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Comparison of a three-peptide separation by capillary electrochromatography, voltage-assisted liquid chromatography and nano-high-performance liquid chromatography

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Abstract

A mixture of three peptides was separated by capillary electrochromatography (CEC), nano-HPLC and voltage-assisted LC. In the latter case the charged analytes migrate through a neutral stationary phase driven by electrophoresis while their interaction with the stationary phase provides the basis for a chromatographic separation. The stationary phases used were poly(glycidyl methacrylate-co-ethylene dimethacrylate)-based monoliths that could be used directly as neutral "C1"-type columns for voltage-assisted LC and nano-HPLC, while their application in CEC became possible after derivatization of the epoxy groups with ionogenic *N*-ethylbutylamine functions. The separation of the peptide mixture was possible in all three modes. Highest plate numbers and resolutions were obtained under voltage-assisted conditions. The elution order showed dependencies on the charge density but also on the hydrophobicity of the peptides and was different in the three investigated chromatographic modes. The effect of changes in the ionic strength and the organic solvent content of the mobile phase on the resolution and the migration behavior of the peptides was investigated and showed the expected behavior. Voltage-assisted LC is suggested as an alternative to CEC for the separation of charged analytes by electrochromatography.

Keywords: Electrochromatography; Voltage-assisted liquid chromatography; Angiotensins; Methionine enkephalin; Peptides

1. Introduction

Capillary electrochromatography (CEC) continues to intrigue the analytical community. The potential for the development of a versatile methodology for high resolution microanalysis is there, yet not much progress has been made in resolving the theoretical and technical difficulties recognized since more than a decade ago especially in the case of charged analytes [1]. In the beginning often seen as a mere hybrid of capillary electrophoresis (CE) and HPLC, CEC has demonstrated the potential to transcend both and become an analytical tool in its own right and with its own array of applications. The potential of CEC has been amply demonstrated by a burst of exemplary separations and applications [2–4].

While the analysis of uncharged compounds by CEC poses few problems and the results can usually be interpreted by established chromatographic theory [5], this is not the case for charged analytes, i.e. most molecules of biological interest [2,6]. The reasons for this lack are mostly related to the complex contribution of different factors to the observed separation. The migration velocity of a charged analyte in CEC will always depend on a mixture of chromatographic, electrophoretic and electroosmotic phenomena, even though under certain conditions one of these can predominate. Moreover, since the stationary phase in CEC has to bear charges in order to produce the electroosmotic flow (EOF), any chromatographic mode save perhaps for pure ion-exchange chromatography, will be based on a mixed mode type of interaction. The use of silica-based (charged) reversed phase stationary phases in CEC is a classic in this context.

The knowledge basis for such separations is to date only poorly developed. Only recently have the first theoretical models for concomitant electrokinetic mass transport and adsorptive interaction of charged analytes been published

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[6–8]. Systematic experimental studies of these theoretical predictions are to date still rare. The same is true for systematic comparisons between CEC and related separation modes such as capillary zone electrophoresis (CZE), nano-LC and voltage-assisted LC.

Walhagen et al. investigated the influence of temperature on the behavior of small linear peptides in CEC [9]. A column packed with *n*-octadecyl bonded silica particles was used as the stationary phase. The EOF as well as the column efficiencies were found to increase with increasing temperature, whereas the retention coefficients of the peptides decreased. The same group compared the CEC separation of four synthetic peptides on columns packed with *n*-octyl and *n*-octadecyl silica particles, as well as a mixed mode C_{18}/strong cation-exchange (SCX) phase to CZE and HPLC [10]. They observed different elution patterns between the various techniques, but also between CEC separations on the reversed-phase packing compared to the mixed-mode packing, and even between two kinds of mixed-mode packings from different manufacturers. The separation mechanism was said to be a mixture between electrophoretic migration and chromatographic interaction. These results were confirmed in a further study separating cyclic and linear peptides on the same stationary phases [11].

Zhang et al. prepared a 'tentacle'-type anion exchanger, by derivatizing 5 μm silica beads with quaternary ammonium groups through hydrophilic spacers [12]. Proteins were eluted at a pH above their isoelectric point. The separation of the proteins was described as principally governed by ion-exchange interactions with superimposed electrophoretic migration of the analytes in solution. Assuming strict additivity of the two contributions, an electrochromatographic resolution equation was derived. Ye et al. investigated the separation of peptides on columns packed with a strong cation-exchange material [13]. They achieved the isocratic separation of ten short peptides in less than 3.5 min, with plate numbers of up to 460 000/m. They also noted selectivity differences in their system compared to RP-CEC and CZE. However, all peptides included in the experiments bore only a single positive charge at the amino terminus and did not contain any charged side chains.

The group of Horváth has published series of papers comparing various monolithic stationary phases for use in HPLC and CEC [14–16]. Counter-directional separation of proteins and peptides was performed at different ionic strengths (pH 2.5) and organic phase contents. Selectivity was mainly modulated by the acetonitrile content of the mobile phase [15]. Interestingly, the retention factors of the proteins decreased with increasing acetonitrile content, whereas the retention factors of the peptides increased. Under optimum conditions separations of the peptide and protein mixtures were possible in less then 10 min. A temperature increase from 25 to 55 °C resulted in an almost 2-fold increase in the speed of the analysis [16].

Adam and Unger reported about reversed phase gradient nano-liquid chromatography for the separation of peptides [17] where the efficiency of the separation could be improved by applying an electric field (EOF generation). A similar approach using a mixed-mode stationary phase containing both octadecylsilanes and dialkylamines for EOF production, was presented by Huang et al. [18]. Depending on the magnitude of the applied voltage (in addition to some pressure), significant variations in the retention and the selectivity of the peptides were observed. In an attempt to overcome the pH dependency of the EOF typically seen with most silica-based materials, the group of Hjertén described a sophisticated system, where continuous beds were adapted to CEC with gradient elution of proteins [19]. The acrylamide based polymers were derivatized with C_{18} and ammonium groups. An HPLC gradient system was connected to the inlet of the CEC system. The directions of the electroosmotic flow and the electrophoretic migration of the proteins respectively could be modulated independently through the ammonium group-content on the stationary phase for the former and the polarity of the applied voltage and the mobile phase composition for the later. Thus normal-flow and counterflow gradients could be produced with the same system.

Wu et al. suggested using monolithic columns without EOF generating moieties [20]. Charged compounds elute electrophoretically from such columns, while interacting chromatographically with the neutral hydrophobic stationary phase. A set of peptides was successfully separated on the basis of their differences in electrophoretic mobility and chromatographic retention. Some peptide isomers (Trp–Ala and Ala–Trp), which could not be separated by CZE, were successfully separated by this method.

A few studies on the behavior of other charged analytes than proteins and peptides have also been reported. In the context of a separation of chiral pairs of derivatized amino acids, Lämmerhofer et al. investigated general aspects of the influence of the stationary phase and the chromatographic conditions on separation efficiency and selectivity [21,22]. Positively charged quinidine functionality served equally as EOF generator and chiral selector. An increase in the counterion concentration (e.g. by high acetic acid content in the mobile phase) improved the efficiency without affecting the selectivity. However, such an increase was limited to the point, where Joule heat production caused unstable currents and poor reproducibility. Comparing organic and hydroorganic mobile phases, they found the latter to be superior in terms of efficiency and effective retention factors, presumably due to the higher elution strength of the more polar aqueous medium. An efficiency increase could also be obtained by increasing the polarity of the stationary phase, i.e. by replacing the glycidyl methacrylate (GMA) with the more polar 2-hydroxyethyl methacrylate (HEMA). This effect was attributed to the reduction of non-specific interactions. A monolithic stationary phase of even more hydrophilic character (weak and strong anion exchanger) was prepared by the same group [23]. These stationary phases were used for the CEC separation of various organic anions. The elution order resembled largely that of normal-phase chromatography.

The basis for the separation of charged proteins and peptides by differential migration under CEC conditions is obviously highly convoluted. As a contribution to a better understanding of the involved phenomena we would like to report in this paper on a recent systematic investigation of the separation of a peptide mixture under CEC, voltage-assisted and nano-HPLC conditions.

2. Materials and methods

2.1. Materials

1-Heptanol, α,α′-azoisobutyronitrile (AIBN) (>98%), cyclohexanol, analytical-reagent grade monobasic and dibasic sodium phosphates, dimethyl sulfoxide (DMSO) (99%), ammonium sulfate (99%), phosphoric acid (85%), sodium hydroxide (98.8%), acetonitrile (HPLC grade), methanol (HPLC grade), acetone (HPLC grade) and fuming hydrochloric acid (37%) were from Fluka (Buchs, Switzerland). Methyl methacrylate (99%), glycidyl methacrylate (97%) and *N*-ethylbutylamine (99%) were from Aldrich (Milwaukee, WI, USA), ethylene dimethacrylate (98%), γ-methacryloxypropyltrimethoxysilane (98%), angiotensin I (acetate salt), angiotensin II (acetate salt), and methionine enkephalin (acetate salt) were from Sigma (St. Louis, MO, USA). All chemicals including the monomer were used without further purification. Water was purified and deionized with a SG ultrapurification system (Barsbüttel, Germany). The fused-silica capillaries (75 μm i.d. × 360 μm o.d.) were from Polymicro Technologies (Phoenix, AZ, USA). The effective length of the capillary columns was 28 cm (inlet-to-detector, corresponding to a total length of 37 cm) for CEC and voltage-assisted LC and 15 cm for nano-HPLC applications.

2.2. Instrumentation

A Hewlett-Packard ^{3D}CE system (Hewlett-Packard, Waldbronn, Germany) upgraded for operation in the CEC mode (12 bar pressure option) was used for CEC and voltage-assisted LC. The nano-HPLC system was an Ultimate Capillary HPLC system (LC Packings, Amsterdam, The Netherlands). The connecting tubings and the calibrator cartridge (ULT-NAN-75) were adapted for nano-flow conditions (columns of 75 μm i.d.). An internal injection loop (10 nL) from Valco (Schenkon, Switzerland) was used for manual sample injection. An Ultimate detector with a U-Z capillary flow cell (3 nL volume) was used for detection.

2.3. Stationary phase preparation

The first step in the preparation of the monolithic stationary phases was the silanization of the fused silica capillaries. For this purpose capillaries were flushed with 0.2 M NaOH (30 min), 0.2 M HCl (30 min) and water (30 min). Then a 30% (v/v) solution of γ-methacryloxypropyl-trimethoxysilane in acetone was pumped through for 15 min. Afterwards the capillary ends were sealed with GC septa and kept overnight at room temperature. Just before introduction of the polymerization mixture, the capillary was flushed with methanol. For the synthesis of the actual stationary phase a solution containing 10% (v/v) each of glycidyl methacrylate and methyl methacrylate, 20% (v/v) of ethylene dimethacrylate, 50% (v/v) of 1-heptanol and 0.3% (w/v) of the initiator (AIBN) was prepared and degassed with nitrogen for 15 min. Then the "porogen" 10% (v/v) cyclohexanol (bad solvent for the polymer) was added to the solution, which was again degassed with nitrogen for 15 min. The final solution was filled into the silanized capillary by means of a water-jet pump. After both ends had been sealed with GC septa, the capillary was heated to 60 °C for 16 h (oven: UM 200, Memmert, Schwabach, Germany). Subsequently, the capillary was washed with methanol for 1 h by means of an HPLC pump (433, Kontron, Watford, UK) and then heated for 2 h at 80 °C in a nitrogen stream. The drying step is necessary to reduce the number of hydrolyzed epoxy groups.

Stationary phases intended for CEC were further derivatized with ionogenic groups. For this purpose the capillary containing the porous monolithic support was filled with *N*-ethylbutylamine. Both ends were sealed, then the capillary was heated at 70 °C overnight and washed with methanol.

The detection window was created by burning off a section of the outer polyimide coating and at the same time locally pyrrolyzing the monolith inside [24]. Before, during and after the creation of the detection window, the capillaries were flushed with methanol for a total of about 2 h, applying flow rates of 0.045 mL/min, in order to remove the non-reacted monomers and the ashes produced during pyrrolyzation. Before use, every column was inspected under the microscope for uniformity of the stationary phase. Columns, which showed irregularities were discarded.

2.4. Separation conditions

The following conditions were generally used in the CEC experiments. The mobile phase was a 10–60 mM aqueous sodium phosphate buffer, pH 7.0, containing the indicated amount of acetonitrile. DMSO (2 μL/ml in water) was used as EOF marker. The sample contained 1 mg/mL each of angiotensins I and II and methionine enkephalin in 30 mM sodium phosphate buffer, pH 7.0. The peaks were assigned by spiking the sample with the compound of interest. For voltage-assisted LC the mobile phase was a 5–60 mM aqueous sodium phosphate buffer, pH 2.5, containing again the indicated amount of acetonitrile. The sample contained 1 mg/mL of each peptide in a 30 mM sodium phosphate buffer, pH 2.5. The elution order was again determined by spiking. Mirroring conditions were used in terms of applied voltage in CEC and voltage-assisted LC (parameters in parentheses). If not otherwise mentioned, the applied

voltage was −25 kV (25 kV) over a total capillary length of 37 cm. Before the measurements and upon every change of the mobile phase, the stationary phase was conditioned by flushing with the new mobile phase at 11 bar for 30 min, while applying a voltage of −7 kV (7 kV), followed by voltage equilibration at −25 kV (25 kV) for another 20 min. Between runs the column was rinsed with acetonitrile for 7 min followed by voltage equilibration at −25 kV (25 kV) with the mobile phase for 20 min at 11 bar inlet pressure. The samples were injected electrokinetically at −7 kV (7 kV) for 3 s. The temperature was 25 °C and the detection was performed at 214 nm.

The electrochromatographic retention coefficient k'_{CEC} was calculated as indicated in Eq. (1):

$$k'_{CEC} = \frac{t_R - t_0}{t_0} \quad (1)$$

with t_R the migration time of the compound in question and t_0 the migration time of the neutral and non-retained tracer ('EOF' marker).

The column efficiency (height of a theoretical plate) H was calculated as L/N from the number of theoretical plates per meter:

$$N = 5.54\left(\frac{t_R}{w_{0.5}}\right)^2 \quad (2)$$

with L column length and $w_{0.5}$ peak width at half height.

Plate heights were routinely evaluated as a function of the mobile phase flow rate u, yielding the so-called Van Deemter curves. The general form of the relationship was assumed to be:

$$H = \frac{B}{u} + A + Cu \quad (3)$$

With B being related to molecular diffusion, A to various constant contributions to the peak dispersion and C related to mass transfer phenomena such as pore diffusion and adsorption kinetics.

For the capillary free zone electrophoresis (CZE) experiments the following conditions were used. Prior to the first use each capillary was rinsed with 1.0 M NaOH for 10 min (10 MPa), 0.1 M NaOH for 10 min (10 MPa), water for 10 min (10 MPa) and finally running buffer for 3 min (10 MPa). Afterwards the capillary was equilibrated at a voltage of 10 kV for 5 min. Prior to each run the capillary was rinsed with water for 3 min (10 MPa) followed by a rinse with the running buffer also for 3 min (10 MPa).

For the nano-HPLC experiments the following conditions were used: column: porous methyl methacryl monolith (C1), length 15 cm; flow rate: 0.2 μL/min; detection: 214 nm; sample: 1 mg/mL of methionine enkephalin, angiotensin II, and angiotensin I; mobile phase isocratic elution: 10 mM sodium phosphate buffer pH 7.0 containing 1.5 M ammonium sulfate, mobile phase gradient elution: buffer A 10 mM sodium phosphate pH 7.0 containing 1.0 M ammonium sulfate, buffer B 10 mM sodium phosphate pH 7.0 containing 15 mM ammonium sulfate, gradient from 0% B to 100% B in 10 min.

3. Results and discussion

3.1. Characterization of the stationary phases

The poly(glycidyl methacrylate–co-ethylene dimethacrylate) chemistry has become very popular for the preparation of monolithic stationary phases in general [15,16,25–29]. A major advantage of this method from a practical point of view is the presence of chemically reactive epoxy groups in the polymer. These epoxy groups can be used in a variety of derivatization reactions for the introduction of additional ligands. Horváth et al. have, e.g., suggested to use this approach for the introduction of the charged moieties required for EOF production into their monolithic stationary phases intended for application in CEC [15,16]. In our case, it presented an elegant approach for the preparation of essentially identical (in terms of general morphology, average pore size and pore size distribution) charge bearing and non-charge bearing ('neutral') stationary phases. The former type could be used for CEC, the latter for voltage-assisted LC and nano-HPLC. For the introduction of charges a derivatization with *N*-ethylbutylamine was carried out as described in [15] and in the materials and methods section, while the original methyl methacrylate based polymer was used without further derivatization as 'C1'-type stationary phase in voltage-assisted LC and nano-HPLC. The *N*-ethylbutylamine groups introduced for the purpose of EOF generation in the stationary phases intended for application in CEC are ionogenic, and therefore the columns' zeta-potential and EOF depend on the pH [15]. The EOF velocity is highest at low pH and decreases somewhat as the pH is elevated, a diminution by roughly 50% has been reported between pH 2.5 and 7.0 for similar stationary phases [15].

When introduced into the nano-HPLC system, all columns produced for these investigations showed a linear increase of the backpressure with the flow-rate for a variety of mobile phases (e.g. methanol, acetonitrile and water) for flow-rates ranging from 0.1 to 0.7 μL/min. Backpressures up to 180 bar were measured during these experiments. Neutral and derivatized columns showed roughly the same behavior under otherwise similar conditions. No extrusion or apparent damage of the monolithic column was observed. It can hence be assumed that the monolith is rigid and that mechanically the capillary columns are sufficiently stable for application in nano-HPLC, voltage-assisted LC and CEC. The absence of an EOF in the non-derivatized stationary phase was demonstrated by the fact that a non-charged inert tracer (DMSO) was not eluted from these columns within several hours of time although a field of several kV was applied.

Fig. 1 shows the Van Deemter curves recorded for DMSO (non-retained tracer) in the CEC mode (charged

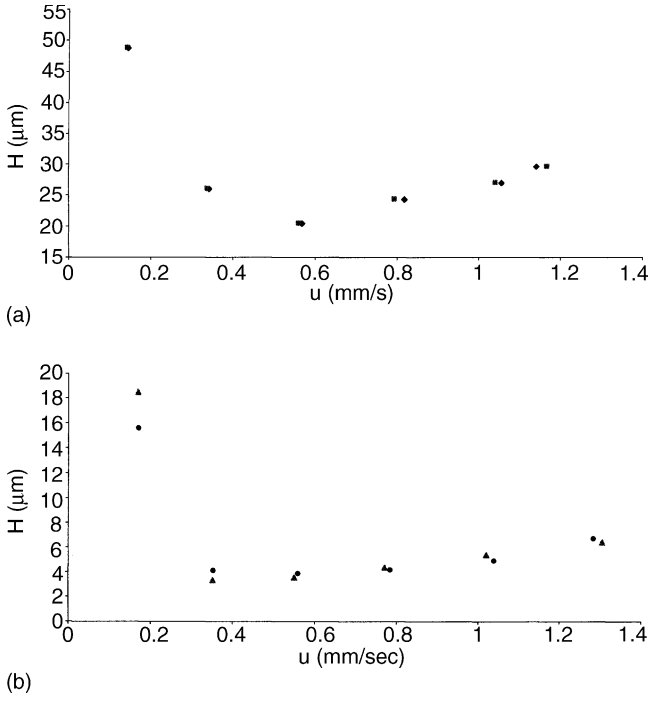

Fig. 1. (a) Van Deemter curve recorded for DMSO (unretained tracer) under CEC conditions. Mobile phase: 40 mM phosphate buffer pH 7.0 containing 30% (v/v) acetonitrile, stationary phase: methyl methacrylate monolith bearing ionogenic groups, column length: 37 cm (effective length 28 cm). Measurements were carried out in duplicate. (b) Van Deemter curve recorded for angiotensin I (retained tracer) under voltage-assisted LC conditions. Mobile phase: 40 mM phosphate buffer pH 2.5 containing 30% (v/v) acetonitrile, stationary phase: neutral methyl methacrylate monolith, column length: 37 cm (effective length 28 cm). Measurements were carried out in duplicate.

stationary phase surface) and for angiotensin I (retained analyte) in the voltage-assisted LC mode (neutral stationary phase). Peak dispersion in CEC is mainly determined by longitudinal molecular diffusion [30] and the curves hence show the expected general shape i.e., a low C-term and a relationship largely dominated by the A- and B-terms. Interestingly, plate heights determined for the retained analyte under voltage-assisted LC conditions were even lower than those recorded for the unretained tracer under CEC conditions, while the optimum plate height was found at roughly the same flow rate. A direct comparison of either the non-retained or the retained compound under both CEC and voltage-assisted LC conditions was unfortunately not possible, since the elution of the non-retained (uncharged) molecule would not have been possible under voltage-assisted conditions, while the retained analyte would have been subjected to both chromatographic and electrorheophoretic[1] forces under CEC conditions.

[1] The term electro*rheo*phoresis has recently been introduced by Rathore and Horváth [5]. It allows differing between electrophoresis under EOF-mediated *flow* conditions, i.e. electro*rheo*phoresis, and electrophoresis as such. We find this a very elegant way of making this important distinction and will continue to use the term throughout this text.

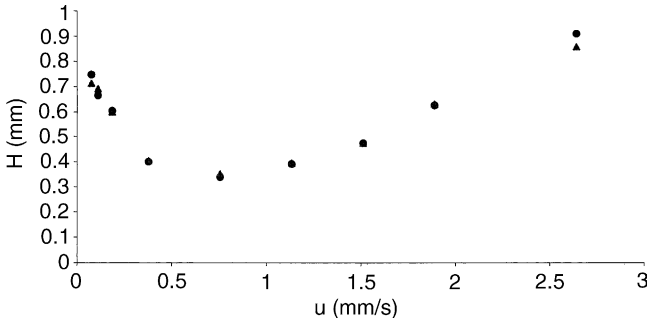

Fig. 2. Van Deemter curve recorded for DMSO (unretained tracer) under nano-HPLC conditions. Mobile phase: water, stationary phase: neutral methyl methacrylate monolith, column length: 15 cm. Measurements were carried out in duplicate.

Monolithic stationary phases in general are expected to have low C-terms [31,32]. However, as Fig. 2 demonstrates, the optimum plate heights recorded for the neutral columns under nano-HPLC conditions for DMSO were roughly one-two order magnitudes larger than when the column was operated in the CEC or voltage-assisted LC mode. A similar effect was observed for the derivatized column. The deterioration at elevated flow rates is also more pronounced under nano-HPLC conditions. This has been observed before [14,33] and can partly be attributed to the lower efficiencies to be expected for the nano-HPLC column due to the parabolic flow profile typical for the pressure driven mode. In addition, system inherent factors like the manual injection or other extra-column effects may also contribute.

3.2. Separation of the peptide mixture in the CEC mode

A mixture of three biologically active peptides, namely angiotensins I and II, and methionine enkephalin, was chosen for the systematic investigation of the effect of a variety of mobile phase parameters on the migration/retention behavior in CEC as well as for the subsequently intended comparison of these results with the voltage-assisted and nano-HPLC mode. The characteristics and amino acid sequences of the peptides are summarized in Table 1.

The mobile phase pH exerted a strong influence on the CEC-separation. At pH 2.5 the peptide mixture could not be separated. At pH 4.0 the separation of the peptides was possible albeit only poorly so for mobile phases containing more than 15% (v/v) acetonitrile. Base line separation was possible at pH 7.0, Fig. 3. A pH of 7.0 is above the isoelectric point of all peptides included in the sample mixture, the analytes can hence be presumed to be slightly negatively charged, while the stationary phase surface is positively charged. Both the analytes and the EOF move into the same direction and a negative field strength of −25 kV was suitable for separation and of −7 kV for sample injection. In spite of their charge, the peptides eluted after the EOF marker, a clear demonstration of their retention by the stationary phase. It should be noted that the elution order of

Table 1
Specifics of the peptides used in the separation experiments

	Angiotensin I	Angiotensin II	Methionine enkephalin
Isoelectric point	6.41	6.32	5.76
Average hydrophobicity index			
pH 2.0 [34]	24.6	23.5	43.0
pH 7.0 [35]	33.6	28.9	47.4
Amino acid sequence	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe–His–Leu	Asp–Arg–Val–Tyr–Ile–His–Pro–Phe	Try–Gly–Gly–Phe–Met
Mass (free base)	1296.5	1056.2	537.7
Net charge (pH 2.5)	4	3	1

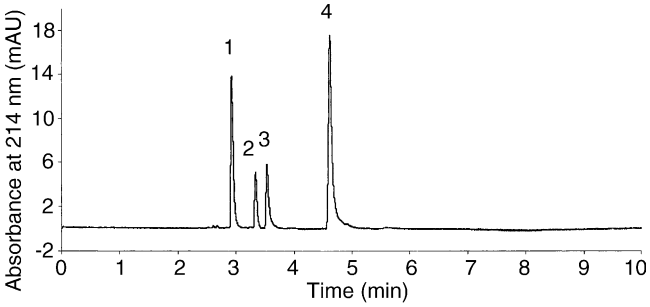

Fig. 3. Separation of the three peptides under CEC conditions. Mobile phase: 50 mM phosphate buffer pH 7.0 containing; 40% (v/v) acetonitrile, sample: (1) DMSO, (2) angiotensin II, (3) angiotensin I, (4) methionine enkephalin 1 mg/mL each.

the three peptides correlates more with their hydrophobicity than with their net charge or charge density.

The strength of the EOF but also the resolution of the peptide mixture depends on both the acetonitrile content and the sodium phosphate concentration of the mobile phase. When the acetonitrile content of a 40 mM aqueous phosphate buffer, pH 7.0, was varied from 10 to 60% (v/v) the EOF velocity changed from 1.78 to 1.36 mm/s, Fig. 4a. Concomitantly, when the sodium phosphate concentration of a mobile phase containing 30% (v/v) acetonitrile was varied in the range of 10 to 60 mM the EOF velocity decreased from 1.8 to 1.2 mm/s, Fig. 4b. Fig. 5 shows four separations of the peptide mixture carried out at increasing acetonitrile content of the mobile phase under otherwise identical conditions. The corresponding retention times (R.S.D. < 1%) and plate numbers are compiled in Table 2. The corresponding changes in the migration velocity of the EOF marker and the peptides as well as in the electrochromatographic migration

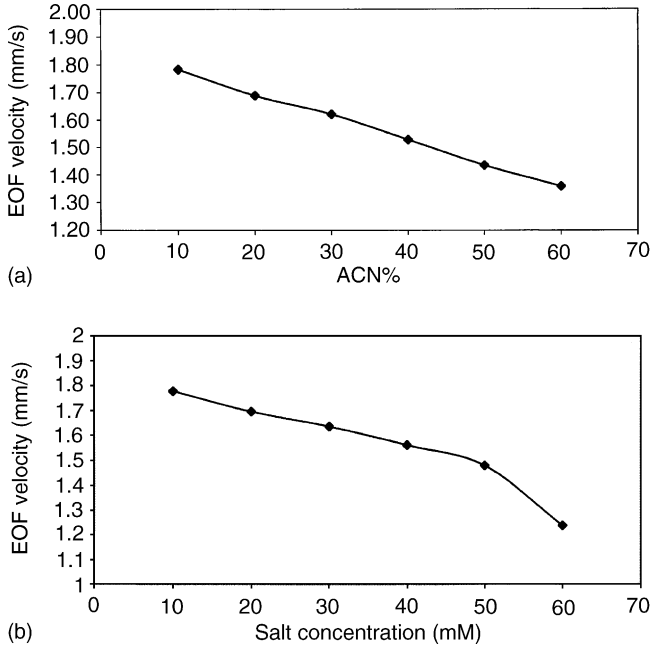

Fig. 4. (a) Plots of the EOF velocity measured with DMSO as unretained tracer as a function of the acetonitrile content of the mobile phase. Mobile phase: 40 mM phosphate buffer pH 7.0 containing the indicated amount of acetonitrile, stationary phase: methyl methacrylate monolith with ionogenic groups, column dimensions: 37 cm (effective length 28 cm) × 75 μm i.d., applied voltage: −25 kV; detection: 214 nm. (b) Plot of the EOF velocity measured with DMSO as unretained tracer as a function of the ionic strength of the mobile phase. Mobile phase: phosphate buffer pH 7.0 of the indicated concentration 30% (v/v) acetonitrile, stationary phase: methyl methacrylate monolith with ionogenic groups, column dimensions: 37 cm (effective length 28 cm) × 75 μm i.d., applied voltage: −25 kV; detection: 214 nm.

Table 2
Column efficiencies and retention times measured for the three peptides in the CEC experiments

Peptide	30% ACN		40% ACN		50% ACN		60% ACN	
	t (min)	*N*	*t* (min)	*N*	*t* (min)	*N*	*t* (min)	*N*
Angiotensin I	3.51	17088	3.849	3194	4.032	4295	4.271	11709
Angiotensin II	3.209	9851	3.451	7829	3.701	16459	3.984	16233
Methionine enkephalin	3.6	20281	4.074	13877	3.984	15897	4.877	17449

Conditions: column length: 37 cm (effective length 28 cm), stationary phase: methyl methacryl monolith with *N*-ethylbutyl moieties, mobile phase: 40 mM sodium phosphate buffer pH 7.0 containing the indicated amount of acetonitrile (ACN), applied voltage: −25 kV.

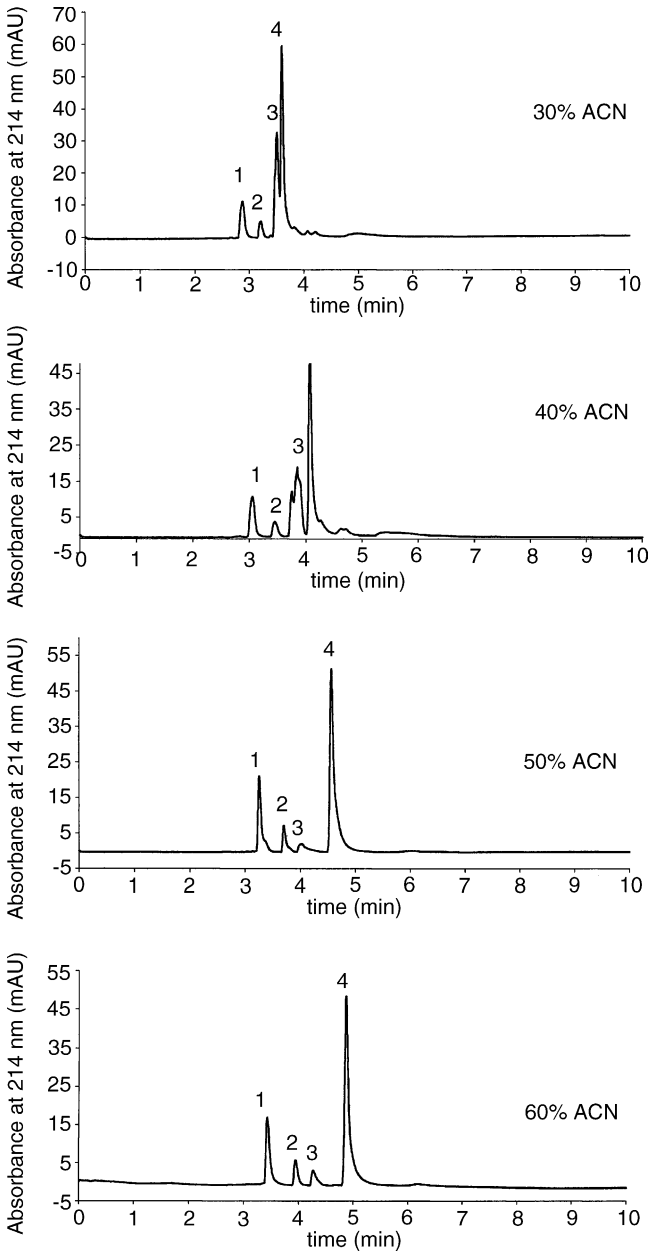

Fig. 5. Resolution of the peptide mixture under CEC condition as a function of the acetonitrile content of the mobile phase. Mobile phase: 40 mM phosphate buffer pH 7.0 containing the indicated amount of acetonitrile, stationary phase: methyl methacrylate monolith with ionogenic groups, column dimensions: 37 cm (effective length 28 cm) × 75 μm i.d., applied voltage: −25 kV, detection: 214, sample: (1) DMSO, (2) angiotensin II, (3) angiotensin I, (4) methionine enkephalin.

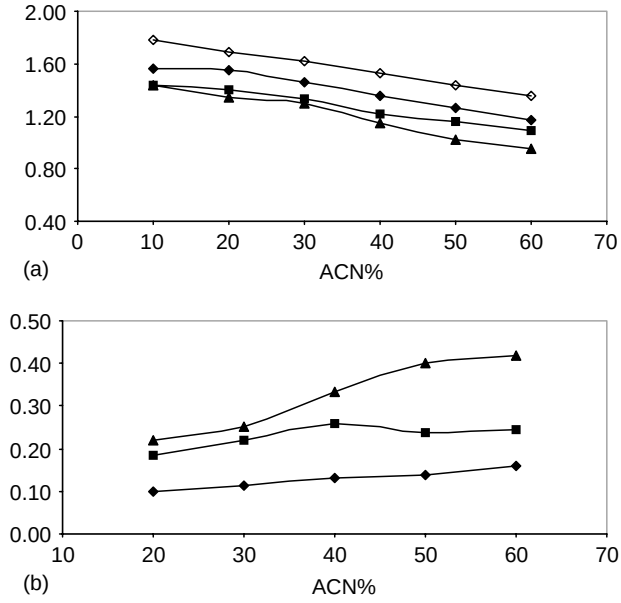

Fig. 6. (a) Plots of the migration velocity of DMSO (◇) and the peptides as a function of the acetonitrile content of the mobile phase. Mobile phase: 40 mM phosphate buffer pH 7.0 containing the indicated amount of acetonitrile, stationary phase: methyl methacrylate monolith with ionogenic groups, column dimensions: 37 cm (effective length 28 cm) × 75 μm i.d., applied voltage: −25 kV, detection: 214 nm, sample: (◇) DMSO, (υ) angiotensin II, (■) angiotensin I, (σ) methionine enkephalin. (b) Plots of the migration factors, k'_{CEC}, of the peptides as a function of the acetonitrile content of the mobile phase. Mobile phase: 40 mM phosphate buffer pH 7.0 containing the indicated amount of acetonitrile, stationary phase: methyl methacrylate monolith with ionogenic groups, column dimensions: 37 cm (effective length 28 cm) × 75 μm i.d., applied voltage: −25 kV, detection: 214 nm, sample: (υ) angiotensin II, (■) angiotensin I, (σ) methionine enkephalin.

factor, also called electrochromatographic retention factor [5,10,15], k'_{CEC}, against the acetonitrile concentration in the eluent are presented in Fig. 6. The apparent "retention" of the peptides increases with increasing acetonitrile concentration, an effect that is only partially due to the decrease in the EOF as the changes in k'_{CEC} show. The increase of the retention with increasing acetonitrile content was surprising given the fact that the hydrophobicity was found to determine the elution order. However, an increase in the acetonitrile content of the mobile phase while reducing hydrophobic interactions will also increase the importance of the electrostatic interactions, which could very well contribute to the observed behavior. The prediction the chromatographic behavior of charged analytes in CEC remains difficult due to the possibility of mixed-mode, i.e. hydrophobic and electrostatic, interactions.

3.3. Voltage-assisted LC

The principle difference between CEC and voltage-assisted LC is the absence of the EOF in the latter. In voltage-assisted LC charged analytes move through the column solely by electrophoresis while being retained by the stationary phase. Since no charges are required for EOF generation, the chromatographic interaction may be a single mode one, while due to the absence of the EOF only electrophoresis takes place instead of electrorheophoresis. In our case voltage-assisted LC was possible by preparing a neutral hydrophobic column by the simple measure of eliminating the derivatization step from our stationary phase preparation protocol. The thereby produced "C1"-column was used in the voltage-assisted but also the nano-HPLC mode.

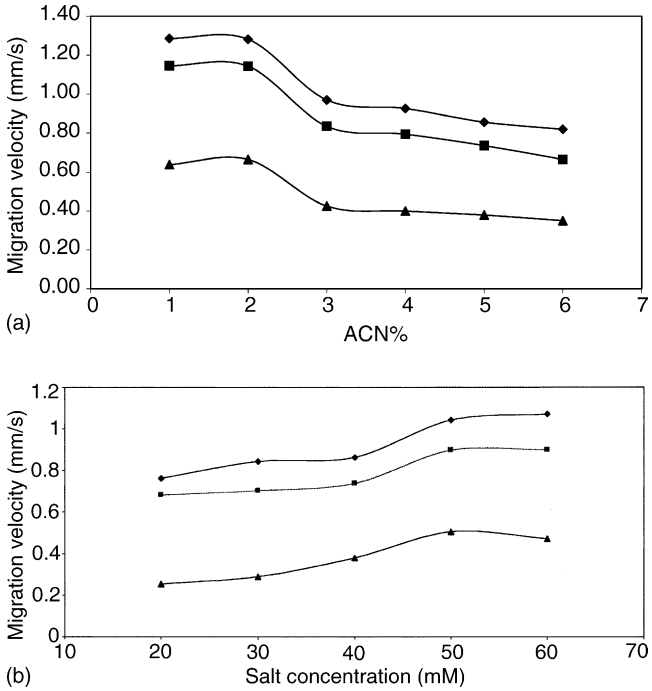

Fig. 7. (a) Plots of the migration velocity of the peptides as a function of the acetonitrile content of the mobile phase under voltage-assisted LC conditions. Mobile phase: 40 mM phosphate buffer pH 2.5 containing the indicated amount of acetonitrile, stationary phase: neutral methyl methacrylate monolith, column dimensions: 37 cm (effective length 28 cm) × 75 μm i.d., applied voltage: 25 kV, detection: 214 nm, sample: (υ) angiotensin I, (■) angiotensin II, (σ) methionine enkephalin. (b) Plots of the migration factor of the peptides as a function of the salt concentration in the mobile phase under voltage-assisted LC conditions. Mobile phase: phosphate buffer pH 2.5 of the indicated strength containing 30% (v/v) acetonitrile, stationary phase: neutral methyl methacrylate monolith, column dimensions: 37 cm (effective length 28 cm) × 75 μm i.d., applied voltage: 25 kV, detection: 214 nm, sample: (υ) angiotensin I, (■) angiotensin II, (σ) methionine enkephalin.

Compared to the CEC-type stationary phase, this column should be slightly less hydrophobic due to the absence of the *N*-ethylbutyl functions, but similar in general morphology.

In voltage-assisted LC the effect of the mobile phase composition was again investigated. A pH of 2.5 was adjusted in this case in order to maximize the charge density of the peptides, which will carry a positive net-charge under these conditions. As a consequence a positive field of 25 kV was applied during separation (7 kV during sample injection). Fig. 7 compiles the changes of the migration velocity of the three peptides as a function of the acetonitrile and salt concentration in the mobile phase. Fig. 8 shows the chromatograms of the peptide mixture recorded for increasing acetonitrile contents of the mobile phase. Table 3 compiles the retention times of the peptides and the plate numbers calculated for the various peaks. Again the separation was very reproducible with R.S.D. for the migration time of less than 1%. The plate numbers for the 37 cm columns were in the range of 6000–45 000 and hence higher than in CEC. Most importantly, the elution order changed between the

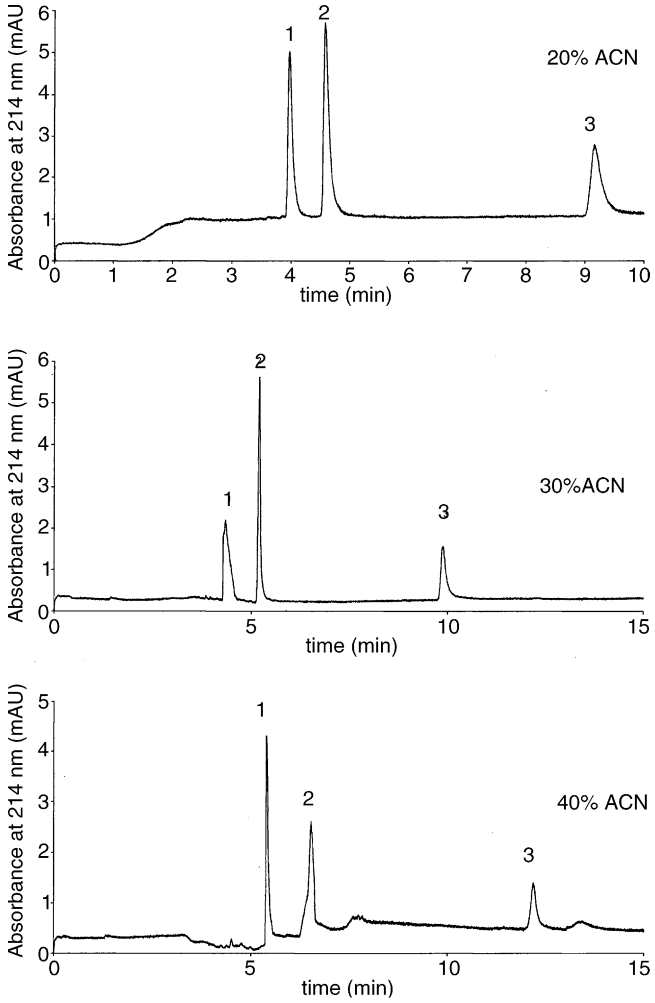

Fig. 8. Separation of the peptide mixture under voltage-assisted LC conditions as a function of the acetonitrile content of the mobile phase. Mobile phase: 60 mM phosphate buffer pH 2.5 containing the indicated amount of acetonitrile, stationary phase: neutral methyl methacrylate monolith, column dimensions: 37 cm (effective length 28 cm) × 75 μm i.d., applied voltage: 25 kV, detection: 214 nm, sample: (1) angiotensin I, (2) angiotensin II, (3) methionine enkephalin.

CEC and the voltage-assisted LC separation mode. In CEC angiotensin II eluted before angiotensin I, in voltage-assisted LC the opposite is the case. Angiotensin I has the higher charge density, but is more hydrophobic than angiotensin II.

Table 3
Column efficiencies and retention times measured for the three peptides in the voltage-assisted LC experiments

Peptide	20% CAN		30% CAN		40% CAN	
	t (min)	*N*	*t* (min)	*N*	*t* (min)	*N*
Angiotensin I	3.983	12819	4.352	6296	5.402	45208
Angiotensin II	4.592	11682	5.213	99942	6.525	13829
Methionine enkephalin	9.169	8601	9.893	19052	12.195	19915

Conditions: column length: 37 cm (effective length 28 cm), stationary phase: neutral methyl methacryl monolith, mobile phase: 60 mM sodium phosphate buffer pH 2.5 containing the indicated amount of acetonitrile (ACN), applied voltage: 25 kV.

Apparently the higher charge is the determining factor in the separation by voltage-assisted LC. Hydrophobic interactions play a more important role in CEC, most likely due to the slightly more hydrophobic stationary phase used in that case.

The migration velocity of the peptides decreases when the acetonitrile concentration in the mobile phase is raised from 5 to 40%. It has been noted before that the apparent pH of mobile phases containing higher percentages of organic solvents are elevated compared to the pH adjusted in the aqueous part [33]. Values above 4 were reached in the cited case for solutions containing 40% acetonitrile. It can hence be assumed that the effective charge density of the peptides is reduced at higher acetonitrile concentration, which would cause the observed reduction in migration velocity. In fact, judging from Fig. 7a significant drop in migration velocity occurs between an acetonitrile content of 10 and 20%, whereas at higher and lower acetonitrile concentrations the mobilities appear almost constant. The effect of the ionic strength of the mobile phase on the separation was studied for a series of aqueous phosphate buffers all containing 30% (v/v) acetonitrile in order to allow a comparison with the conditions previously chosen in the CEC experiments. In this case the retention increased with increasing salt content of the mobile phase an effect that may be caused either by a decrease in the electrophoretic mobility of the peptides perhaps due to an increase in viscosity or caused by an increase of the chromatographic interaction with the stationary phase, i.e. an enforcement of the hydrophobic interactions.

The question of the relative importance of the electrophoretic and the chromatographic contribution to the separation raises itself also for voltage-assisted LC. In this case, however, the separation is relatively simple, as strict additivity of the two contributions can be presumed. In order to investigate the electrophoretic contribution, the peptide mixture was separated under optimized free zone capillary electrophoresis (CZE) conditions (no stationary phase, but EOF), Fig. 9a. Fig. 9b by comparison, shows a separation of the peptide mixture by voltage-assisted LC (stationary phase, no charges/EOF) under otherwise

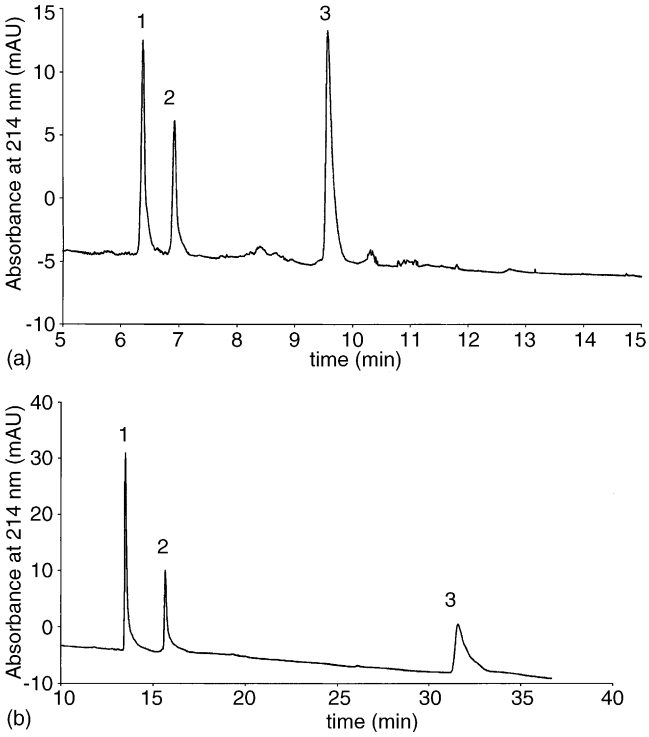

Fig. 9. (a) Separation of the peptide mixture under capillary zone electrophoresis (CZE) conditions. Mobile phase: 40 mM phosphate buffer pH 2.5 containing 30% (v/v) of acetonitrile, column dimensions: 37 cm (effective length 28 cm) × 75 μm i.d., applied voltage: 10 kV, detection: 214 nm, sample: (1) angiotensin I, (2) angiotensin II, (3) methionine enkephalin. (b) Separation of the peptide mixture by voltage-assisted LC and otherwise similar conditions as in Fig. 9a. Mobile phase: 40 mM phosphate buffer pH 2.5 containing the indicated amount of acetonitrile, stationary phase: neutral methyl methacrylate monolith, column dimensions: 37 cm (effective length 28 cm) × 75 μm i.d., applied voltage: 10 kV, detection: 214 nm, sample: (1) angiotensin I, (2) angiotensin II, (3) methionine enkephalin.

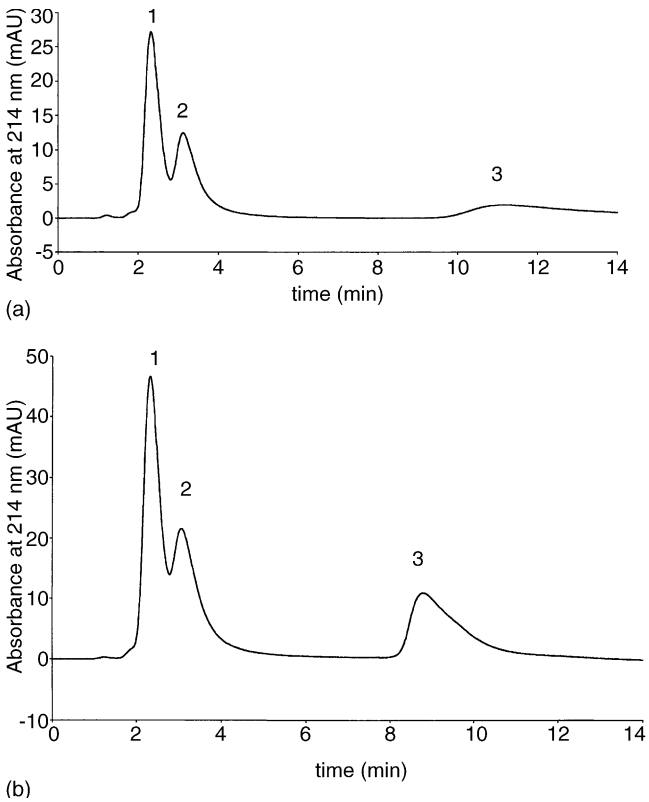

Fig. 10. (a) Isocratic separation of the peptide mixture under nano-HPLC conditions. Mobile phase: 10 mM phosphate buffer pH 7.0 containing in addition 1.5 M ammonium sulfate, flow rate 0.2 μL/min, stationary phase: neutral methyl methacrylate monolith, column dimensions: 15 cm × 75 μm i.d., detection: 214 nm, sample: (1) methionine enkephalin, (2) angiotensin II, (3) angiotensin I. (b) Gradient elution separation of the peptide mixture under nano-HPLC conditions. Buffer A: 10 mM phosphate buffer pH 7.0 containing in addition 1.0 M ammonium sulfate, buffer B: 10 mM phosphate buffer pH 7.0 containing in addition 15 mM ammonium sulfate, gradient: from 0% buffer B to 100% buffer B in 10 min, flow rate 0.2 μL/min, stationary phase: neutral methyl methacrylate monolith, column dimensions: 15 cm × 75 μm i.d., detection: 214 nm, sample: (1) methionine enkephalin, (2) angiotensin II, (3) angiotensin I.

identical conditions. The fact that the elution order is identical in CZE and voltage-assisted LC demonstrates the importance of the electrophoretic contribution to the separation in these modes. The presence of chromatographic retention can be seen in the prolongation of the peptide residence time.

3.4. Nano-HPLC

The neutral monolithic column (C1, no ethylbutyl groups) that had already been used for voltage-assisted LC was further investigated for the separation of the peptide mixture by nano-HPLC. Given the stationary phase chemistry and the physico-chemical nature of the peptides, a separation by a form of hydrophobic interaction chromatography seemed to be most likely. Fig. 10 shows the separation of the peptide mixture under isocratic (Fig. 10a) and gradient elution conditions (Fig. 10b). The mobile phase was an aqueous phosphate buffer containing the indicated amounts of ammonium sulfate in order to promote hydrophobic interactions.

As expected from the Van Deemter curve measurements, a much lower efficiency was observed for the purely pressure driven separation mode compared to either the CEC or the voltage-assisted LC mode. Somewhat sharper peaks are obtained for gradient elution. More importantly, however, the elution order changes once more with methionine enkephalin eluting first followed by the two angiotensins. Just as in CEC Angiotension II elutes before angiotensin I. The neutral monolith hence supports peptide separation by hydrophobic interaction in the nano-HPLC-mode, while the separation on the same stationary phase becomes dominated by electrophoresis in the voltage-assisted-LC mode.

4. Conclusions

The separation and analysis of charged analytes by CEC remains a challenge. In this paper we show how at least four different factors—two types of chromatographic interaction, electrophoresis and electroosmosis—can influence the differential migration behavior of such analytes under CEC conditions. Since changes in the mobile phase such as an increase in the ionic strength or the organic solvent content may influence each of these interactions independently, the design of the separation strategy or even the planning of a simple gradient elution becomes rather complex. Nano-HPLC is not a viable alternative, since compared to CEC existing nano-HPLC systems yield much lower plate numbers and inferior resolution. The voltage-assisted separation mode developed in this work, on the other hand, may constitute a complementary technique for the efficient separation of charged analytes by a simplified form of electrochromatography.

Acknowledgements

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