Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1004 (2003) 195–208
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Investigation of mixed-mode monolithic stationary phases for the analysis of charged amino acids and peptides by capillary electrochromatography

Daniela Hoegger, Ruth Freitag*
Center of Biotechnology, Swiss Federal Institute of Technology Lausanne, 1015 Lausanne, Switzerland

Abstract

The potential of *N*,*N*-dimethylacrylamide–piperazine diacrylamide-based monolithic stationary phases bearing sulfonic acid groups for electroosmotic flow generation is investigated for the separation of positively charged amino acids and peptides. The capillary columns were used under electrochromatographic but also under purely chromatographic (nano-HPLC) conditions and the separations interpreted as the result of possible chromatographic and electrophoretic contributions. The stationary phases were found to be mechanically stable up to pressures of 190 bar and chemically stable towards a wide variety of organic and hydro-organic mobile phases. In order to investigate the retention mechanism, the salt concentration and the organic solvent content of the (hydro-)organic mobile phase were varied in a systematic manner, taking three aromatic amino acids (phenylalanine, tryptophan, histidine) as model analytes. The respective contributions of electrostatic and hydrophobic and/or hydrophilic interactions were further investigated by varying the charge density and the hydrophobicity of the standard stationary phase. The former was done by varying the amount of charged monomer (vinylsulfonic acid) added during synthesis, the latter by (partially) replacing the interactive monomer (*N*,*N*-dimethylacrylamide) by other more hydrophobic monomers. A mixed mode retention mechanism based primarily on electrostatic interactions modified in addition by ''hydrophilic'' ones seems most suited to interpret the behavior of the amino acids, which stands in contradistinction to the previously investigated case of the behavior of neutral analytes on similar stationary phases. Finally the separation of small peptides was investigated. While the separation of Gly–Phe and Gly–Val was not possible, the separation of Phe–Gly–Phe–Gly and Gly–Phe but also of the closely related Gly–His and Gly–Gly–His could be achieved.

Keywords: Mixed-mode electrochromatography; Monolithic columns; Stationary phases, electrochromatography; Amino acids; Peptides

1. Introduction

The potential of capillary electrochromatography (CEC) has been amply demonstrated by a burst of exemplary separations and applications (see Refs. [1–4] for recent reviews). Obviously extremely efficient separations can be achieved in CEC, especially if stacking and focussing effects are exploited [5–7]. The sample volumes can theoretically be reduced to the volume of a single cell and due to its low flow-rates and mobile phases, which are not likely to contaminate the ion source, CEC should be

*Corresponding author. Tel.: +41-21-693-6108; fax: +41-21-693-6030.
E-mail address: ruth.freitag@epfl.ch (R. Freitag).

0021-9673/03/$ – see front matter
doi:10.1016/S0021-9673(03)00563-6

most compatible with electrospray mass spectrometry. Clearly these advantages of CEC match very well the needs of the most demanding and rapidly evolving area of analytics, namely bioanalytics (genomics, proteomics, metabolomics, zoeomics, to give a few key words). However, biocompounds also seem to represent the biggest challenge for CEC, mainly because these compounds tend to be in the charged state.

The separation of uncharged substances poses few problems in CEC and taking into account the difference in the propagation of the mobile phase (electroosmosis rather than pressure), the established chromatographic theory is largely applicable. The separation of charged analytes, on the other hand, is simultaneously governed by chromatographic interaction and electrophoretic migration. Also—depending on the sign of their charges—the analytes may migrate co- or counter-directionally to the electroosmotic flow, EOF. While the latter can lead to an increased selectivity, it may also lead to long elution times [8]. Furthermore, a change in the mobile phase under these conditions not only influences the state of the analytes, or the chromatographic elution power of the mobile phase towards these analytes, but also induces a change in the velocity of the mobile phase itself.

Only recently have the first theoretical models for simultaneous electrokinetic mass transport and adsorptive interaction of charged analytes in the electrochromatographic mode been published [8–12]. One conclusion from these simulations is that CEC is indeed a separation method in its own right and with its own particularities, which cannot be treated as a simple ‘‘hybrid’’ of liquid chromatography and capillary zone electrophoresis. As a consequence, a detailed experimental investigation on the retention behavior, but also the development of stationary phases especially tailored for the needs of electrochromatography, is called for.

To date, several attempts to achieve a separation of charged analytes in CEC have been presented (see Refs. [2,3] for recent reviews). Peptides and proteins served as model analytes in the majority of these reports, although other charged compounds have been considered occasionally. In most cases silica-based particles (originally intended for conventional HPLC) have been used in these experiments. The retention behavior of peptides on alkyl-functionalized silica packings under electrochromatographic conditions has, e.g., been investigated by Adam and Unger [13] and Walhagen et al. [14,15]. However, a problem of using silica-based stationary phases in CEC is the inherent low EOF at low pH due to the neutralization of the charge creating silanol groups. In this context the use of silica-based, mixed-mode [hydrophobic/strong ion exchanger (SCX)] stationary phases presents a more viable alternative, which has been taken into consideration by several research groups [5,16–20]. The group of Unger et al. compared, e.g., the CEC separation of four synthetic peptides on columns packed with *n*-octyl and *n*-octadecyl silica, as well as with a mixed mode C_{18}/SCX stationary phase with separations in the capillary zone electrophoresis (CZE) and HPLC modes [16]. They observed different elution patterns for the various techniques, but also between CEC separations on purely reversed-phase stationary phases compared to mixed-mode ones, and even between mixed-mode phases from different manufacturers. The group of Regnier and co-workers showed preliminary results, where open tubular columns (coated with an organosilane stationary phase) with a low phase ratio were found to yield isocratic separations of a resolution, which was equivalent to that obtainable in gradient HPLC [20,21]. However, since mixed-mode interactions are often not desired in HPLC, the selection of such materials from commercial suppliers remains small. Most silica-based materials intended for reversed-phase HPLC have a very low density of residual silanol groups and therefore are not suited for CEC.

In this context stationary phases based on synthetic organic polymers constitute an interesting alternative, especially monolithic and open-tubular ones, which consequently have also received attention as stationary phases for CEC of charged analytes [22–27]. Such columns have the advantage that they are prepared in situ and hence offer great flexibility in the morphological design. The pore size (distribution), the surface charge density and the accessible chromatographic surface can in principle be freely adjusted within a certain range. Again mostly charged, reversed-phase columns bearing hydrophobic ligands, such as C_4, C_8, C_{12} in addition to the charges were used in the past.

Horváth et al. reported in this context about protein and peptide separations in open-tubular columns clad with a stationary phase of highly cross-linked, porous poly(vinylbenzyl chloride) derivatized with *N*,*N*-dimethyldodecylamine [23], i.e., a positively charged chromatographic phase modified with C_{12} alkyl chains. Counterdirectional separation of basic proteins and peptides was possible at pH 2.5. The same group also compared the behavior of a monolithic stationary phases where the same styrenic polymer was derivatized with *N*,*N*-dimethyloctylamine in the micro (μ) HPLC and the CEC modes [22]. In addition, a mixed-mode weak anion exchanger was prepared from a polyacrylic acid-based polymers derivatized with a secondary amine (*N*-ethylbutylamine) [24]. For this column the counterdirectional separation of proteins and peptides was again investigated at pH 2.5 for different ionic strengths and organic modifier content of the mobile phase. According to the authors, the selectivity was mainly depended on the acetonitrile content in the mobile phase. Surprisingly, however, the retention factors of the proteins decreased with increasing acetonitrile content, whereas that of the peptides increased.

Ericson and Hjertén described a sophisticated system, where continuous beds, previously designed for HPLC applications, were used in gradient CEC [25]. A poly(acrylamide)-based polymer was derivatized with C_{18} (stearyl methacrylate) and ammonium groups (dimethyldiallylammonium). An HPLC gradient system was connected to the CEC system. The flow was regulated by an adjustable splitting valve, in order to deliver a gradient at zero pressure thereby assuring that the gradient was propelled through the capillary solely by electroosmosis. The directions of the electroosmotic flow and the electrophoretic migration of the proteins, respectively, could thus be modulated independently through the ammonium group-content of the stationary phase for the former and through polarity of the applied voltage and the mobile phase composition for the latter. Thus normal-flow and counterflow gradients could be performed with the same system.

Yu et al. reported an attempt to separate peptides on hydrophobic, methacrylic monoliths derivatized with sulfonic acid moieties [26]. The analytes were eluted by the addition of sodium 1-octanesulfonate as an ion-pairing reagent. As an alternative, a non-charged monolithic stationary phase has been presented for the separation of peptides according to their differences in electrophoretic mobility and their ability to interact with a hydrophobic chromatographic surface in the absence of an EOF [28].

Only a few studies have to date been reported on the behavior of charged analytes other than proteins/peptides. Que et al. investigated the separation of bile acids and their conjugates on poly(acrylamide)-based continuous beds coupled to an electrospray ion trap mass spectrometer [7]. They subdivided the analytes into two classes, using a hydrophobic C_{12} stationary phase and positive-ion detection mode for the free and glycine-conjugated bile acids and a hydrophilic amine phase with negative-ion detection mode for a mixture of glycine- and taurine-conjugated solutes. The former exhibited reversed-phase elution patterns, the latter apparently normal-phase ones. However, as charged analytes were involved, the separation was found to depend also on the mass-to-charge ratio and charge sign of the analytes.

In the context of the separation of chiral derivatized amino acids, Lämmerhofer et al. investigated general aspects of the influence of the stationary phase and the chromatographic conditions on the separation efficiency and selectivity [29,30]. The authors noted a dependence of the flow velocity on the pore size of the monolithic stationary phases. The positively charged quinidine moieties served equally as EOF generator and as chiral selector, and selectivity was achieved through an anion-exchange mechanism. An increase in the counterion concentration (e.g., by high acetic acid content in the mobile phase) improved the efficiency without affecting the selectivity. A comparison of purely organic versus hydro-organic mobile phases demonstrated the superiority of the latter in terms of efficiency and retention. An efficiency increase could also be obtained by increasing the polarity of the stationary phase, by replacing the glycidyl methacrylate (GMA) with the more polar 2-hydroxyethyl methacrylate (HEMA). This effect was attributed to the reduction of nonspecific interactions.

Mixed-mode stationary phases has thus given interesting results, to date mostly in a mixed reversed-phase/ion-exchange mode. However, the two interaction modes–hydrophobic and electrostatic–

possible on such mixed-mode columns may be difficult to exploit to advantage in the case of charged analytes. For instance, the high salt buffer needed for elution in ion-exchange chromatography enforces hydrophobic interactions and vice versa, the high organic phase content used for the elution of hydrophobic solutes enhances electrostatic interactions. The elution of a charged biological, e.g., a protein, from such a mixed-mode CEC column may therefore at times be rather difficult. We have recently proposed a monolithic stationary phase for CEC that is based on a hydrophilic rather than a hydrophobic (charged) polymer [31]. The corresponding columns have already given good results for the separation of uncharged analytes. Here such stationary phases were for the first time investigated in the separation of mixtures of charged biologicals such as amino acids and peptides.

2. Experimental

2.1. Materials and equipment

N,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), *N*,*N*-dimethylacrylamide (DMAA), methacrylamide (MAA), 2-hydroxyethyl methacrylate (HEMA, >96%), 2-hydroxyethyl acrylate (HEA, >96%), vinylsulfonic acid (VSA, 30% aqueous solution of the sodium salt), salts for buffer preparation, and methanol were from Fluka (Buchs, Switzerland). Piperazine diacrylamide (PDA) was from Bio-Rad (Hercules, CA, USA). Acetonitrile (HPLC grade) was from Biosolve (Valkenswaard, The Netherlands). Amino acids were from Sigma (St. Louis, MO, USA), peptides from Fluka. All substances were used without further purification. The fused-silica capillaries (75 μm inner diameter, 360 μm outer diameter) were from Polymicro Technologies (Phoenix, AZ, USA).

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 experiments. For column rinsing after the polymerization, an HPLC pump (433, Kontron, Watford, UK) was used. 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.). The injection was performed manually using a four-port valve with an internal injection loop of 10 nl from Valco (Schenkon, Switzerland). An Ultimate UV detector with a U-Z capillary flow cell (3 nl volume) was used for detection. A Porosimeter 2000 WS (Carlo Erba, Milan, Italy) was used to determine the average pore size and the pore size distribution of the polymers. The appearance of the polymers inside the capillary was investigated by scanning electron microscopy (XLF 30 FEG, Philips).

2.2. Stationary phase synthesis

Prior to stationary phase introduction, silica 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 3-(trimethoxysilyl)propyl methacrylate 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 and water. The *C*%, *T*% nomenclature originally proposed by Hjertén [32] was used to describe the composition of the polymerization mixture. The standard column used in these investigations was prepared with DMAA as functional monomer and $T = 15\%$, $C = 52\%$, according to a previously described protocol [31]. Other monomers were used as indicated. Table 1 compiles the compositions of all columns used in these investigations. The effective length of the capillary columns was 27 cm (inlet-to-detector, corresponding to a total length of 35.5 cm) for CEC and 15 cm for μHPLC applications.

For the synthesis, the monomer mixture, the crosslinker and the indicated amount of ammonium sulfate (50 mg/ml unless indicated otherwise) were dissolved in 1 ml of a 50 m*M* phosphate buffer (pH 7). The mixture was degassed by ultrasonication for 10 min, then 10 μl of a 10% (w/v) ammonium persulfate and 10 μl of a 10% (v/v) TEMED solution were added and the solution was briefly mixed. The capillary was completely filled with the mixture and the ends of the capillary were again plugged with the GC septa, The polymerization started

Table 1
Compositions of the monoliths prepared as stationary phases for the CEC experiments

Functional monomer	Monomer (mg)	PDA (mg)	VSA (μl)	$(NH_4)SO_4$	%*T*	%*C*
DMAA	71	78	15	30, 40, 50	15	52
MAA	71	78	15	50	15	52
HEA	175	175	15	30	35	50
HEMA	175	175	15	30	35	50

spontaneously soon after and was allowed to proceed overnight at room temperature. Afterwards, the detection window was created in the capillary by burning off a section of the outer polyimide coating and in the same way locally pyrrolyzing the monolith inside. Before, during and after the creation of the detection widow, the capillaries were flushed with pure water for a total of about 1 h, applying flow-rates of 0.045 ml/min, in order to efficiently remove the non-reacted monomers and the ashes produced during pyrrolization. Before use, every column was inspected under the microscope for uniformity of the stationary phase. Columns, which showed irregularities were discarded.

2.3. Electrochromatography

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 about 30 min, while applying a voltage of 7 kV, followed by pure voltage equilibration (25 kV) for another 10 min. In between runs under identical chromatographic conditions, no conditioning was performed. The elution order in the electrochromatograms was determined by spiking the sample with the individual compounds. The mobile phases were prepared as follows: first, an aqueous stock solution of 100 m*M* ammonium acetate–4 *M* acetic acid was prepared. Then the proportions of (organic phase–water–stock solution) were calculated, in order to obtain a mobile phases where the overall ionic strength was, e.g., kept constant when varying the organic phase content. All samples (amino acids, peptides) were prepared in the respective mobile phase at a concentration of ca. 5 and 1 mg/ml, respectively, followed by dilution to concentrations of ca. 1–5 m*M*. If not otherwise mentioned, the applied voltage was +30 kV over a total capillary length of 35.5 cm. Injection was performed electrokinetically (7 kV for 3 s). The temperature was 25 °C, and the detection was performed at 214 nm. The observed mobility, which for ionic species includes the electrophoretic mobility and the effective mobility, was calculated as follows:

$$\mu_{obs} = u/E$$

where $E = 30\ \text{kV}/35.5\ \text{cm}$; $u = 27\ \text{cm}/t_r$.

The electrochromatographic retention coefficient k_{CEC} was calculated as follows:

$$k_{CEC} = \frac{t_r - t_o}{t_o}$$

With t_r the migration time of the compound in question and t_o the migration time of a neutral and non-retained tracer (''EOF-marker''). DMF was chosen as EOF marker in these investigations. The column efficiency H was calculated as L/N from the number of theoretical plates per meter:

$$n = 5.54 \cdot \left(\frac{t_r}{w_{0.5}}\right)^2$$

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

3. Results and discussion

3.1. Preparation and characterization of the stationary phase

Based on our previous studies on the systematic development of acrylamide based monolithic CEC columns [31], a stationary phase composed of DMAA and VSA as interactive monomers and PDA as crosslinker was chosen for the separation of charged analytes. In particular, the standard column

was prepared with $C = 52\%$, $T = 15\%$, 50 mg/ml of ammonium sulfate as salting out (pore forming) agent, and 15 μl/ml of VSA. According to the mercury porosimetry measurements, such polymers typically had a porosity of 77% and a mean pore radius of 840 nm.

For the preparation of the detection window, the method of burning away a small section of the polymer (simultaneously with the removal of the polyimide coating of the capillary) was used rather than a partial filling of the column with the monomer mixture. This makes on-column detection possible, while assuring a uniform EOF over the whole column length. As a result, better stability upon voltage application could be observed (although no difference was observed during hydrodynamic flushing). It may be that a monolith that fills the entire capillary column save for the small detection window is less prone to EOF reflow or deviations from the plug-flow profile, phenomena discussed by Hjertén [33] and Rathore and Horváth [34], respectively.

When introduced into the nano-HPLC system, the columns showed a linear increase of the backpressure with the flow-rate for a variety of mobile phases (e.g., methanol, acetonitrile, water and mixtures of these) for flow-rates ranging from 0.1 to 1.0 μl/min, Fig. 1a. Backpressures up to 190 bar were measured during these experiments. 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 and CEC. In addition, the backpressure of three DMAA columns, which had been synthesized from monomer mixtures containing 30, 40 and 50 mg/ml ammonium sulfate were compared for a 4:6 mixture of acetonitrile–methanol as mobile phase, Fig. 1b. Again the backpressure was found to increase linearly with increasing flow-rate in the investigated range between 0 and 0.7 ml/min. More importantly however, a clear relation between the ammonium sulfate content and the created backpressure and hence the pore size could be observed. The column prepared with 30 mg/ml ammonium sulfate (mean pore radius 0.15 μm by mercury porosimetry) exhibited the highest backpressure. By comparison, a polymer prepared with 50 mg/ml ammonium sulfate had a mean pore radius of 0.84 μm and for a given mobile phase flow-rate, the backpressure of such a column was less then 50% of that created by the column prepared with 30 mg/ml ammonium sulfate.

When the electroosmotic flow velocity in the columns prepared with different ammonium sulfate concentrations was measured as a function of the applied voltage for a hydro-organic (20 m*M* ammonium acetate, 800 m*M* acetic acid–[40% (v/v) acetonitrile–methanol (8:2)] mobile phase, the relationship was linear (Fig. 2). Moreover, almost no difference could be observed between the three DMAA columns. In our case, the EOF hence appears to be largely independent of the mean pore size of

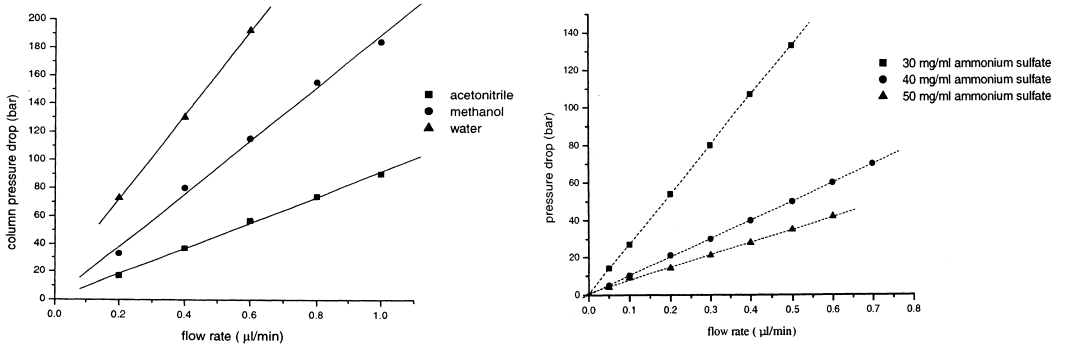


Fig. 1. (a) Column backpressure as a function of the applied volumetric flow-rate for a given DMAA column with acetonitrile, methanol, and water as mobile phase. $T = 29\%$, $C = 52\%$, 50 mg/ml ammonium sulfate, length 29 cm. (b) Column backpressure as a function of the applied volumetric flow-rate for DMAA columns prepared with 30, 40, and 50 mg/ml of ammonium sulfate. $T = 15\%$, $C = 52\%$, length 15 cm, mobile phase acetonitrile–methanol (4:6).

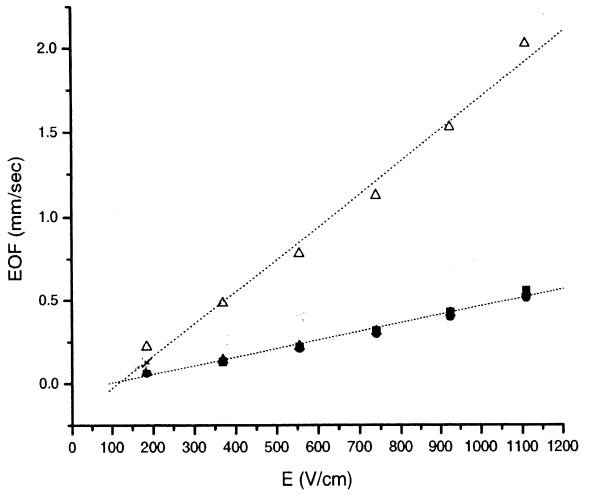


Fig. 2. EOF as a function of the applied electric field strength for DMAA columns prepared with 30 (■), 40 (●), and 50 (▲) mg/ml ammonium sulfate using hydro-organic (20 m*M* ammonium acetate–1 *M* acetic acid in 40% (v/v) (acetonitrile–methanol, 8:2) and (△) a purely organic (acetonitrile–methanol, 8:2) mobile phases (the latter only for the 50 mg/ml ammonium sulfate column).

the column. This stands in some contrast to the findings of other authors [26,35,36]. However, since in our case the pore size was determined in a bulk polymer and in the dry state, this could also indicate that such pore size measurements are not always representative of the actual pore size in the column filled with mobile phase. When a purely organic mobile phase (acetonitrile–methanol, 4:6) was used in case of the stationary phase prepared with 50 mg/ml ammonium sulfate, the slope of the electroosmotic flow velocity as function of the electric field was steeper than with the hydro-organic mobile phase, Fig. 2. This might be due to the difference in the viscosities and the ζ-potentials of the respective mobile phases, although changes in the pore structure of the stationary phase upon changes of the mobile phase cannot be excluded at this point.

The Van Deemter curves recorded for a non-retained (DMF) and a retained but noncharged (phenanthrene) tracer showed the expected general shape [37], i.e., a low C-term and hence a plot largely dominated by the A- and B-terms (Fig. 3). For purely organic mobile phases, optimum plate heights of 16 μm were measured for the retained tracer. When a hydro-organic mobile phase {20 m*M* ammonium acetate–[40% (v,v) ACN–MeOH (8:2)]} was used, even lower plate heights (around 3 μm) were measured, albeit with the optimum at lower flow velocities. In spite of the slightly lower EOF, a hydro-organic mobile phase hence promises more efficient separations by the monolithic columns than a purely organic one. When the column was run in the μHPLC mode optimum plate heights were roughly one order of magnitude larger than under CEC conditions for all investigated cases [38]. This can partly be attributed to a lower efficiency to be expected for the nano-HPLC column due to the

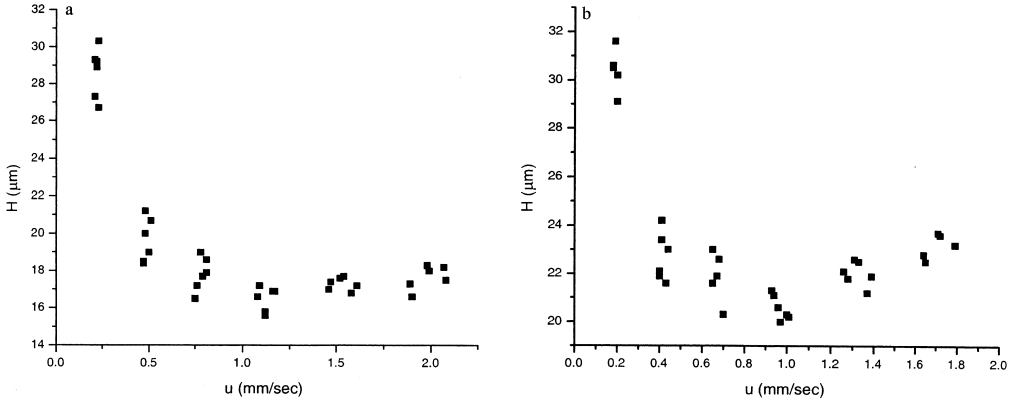


Fig. 3. (a) Van Deemter curve recorded for DMF (unretained marker) in acetonitrile–methanol (4:6) as mobile phase. Stationary phase DMAA column (T=15%, C=52%, 50 mg/ml ammonium sulfate). (b) Van Deemter curve recorded for phenanthrene (retained, but non-charged marker) in acetonitrile–methanol (4:6) as mobile phase. Stationary phase DMAA column (T=15%, C=52%, 50 mg/ml ammonium sulfate).

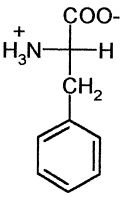
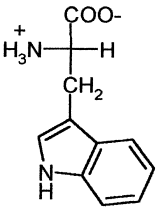
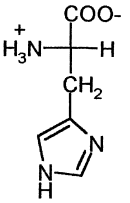
parabolic flow profile typical for the pressure driven mode, but can also be due to system inherent factors like the manual injection or other extra-column effects.

3.2. Investigation of the retention mechanism

In order to obtain an overall view of the retention mechanisms of charged compounds on the proposed stationary phases, the influence of a variety of mobile and stationary phase parameters on the migration–retention were systematically investigated. We were in particular interested in the relative contributions of electrostatic and hydrophobic–hydrophilic interactions to the chromatographic retention and in the relative contributions of the total chromatographic as opposed to the electrophoretic effect on the observed separations. Three UV-active amino acids, namely phenylalanine (Phe), tryptophan (Trp) and histidine (His) were taken as model analytes. Their characteristics and chemical structures are summarized in Table 2. Amino acids are amphiphilic molecules and most of them have an isoelectric point above 5. Low pH conditions are therefore suited for the separation of amino acids by CEC, since they should all bear a positive net charge under these conditions. The classification of the amino acids according to their relative hydrophobicity is less straightforward. Various data on the relative hydrophobicity of amino acids have been published (e.g., Refs. [39,40]), although depending on the experimental conditions significant differences can apparently be observed.

When testing different mobile phase conditions it was noticed that the baseline stability depended strongly on the mobile phase. Mixed aqueous–organic phases gave stable baselines and measurement conditions up to salt concentrations of 100 m*M* when ammonium acetate or ammonium formate, respectively, were chosen as buffering agents (pH 3). Mobile phases based on sodium phosphate (from pH 3 to pH 9), Tris–borate, or tri-ethylammonium

Table 2
Chemical structures, isoelectric points and p*K* values of the amino acids (phenylalanine, tryptophan, histidine) used as model compounds

	pK_{COOH}	$pK_{NH^{3+}}$	$pK_{side\ chain}$	p*I*	Structure
Phenylalanine	1.83	9.13	–	5.48	
Histidine	1.82	9.17	6.00	7.59	
Tryptophan	2.83	9.39	–	5.89	

phosphate buffers, often resulted in noisy, unstable baselines, even at low electrolyte concentrations. Finally, a ternary mixture of acetonitrile, methanol and acetate buffer was used as mobile phase rather than a mixture of acetonitrile and acetate buffer, as an increase of the elution power was observed upon addition of methanol (acetonitrile–methanol, 8:2) to the mobile phase. For the sake of comparability this mobile phase was kept for all the investigations with amino acids.

Fig. 4a shows an exemplary electrochromatogram of the three amino acids and the EOF marker DMF. The amino acids are well separated, His and Phe elute before the EOF marker, Trp after it, indicating the presence of additional effects besides the purely chromatographic ones in the separation. The same capillary column was also used in the nano-HPLC mode, Fig. 4b, where a different elution order (Phe, Trp, His) was observed for the amino acids, which in addition eluted all three after the nonretained tracer. A somewhat different mobile phase was used in the two cases (CEC, nano-HPLC) to allow for some individual optimization of the two separation modes. However, this is not responsible for the observed differences in the elution order. As expected from the Van Deemter curve measurements, a much lower efficiency was observed for the pressure driven compared to the electrochromatographic mode.

Table 3
Observed mobilities μ_{obs} (10^{-5} cm^2 V^{-1} s^{-1}) of Phe, Trp, His and the EOF marker DMF as a function of the cation content of the hydro-organic phase for three mobile phases containing 20, 40 and 60% of an 8:2 mixture of acetonitrile and methanol and as a function of the organic content (acetonitrile–methanol, 8:2) for three mobile phases containing 4/160, 10/400, and 20/800 m*M* ammonium acetate–acetic acid, respectively

		20%	40%	60%
4 m*M*	DMF	5.8	6.3	7.0
	Phe	4.9	5.6	6.4
	His	–	–	–
	Trp	2.2	3.4	4.2
10 m*M*	DMF	6.5	6.8	7.1
	Phe	6.6	7.2	7.8
	His	13.	11.2	9.9
	Trp	3.6	5.1	6.8
20 m*M*	DMF	6.9	6.7	6.0
	Phe	8.0	7.9	7.4
	His	19.	15.3	13.5
	Trp	5.3	6.6	6.9

Based on this first separation, the electrochromatographic parameters were systematically varied. Table 3 compiles the mobilities μ_{obs} of the three amino acids as a function of the salt concentration (m*M*) and the organic content (%, v/v) of the mobile phase (three data points each). From these data some general tendencies can be deduced. All mobilities

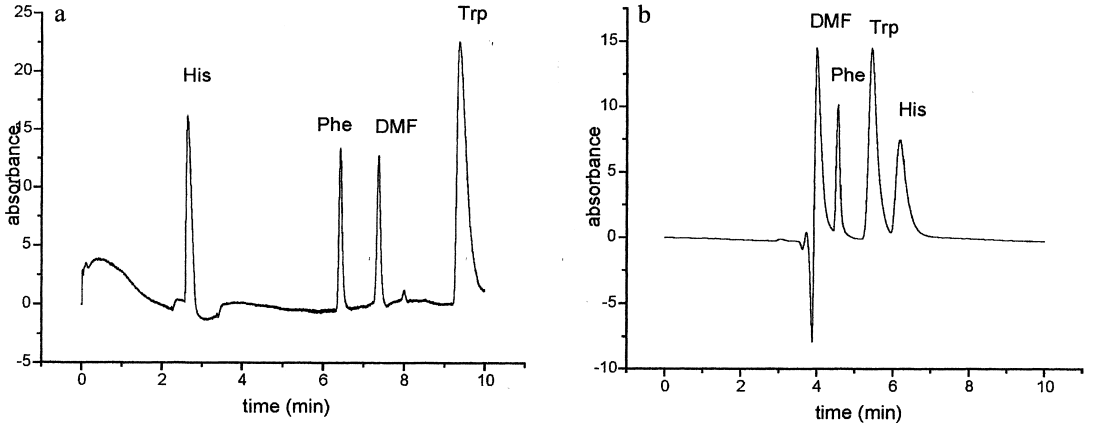


Fig. 4. (a) Electrochromatographic separation of His, Phe, DMF, and Trp on DMAA standard column. Mobile phase: 20 m*M* ammonium acetate–800 m*M* acetic acid in 20% (acetonitrile–methanol, 8:2). Voltage, 30 kV. Electrokinetic injection, 7 kV, 3 s. Detection, 214 nm. (b) Chromatographic separation of Phe, His, Trp on DMAA standard column. Mobile phase: 14 m*M* ammonium acetate–560 m*M* acetic acid in 30% acetonitrile. Column length, 15 cm. Flow-rate, 0.2 ml/min. Detection, 214 nm.

were calculated from retention data with a typical standard deviation of less than 1.5% (run-to-run reproducibility).

Generally, an increase in mobility is observed with increasing salt concentration in the mobile phase for any given amount of organic solvent. The effect is least pronounced for the highest investigated organic solvent content (60%, v/v) and for the EOF marker DMF. Phe and Trp show intermediate behavior, whereas His does not elute at all at 4 m*M* salt, but elutes first for the other two salt concentrations. At low salt all amino acids elute after the EOF marker (or not at all), while at least His and Phe tend to overtake the EOF marker at the higher salt concentration. Some deviation from these tendencies is observed at the highest investigated salt (20 m*M*) and organic solvent content (60%, v/v), where the mobilities of DMF and Phe are found to decrease compared to 10 m*M* salt–60% (v/v) solvent, while the mobility of Trp stabilizes and only that of His is still observed to increase.

If the effect of an increase in organic content is followed for a given salt concentration, at 4 m*M* salt, all mobilities increase with increasing organic solvent content (save for His, which could not be eluted/measured at all). A similar tendency for mobility increase is observed for DMF, Phe, and Trp at 10 m*M* salt, while the mobility of His decreases under these circumstances with increasing organic solvent content. At 20 m*M* salt, the mobilities of all investigated substances save Trp decrease with increasing organic solvent content. As a result, all amino acids elute in front of the EOF marker DMF when a mobile phase with 20 m*M* salt and 60% (v/v) organic solvent is used.

The interpretation of the observed behavior can largely rely on the assumption of a retention mechanism, which is principally governed by ion-exchange phenomena, superposed by differential electrokinetic mobility. At low organic solvent content (and low salt concentration) all substances are well retained, the EOF is also lowest (measured by the marker DMF). As the salt content of the mobile phase increases, the electrostatic interactions are reduced and the retention of all substances decreases; the first analytes start to overtake the non-retained EOF marker. As all analytes are charged, they are accelerated in the electric field when in the mobile phase, an effect, which becomes more determining as the retention is reduced. The effect is strongest for histidine, hence the tendency to elute first at high salt concentrations. As a consequence, at low salt (‘‘chromatographic domain’’) the retention of the amino acids on the stationary phase surface gives them a lower mobility than the noncharged EOF marker. At higher mobile phase salt concentration (‘‘electrophoretic domain’’) the charged analyte molecules spend sufficient time in the mobile phase to be accelerated beyond the speed of the EOF by the additional effect of electrophoresis.

The effect of the organic solvent content of the mobile phase for a given salt concentration is more difficult to explain. At low salt concentration, an increase in the organic solvent content also reduces retention, i.e., the behavior is reminiscent of reversed-phase chromatography. At higher salt concentration, an increase of the organic solvent content may have a positive or a negative effect on the mobility of the analytes. Especially at 60% solvent, the mobilities of some analytes go through an optimum as a function of the salt content, which is reminiscent of the behavior observed by Gusev et al. [22]. However, this is in our case largely due to a corresponding change in the strength of the EOF. When the capacity factors themselves were calculated, these values were found to either increase or decrease consistently as a function of the salt and/or organic solvent content.

An elevated content of organic solvent in the mobile phase will reduce hydrophobic interactions and increase the importance of electrostatic but also hydrophilic ones (such as H-bridges). Putative changes in the charge status of the analytes have to be taken into account in this context. Even though a pH of 3.0 was measured for the ammonium acetate–acetic acid stock solution in water, the apparent pH* measured in organic phase were pH* = 3.5 in 20%, pH* = 4.0 in 40% and pH* = 4.5 in 60% organic phase. The latter approaches the isoelectric point of Trp and Phe. At low salt concentration, the organic solvent thus acts as eluent. At intermediate salt concentration, it start at least in the case of His to enforce some other type of interaction instead, hence the reduced mobility of this compound. At high salt, this enforcing rather than eluting effect seems to be active in the case of Phe as well and only Trp is

becoming more mobile as the organic solvent content is increased. Although the hydrophilicity of the analytes investigated here seems to be insufficient to make hydrophilic interaction dominate the chromatographic behavior–as had previously been observed for uncharged aromatic compounds [31]–it may nevertheless be able to influence the chromatographic behavior of such substances at high organic solvent–salt content.

The presence of a mixed-mode interaction mechanism is also evidenced in Fig. 5, where the shape of the histidine elution peak is shown as a function of the mobile phase composition. A high salt content (reduced electrostatic interactions) combined with a low organic solvent content (low hydrophilic–high hydrophobic interactions) results in the sharpest peak (Fig. 5d) and the highest mobility. If the salt content is reduced while the organic solvent content remains low (Fig. 5c), the peak shape deteriorates dramatically. Intermediate salt and organic solvent content (Fig. 5b) result again in a reasonable peak shape, albeit with lower plate numbers. At intermediate salt and high organic solvent content (Fig. 5a), the peak shape deteriorates once more. Thus the peak shape improves when the buffer concentration in the mobile phase is high enough to reduce the electro-

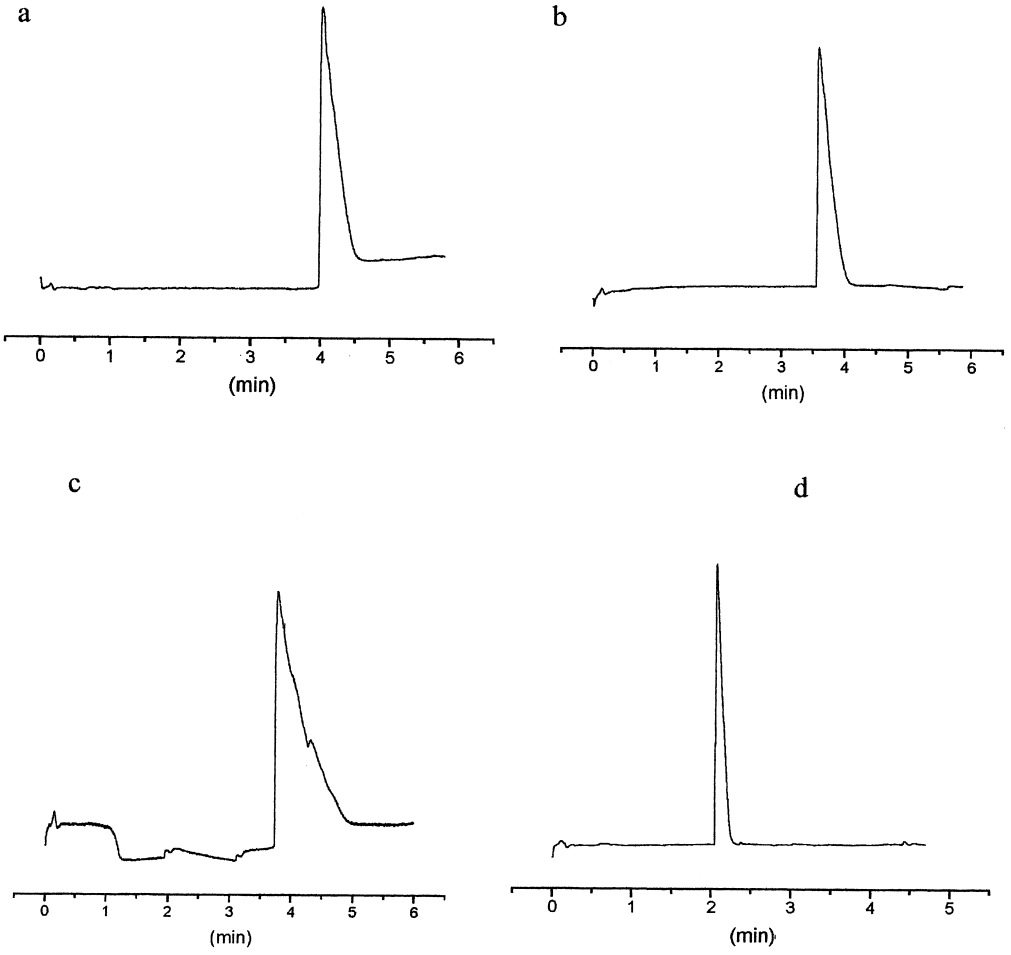


Fig. 5. Comparison of the peak shape of histidine as a function of the mobile phase composition. Stationary phase: DMAA standard column: (a) 20 m*M* ammonium acetate–800 m*M* acetic acid in (acetonitrile–methanol, 8:2)–water (6:4); (b) 20 m*M* ammonium acetate–800 m*M* acetic acid in (acetonitrile–methanol, 8:2)–water (4:6); (c) 13.3 m*M* ammonium acetate–800 m*M* acetic acid in (acetonitrile–methanol, 8:2)–water (2:8); (d) 40 m*M* ammonium acetate–800 m*M* acetic acid in (acetonitrile–methanol, 8:2)–water (2:8).

static interactions between the charged functionalities of the stationary phase and the analytes, and if the aqueous content of the mobile phase is sufficient to reduce the hydrophilic interactions.

In order to further investigate the influence of the two phenomena, stationary phases were subsequently synthesized, where either the surface charge density was varied or where the DMAA was (partially) replaced by a more hydrophobic monomer (Table 1). Evidently a change in the surface charge density produces changes in the overall flow velocity, i.e., the EOF, as well as changes in the retention behavior. Fig. 6 shows the dependency of the mobilities of the EOF marker and the amino acids on the amount (μl) of VSA added to the polymerization mixture. As expected, the flow velocity increased with the addition of VSA (increase in the mobility of the EOF marker), albeit in a nonlinear fashion. Incidentally, when no VSA was added, no EOF was observable, hence a complete coverage of the silica surface by the monolithic column can be assumed. When normalized for the increase in EOF, the mobilities of all investigated amino acids and most pronouncedly His were reduced (increased retention) as the amount of charged monomers used for column preparation and hence presumably the charge density of the stationary phase was increased. This clearly speaks for an involvement of the surface charges in the retention of all investigated amino acids.

Subsequently columns were produced where the DMAA was substituted by comonomers such as MAA, HEMA and HEA. The compositions of the polymer mixtures were adapted in each case, in order to obtain polymers of similar physical characteristics (mechanical strength, porosity). When these columns were subjected to similar tests of the influence of the mobile phase composition on the separation of charged amino acids, retention was consistently strongest on the HEMA column, followed by MAA. HEA and DMAA columns showed a somewhat lower and very similar retention potential (Fig. 7). Overall this influence of the stationary phase chemistry can be interpreted as a direct effect of the monomer hydrophilicity, which changes in the order of HEMA>MAA>HEA–DMAA.

3.3. Separation of charged peptides

Based on the experience gained with the amino acids, first peptide separations were attempted. The CEC separation of Gly–Phe and Phe–Gly–Phe–Gly is shown in Fig. 8a. A mobile phase containing 20 m*M* ammonium acetate and 20% (v/v) organic solvent was suitable in this case. Both peptides

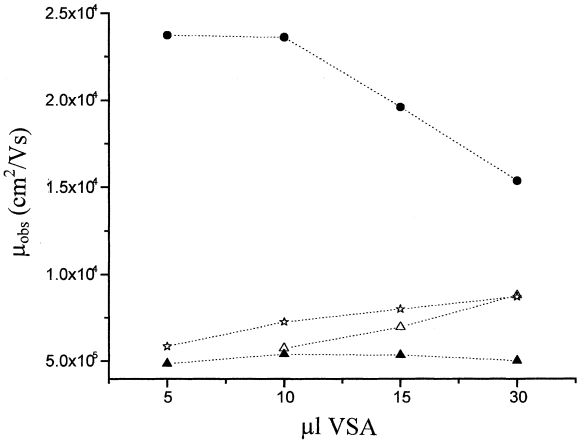


Fig. 6. Observed mobilities of (△) DMF, (☆) Phe, (●) His, (▲) Trp in function of the amount of vinyl sulfonic acid added to the monomer mixture during the synthesis of a DMAA stationary phase. Mobile phase: 20 m*M* ammonium acetate–800 m*M* acetic acid in 20% (acetonitrile–methanol, 8:2).

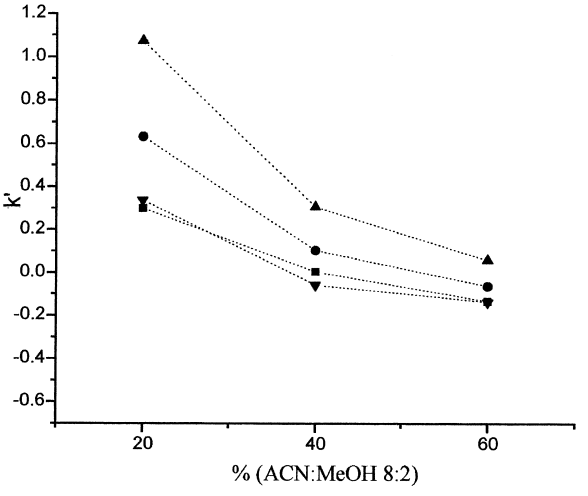


Fig. 7. Comparison of the retention factors of Tryptophan measured on (■) DMAA, (▼) HEA, (▲) HEMA, and (●) MAA columns as a function of the organic solvent content in a mobile phase containing 20 m*M* ammonium acetate–800 m*M* acetic acid.

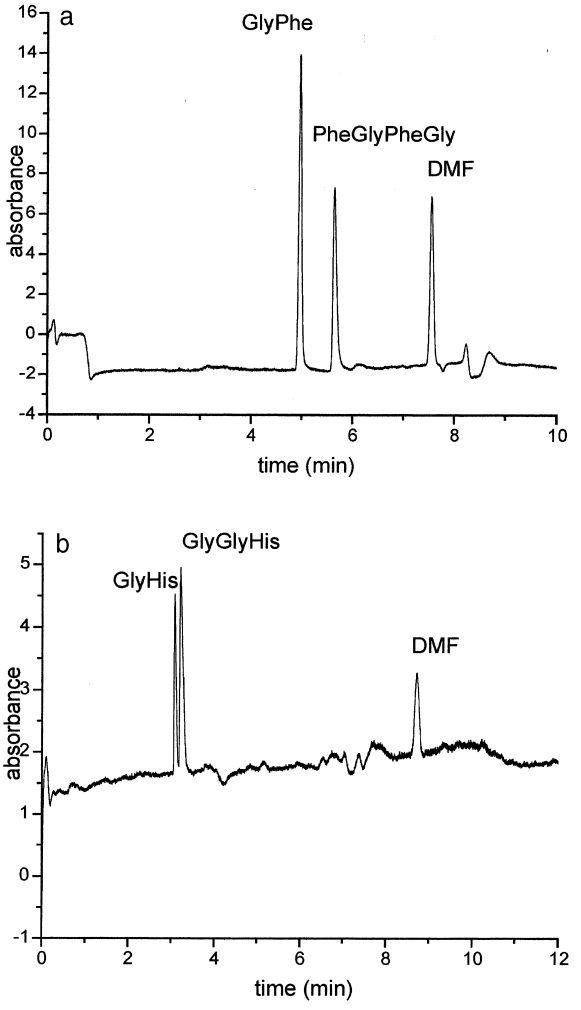


Fig. 8. (a) Electrochromatographic separation of Phe–Gly and Phe–Gly–Phe–Gly. Stationary phase: DMAA; mobile phase: 20 m*M* ammonium acetate–800 m*M* acetic acid in 20% (acetonitrile–methanol, 8:2). (b) Electrochromatographic separation of Gly–His and Gly–Gly–His. Stationary phase: DMAA (T=15%, C=52%); mobile phase: 40 m*M* ammonium acetate–800 m*M* acetic acid in 20% (acetonitrile–methanol, 8:2).

eluted before the EOF marker, with Phe–Gly–Phe–Gly eluting after Gly–Phe. While the separation of Gly–Phe and Gly–Val was not possible, the separation of the closely related Gly–His and Gly–Gly–His could be achieved (Fig. 8b). For this separation, a mobile phase with a higher ammonium acetate concentration (40 m*M*) and 20% (v/v) organic solvent was necessary. However, this salt concentration was not sufficient for the elution of multiply charged peptides, e.g., the triply charged His–Lys.

4. Conclusions

In hydrodynamically driven chromatography we have become accustomed to single mode interactions. Unfortunately, this is nearly impossible if chromatographic interactions are to be combined with electroosmotic propagation of the mobile phase (CEC). The necessity of the presence of charges for generation of the EOF leads to mixed mode interactions on most CEC columns. At present this tends to complicate the separation especially of charged analytes. In this paper a monolithic column capable of supporting both electrostatic and hydrophilic interactions was investigated for the separation of charged amino acids and peptides. An ion-exchange mode superposed with electrokinetic migration was found to be the dominant mode. However, the uncharged functionalities appeared to fine-tune the separation. Best results were obtained with mobile phases where the strength of both kinds of interactions was either low or intermediate. The development of monoliths of more pronounced hydrophilicity is currently under investigation in our group.

Acknowledgements

This work was supported by the Swiss National Science Foundation (grant No. 20-58879.99 to RF).

References

[1] F. Svec, E.C. Peters, D. Sykora, J.M.J. Frechet, J. Chromatogr. A 887 (2000) 3.
[2] I.S. Krull, A. Sebag, R. Stevenson, J. Chromatogr. A 887 (2000) 137.
[3] K. Walhagen, K.K. Unger, M.T.W. Hearn, J. Chromatogr. A 887 (2000) 165.
[4] Z. Deyl, F. Svec, in: Capillary Electrochromatography, Elsevier, Amsterdam, 2001.
[5] M.L. Ye, H.F. Zou, Z. Liu, J.Y. Ni, J. Chromatogr. A 869 (2000) 385.
[6] N.W. Smith, M.B. Evans, Chromatographia 41 (1995) 197.
[7] A.H. Que, T. Konse, A.G. Baker, M.V. Novotny, Anal. Chem. 72 (2000) 2703.

[8] R. Xiang, Cs. Horváth, Anal. Chem. 74 (2002) 762.
[9] M.L. Ye, H.F. Zou, Z. Liu, J. Zhu, J.Y. Ni, Y.K. Zhang, Sci. China Ser. B Chem. 42 (1999) 639.
[10] J. Stahlberg, J. Chromatogr. A 887 (2000) 187.
[11] J. Stahlberg, J. Chromatogr. A 892 (2000) 291.
[12] B.A. Grimes, A.I. Liapis, J. Chromatogr. A 919 (2001) 157.
[13] T. Adam, K.K. Unger, J. Chromatogr. A 894 (2000) 241.
[14] K. Walhagen, K.K. Unger, M.T.W. Hearn, J. Chromatogr. A 893 (2000) 401.
[15] K. Walhagen, K.K. Unger, M.T.W. Hearn, Anal. Chem. 73 (2001) 4924.
[16] K. Walhagen, K.K. Unger, A.M. Olsson, M.T.W. Hearn, J. Chromatogr. A 853 (1999) 263.
[17] Y. Liu, D.J. Pietrzyk, Anal. Chem. 72 (2000) 5930.
[18] P.Q. Huang, X.Y. Jin, Y.J. Chen, J.R. Srinivasan, D.M. Lubman, Anal. Chem. 71 (1999) 1786.
[19] J. Zhang, X. Huang, S.H. Zhang, Cs. Horváth, Anal. Chem. 72 (2000) 3022.
[20] W.S. Xu, F.E. Regnier, J. Chromatogr. A 853 (1999) 243.
[21] B. He, J.Y. Ji, F.E. Regnier, J. Chromatogr. A 853 (1999) 257.
[22] I. Gusev, X. Huang, Cs. Horváth, J. Chromatogr. A 855 (1999) 273.
[23] X. Huang, J. Zhang, Cs. Horváth, J. Chromatogr. A 858 (1999) 91.
[24] S.H. Zhang, X. Huang, J. Zhang, Cs. Horváth, J. Chromatogr. A 887 (2000) 465.
[25] C. Ericson, S. Hjertén, Anal. Chem. 71 (1999) 1621.
[26] C. Yu, F. Svec, J.M.J. Frechet, Electrophoresis 21 (2000) 120.
[27] R. Shediac, S.M. Ngola, D.J. Throckmorton, D.S. Anex, T.J. Shepodd, A.K. Singh, J. Chromatogr. A 925 (2001) 251.
[28] R.A. Wu, H.F. Zou, M.L. Ye, Z.D. Lei, J.Y. Ni, Anal. Chem. 73 (2001) 4918.
[29] M. Lämmerhofer, F. Svec, J.M.J. Frechet, Anal. Chem. 72 (2000) 4623.
[30] M. Lämmerhofer, E.C. Peters, C. Yu, F. Svec, J.M.J. Frechet, Anal. Chem. 72 (2000) 4614.
[31] D. Hoegger, R. Freitag, J. Chromatogr. A 914 (2001) 211.
[32] S. Hjertén, Arch. Biochem. Biophys. Suppl. 1 (1962) 147.
[33] S. Hjertén, K. Kubo, Electrophoresis 14 (1993) 390.
[34] A.S. Rathore, Cs. Horváth, Anal. Chem. 70 (1998) 3271.
[35] E.C. Peters, M. Petro, F. Svec, J.M.J. Frechet, Anal. Chem. 70 (1998) 2288.
[36] M. Lammerhofer, F. Svec, J.M.J. Frechet, Anal. Chem. 72 (2000) 4623.
[37] M.M. Dittmann, G.P. Rozing, J. Chromatogr. A 744 (1996) 63.
[38] D. Hoegger, Thesis No. 2464, Ecole Polytechnique Fédérale de Lausanne, Lausanne, 2001.
[39] M.C.J. Wilce, M.-I. Aguilar, M.T.W. Hearn, Anal. Chem. 67 (1995) 1210.
[40] T. Yoshida, T. Okada, T. Hobo, R. Chiba, Chromatographia 52 (2000) 418.