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Application of hydrophobic anion-exchange phases in capillary electrochromatography

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Abstract

Capillary electrochromatography (CEC) requires stationary phases that enable appropriate electroosmotic propel under various conditions. Analyte retention can be controlled through hydrophobic or electrostatic interaction with the packing material. The development and characterization of new strong anion-exchange materials with additional hydrophobic moieties (SAX/C_{18} mixed-mode phases) is described. The synthesis was based on polymer encapsulation of porous silica. The phases were systematically characterized by means of elemental analyses, HPLC frontal analyses and CEC experiments. The studies focused on the influence of various parameters (e.g., pH, kind of buffer, capillary wall) on the electroosmotic flow (EOF). Phases with high anion-exchange capacity generated a fast and constant EOF over a wide pH range. Long-time stability of EOF and hydrophobic retention under CEC conditions were demonstrated within the course of 100 consecutive injections. The applicability of the SAX/C_{18} phases in appropriate buffer systems is demonstrated for neutral, acidic and basic compounds.

Keywords: Stationary phases, electrochromatography; Mixed-mode chromatography; Electroosmotic flow; pH effects; Stability studies; Capillary electrochromatography

1. Introduction

Capillary electrochromatography (CEC) is a hybrid microseparation technique combining the excellent efficiency of capillary electrophoresis (CE) and the high selectivity of micro-high-performance liquid chromatography (μ-HPLC). The concept of using the electroosmotic flow (EOF) to transport a mobile phase through a particle-packed bed was introduced by Pretorius et al. in 1974 [1]. Due to the plug-like flow profile of the EOF, the separation efficiency obtained in CEC is higher compared to μ-HPLC, where pressure gradients are applied to drive the mobile phase [2–4].

Most of the work done in CEC by now focused on silica-based ODS-type stationary phases originally developed for HPLC [3,5–10]. A major drawback of these stationary phases is the strong pH dependence of the EOF, which disables fast separations under acidic pH conditions. In order to perform fast CEC separations even under acidic conditions strong cation-exchange (SCX) [3,11–14] or SCX/alkyl chain mixed-mode phases [15–20] have been used. The number of publications dealing with strong anion-exchange (SAX) or SAX/alkyl chain mixed-mode packings for CEC is still smaller. P. Huang et al. used a commercial anion-exchange/reversed-phase mixed-mode stationary phase, which must be considered a weak anion-exchanger (WAX), for the

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separation of peptide mixtures in pressurized capillary electrochromatography (pCEC) [21]. A secondary amine group provided the positive charges necessary for anion-exchange. As the ionization state of secondary amines decreases significantly in the neutral pH range, a constant and fast anodic EOF was generated between pH 2 and pH 5. At higher pH values the EOF velocity dropped dramatically. X. Huang et al. employed porous-layer open-tubular (PLOT) CEC columns with a positively charged chromatographic surface to analyze basic proteins and peptides [22]. The highly cross-linked porous layer was prepared by in situ polymerization of vinylbenzyl chloride and divinylbenzene in the presence of a porogen inside a fused-silica capillary. To obtain the positive surface charges, the chloromethyl functions of the porous layer were reacted with a tertiary amine. Zhang et al. utilized CEC columns packed with a silica-based SAX phase to separate a mixture of acidic proteins [23]. The synthesis of this SAX phase was a two-step reaction. First the silica beads were reacted with a heterobifunctional silanizing reagent. Then a vinyl monomer containing a quaternary ammonium group was covalently attached. CEC columns packed with these SAX particles showed constant EOF velocities from pH 2.5 to pH 7. Another approach to modify the surface of silica particles was described by Suzuki et al. [24]. A chemically bonded SAX phase was prepared in-column by pumping a special silylating reagent through a CEC capillary packed with silica particles. The silylating reagent provided positive surface charges (quaternary ammonium functions) as well as hydrophobicity (C_{18} alkyl chains). The resulting stationary phase was employed for the separation of alkylbenzoates and 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives of some monosaccharides. Ye et al. [25] separated different aromatic acids via anion-exchange CEC using a commercially available SAX material as stationary phase. Klampfl et al. [26] described the separation of acidic, basic and neutral organic compounds as well as inorganic ions using a commercial SAX/C_6 mixed mode stationary phase. The EOF generated by this stationary phase showed a severe decrease when the pH of the mobile phase was raised from 6 to 8.5. Hilder et al. [27] employed a commercial polymer-based anion-exchanger for CEC separations of inorganic anions by generating the mobile phase flow by pure electroosmosis as well as by an additional pressure gradient.

In this paper we present the synthesis of new polymer encapsulated SAX/C_{18} mixed-mode phases. The resulting stationary phases were characterized for hydrophobic properties and the ion-exchange capacities were measured by means of HPLC frontal analyses. Both values varied over a wide range, thus enabling systematic studies on the influence of these parameters on their behavior under CEC conditions. In order to investigate the correlation between anion-exchange capacity and EOF velocity, the SAX/C_{18} phases were characterized with different buffer systems at various pH values. It will be shown that high ion-exchange capacity of the SAX/C_{18} phases generally leads to pH-independent EOF. The long-time stability of EOF and hydrophobic retention measured on these mixed-mode phases were studied and different buffers were compared. Furthermore, the applicability of the phases for highly efficient CEC separations was demonstrated in the analysis of different test mixtures. Both neutral and charged analytes were employed.

2. Materials and methods

2.1. Materials and reagents

Fused-silica capillaries (100 μm I.D.×365 μm O.D. and 50 μm I.D.×365 μm O.D.) were obtained from Polymicro Technology (Phoenix, AZ, USA). The ProntoSIL 120-5 silica (pore width 12 nm, particle diameter 5 μm) was purchased from Bischoff Chromatography (Leonberg, Germany). Spherisorb S5 (pore width 8 nm, particle diameter 5 μm) was purchased from Waters Phase Separations (Milford, MA, USA). A commercial C_{18} phase containing a quaternary ammonium function (Stability 100 BS C23; pore width 10 nm, particle diameter 5 μm) was kindly donated by Dr Maisch (High Performance LC, Ammerbuch, Germany).

Vinylbenzyl chloride was obtained from Polysciences (Warrington, PA, USA). Triethylamine (≥99.6%), phosphoric acid (85%) and toluene (≥99.6%) were purchased from Merck (Darmstadt, Germany). Vinyltrichlorosilane (≥97%), α,α′-azo-isobutyronitrile (AIBN, ≥98%), thiourea, *p*-ethyl-

aniline, ethylbenzene, monobasic, dibasic and tribasic sodium phosphate were obtained from Fluka (Deisenhofen, Germany). Octadecyl acrylate (97%), salicylic acid (≥98%), acetylsalicylic acid (≥99%), 2,5-dihydroxybenzoic acid (97%) and 2-hydroxyhippuric acid (97%) were purchased from Aldrich (Milwaukee, WI, USA). Acetone (≥99.5%) was purchased from Riedel-de Haen (Seelze, Germany). Gradient-grade acetonitrile (ACN) was received from Bischoff Chromatography. Tris(hydroxymethyl)-amino methane (Tris, ultrapure) was received from ICN Biomedicals (Aurora, OH, USA). Water was purified and deionized using a Milli-Q system (Millipore, Bedford, MA, USA). The pH values of the phosphate buffers were adjusted by mixing appropriate volumes of phosphoric acid with solutions of sodium dihydrogenphosphate or disodium hydrogenphosphate. The pH values were measured in the corresponding aqueous buffers before acetonitrile was added.

2.2. Instrumentation

All experiments were carried out using an Agilent CE system (Agilent Technologies, Waldbronn, Germany) with pressurization option. A nitrogen pressure of 6 bar was applied to both inlet and outlet vial during analysis in order to prevent bubble formation. Data were processed using Agilent Chemstation Software (Rev. A. 06.03). Separations were carried out at 25°C. Samples were injected electrokinetically at −3 kV for 3 s. The absorbances were measured at 210 nm with a bandwidth of 20 nm.

2.3. Synthesis of SAX/C_{18} mixed-mode phases

The synthesis of the SAX/C_{18} mixed-mode phases is depicted schematically in Fig. 1. The pure silica is initially treated with 5 *M* hydrochloric acid in order to activate the surface silanol groups. In the first step of the synthesis, the activated silica is brought to reaction with vinyltrichlorosilane resulting in a vinylsilica. This reaction is carried out in dry toluene and triethylamine is added to neutralize the hydrochloric acid formed in this process. In a second step the vinylsilica is transformed to the corresponding SAX/C_{18} mixed-mode phases via radical polymerization in solution using octadecyl acrylate (AODE) and triethylammonium methylstyrene chloride (TAMS), which was synthesized in a nucleophilic substitution of vinylbenzyl chloride and triethylamine, as reactive monomers and AIBN as radical initiator. This second transformation can either be done as a copolymerization (see Fig. 1) or the monomers can be fixed to the surface of the vinylsilica subsequently by two separate polymeri-

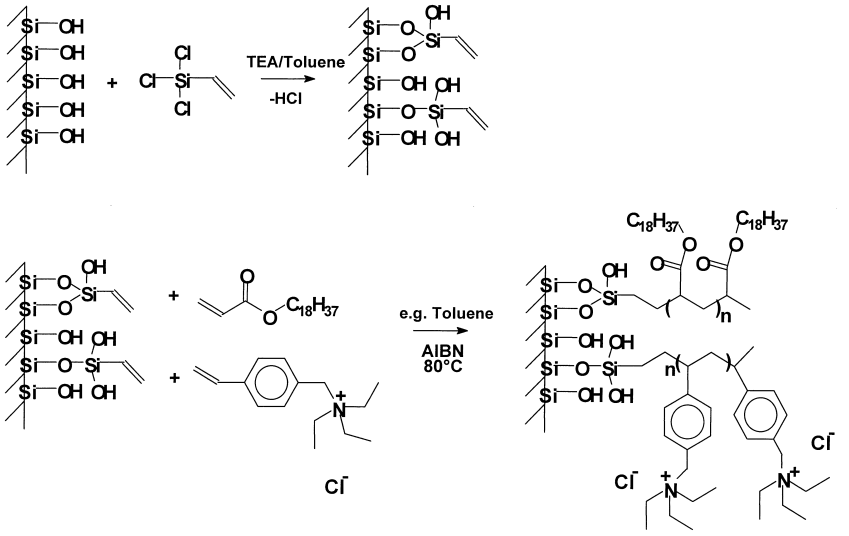

Fig. 1. Synthesis scheme for SAX/C_{18} mixed-mode phases.

zation steps, eventually with solvent variation. For a more detailed description of this so-called polyencap technology the original literature should be taken into account [28]. The ratio of AODE/TAMS and the selection of the solvent used in the polymerization reaction enable the variation of hydrophobicities and ion-exchange capacities of the mixed-mode phases.

2.4. Preparation of the CEC capillaries

We were unable to produce stable frits from the synthesized mixed-mode phases by hydrothermal treatment. Therefore the new fritless column technology taking advantage of the so-called keystone effect recently published by Rapp and Bayer [29] was employed. An internal taper served as inlet retainer and the outlet frit was replaced by a dead volume free connection of the packed capillary to an internally tapered detection capillary. For detailed information about this column technology the original paper should be considered.

The capillaries were packed with different SAX/C_{18} mixed-mode phases using the slurry packing technique [30,31]. As the capillary to be packed (I.D. 100 μm) was internally tapered before the packing procedure, it was not necessary to use any initial frit in order to retain the packing particles in the fused-silica capillary. After the capillary was fully packed, it was cut to the desired length of 25 cm and connected to an internally tapered detection capillary (I.D. 50 μm) via a polytetrafluoroethylene/perfluoroethylenepropylene (PTFE/FEP) dual shrinkage tube. It is important that the endings of both capillaries are absolutely plain to achieve a dead volume free connection of the capillaries.

After an initial training period, column to column reproducibility for CEC columns produced by this technique was satisfactory. The corresponding EOF velocities varied less than 6%.

3. Results and discussion

3.1. Characterization of SAX/C_{18} mixed-mode phases prior to investigation under CEC conditions

Before the synthesized mixed-mode phases were investigated under CEC conditions several characteristic parameters were determined. Ion-exchange capacity and hydrophobicity of a stationary phase play an important role for the application in CEC. In the case of unpolar analytes the retention factors will increase with increasing hydrophobicity (carbon content) of the stationary phase and its ion-exchange capacity will influence the magnitude of the EOF. In order to estimate the hydrophobicities of the different phases elemental analyses were carried out. It can be seen in Table 1 that the carbon contents of the phases vary between 13.1% (TAMS 1, SAX/C_{18} mixed-mode phase) and 5.2% (TAMS 3, pure SAX phase). The nitrogen percentages achieved from elemental analyses can only give indications about the amount of ammonium functions bound to the different silica surfaces. As the nitrogen contents are rather low these values must be considered error prone. To determine the anion-exchange capacities of these phases under chromatographic conditions, the phase materials were packed into HPLC columns and frontal analyses have been carried out. The ion-exchange capacities were calculated from nitrate breakthrough measurements. The corresponding numbers are also included in Table 1 and vary over a wide range. Comparing these ''chromatographic''

Table 1
Characterization of the different phases

Phase	Surface modification	Specific surface area (m^2/g)	% C	% N	Ion-exchange capacity (μmol/g)	
					Calculated from %N	By frontal analysis
TAMS 1	SAX/C_{18}	300	13.1	0.52	308	131.3
TAMS 2	SAX/C_{18}	220	8.2	0.35	216	98.8
TAMS 3	SAX	220	5.2	0.24	136	30.1
TAMS 4	SAX/C_{18}	220	10.3	0.27	159	49.9
Stab. BS C23	SAX/C_{18}	280	11.0	0.64	414	85.4

ion-exchange capacities with the values calculated from the nitrogen contents it becomes obvious that not all the surface charges are chromatographically accessible. This may be due to steric hindrance. Table 1 also provides an overview of other important phase parameters like the specific surface areas of all the materials employed in these investigations.

3.2. pH dependency of the EOFs generated by different SAX/C_{18} mixed-mode phases

The pH dependencies of the EOFs measured on the synthesized phases TAMS 2, TAMS 3 and TAMS 4, based on the same Sperisorb silica, are presented in Fig. 2. All experiments were carried out in a 5 m*M* phosphate buffer with pH values between 3 and 9. Thiourea was used as an inert marker. In addition to mobile phase solvent properties (ratio of dielectric constant to viscosity) the velocity of the EOF is also controlled by the nature of the stationary phase in equilibrium with the mobile phase buffer (surface charge density, ξ-potential). The net charge of the stationary phase depends on the pH of the mobile phase. On the one hand, the quaternary ammonium functions of the SAX/C_{18} mixed-mode phases carry positive charges over the whole pH range. On the other hand, the deprotonation of residual silanol functions on the packing particles is strongly dependent on the pH of the mobile phase. Silica is a moderate acid with a pK_a of about 5. Consequently, at pH 3 residual silanol functions can be considered almost undissociated and can no longer contribute to surface charge. In the neutral and alkaline pH range the residual surface silanols are fully deprotonated and thus negatively charged. The counterplay between these negative charges and the positive charges resulting from the surface modification of the SAX/C_{18} mixed-mode phases can be monitored by the curves presented in Fig. 2. The pure SAX phase (TAMS 3) is characterized by the lowest ion-exchange capacity and shows the smallest anodic EOF velocity which is constant between pH 3 and pH 7. At higher pH values the EOF velocity drops about 20% (pH 9), obviously due to partial compensation of the positive excess charges resulting from the SAX functionalities by the deprotonated residual silanol groups of the stationary phase. The mixed-mode phase with the highest hydrophobicity (TAMS 4) and a moderate anion-exchange capacity of 49.9 μmol/g generated the fastest EOF, which was more or less constant between pH 3 and pH 6. From pH 6 to pH 9 the EOF velocity observed for this phase decreased by a factor of 2. This stronger decrease compared to the phase TAMS 3 cannot be explained by differences in the concentration of residual silanol groups between both phases as they are based on the same vinyl-silica. Due to the increased hydrophobicity of the TAMS 4 phase, the irreversible adsorption of buffer ions ($H_2PO_4^-$, HPO_4^{2-}) on this SAX/C_{18} phase may explain the strong decrease of the EOF velocity at alkaline pH values. Such problems encountered with phosphate buffers are also known in RP–HPLC [32]. In contrast to theory, the Sperisorb based SAX/C_{18} mixed-mode phase with the highest ion-exchange capacity (TAMS 2, 98.8 μmol/g) did not generate the fastest EOF. But the EOF velocity of this phase was almost independent of the eluent pH. Even at pH 9 the measured EOF velocity of 1.22 mm/s was only 7% smaller than the value measured at pH 3.

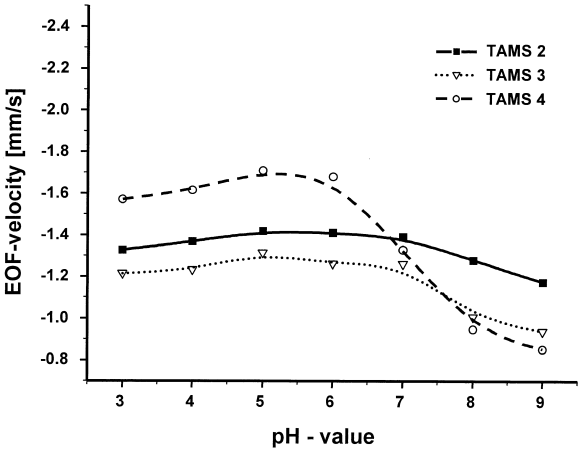

Fig. 2. Comparison of the EOF velocities generated by SAX/C_{18} phases (all phases based on the same Spherisorb silica). Buffer: 5 m*M* phosphate, pH variable–water–ACN (10:20:70); voltage: −25 kV; injection: −3 kV, 3 s; detection: UV, 210 nm; inert marker: thiourea; capillary: 100 μm I.D. packed with TAMS phases connected to 50 μm I.D. detection capillary ($L_{pac}/L_{det}/L_{total}$=25/26.4/34.9 cm).

In order to investigate whether high anion-exchange capacities tend to result in less pH-dependent EOF, two further materials of high ion-exchange capacities were packed into capillaries and run under

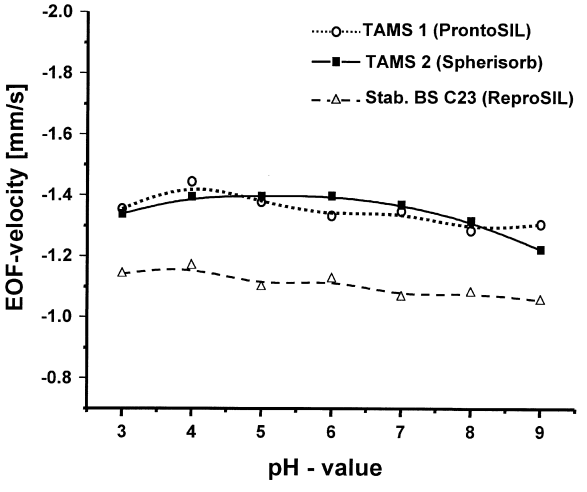

Fig. 3. Comparison of the EOF velocities generated by SAX/C_{18} phases (all phases based on different silicas). Buffer: 5 m*M* phosphate, pH variable–water–ACN (10:20:70) voltage: −25 kV; injection: −3 kV, 3 s; detection: UV, 210 nm; inert marker: thiourea; capillary: 100 μm I.D. packed with corresponding phases connected to 50 μm I.D. detection capillary ($L_{pac}/L_{det}/L_{total}$=25/26.5/34.9 cm).

different pH conditions (see Fig. 3). The ProntoSIL-based synthesized SAX/C_{18} mixed-mode phase (TAMS 1, ion-exchange capacity 131.3 μmol/g) and the commercially available phase Stability BS C23 (ion-exchange capacity 84.5 μmol/g) both exhibited an EOF-velocity almost independent of the eluent pH. Unlike all the laboratory-synthesized phases, Stability BS C23 is a brush-type stationary phase. This phase can be considered as a hydrophobic anion-exchanger. A dimethyloctadecylammonium group is covalently bound to an ultrapure silica via a propyl spacer and the activity of residual silanol groups is minimized by an endcapping procedure. The curve corresponding to the TAMS 2 phase is shown again in Fig. 3. The phases TAMS 1 and TAMS 2 were prepared under identical reaction conditions and the only difference was the silica. Since the synthesis is reproducible, two SAX/C_{18} mixed-mode phases with almost identical surface charge densities (only 2% deviation) resulted. Due to these identical surface charge densities both phases showed almost the same EOF–pH behavior from pH 3 to pH 9 in a phosphate buffer.

3.3. Influence of the buffer on EOF–pH characteristics

In order to get further information about the influence of the buffer components in the mobile phase, the SAX/C_{18} mixed-mode phases TAMS 2 and TAMS 4 were characterized in terms of their EOF velocities in a Tris buffer system at three different pH values where Tris provides adequate buffering capacity. The measured EOF velocities are depicted in Fig. 4. Compared to the EOF velocities observed in the phosphate buffer system, the decrease of the EOF velocity from pH 7 to pH 9 is less pronounced in the Tris-buffered eluent. The EOF velocities obtained for the TAMS 2 phase were not significantly different from the phosphate buffer system. On the other hand, the TAMS 4 phase driven in a Tris buffer showed constant EOF velocities from pH 7 to pH 9, which is an advantageous contrast to the behavior of the same phase in a phosphate system. Consequently, working in Tris-buffered mobile phases allows fast separations even under alkaline conditions using TAMS 4 as stationary phase. The curves measured for TAMS 4 underline the assumption that irreversible adsorption of negatively charged buffer ions may be the reason for the severe decrease of the EOF velocity under alkaline conditions using phosphate buffers. It should be mentioned that the ionic strength of the Tris buffers used in these investigations varied slightly as the pH values were adjusted by adding different amounts of hydrochloric acid.

3.4. Long-time stability of the SAX/C_{18} mixed-mode phases

In order to monitor the long time stability of the mixed-mode phases with respect to EOF velocity and hydrophobicity, 100 consecutive injections were carried out using different mobile phases. As already mentioned problems arise due to irreversible adsorption of phosphate buffer ions, especially under alkaline conditions. Therefore an acidic phosphate buffer (10 m*M* phosphate, pH 4–water–ACN, 10:20:70) was employed in these investigations of the TAMS 1 phase. The corresponding elution times of thiourea (EOF tracer) and the retarded toluene are shown in Fig. 5a). The RSD values for the elution

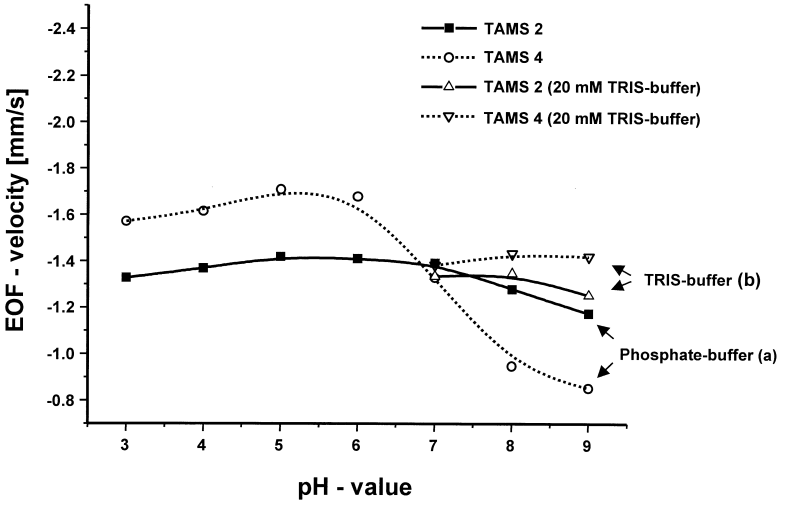

Fig. 4. EOF velocities generated by SAX/C_{18} phases in different buffer systems. Buffers: (a) 5 m*M* phosphate, pH variable–water–ACN (10:20:70); (b) 20 m*M* Tris, pH variable–water–ACN (10:20:70). Voltage: −25 kV; injection: −3 kV, 3 s; detection: UV, 210 nm; inert marker: thiourea; capillary: 100 μm I.D. packed with TAMS phases connected to 50 μm I.D. detection capillary ($L_{pac}/L_{det}/L_{total}$ 25/26.4/34.9 cm).

times of thiourea and toluene over 100 injections were 3.4% and 4.2% and both elution times showed slightly increasing tendencies. Looking at the retention factor of toluene, it becomes obvious that the hydrophobicity of the TAMS 1 phase increased with operation time when a phosphate buffer was used. The retention factor of toluene increased by 22% within the course of the first 50 injections. Then the retention factor of toluene remained more or less constant within the next 50 injections.

When the phosphate buffer was replaced by a Tris buffer system (20 m*M* Tris, pH 7–water–ACN, 10:20:70) the SAX/C_{18} phases showed excellent long-time stability of EOF velocity and hydrophobic retention. The elution times of thiourea and toluene as well as the retention factor of toluene measured on the TAMS 4 phase using the Tris buffer are shown in Fig. 5b. The elution times of thiourea and toluene were constant over the range of 100 injections and the corresponding RSD values were 1.2% and 1.5%. The retention factor of toluene was also constant and its RSD value of 1.4% was comparable to HPLC reliability.

This observation can be explained again by irreversible adsorption of buffer ions ($H_2PO_4^-$) which obviously occurs also at a pH of 4. This adsorption leads to a decrease in the positive surface charge density and thus increases the hydrophobicity of the SAX/C_{18} mixed-mode phase.

Thus the Tris buffer proved to be superior to the phosphate buffer in terms of the long-time stability of electrochromatographic performance with the described SAX/C_{18} mixed-mode phases.

3.5. Influence of the connected detection capillary on the EOF

The potential contribution of negative capillary surface charges on the overall EOF in packed column CEC led to controversial discussions in literature.

Zhang and El Rassi [16] observed higher EOF velocities for octadecyl sulfonated silica particles packed into fused-silica capillaries with a hydrophilic sulfonated coating than for the same particles packed into untreated fused-silica capillaries. The difference in the EOF velocities was most pronounced in the acidic pH range.

On the other hand Dittmann and Rozing [15] as well as Smith and Evans [19] proved that in packed

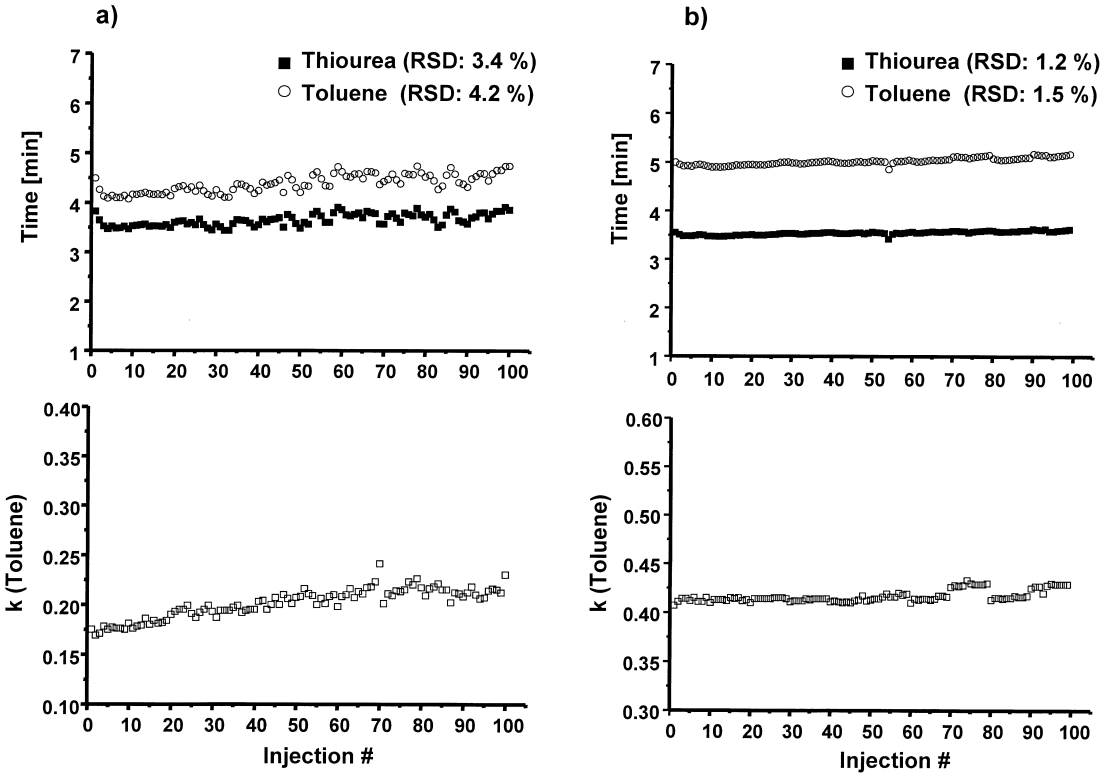

Fig. 5. Long-time stability of EOF and hydrophobic retention on of SAX/C_{18} mixed-mode phases. Buffers: (a) 10 m*M* phosphate, pH 4–water–ACN (10:20:70); (b) 20 m*M* Tris, pH 7–water–ACN (10:20:70); Voltage: −25 kV; injection: −3 kV, 3 s; detection UV, 210 nm; inert marker: thiourea; capillary: 100 μm I.D. packed with TAMS 1 (a) and TAMS 4 (b) connected to 50 μm detection capillary ($L_{pac}/L_{det}/L_{total}$=25/26.4/34.4 cm).

column CEC the EOF is almost exclusively generated by the particles. Dittmann and Rozing [15] separated a neutral test mixture using an ODS-type stationary phase and a Tris-buffered mobile phase (pH 8). This separation was carried out under different capillary wall conditions: The stationary phase was packed in a poly(vinyl alcohol)-coated fused-silica capillary (suppressed EOF because of shielded silanol groups) and in a bare fused-silica capillary. Neither the elution times of the test solutes nor the EOF velocity changed with different capillary wall properties.

Smith and Evans [19] carried out comparable experiments and concluded also that the packing material contributes predominantly to the EOF in packed column CEC.

One significant difference between these experiments described in literature and our work lies in the sign of the ξ-potentials of the packing particles and the capillary walls. All the authors mentioned above worked in CEC systems where the surface of the packing particles and the capillary walls were both negatively charged. In our experiments (Tris buffer, pH 7) the capillary wall is negatively charged whereas the packing particles have positive ξ-potentials.

Smith reported also that the use of columns packed with SAX materials in CEC was only possible either under acidic pH conditions or in the combination with coated capillary walls [33]. A possible reason for the problems Smith encountered with his SAX phases may be a low anion-exchange capacity of these packings. This would favor an influence of the negative wall charges located on the fused-silica capillary wall.

As already mentioned, all the SAX/C_{18} mixed-

mode phases employed in this study generated an EOF directed towards the anode when a mobile phase pH between 3 and 9 was applied. The anion-exchange stationary phases were packed into unmodified fused-silica capillaries. The wall charge of the fused-silica capillary is strongly dependent on the pH of the mobile phase. In the acidic pH range the surface silanols are only deprotonated to a low degree. If the pH is increased to the neutral and alkaline range, the silanol groups of the capillary wall get more and more deprotonated. Consequently, the negative surface charge density and thus the ξ-potential of the capillary wall adopt higher values. In order to investigate whether the negative ξ-potential of the capillary wall affects the EOF mobility of the capillaries packed with the SAX/C_{18} mixed-mode phases (positive ξ-potential) the following experiments have been designed. The phases TAMS 2 (high ion-exchange capacity) and TAMS 3 (lowest ion-exchange capacity) were packed into fused-silica capillaries (100 μm I.D.) of about 24 cm length. Usually these packed capillaries were connected to 10-cm-long internally tapered detection capillaries (50 μm I.D.). To increase the potential effect of the capillary wall charges these short detection capillaries were replaced by ~29-cm-long detection capillaries. Thus the overall lengths of the capillary assemblies were ~53 cm. Detection windows were initially located about 44 cm away from the capillary inlet, which implied 20 cm of open fused-silica capillary between the end of the packed bed and the detection window. The EOF mobilities of both phases (TAMS 2 and TAMS 3) were measured using a Tris buffer at neutral pH and the column geometries described above. The EOF mobilities calculated from five consecutive injections are depicted graphically in Fig. 6. These capillaries were then shortened by ~20 cm (in the section of the detection capillary) and new detection windows were produced closer to the end of the packed bed. Again the mobilities were measured under identical mobile phase conditions. The corresponding EOF mobilities are also shown in Fig. 6. Comparing the ''long'' and the ''short'' column geometries for TAMS 2 and TAMS 3, one can see that the EOF mobilities are of comparable magnitude in both cases. For the TAMS 2 phase the EOF mobility was 5.6% higher in the ''short'' column geometry, whereas in the case of the TAMS 3 phase the EOF mobility measured in the ''short'' column geometry was 10% lower. This indicates that the negative charges of the fused-silica walls do not have a significant influence on the overall EOF mobility. If these negative charges played a major role, the EOF mobilities measured in the ''long'' column geometry should have been significantly smaller than those obtained in the ''short'' column geometry due to the higher amount of surface silanols. These results indicate that the positive charges located in the packed bed overcompensate the negative charges of the capillary wall even if the detection capillary is extraordinarily long. No problems with stability of current or EOF were observed although the ξ-potentials of the packing particles (SAX/C_{18}, SAX) and the capillary walls were of opposite signs.

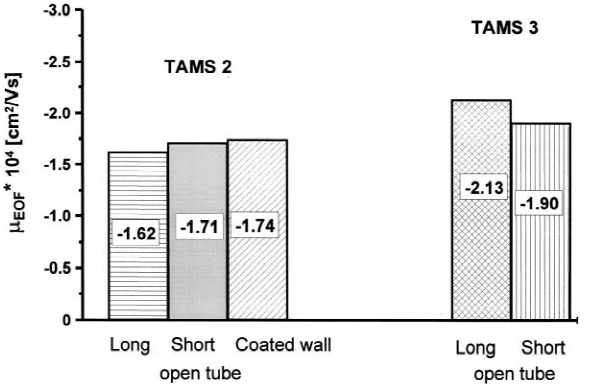

Fig. 6. Influence of the capillary wall charges on the mobility of the EOF. Buffer: 20 m*M* Tris, pH 7–water–ACN (10:20:70); voltage: −25 kV; injection: −3 kV, 3s; detection: UV, 210 nm; inert marker: thiourea; capillary: 100 μm I.D. packed with TAMS 2 and TAMS 3 connected to 50 μm I.D. detection capillaries of different lengths: TAMS 2 (long open tube): ($L_{pac}/L_{det}/L_{total}$=24/44.6/53.1 cm); TAMS 2 (short open tube): ($L_{pac}/L_{det}/L_{total}$=24/27.1/36 cm); TAMS 2 (coated wall open tube): ($L_{pac}/L_{det}/L_{total}$=25.1/26.5/35 cm); TAMS 3 (long open tube): ($L_{pac}/L_{det}/L_{total}$=23/42.5/51 cm); TAMS 3 (short open tube): ($L_{pac}/L_{det}/L_{total}$=23/25.5/34 cm).

In order to complete our studies on the potential influence of the capillary wall charges the phase TAMS 2 was packed into a fused-silica capillary carrying covalently fixed ammonium functions. The wall of the connected detection capillary was also covalently coated with positively charged TAMS groups. In that case both the capillary walls and the

packing particles have positive ξ-potentials and generate an EOF directed towards the anode. The EOF mobility measured in this capillary setup (-1.74 cm^2/V s) is comparable to the EOF mobility measured with the same packing particles packed into unmodified fused-silica capillaries (-1.71 cm^2/V s) (see Fig. 6).

Our experimental observation of a negligible contribution of the capillary inner wall to the overall EOF mobility contrasts the results of Zhang and El Rassi [16] and the SAX results of Smith [33]. On the other hand it is not surprising that the capillary wall charges do not significantly affect the overall EOF mobility as the surface area of the packing particles is almost seven times higher than the capillary wall surface even if the pores are neglected. This calculation is based on the assumption of a closely packed capillary, where 78% of the volume is filled by the particles. Nevertheless, charges located in larger pores may additionally contribute to the overall EOF.

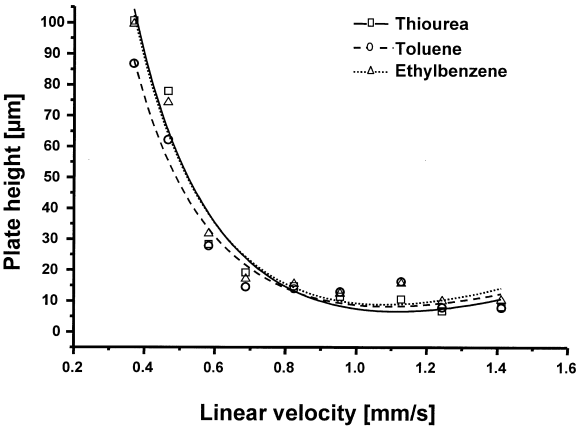

Fig. 7. Van Deemter plot of an SAX/C_{18} mixed-mode phase. Buffer: 5 m*M* phosphate, pH 4–water–ACN (10:20:70); voltage: variable, -5 to -30 kV; injection: -3 kV, 3 s; detection: UV, 210 nm; samples: thiourea; toluene, ethylbenzene; capillary: 100 μm I.D. packed with TAMS 1 connected to 50 μm I.D. detection capillary ($L_{pac}/L_{det}/L_{total}$=25/26.4/34.4 cm).

3.6. Electrochromatographic characterization of the SAX/C_{18} mixed-mode phases

3.6.1. Van Deemter plot

In order to investigate the dependence of the separation efficiency on the linear velocity of the mobile phase, a Van Deemter curve was measured. The test mixture contained thiourea, toluene and ethylbenzene. The SAX/C_{18} phase TAMS 1 was used in combination with an acidic phosphate buffer. As can be seen in Fig. 7 plate heights as low as 7 μm (reduced plate height 1.4) in the minimum could be reached. The corresponding linear velocities were in the range around 1.1 mm/s. The low plate heights could demonstrate that the synthesized SAX/C_{18} mixed-mode phases are suitable for highly efficient CEC separations.

3.6.2. Standard phase test under acidic and alkaline conditions

A test mixture consisting of thiourea, *p*-ethylaniline, toluene and ethylbenzene was separated under acidic and alkaline conditions using a capillary packed with the TAMS 1 phase (see Fig. 8). As the EOF velocity of the TAMS 1 phase is almost independent of the eluent pH, the separations at pH 3 and pH 9 can be carried out in comparable analysis time. The retention factors of the test analytes and the corresponding plate numbers are given in Table 2. The observed separation efficiencies were excellent at both pH values. The hydrophobic compounds toluene and ethylbenzene show 1.7 times higher retention factors at pH 9. In the case of the hydrophobic analytes the stronger retention under alkaline conditions may be due to reduced positive excess surface charge on the packing particles because of the deprotonation of residual silanol functions. The result is a more hydrophobic stationary phase. This model cannot explain the constancy of the EOF velocity. According to these assumptions, the EOF velocity should decrease with decreasing positive excess surface charges of the packing material. The effect of stronger retention under alkaline conditions is even more pronounced for the weak base *p*-ethylaniline where the retention factor at pH 9 is twice as high as under acidic conditions. In that case the effect can be explained by partial exclusion of the positively charged *p*-ethylaniline from the SAX/C_{18} phase which carries also positive charges.

3.6.3. Hydrophobic retention on SAX/C_{18} mixed-mode phases

In RP chromatography the retention factors of

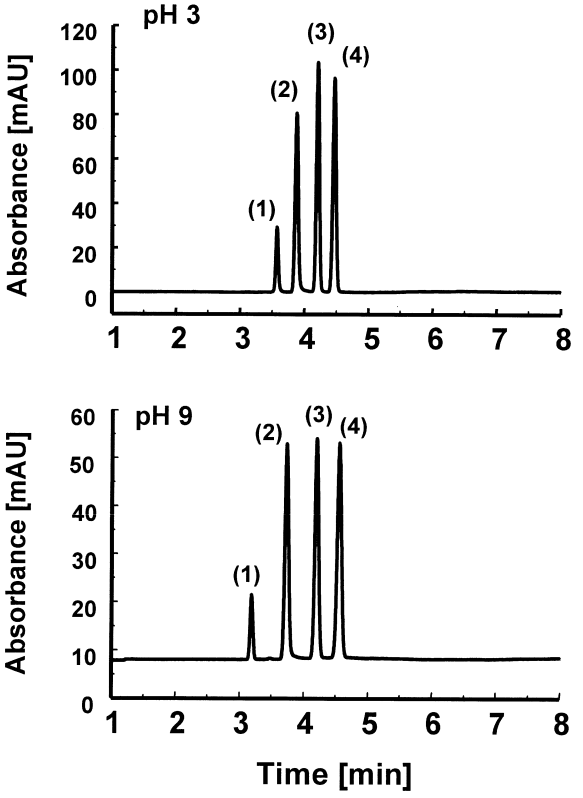

Fig. 8. Separation of a test mixture under acidic and alkaline conditions. Buffers: 5 m*M* phosphate, pH 3 and pH 9–water–ACN (10:20:70); voltage: −25 kV; injection: −3 kV, 3 s; detection: UV, 210 nm; samples: (1) thiourea, (2) *p*-ethylaniline, (3) toluene, (4) ethylbenzene; capillary: 100 μm I.D. packed with TAMS 1 connected to 50 μm I.D. detection capillary ($L_{pac}/L_{det}/L_{total}$=23.3/24.7/33 cm).

hydrophobic compounds increase linearly with the carbon content of the stationary phase.

In order to investigate whether such a correlation exists also in the case of SAX/C_{18} phases the retention factor of toluene was determined for all the phases mentioned in Table 1. All experiments were carried out using an acidic phosphate buffer as eluent. In Fig. 9a the retention factor of toluene is plotted versus the carbon content of the different phases. It can be seen that there is no obvious correlation between the retention factor of toluene and the carbon content. For the phase with the highest carbon content (TAMS 1; 13.1%) the *k* value of toluene is 58% smaller than in the case of the TAMS 4 phase (10.3% C).

Table 2
Retention factors and plate numbers of the test compounds

Analyte	pH 3		pH 9	
	k value	*N* per column	*k* value	*N* per column
Thiourea		31700		23900
p-Ethylaniline	0.084	24400	0.171	15700
Toluene	0.176	31900	0.318	20000
Ethylbenzene	0.248	31900	0.419	19500

If the retention factor of toluene is plotted as a function of the carbon content divided by the nitrogen content a more or less linear increase can be observed for the synthesized phases (see Fig. 9b). The smaller the nitrogen content of an SAX/C_{18} phase the more hydrophobic is the chromatographic surface as the surface charge density decreases with decreasing nitrogen content. The commercially available phase Stability BS C23 does not follow the trend of the synthesized phases, obviously due to its completely different surface chemistry.

3.6.4. Separation of anionic analytes by anion-exchange CEC

A mixture of acetyl salicylic acid and three of its metabolites was separated under alkaline conditions using TAMS 1 as stationary phase (see Fig. 10). In Fig. 10a the separation obtained on a packed bed of 25 cm length is shown. Although the four acids could be separated within 13 min, the separation efficiency was not satisfactory. Only for the inert marker a narrow and symmetrical peak could be obtained. Acetylsalicylic acid, carrying only one negative charge, eluted first. With increasing negative charge (the other acids carry two negative charges; the 5-hydroxy group of 2,5-dihydroxybenzoic acid is almost undissociated under the experimental conditions) not only the retention factor but also the peak asymmetry increased. The fact that the most hydrophobic compound (2-hydroxyhippuric acid) eluted much later than the other two-fold negatively charged acids indicates that the overall retention mechanism is governed by anion-exchange interactions as well as hydrophobic interactions.

In order to elute all these negatively charged analytes it was necessary to apply a mobile phase of relatively high ionic strength. In this case a 100 m*M* Tris buffer was used. The elution strength of the

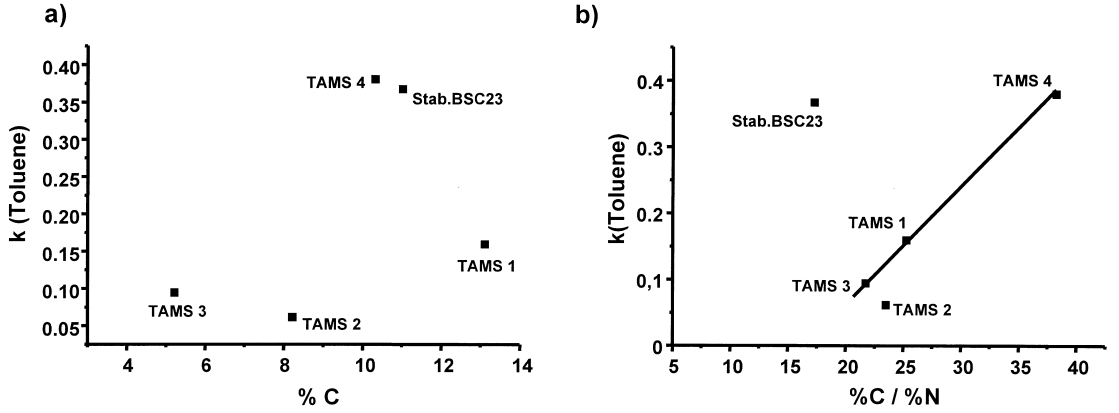

Fig. 9. Hydrophobic retention on different SAX/C_{18}mixed-mode phases. Buffer: 5 m*M* phosphate, pH 3–water–ACN (10:20:70); voltage: −25 kV; capillaries: 100 μm I.D. packed with different phases connected to 50 μm I.D. detection capillary ($L_{pac}/L_{det}/L_{total}$=25/26.4/34.9 cm); (a) *k*(toluene)=*f*(carbon content); (b) *k*(toluene)=*f*(carbon content/nitrogen content).

buffer resulted from the chloride ions which were added as hydrochloric acid to adjust the pH.

With a 20 m*M* Tris buffer only acetylsalicylic acid could be eluted with severe tailing whereas all other aromatic acids could not be detected within 1 h.

In Fig. 10b the separation of the aromatic acids obtained on a packed bed of 7 cm length is shown. Using identical buffer conditions as in the case of Fig. 10a the four acids could be baseline resolved within 1.8 min with sufficient chromatographic resolution.

4. Conclusions

The way to appropriate phases adopted to the requirements of CEC turns out to be long and troublesome. Within this course SAX mixed-mode

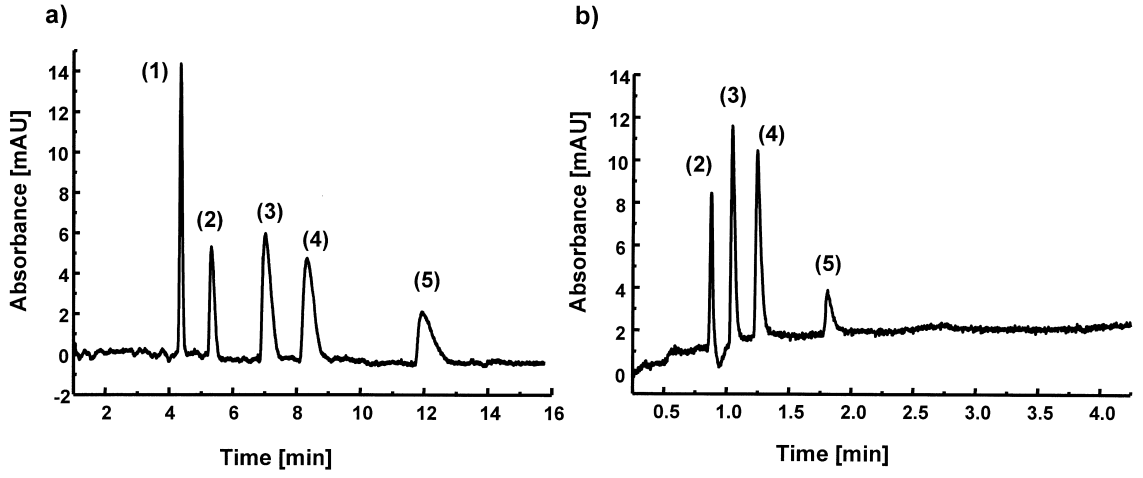

Fig. 10. Separation of acetylsalicylic acid and three of its metabolites. (a) Buffer: 100 m*M* Tris, pH 8.5–ACN (30:70); voltage: −25 kV; injection: −3 kV, 3 s; detection: UV, 210 nm; capillary: 100 μm I.D. packed with TAMS 1 connected to 50 μm I.D. detection capillary ($L_{pac}/L_{det}/L_{total}$=25/26.4/34.6 cm); (b) Same buffer as in (a), but ''short end'' of the capillary packed; voltage: 25 kV; injection: 0.5 kV, 3 s (inverse separation direction); capillary: 100 μm I.D. packed with TAMS 1 connected to 50 μm I.D. detection capillary ($L_{pac}/L_{det}/L_{total}$=7/8.5/31.9 cm). Analytes: (1) thiourea, (2) acetylsalicylic acid, (3) salicylic acid, (4) 2,5-dihydroxybenzoic acid, (5) 2-hydroxyhippuric acid.

phases should be considered a promising approach. Phases with elevated ion-exchange capacity proved to generate a fast and almost constant EOF from pH 3 to pH 9. It could be demonstrated that the charges located on the capillary wall do not significantly contribute to the EOF mobility in packed column CEC. The materials showed good compatibility with neutral, acidic and weakly basic analytes. The kind of buffer is a very crucial parameter for the long-time stability and pH dependence of the EOF. Tris appeared to be superior to phosphate, even though its buffering range is limited. From our point of view, CEC systems able to compete with HPLC still require considerable further development.

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