, ALEA, Des Tyr LE and Dalargin) were purchased from Biovalley (American Peptides, Sunnyvale, CA, USA). Analytical concentrations for HPLC experiments were 0.025–0.1 mg/mL for each peptide and 0.42 mg/mL for thiourea, diluted in the mobile phase.

2.2. Instrumentation

A 2510 HPLC pump (Varian, Walnut Creek, CA, USA) coupled to a 484 UV detector (Waters, Milford, MA, USA) and a Rheodyne 7725i injector (Cotati, CA, USA) with a 20 μL (C18) or 10 μL (BS-C23) loop were used in all experiments. Data acquisition and evaluation were performed by the EZCHROM elite HPLC software (Scientific Software, Pleasanton, CA, USA). A column thermostat 2027 (Jones Chromatography, Llandbradach, Mid Gladmorgan, UK) was used to maintain the column at 30 °C. The column containing a permanent positive charge was an end-capped Stability BS-C23 (250 mm × 2 mm I.D.; particle size, 5 μm; pore size, 300 Å) (CIL Cluzeau, Sainte Foy la Grande, France) and the reference RP column was an Interchrom Kromasil C18 (250 mm × 4.6 mm I.D.; particle size, 5 μm; pore size, 100 Å) (Eka Nobel, Bohus, Sweden), as previously described [32].

The buffers were first prepared with deionized water from a Direct-Q 3 UV purification system (Millipore, Milford, MA, USA) and ACN was added thereafter to prepare the final mobile phase. All pH values reported in the text are those measured for solutions in pure water. Buffer pH and concentration calculations were performed using the Phoebus program (Analis, Suarlée, Belgium). A pH-meter 730 (WTW Inolab, Weilheim, Germany) was used to check pH values. All solvents were filtered through a 0.22 μm membrane and degassed before use.

2.3. Chromatographic conditions

HPLC experiments were performed at 1.0 or 0.3 mL/min flow rates for Kromasil C18 and BS-C23, respectively. Detection was carried out at 205 nm. Column void volume was measured by injection of thiourea. The retention times are the average of triplicate determinations.

To study the effects of buffer pH and type on ENK retention, the mobile phases were composed of either phosphate or TRIS buffer (ionic strength of 37 mM, pHs ranging from 2.5 to 7.5) mixed with ACN, 75:25 (v/v), (BS-C23) or 80:20 (Kromasil C18) (v/v) ratios. Sodium phosphate buffers were prepared with phosphoric acid and sodium hydroxide. TRIS buffers were prepared with TRIS and hydrochloric acid.

To investigate the effect of eluent anion type and concentration on retention, the eluent composition was ACN/buffer solution (10:90, v/v) at pH 2.5 or (25:75, v/v) at pH 7.5. Concerning the study of phosphate concentration effect, phosphate buffers were prepared in order to obtain the appropriate pH and concentration (from 10 to 80 mM pH 2.5, from 4 to 60 mM pH 7.5). For the study of effects of chloride and perchlorate anion addition, the aqueous part of the mobile phase was first buffered with 10 mM (pH 2.5) or 4 mM (pH 7.5) phosphate concentration, in order to obtain the same ionic strength (10 mM) at the two pH values studied. Mobile phases were then prepared by adding the appropriate quantities of the sodium salts ($NaClO_4$, NaCl) to the buffers to obtain the desired concentration 0–90 mM; pH 2.5, and 0–60 mM; pH 7.5.

The study of ACN content effects was performed at two pH values (2.5 and 7.5) with phosphate or perchlorate anions. At pH 2.5, ACN content varied from 5% to 20% when using phosphate anions (20 and 60 mM) or from 10% to 25% when using perchlorate anions (20 and 60 mM). At pH 7.5, ACN content varied from 25% to 40% when using phosphate anions (4 and 24 mM) or from 20% to 30% when using perchlorate anions (20 mM). The preparation of the mobile phase was as previously described.

3. Results and discussion

We investigated the effects of various mobile phase experimental factors, such as buffer pH, anion type and concentration, and ACN content, on ENK retention with the quaternary ammonium-embedded stationary phase. The physicochemical properties (p*I*, structure, acidic and basic ionisable groups) of ME, LE and their analogues are summarized in Table 1. Depending on the groups that might be ionised in the studied pH range, ENKs could be divided into three categories

Group (1): ENKs that possess free C-carboxyl and N-amine terminal groups [LE, ME, ALE, AME, Des Tyr ME and Des Tyr LE].
Group (2): ENKs that possess a free N-amine terminal group but have an amidated C-terminal group [C-carboxyl terminals are amide groups] [LEA, MEA, AMEA and ALEA].
Group (3): ENKs that possess a free C-carboxyl terminal and two amines (N- terminal and one lateral residue) groups [Arg ME and Dalargin].

3.1. Effect of buffer pH

Firstly, the buffer pH was varied to assess the effect of the ionic state of ENKs on their selectivity on BS-C23. To further understand the mechanisms involved in ENK retention on BS-C23, additional experiments were performed on a C18 column (Kromasil C18).

On the basis of tabulated p*I* values, all peptides belonging to the same group should have identical ionic states over the pH-range studied. Their retention should therefore follow the same trend as the pH varies. Possible correlations between retention factors obtained on BS-C23 and C18, for the three groups of ENKs were evaluated (Fig. 1). Two main observations could be drawn from this figure:

(i) The retention factor on the embedded stationary phase increased by raising the buffer pH for the three groups of ENKs. This effect was more pronounced for the ENKs of group (1) possessing a free C-carboxyl and a N-amine terminal group.
(ii) On the C18 stationary phase, the retention behaviour of group (1) was the opposite of that found on BS-C23; a decrease in ENK retention factor was observed with increasing pH, while for ENK groups (2) and (3), the change in retention was similar to that observed on BS-C23.

As all the peptides studied were positively charged at acidic pH, the ion-exclusion phenomenon was predominant for the three groups on BS-C23, because of electrostatic repulsion between peptides and the embedded quaternary ammonium (leading to negative retention factors) [35,36]. However, on C18 at acidic pH, retention factors were higher than those observed on BS-C23, and this was true for the three groups, due to a predominant RP mechanism. By increasing the pH, retention factors were increased for ENK group (1) on the BS-C23, leading to retention factors higher than those obtained for these ENKs on C18 at neutral pH (Fig. 1A). This

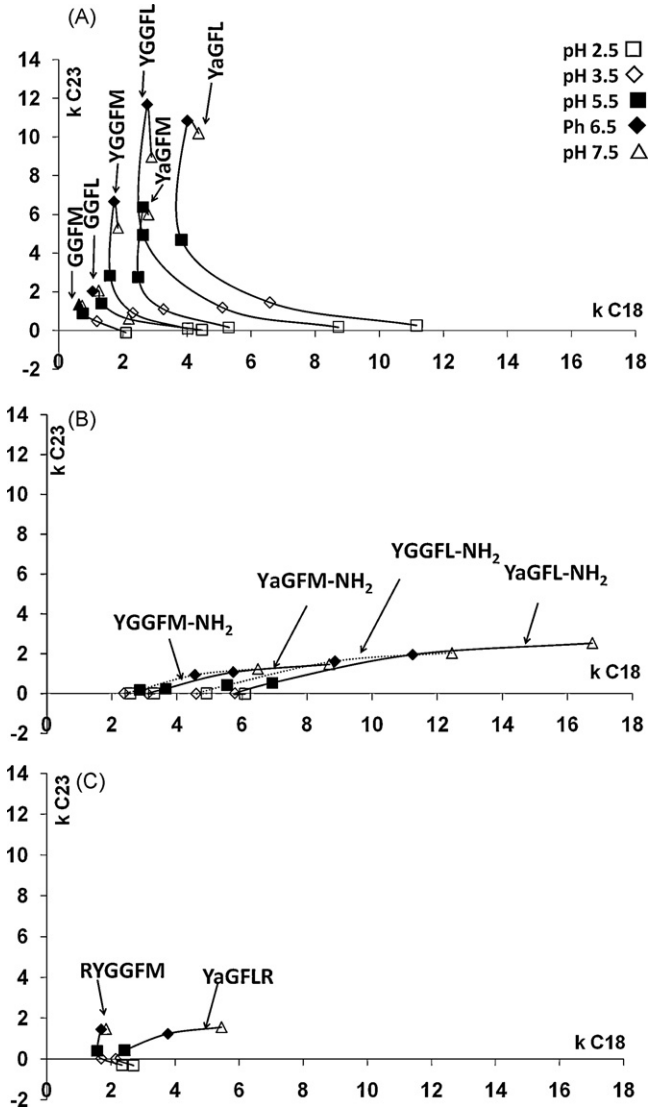

Fig. 1. Correlation between ENK retention factors (k) on BS-C23 and C18 for the three groups of ENKs: (A) group (1), (B) group (2) and (C) group (3), at all tested buffer pH values, □: pH 2.5, ◇: pH 3.5, ■: pH 5.5, ♦: pH 6.5, △: pH 7.5. Column: stability BS-C23, 250 mm × 2 mm, d_p = 5 μm, mobile phase: phosphate buffer (37 mM)/ACN, 75:25 (v/v), flow rate: 0.3 mL/min, injection volume: 10 μL. Column: Kromasil C18, 250 mm × 4.6 mm, d_p = 5 μm, mobile phase: phosphate buffer (37 mM)/ACN, 80:20 (v/v), flow rate: 1 mL/min, injection volume: 20 μL. Temperature: 30 °C, λ: 205 nm.

could be attributed to an electrostatic attraction (between the ionized C-terminal group and the embedded quaternary ammonium) combined with RP mechanisms. We can see from Fig. 1A that the retention factor increases were more significant with ENKs exhibiting a higher hydrophobicity (*e.g.* ALE containing a hydrophobic Leu residue *vs.* AME with a Met one).

The ENKs of groups (2) and (3) were more retained on C18 than on BS-C23 over the pH range studied (Fig. 1B and C). This could be explained by the predominance of electrostatic repulsion between these peptides and the positively charged embedded group in the BS-C23. By increasing the pH, the decrease of their net positive charge led to a growing contribution of the RP mechanism in their retention. However, this contribution was still less on BS-C23 than on C18 due to its lower hydrophobicity [32], thereby, lowering retention factors for these peptides on BS-C23.

Ionic interactions between ENKs and the quaternary ammonium-embedded group, led to a selectivity differing from that with C18. Indeed, as depicted in Table 2, we can observe

Table 2
Comparison of ENK selectivities ($\alpha_{A/B}$, between peptides A and B) on stability BS-C23 and Kromasil C18 at two buffer pH values: pH 6.5 and pH 7.5.

Pair of peptides (A/B)	$\alpha_{A/B}$			
	pH 6.5		pH 7.5	
	C23	Kromasil	C23	Kromasil
LE/LEA	7.20	0.24	4. 40	0.17
ALE/ALEA	5.52	0.45	4.02	0.35
ME/MEA	7.11	0.38	4.26	0.28
AME/AMEA	5.93	0.46	4.06	0.32

$\alpha_{A/B}$ is always calculated as k_A/k_B.

an inversion of elution order between the ENKs of group (1) and their structurally related peptides from group (2) between the two columns. This was related to the modification of the C-carboxyl terminal group of these peptides (–COO^- for group (1) and –CO–NH_2 for group (2)), demonstrating the influence of the electrostatic attraction on retention of ENKs from group 1 with the BS-C23 column.

3.2. Effect of buffer type

A previous study demonstrated a decrease of retention factors for several peptides above pH 7.0 on the BS-C23 [32]. Different explanations were proposed, in particular the increase in overall negative charge of the buffer phosphate ions. The same trend was observed in this study for ENKs from group (1). To understand this phenomenon, the effect of the buffer composition on ENK retention was assessed on BS-C23. TRIS was chosen as the buffer because the valences of the anion and the cation constituting this buffer remain constant at the different pHs studied. Consequently, the variation of peptides retention would essentially be related to the peptide ionic states.

In the pH range 3.5–6.5, the comparison of the retention factors obtained with these two buffers showed the same retention behaviour with no change in selectivity between ENKs (data not shown). In contrast, at pH 7.5, the decrease of ENK retention factors, observed with phosphate buffer and described in the previous section, did not occur with TRIS buffer. However, as reported by several authors [37,38], the addition of ACN would change the pKa of the phosphate salts, leading to a much higher pH as compared to TRIS. Therefore, the proportion of ionized amino groups of enkephalins would decrease when using ACN/phosphate mixture, increasing their retention factors. However, this was not observed, we can hypothesis therefore that the retention decrease of enkephalin group 1, observed at pH 7.5, is related to the nature of the phosphate buffer itself [32]. Thus, the increase in negative charge of the phosphate anions, by increasing the pH from 6.5 to 7.5, increased interactions between these anions and the quaternary ammonium groups of the sorbent, preventing potential electrostatic interactions with the negatively charged ENKs.

3.3. Effect of type and concentration of eluent anion

We supposed that the ionic medium surrounding the quaternary ammonium group could impact on the interactions between the ENKs and the stationary phase, as observed with phosphate buffer at pH 7.5 (Section 3.2). To test this assumption, the effects of three anions (perchlorate, chloride, phosphate) were investigated at two pH values (2.5, 7.5). Phosphate buffer was always used to fix the pH at 2.5 and 7.5. To extend this study, we have then evaluated the effects of ACN content on the retention of ENKs comparing results obtained with phosphate or perchlorate anions at different concentration.

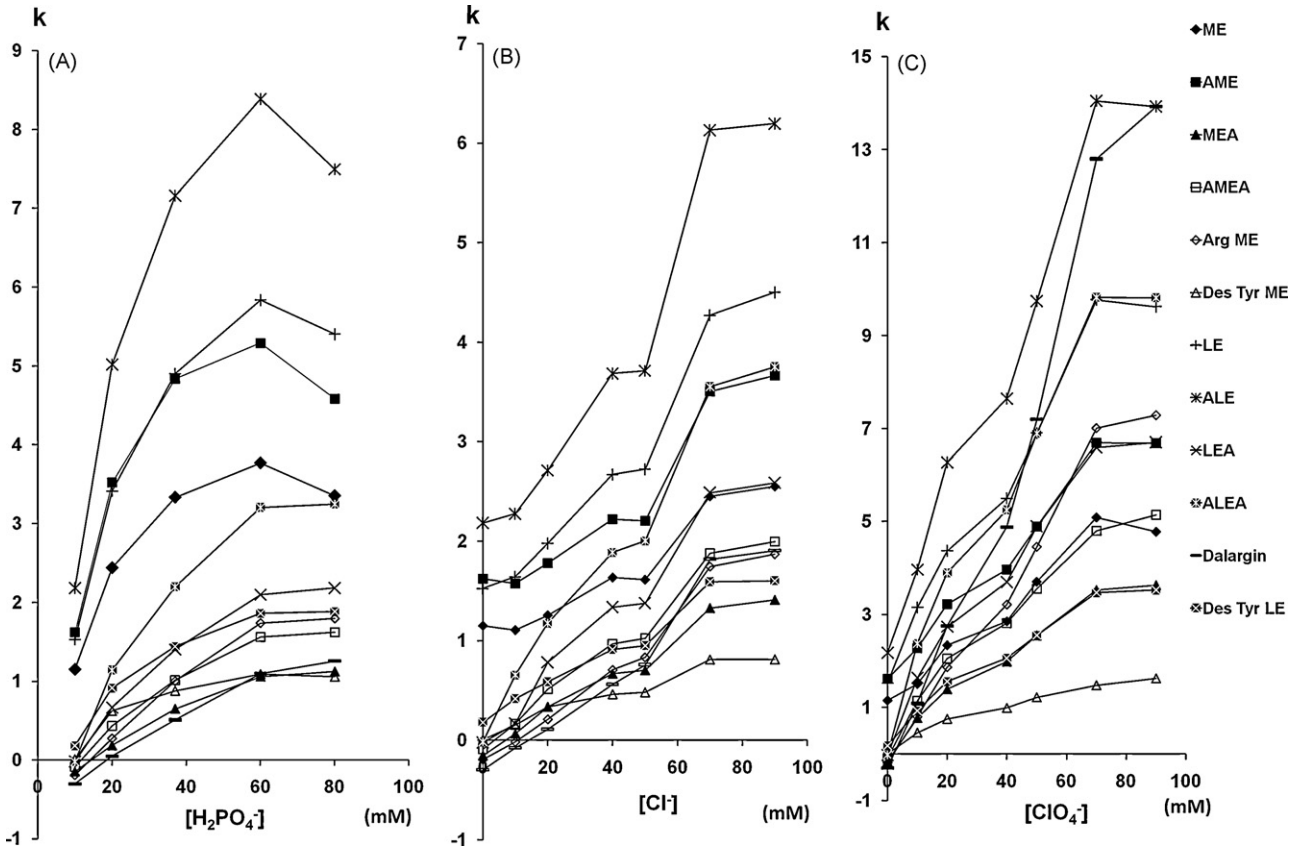

Fig. 2. Plot of ENK *k vs*. dihydrogen phosphate anion (A), chloride anion (B), perchlorate anion (C) concentration [mM]. Column: BS-C23. Phosphate anions, mobile phase: phosphate buffer pH 2.5 at various concentrations/ACN, 90:10 (v/v). Perchlorate and chloride anions, initial conditions of mobile phase: sodium phosphate buffer (10 mM) pH 2.5/ACN, 90:10 (v/v). Other experimental conditions as mentioned in Fig. 1.

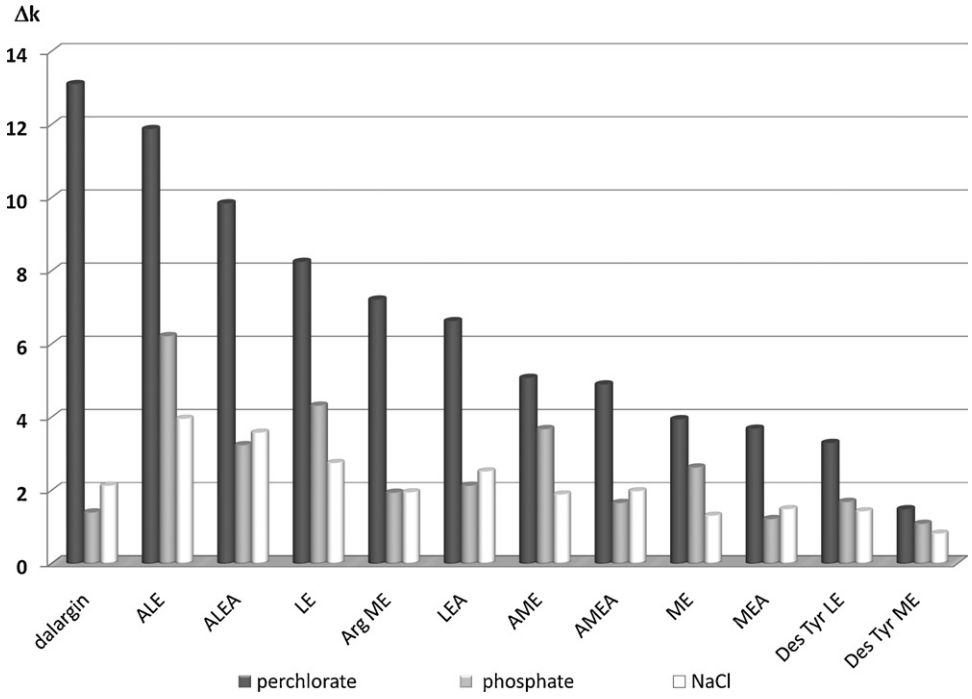

Fig. 3. Absolute increase in retention factors (Δk) using the three eluent anions (phosphate, chloride and perchlorate). Δk is the difference between the maximal retention factor and the retention factor in initial conditions (10 mM of phosphate buffer pH 2.5/ACN, 90:10, v/v) for the investigated peptides.

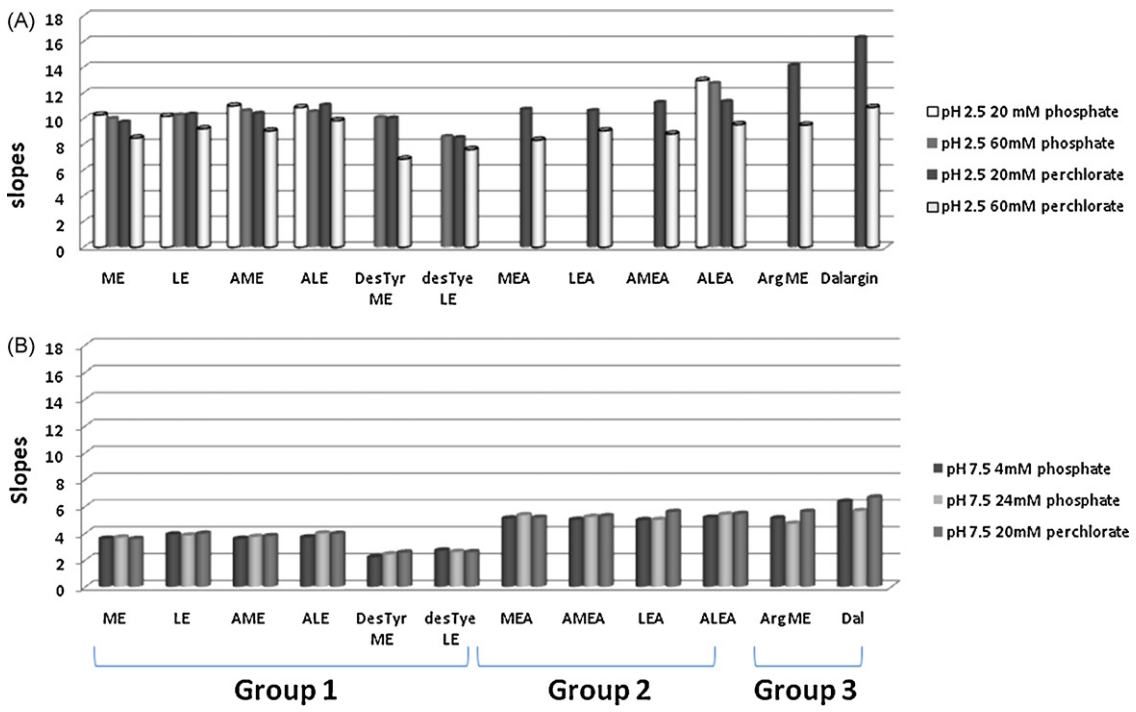

Fig. 4. Comparison of ENK slopes obtained from the regression of log *k* *vs.* volume fraction of ACN in the mobile phase at two pH values (A) pH 2.5; phosphate anion concentration (20 and 60 mM), perchlorate anion concentration (20 and 60 mM) and (B) pH 7.5; phosphate anion concentration (4 and 24 mM), perchlorate anion concentration (20 mM). Column: BS-C23. Other experimental conditions as mentioned in Fig. 1.

3.3.1. pH 2.5

At low pH, due to the cation exclusion mechanism, an early elution of the protonated ENKs was observed (Fig. 2). As expected, the increase of phosphate, chloride or perchlorate ion concentration produced an increase in the peptide retention factor, for all the ENKs until a constant value was reached, around 70 mM. This concentration may correspond to a saturation of the electrostatic interactions between the anions and the cationic embedded

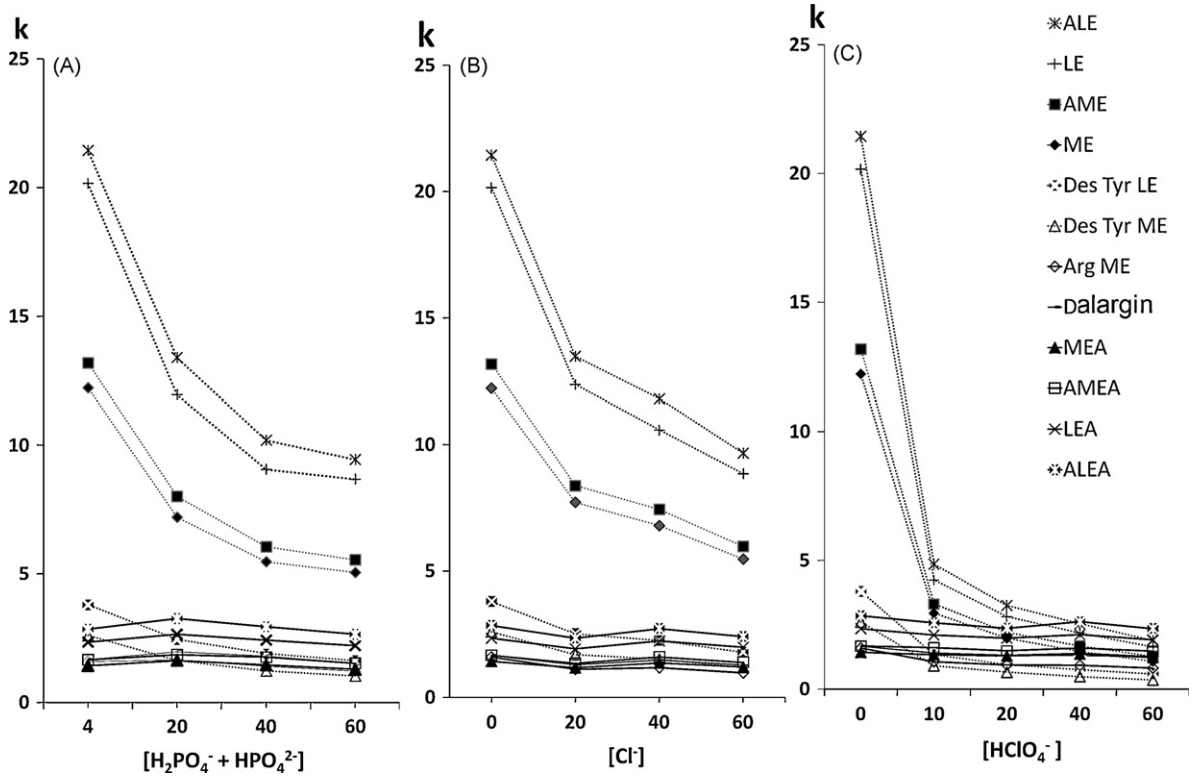

Fig. 5. Plot of ENK *k* *vs.* phosphate anion (A), chloride anion (B), perchlorate anion (C) concentration [mM]. Column: BS-C23. For phosphate anions, mobile phase: phosphate buffer pH 7.5/ACN, 75:25 (v/v). For perchlorate and chloride anions, initial conditions of mobile phase: phosphate buffer (4 mM) pH 7.5/ACN, 75:25 (v/v). Other experimental conditions as mentioned in Fig. 1.

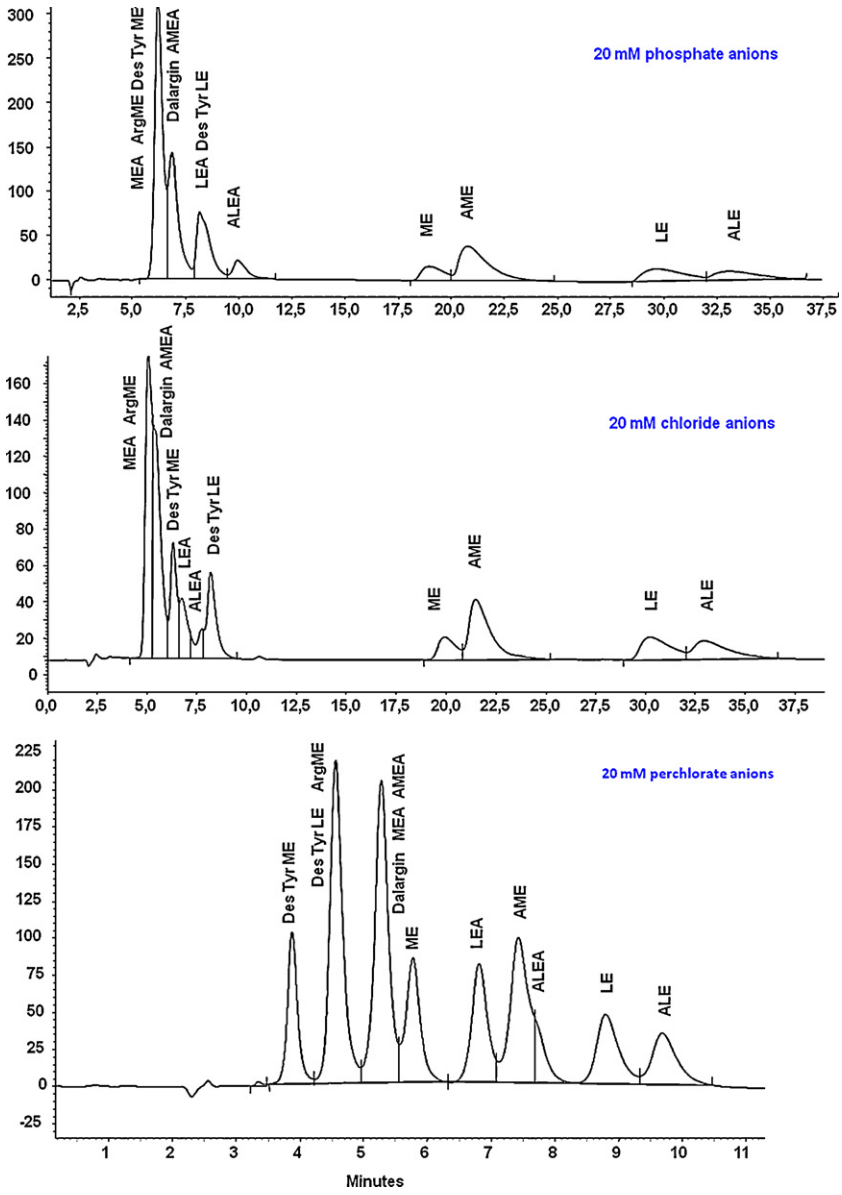

Fig. 6. Separation of a mixture of the twelve enkephalins using mobile phases containing 20 mM of phosphate anion (A), chloride anion (B), perchlorate anions (C). Column: BS-C23. Other experimental conditions as mentioned in Figs. 4 and 1.

group and /or with the ENKs. The increase of retention factor is probably due to the reduction of the repulsive forces between the peptides and the cationic embedded group, due to the electrostatic screening effect which occurs as ionic strength increases [39]. However, to better understand the effects related to the presence of these anions, we compared graphically the absolute increases in retention factors (Δk) obtained with these anions (Fig. 3), where Δk is the difference between the retention factor measured under initial mobile phase conditions (1) (ACN/10 mM phosphate buffer, 10:90, v/v) and k_{max} obtained with conditions (1) supplemented with the addition of eluent anion. Perchlorate anions led to Δk two or four fold higher than phosphate or chloride. This was consistent with the theory of chaotropicity which classifies the anions in order of increasing chaotropicity: $H_2PO_4^- < HCOO^- < CH_3SO_3^- < Cl^- < NO_3^- < CF_3COO^- < BF_4^- < ClO_4^- < PF_6^-$ [40–44]. Indeed, chaotropic anions such as perchlorate, which are less polar than water, destroy the network of hydrogen bridges (chaotropic order-breaking), and also the solvation shell surrounding the positively charged ENKs in aqueous media. This leads to their desolvation and consequently increases their hydrophobicity, resulting in retention increase due to the predominant RP mechanism. In addition to the chaotropic effects, an ion-pairing phenomenon between the additive anions and the positively charged ENKs could be proposed. Perchlorate ions possess a higher ability to establish ion-pairing interactions with cationic compounds as compared to phosphate and chloride anions [45–47]. Perchlorate induces therefore a dramatic increase of ENK hydrophobic interactions with the alkyl chain of BS-C23, leading to higher retention factors.

This retention enhancement observed with perchlorate anions depended however on the number of positively charged groups and the hydrophobicity of the ENKs. As an example, retention increase of Dalargin (two amino groups) was greater than that of ALE (one amino group only) and Arg ME (Arg ME has two amino groups but is less hydrophobic than Dalargin). This confirms the hypothesis that peptide hydrophobicity plays an important role in determining the extent to which chaotropic anions affect the retention of these protonated peptides. For chloride and phosphate (two hydrophilic anions), the increase in Δk only reflected the hydrophobicity of ENKs. From these observations, we concluded

that the improvement of separation and selectivity of ENKs, at acidic pH, significantly depends on the type and concentration of eluent anion.

In order to further understand the influence of the additive anions towards the reversed-phase partitioning contribution, the effect of ACN content on ENK retention has been investigated at different concentrations of phosphate or perchlorate anions. The chloride effect is not reported here as it leads to a similar behaviour than phosphate. The log of ENK retention factors were plotted *vs*. volume fraction of ACN in the mobile phase. The log *k* decreased linearly with the increase of ACN content, indicating the contribution of a reversed-phase partitioning in ENK retention. Fig. 4A presents the slopes of ENKs obtained from this regression. However, when using phosphate anions, some ENKs from groups (2) and (3) exhibited a weak retention rendering slopes estimation difficult.

With perchlorate anions, we observed, for all the peptides, that an increase of eluent anion concentration led to a decrease of their slopes. These results could be explained by an ion-pairing phenomenon between peptides and perchlorate anions leading to lower slope values at the concentration of 60 mM. This is in agreement with the hypothesis proposed by Wang and Carr [48]. This effect which was more pronounced with ENKs from group (3) (due to their two positive amino groups) supports this hypothesis.

3.3.2. pH 7.5

At pH 7.5, the carboxyl terminal group of ENKs is ionized but the ENKs still have positively charged groups (amine lateral and/or amine terminal). Therefore, when we increased the anion concentration, we supposed that two processes could occur for the retention of the ENKs; (i), interactions between ENK positive charges and the eluent anions, by the mechanisms described at pH 2.5, which should increase their retention, and (ii) anion-exchange mechanisms resulting from the competition between peptide negative charges and eluent anions, which would reduce the peptide interactions with the embedded cationic group and decrease the retention factors. In addition, as the concentration of sodium ions increased by increasing eluent anion concentration, another mechanism related to the presence of the residual silanols could be involved in the retention of ENKs.

At this neutral pH, an increase of phosphate, chloride or perchlorate ion concentration in the mobile phase led to a decrease in the retention factors of ENKs belonging to group (1) and to a slight modification of those of ENKs from groups (2) and (3) (Fig. 5). However, the retention decreases of ENK from group (1) were sharper with perchlorate than with chloride and phosphate ions. Indeed, perchlorate anions, comparatively large ions with a symmetric charge distribution, establish strong interactions with the positively charged groups in the stationary phase, compared to chloride and phosphate anions. Strong interactions of perchlorate ions were also reported with inner positive charges on zwitterionic stationary phases [49,50]. This agrees with the retention order of these anions ($ClO_4^- > Cl^- > H_2PO_4^-$) on anion-exchange materials [51]. These decreases highlighted the significant contribution of the anion-exchange mechanism in the retention of these peptides, negatively charged at pH 7.5. In accordance to the simple empirical stoichiometric displacement model of the ion-exchange mechanisms [52], log *k* of ENKs from group (1) was plotted against log of anion concentration. ENK group (1) exhibited similar slope values with both phosphate and chloride anions (−0.3), while with perchlorate, higher slopes (between −0.4 and −0.5) were obtained (data not shown). Perchlorate anions also had a greater effect on improvement of peak symmetry and column efficiency than chloride and phosphate anions did (data not shown). The effects of ACN content on the retention of ENKs have been finally investigated at pH 7.5 in the presence of different concentration of phosphate or perchlorate anions. The log of retention factors of ENKs were plotted *vs*. volume fraction of ACN in the mobile phase. As for pH 2.5, these logs decreased linearly with the increase of ACN content, demonstrating a contribution of reversed-phase mechanism in ENK retention on this mixed column. The obtained slopes from these regressions were compared for the 12 ENKs (Fig. 4B). Interestingly, we observed that slopes were quite similar with the different type of anion, demonstrating that the ion-pairing between the peptide positive charges and the perchlorate anions was negligible at pH 7.5. This could be explained by the fact that these peptides possess less positive charges at pH 7.5 than at pH 2.5.

Thus, the various anions differ in their ability to establish an anion-exchange mechanism, leading to a difference in selectivity. To illustrate the significant role of the eluent anion, the separation of a complex mixture of the twelve ENKs, at pH 7.5, with 20 mM concentrations of phosphate, chloride or perchlorate ions is shown in Fig. 6. The elution order of ENKs depends on the type of anion used in the eluent, the perchlorate anion offering an improved separation combined with a shorter analysis time.

4. Conclusion

This study showed the benefits of the unique selectivity, previously observed on the mixed-mode stationary phase (Stability BS-C23) in comparison to a RP column, for the simultaneous separation of peptides, sharing structural similarities (*e.g.* enkephalins). The separation mechanism is a superimposition of reversed-phase and electrostatic interactions with this stationary phase. We demonstrated that the extent to which each mechanism contributes to the enkephalin retention is mainly dependent on the buffer pH, but also on the nature and concentration of eluent anions. Experiments comparing the effect of different anions such as phosphate, chloride, and perchlorate showed that various concepts were involved in the peptide retention including ion-pairing and chaotropic effects, at acidic pHs, and anion-exchange properties, at neutral pHs. This study is useful for predicting the effect of mobile phase composition during the development of peptide separation protocols with this mixed-mode stationary phase (BS-C23).

Acknowledgements

We thank Tichrine-University of Syria for providing Ayat ABBOOD's scholarship. This work was supported in part by the FW6 Neurotas project.

References

[1] N. Sewald, H.-D. Jakubke, Peptides: Chemistry and Biology, Wiley-VCH Verlage, Weinheim, 2002, p. 61.
[2] R.J. Bodnar, Peptides 29 (2008) 2292.
[3] J.-M. Scherrmann, J. Temsamani, J. Pharmaco, Exp. Ther. 306 (2003) 371.
[4] V. Kašička, Z. Prusik, J. Pospíšek, J. Chromatogr. 608 (1992) 13.
[5] V. Pacáková, J. Suchánková, K. Štulík, J. Chromatogr. B 681 (1996) 69.
[6] I.M. Lazar, E.D. Lee, A.L. Rockwood, M.L. Lee, J. Chromatogr. A 829 (1998) 279.
[7] V. Šolínová, V. Kašička, T. Barth, L. Hauzerová, S. Fanali, J. Chromatogr. A 1081 (2005) 9.
[8] J.R. Catai, G.W. Somsen, G.J. de Jong, Electrophoresis 25 (2004) 817.
[9] J. Jiskra, V. Pacáková, M. Tichá, K. Štulík, T. Barth, J. Chromatogr. A 761 (1997) 285.
[10] A. Fürtös-Matei, J.J. Li, K.C. Waldron, J. Chromatogr. B 695 (1997) 39.
[11] T. Stroink, G. Wiese, J. Teeuwsen, H. Lingeman, J.C.M. Waterval, A. Bult, G.J. de Jong, W.J.M. Underberg, Electrophoresis 24 (2003) 897.
[12] T. Stroink, P. Schravendijk, G. Wiese, J. Teeuwsen, H. Lingeman, J.C.M. Waterval, A. Bult, G.J. de Jong, W.J.M. Underberg, Electrophoresis 24 (2003) 1126.
[13] S. Mousa, D. Couri, J. Chromatogr. 267 (1983) 191.
[14] S. Shibanoki, S.B. Weinberger, K. Ishikawa, J.L. Martinez, J. Chromatogr. 532 (1990) 249.
[15] M. Ohno, M. Kai, Y. Ohkura, J. Chromatogr. 421 (1987) 245.
[16] V.-P. Ranta, A. Urtti, S. Auriola, J. Chromatogr. A 766 (1997) 85.
[17] V.-P. Ranta, K.M. Hämäläinen, S. Auriola, A. Urtti, J. Chromatogr. B 709 (1998) 1.
[18] T.A. Ivandini, B.V. Sarada, C. Terashima, T.N. Rao, D.A. Tryk, H. Ishiguro, Y. Kubota, A. Fujishima, J. Chromatogr. B 791 (2003) 63.

[19] K. Soukupová, E. Krafková, J. Suchánková, E. Tesařová, J. Chromatogr. A 1087 (2005) 104.
[20] T.L. Lisi, K.A. Sluka, J. Neurosci. Methods 150 (2006) 74.
[21] R. Nogueira, M. Lämmerhofer, W. Lindner, J. Chromatogr. A 1089 (2005) 158.
[22] F. Progent, M. Taverna, A. Banco, A. Tchapla, C. Smadja, J. Chromatogr. A 1136 (2006) 221.
[23] R. Nogueira, D. Lubda, A. Leitner, W. Bicker, N.M. Maier, M. Lämmerhofer, W. Lindner, J. Sep. Sci. 29 (2006) 966.
[24] N.H. Davies, M.R. Euerby, D.V. McCalley, J. Chromatogr. A 1138 (2007) 65.
[25] J. Li, S. Shao, M.S. Jaworsky, P.T. Kurtulik, J. Chromatogr. A 1185 (2008) 185.
[26] X. Liu, C.A. Pohl, J. Weiss, J. Chromatogr. A 1118 (2006) 29.
[27] E. Apfelhalter, W. Bicker, M. Lämmerhofer, M. Sulyok, R. Krska, W. Lindner, R. Schuhmacher, J. Chromatogr. A 1191 (2008) 171.
[28] J.S.O. McCullagh, D. Juchelka, R.E.M. Hedges, Rapid Mass Spectrom. 20 (2006) 2761.
[29] R. Bischoff, L.W. McLaughlin, J. Chromatogr. 296 (1984) 329.
[30] W.S. Hancock, J.T. Sparrow, J. Chromatogr. 206 (1981) 71.
[31] M. Michel, T. Baczek, S. Studzinska, K. Bodzioch, T. Jonsson, R. Kaliszan, B. Buszewski, J. Chromatogr. A 1175 (2007) 49.
[32] A. Abbood, C. Smadja, C. Herrenknecht, Y. Alahmad, A. Tchapla, M. Taverna, J. Chromatogr. A 1216 (2009) 3244.
[33] F. Progent, M. Taverna, J. Chromatogr. A 1052 (2004) 181.
[34] C. Stella, S. Rudaz, J.-Y. Gauvrit, P. Lantéri, A. Huteau, A. Tchapla, J.-L. Veuthey, J. Pharm. Biomed. Anal. 43 (2007) 89.
[35] M.R. Euerby, P. Petersson, J. Chromatogr. A 994 (2003) 13.
[36] N.S. Wilson, J. Gilroy, J.W. Dolan, L.R. Snyder, J. Chromatogr. A 1026 (2005) 91.
[37] S. Espinosa, E. Bosch, M. Rosés, Anal. Chem. 72 (2000) 5193.
[38] L.G. Gagliardi, C.B. Castells, C. Ràfols, M. Rosés, E. Bosch, Anal. Chem. 79 (2007) 3180.
[39] J. Ståhlberg, J. Chromatogr. A 855 (1999) 3.
[40] R. LoBrutto, A. Jones, Y.V. Kazakevich, J. Chromatogr. A 913 (2001) 189.
[41] A. Jones, R. LoBrutto, Y. Kazakevich, J. Chromatogr. A 964 (2001) 179.
[42] R. LoBrutto, A. Jones, Y.V. Kazakevich, H.M. McNair, J. Chromatogr. A 913 (2001) 173.
[43] J.M. Roberts, A.R. Diaz, D.T. Fortin, J.M. Friedle, S.D. Piper, Anal. Chem. 74 (2002) 4927.
[44] J. Flieger, J. Chromatogr. A 1113 (2006) 37.
[45] T.J. Sereda, C.T. Mant, R.S. Hodges, J. Chromatogr. A 776 (1997) 153.
[46] J. Dai, P.W. Carr, J. Chromatogr. A 1072 (2005) 169.
[47] M. Shibue, C.T. Mant, R.S. Hodges, J. Chromatogr. A 1080 (2005) 49.
[48] X. Wang, P.W. Carr, J. Chromatogr. A 1154 (2007) 165.
[49] H.A. Cook, W. Hu, J.S. Fritz, P.R. Haddad, Anal. Chem. 73 (2001) 3022.
[50] E.P. Nesterenko, P.N. Nesterenko, B. Paull, J. Chromatogr. A 1178 (2008) 60.
[51] J.S. Fritz, D.T. Gjerde, Ion Chromatography, Wiley-VCH, Weinheim, 2000.
[52] W. Kopaciewicz, M.A. Rounds, J. Fausnaugh, F.E. Regnier, J. Chromatogr. 266 (1983) 3.