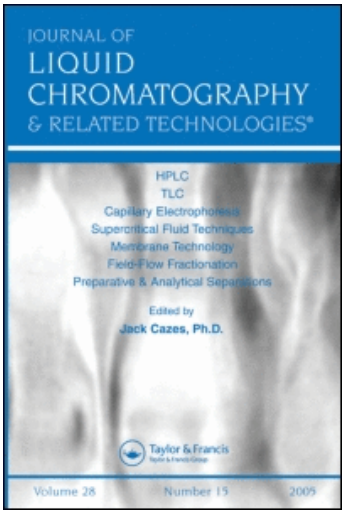


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# Capillary Electrochromatography of Charged Biomolecules with Mixed-Mode Stationary Phases

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# Capillary Electrochromatography of Charged Biomolecules with Mixed-Mode Stationary Phases

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**Abstract:** Capillary electrochromatography (CEC) is a relatively new hybrid separation technique that combines the selectivity of high performance liquid chromatography with the high efficiency of capillary electrophoresis. Due to its potential, CEC is gathering increasing attention as a powerful separation technique for the complex mixture of biomolecules. However, CEC with traditional silica-based reversed phase stationary phase (e.g., $C_{18}$) has some limitations in the separation of ionizable analytes.

Recently, the mixed-mode stationary phase addressed the problems and became an attractive alternative in CEC analysis of biological compounds. This review will introduce several mixed-mode stationary phases for the CEC separation of charged biomolecules, especially of peptides.

**Keywords:** Capillary electrochromatography, Mixed-mode stationary phase, Charged biomolecules, Peptides

## INTRODUCTION

Capillary electrochromatography (CEC), which combines the features of capillary zone electrophoresis and high performance liquid chromatography

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(HPLC), is a powerful separation technique with high efficiency, high resolution, and low sample consumption.[1,2] The focus of initial CEC researches has been concentrated on the separation of neutral pharmaceuticals and aromatic hydrocarbons that were well studied in reversed phase (RP)-HPLC. With the development of CEC column technologies and the understanding of electrochromatographic separation process based on a burst of fundamental studies, CEC applications have been expanded to the separation of relatively complex biomolecules, such as synthetic peptides, protein digests, and nucleosides.[3–6]

To date, several attractive attempts to achieve a CEC separation of charged analytes, e.g., peptides, have been described.[7,8] In the case of peptides, the silica-based particles for RP-HPLC (e.g., $C_{18}$) have also been used in combination with acidic mobile phases to prevent the electrostatic interaction between the positively charged sites of peptides and the negatively charged silanol groups on the particles. However, the use of traditional silica-based RP stationary phases in CEC has the disadvantage of a marked reduction in electroosmotic flow (EOF) at low pH due to the protonation of silanol groups. Also, charged analytes are hardly retained on the RP stationary phases. Although there are some examples of CEC separations using ion-exchange materials instead of $C_{18}$ silica, these stationary phases are not appropriate for the separation of peptides, since there are no long carbon chains to retain them. In order for further growth of the CEC of charged biomolecules, specially designed stationary phases, which support a strong EOF over a wide range of pH and retain the charged analytes according to their hydrophobicity, are essential.

From these standpoints, a research trend in CEC of charged analytes is directed toward the development of mixed-mode stationary phases that combine both ion-exchange and hydrophobic sites on the chromatographic support involving particular-type or monolithic-type format. The permanently charged ion-exchange functional groups provide an increased cathodic (strong-cation-exchange [SCX]/RP) or an anodic (strong-anion-exchange [SAX]/RP) EOF, and also attract or repulse the charged analytes. The hydrophobic group contributes to the interaction with the hydrophobic part of charged analytes. Therefore, the separation mechanism in CEC with mixed-mode stationary phases is a hybrid of electophoretic migration and chromatographic retention involving hydrophobic and electrostatic interactions, which leads to its different selectivity from that of RP-HPLC and RP-CEC. Actually, these mixed-mode stationary phases have addressed the problems in RP-CEC of charged analytes and become an attractive alternative.

The most promising area for further development of CEC now lies in the separation of charged biomolecules. Among the biological analyses, protein mapping is now widely employed in protein characterization for proteomic applications. Although HPLC is traditionally utilized for peptide mapping, new techniques, which permit faster analysis of samples with higher efficiency, are required. In this context, CEC attracts increasing attention as

a powerful separation technique for complex mixture of peptides because of its potential to simultaneously separate neutral and charged, hydrophilic and hydrophobic analytes in the same system.[3,9] This short review is focused on some approaches for the separation of charged biomolecules, especially of peptides, by CEC with mixed-mode stationary phases.

## SCX/RP MIXED-MODE STATIONARY PHASE

The use of SCX/RP mixed-mode stationary phases in CEC is a straightforward process to overcome the disadvantages in traditional silica-based RP stationary phases. However, the mixed-mode stationary phases specially designed for CEC are not commercially available and, thus, some attempts were carried out by mixing octadecyl silica and SCX materials.[10] In addition, mixed-mode stationary phases, which were used in HPLC, containing chemically co-bonding propylsulfonic acid and *n*-alkyl (hexyl, octyl or octadecyl) groups on the silica surface have been proposed for the separation of peptides.[11–14] These mixed-mode stationary phases exhibited a significant EOF over a wide range of pH values because of the permanently ionized sulfonic acid groups (Figure 1).[11,12] Walhagen et al. investigated two commercial mixed-mode phases (Spherisorb $C_{18}$/SCX and Hypersil mixed-mode) with the synthetic peptides. The different elution order of the model peptides on these columns was obtained probably due to their charge density of the surface.[12] They have also studied the retention behavior of cyclic and linear peptides by CEC with the same stationary phases.[13] More recently, Yang et al. characterized the performance of columns packed with 3 μm SCX/$C_{18}$ mixed-mode phases, employing a thrombin receptor antagonistic peptide (TRAP-1) and its analogues (TRAP 2-6) as a probe. Their resolution by RP-CEC proved to be difficult because of their similar amino acid sequences and mass to charge status.[14] Based on the understanding of the complex electrochromatographic process, a successful separation of the six TRAP peptides could be achieved, as illustrated in Figure 2A. In comparison, application of capillary zone electrophoresis (CZE) under the same conditions as CEC results in the six TRAP peptides being separated into two peak zones, because of their very close charge-mass ratios (Figure 2B).

In contrast to the use of commercially available mixed-mode phases, some efforts have been invested in the area of stationary phases that are tailor made for CEC. Zhang et al. specially designed an octadecyl-sulfonated silica stationary phase (ODSS, Figure 3), which was composed of a hydrophilic, negatively charged sublayer and a nonpolar top layer containing octadecyl ligands.[15] Due to the presence of permanently charged sulfonic acid groups in the sublayer, the hydrophilic nature of the sublayer and the hydrophobic character of the top octadecyl layer, retention and selectivity of charged and relatively polar nucleosides and bases on the ODSS stationary phase are based on electrostatic, hydrophilic, and hydrophobic interactions

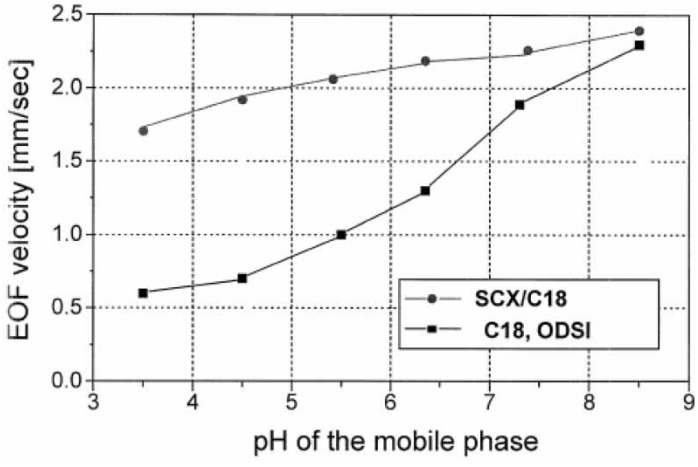


***Figure 1.*** Dependence of EOF on the pH of eluent for a *n*-octadecyl bonded silica and a mixed mode bonded silica (sulphonic acid-*n*-octadecyl). Capillary column: 25 cm × 100 μm I.D. packed capillaries with Waters Spherisorb SCX/$C_{18}$, ODS-Hypersil. CEC conditions: mobile phase, 25 mM phosphate/acetonitrile (20/80, v/v %); injection, electrokinetic for 6 s at 5 kV; UV detection, 254 nm; temperature, 20°C. Sample: thiourea. (From Ref.[11] with permission.)

(Figure 4).[16] Also, this stationary phase was successfully exploited, using an ion-pairing reagent, in the separation of small (e.g., mono- and di-nucleosides) and large nucleic acids (t-RNA).[17] A CEC column packed with sulfonated naphthalimido-modified silyl silica (SNAIP, Figure 5) was prepared by Ohyama et al.[18] The elution process of charged analytes on this column was dominated by a combination of both the electrophoretic process and chromatographic process involving hydrophobic, electrostatic, as well as $\pi$-$\pi$ interactions. As mentioned by Fu et al.,[9] multiply charged peptides cannot be eluted, or eluted in a long time, from SCX/RP mixed-mode stationary phases because of the strong electrostatic interaction. In general, although the electrostatic interaction could be suppressed by using the buffers with high ionic strength, the bubbles are generated within a capillary column due to excessive Joule heating when the buffer concentration is too high. In this context, using this column without the pressurization, electrochromatography could be applied to the separation with high current, which is unavoidable when employing higher buffer concentration for earlier elution of peptides by SCX/RP mixed mode. Furthermore, the triply charged peptides, Lys-Lys, could be eluted from the SNAIP column even with a moderate buffer concentration.[19] A later publication described that the separation of peptides on the SNAIP column could be improved and accelerated by a stepwise gradient of buffer concentration (Figure 6).[20] In addition, the application scope of the same column was expanded to the separation of nucleosides and nucleic acid bases with highly aqueous mobile phase.[21]

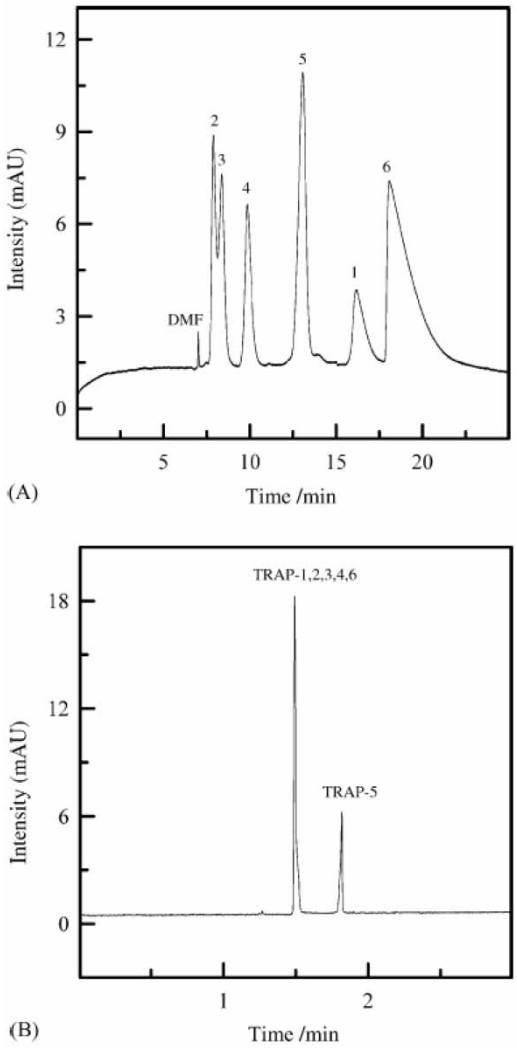


***Figure 2.*** (A) Separation of TRAP peptides on an SCX/$C_{18}$ capillary column. CEC conditions: mobile phase, acetonitrile/100 mM phosphate buffer (pH 6.5)/water (32/10/58, v/v/v %); voltage, 20 kV; UV detection, 214 nm. (B) Separation of TRAP peptides on a bare-fused silica capillary (total length, 34.0 cm; effective length, 25.5 cm; 100 μm I.D.). CZE conditions are same as CEC. (From Ref.[14] with permission.)

CEC with packed columns have some technical difficulties: the packing of particles into a capillary, the difficult fabrication of frits within a capillary, and the bubble formation within a capillary due to the existence of frits. Recently, monolithic stationary phases constituted an interesting alternative, which consequently have received attention as stationary phases

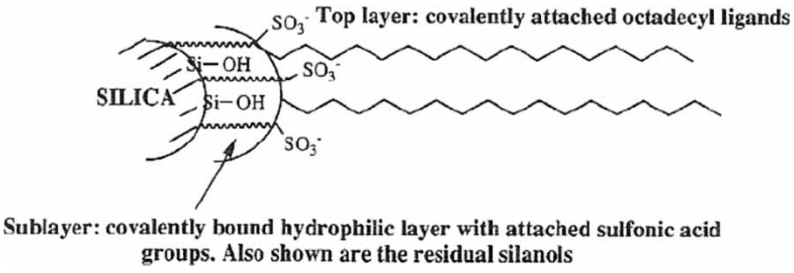


***Figure 3.*** Skeleton structure of the ODSS stationary phase. (From Ref.[15] with permission.)



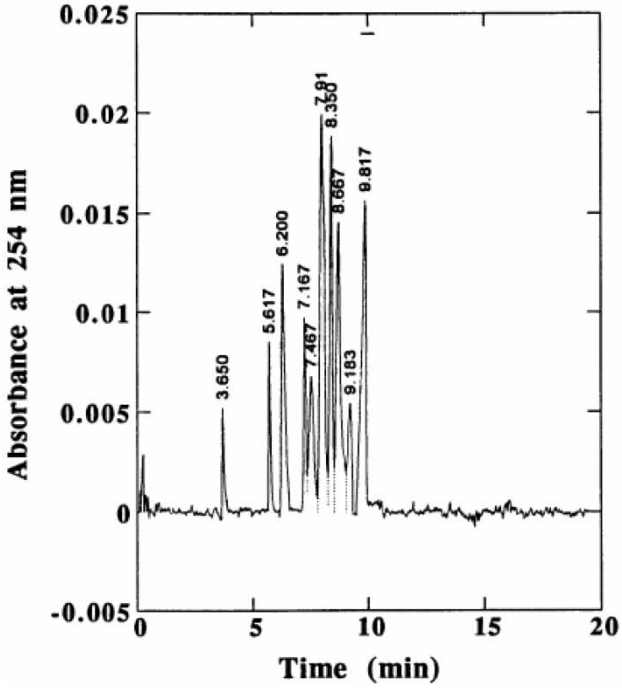


***Figure 4.*** Separation of a mixture of purine and pyrimidine bases and their nucleosides on the ODSS capillary column. Capillary column: total length, 27 cm; effective length, 20.5 cm; 100 μm I.D. CEC conditions: mobile phase, 4.8 mM sodium acetate (pH 4.5) containing 40% v/v acetonitrile; voltage, 20 kV; injection, electrokinetic for 2 s at 1 kV. Peaks: 1, uracil; 2, uridine; 3, thymine; 4, cytosine; 5, cytidine; 6, inosine; 7, adenine; 8, guanine; 9, adenosine; 10, guanosine. (From Ref.[16] with permission.)

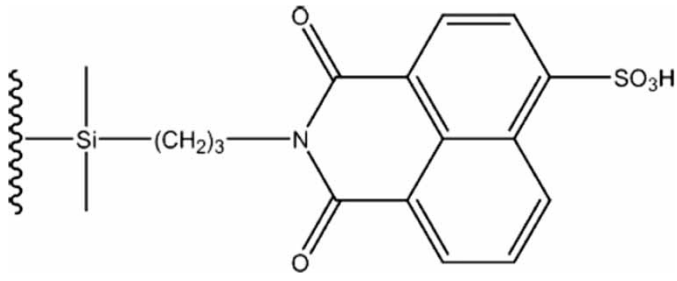


***Figure 5.*** Structure of 3-(4-sulfo-1,8-naphthalimido)propyl-modified silyl silica gel.

for CEC.[22–24] Monolithic stationary phases are subdivided into two main categories, i.e., silica- and polymer-based materials. An interesting feature of polymer-based monoliths is that a wide variety of monomers are available for their preparation, and the functionality of the monoliths can be tuned for specific applications. They are prepared *in situ* and offer great flexibility in the morphological design. The pore size, the surface charge density, and the accessible chromatographic surface can, in principle, be freely adjusted within a certain range. Monolithic CEC columns are increasingly popular, especially in proteomic research (i.e. the separation of complex peptide mixtures obtained from protein digestion).

Svec and Fréchet first demonstrated the suitability of incorporating ion-exchange moieties in polymethacrylate monoliths to generate a stable EOF.[25] Subsequently, Peters et al. reported the use of the monomers, ethylene dimethacrylate, butylmethacrylate, and 2-acrylamido-2-methyl- 1-propanesulfonic acid (AMPS), to produce a methacrylate-based monolith.[26] The presence of a butyl moiety and the EOF-generating AMPS produced a mixed-mode phase. Yu et al. reported the separation of a mixture of peptides on methacrylic monoliths, which were prepared by photoinitiated free radical polymerization and modified with AMPS,[27] however, the peptides were not eluted from the CEC column under separation conditions similar to those used for the separation of uncharged analytes, because of strong Coulombic interactions between the amino group of peptides and the sulfonic functionalities of the monolith. However, a baseline separation of those peptides was obtained using a mobile phase containing an ion-pairing reagent of 1-octanesulfonate. Wu et al. prepared the capillary monolithic column by *in situ* copolymerization of 2-(sulfooxy)ethyl methacrylate (SEMA) and ethylene dimethacrylate in the presence of porogens.[28] The sulfonate group provided by the SEMA monomer was used for the generation of EOF and served as a SCX stationary phase. A mixed-mode retention mechanism consisting of hydrophobic and electrostatic interactions was confirmed through the investigation of the influence of mobile phase composition on the retention coefficients. As shown in Figure 7, a separation of ten peptides in acidic condition was obtained with column efficiencies up to 110,000 plates/m.

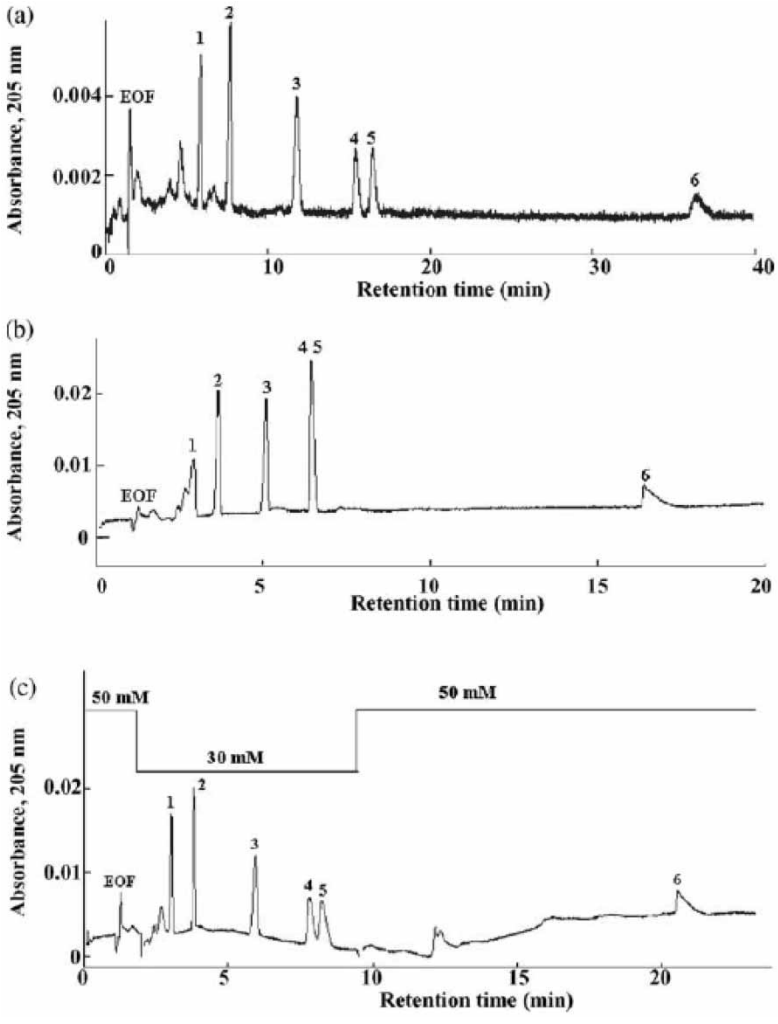


***Figure 6.*** Separation of model peptides on SNAIP capillary column. CEC conditions: mobile phase, (a) 30 mM phosphate buffer (pH 3.8)/methanol (60/40, v/v %); (b) 50 mM phosphate buffer (pH 3.8)/methanol (60/40, v/v %); (c) phosphate buffer (pH 3.8)/methanol (60/40, v/v %) (initial 50 mM for 2 min, intermediate 30 mM for 7.5 min and final 50 mM): voltage, 20 kV; injection, electrokinetic for 8 s at 20 kV. Peaks: 1, Gly-Val; 2, Gly-Ile; 3, Gly-Phe; 4, Gly-Lys; 5, Gly-His; 6, Lys-Lys. (From Ref.[20] with permission.)

Although, SCX/RP mixed-mode phases have been successfully applied to the separation of certain basic biomolecules, difficulties with the elution of basic analytes from such stationary phases because of the strong electrostatic interaction is often highlighted. In this context, the decrease in

protonation of basic analytes at moderately high pH values should facilitate their separation with minimal electrostatic interaction. Based on the stability of the polymeric stationary phases in a high pH environment, Adu et al. attempted the separation of relatively complex therapeutic peptides, some of which contain highly basic amino acids, at a high pH value (pH 9.5) with suppression of the electrostatic interaction.[29] As shown in Figure 8, good resolution was achieved at a high pH value, whereas the peptides were not completely separated at a low pH value (pH 2.8). Separations with efficiencies up to 500,000 plates/m and stability to repeated injections of a mixture of peptides were demonstrated.

The change of mobile phase composition reciprocally influences the two interaction modes, hydrophobic and electrostatic interactions, on an SCX/RP stationary phase; for instance, the high concentration buffer needed for elution in ion-exchange chromatography enforces hydrophobic interaction and the high organic content in mobile phase used for the elution of hydrophobic analytes enhances electrostatic interaction. Hoegger et al. proposed a mixed-mode monolithic stationary phase that is based on a hydrophilic rather than a hydrophobic polymer for the CEC of



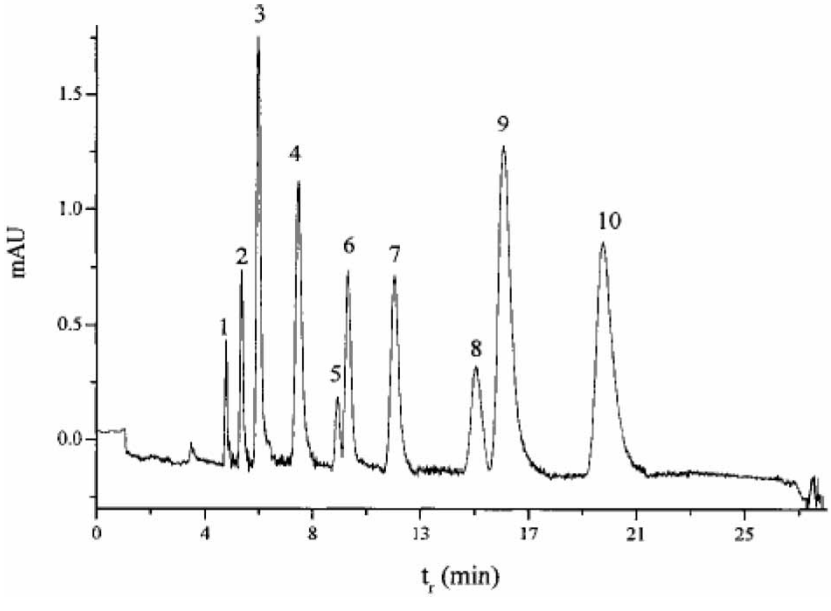


***Figure 7.*** Separation of peptides on the mixed-mode CEC. Capillary column: total length, 30 cm; effective length, 9.5 cm; 100 μm I.D. CEC conditions: mobile phase, 50% acetonitrile in 8 mM phosphate buffer, pH 3.0; temperature, 25°C; voltage, 5 kV; injection, electrokinetic for 2 s at 2 kV; UV detection, 214 nm. (From Ref.[28] with permission.)

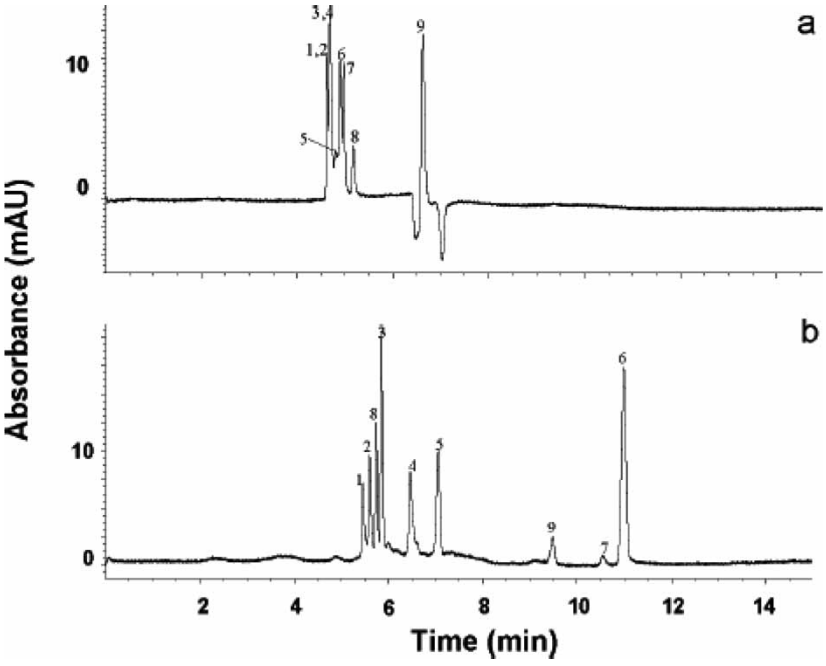


***Figure 8.*** Separation of a nine peptide mix by CEC on a BMA-based mixed-mode monolithic capillary under (a) acidic condition (pH 2.8) and (b) basic condition (pH 9.5). Capillary column: total length, 33.5 cm; effective length, 25 cm; 100 μm I.D. CEC conditions: mobile phase, (a) acetonitrile/water/50 mM ammonium formate buffer (70/10/20, v/v/v %), (b) acetonitrile/wate/50 mM sodium borate buffer (70/10/20, v/v/v %); voltage, 10 kV; injection, electrokinetic for 2 s at 5 kV/; temperature, 20°C; UV detection, 206 nm; 6 bars pressure in both vials. This figure combined the two figures in Ref.[29] for this review. (From Ref.[29] with permission.)

amino acids and peptides.[30] This stationary phase was *N,N*-dimethacylamide-piperazine diacrylamide-based monolith derivatized with vinylsulfonic acid. On this monolithic column, some closely related peptides could be separated.

## SAX/RP MIXED-MODE STATIONARY PHASE

As an alternative to SCX/RP mixed-mode stationary phases, strong anion exchangers with hydrophobic sites have also been described for generating an anodic EOF, as well as avoiding the peak tailing of charged biomolecules by the Coulombic repulsion. Huang et al. used a commercially available mixed-mode (SAX/RP) stationary phase, which consisted of a high purity

spherical silica bonded with both $C_{18}$ and dialkylamine, for the pressurized CEC (pCEC) of peptide mixtures under acidic conditions.[31] Compared with $C_{18}$ reversed phase, the mixed-mode phase provided similar column efficiency, but with a different elution pattern of the peptides, in the pCEC separation of tryptic digests from horse heart myogobin. The mixed-mode phase demonstrated an attractive feature, that a constant EOF can be obtained over pH 2-5 compared with $C_{18}$ reversed phase. The elution order of the peptides on the mixed-mode phase dramatically changed with the increased pH (Figure 9). The amino groups on the stationary phase can repulse the positively charged peptides and prevent them from approaching the residual silanol group, which made the peak shapes good at $pH > 4$. Progent et al. evaluated the potential of a stationary phase (Stability BS-C23, Figure 10) with a permanent positive charge, by performing peptide separation without pressurization or gradient elution.[32] A quaternary ammonium group is covalently bonded via a spacer at the surface of silica support and forms a relatively hydrophilic and charged sublayer, while a non-polar upper layer of octadecyl functionalities plays the part of a hydrophobic binding layer. The anodic EOF was found to be independent of pH over 2-12, as well as of the organic solvent content in the mobile phase. The stability of the stationary phase was demonstrated at extreme pH values (2.5 and 9.1). In acidic conditions, the electrokinetic contribution was predominant compared to the chromatographic one. Chromatographic interactions (i.e., hydrophobic interaction and anion-exchange) were involved in the separation of peptides, whereas repulsive electrostatic interaction could be considered as negligible. Finally, a tentative peptide mapping of $\beta$-lactoglobulin and human growth hormone with isocratic elution was performed, as illustrated in Figure 11.

Several efforts have been made on the special design of SAX/RP mixed-mode stationary phases for CEC. Huang et al. reported the separation of proteins and peptides in open tubular columns with a stationary phase of highly cross-linked, porous poly(vinylbenzyl chloride) derivatized with *N,N*-dimehyldodecylamine.[33] To obtain the positive surface charges, the chloromethyl functions of the porous layer were reacted with a tertiary amine. This system enabled the isocratic separation of proteins and exhibited an increased elution time at higher acetonitrile content. The same groups also compared the behavior of a monolithic stationary phase, where the styrenic polymer was derivatized with *N,N*-dimehyloctylamine in the micro-HPLC and CEC.[34] Insulin and synthetic angiotensin-type peptides were separated isocratically by counterdirectional CEC with reversed polarity. The electrochromatogram in Figure 12 shows that both acidic and basic polypeptides were separated in a single run at pH 3.0, in the order of increasing basicity. The strong retention of strongly basic peptides by the strongly basic stationary surface was due to an interplay of the electophoretic process (EOF and electrophoretic mobility of analytes) and chromatographic process (hydrophobic retention and Coulombic repulsion). In addition, a

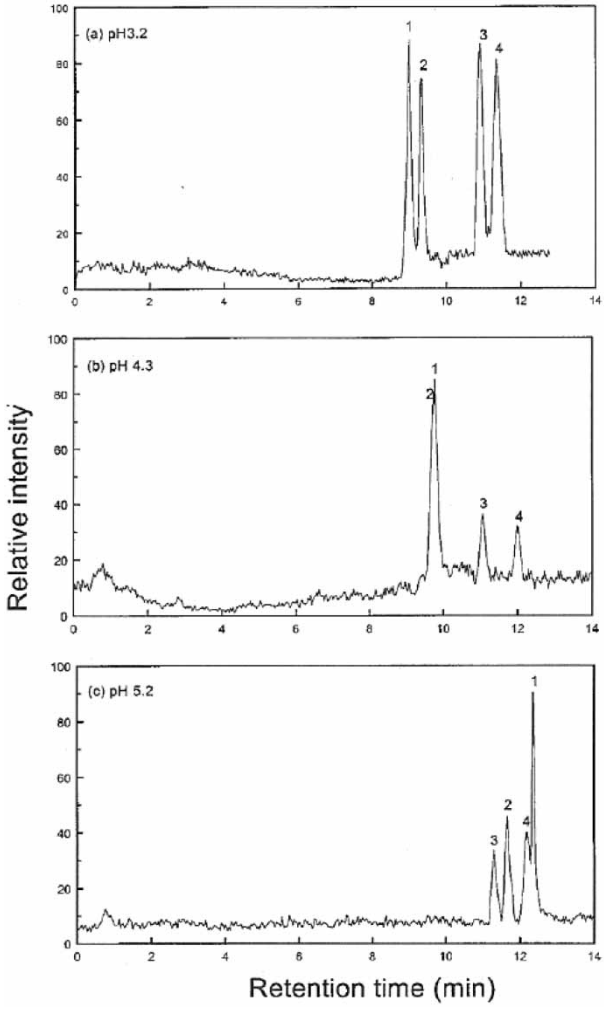


***Figure 9.*** Effect of pH on the separation of four peptides. Capillary column: 120 mm × 150 μm I.D. CEC conditions: mobile phase, equilibration with 100% solvent A (4 mM acetic acid-ammonium acetate in aqueous solution) and the elution of sample with 25% solvent B (equal amount of the electrolyte in acetonitrile); voltage, 4 kV; pressure, 80 bar. Peaks: 1, angiotensin I; 2, methionine enkephalin-Arg-Phe; 3, methionine enkephalin; 4, leucine enkephalin. (From Ref.[31] with permission.)

mixed-mode weak anion exchanger was prepared from polyacrylic acid-based polymers derivatized with a secondary amine (*N*-ethylbutylamine).[35] For this column, the counterdirectional CEC separation of proteins and peptides was investigated at pH 2.5. In the case presented here, solvophobic interactions

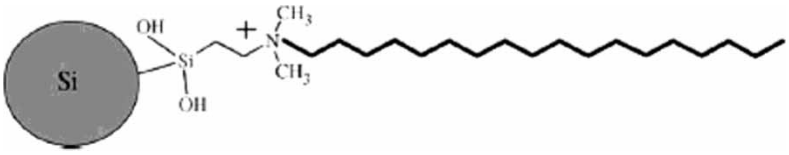


***Figure 10.*** Structure of the Stability BS-C23 stationary phase. (From Ref.[32] with permission.)

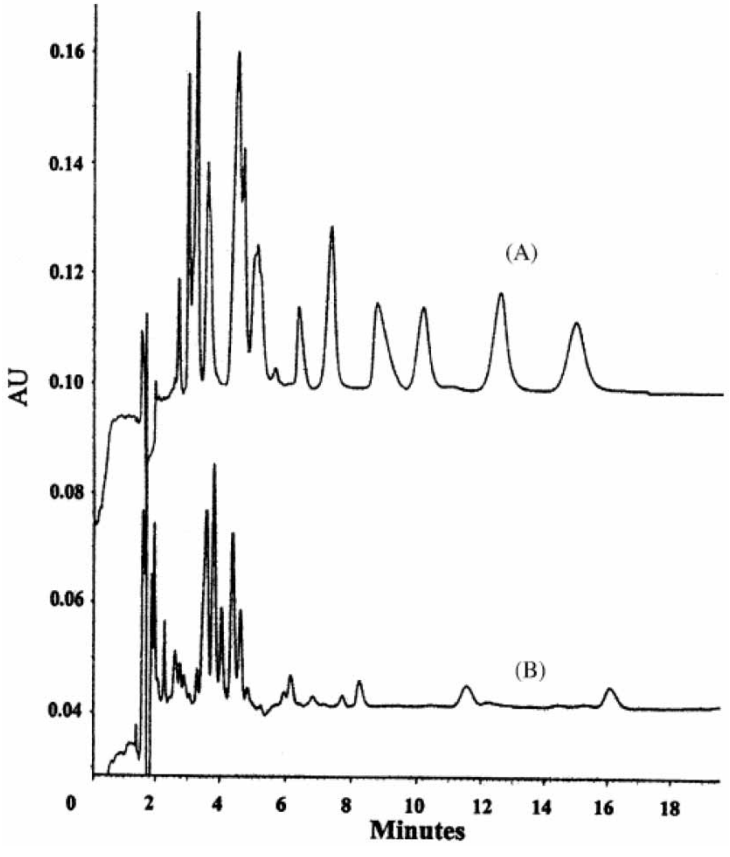


***Figure 11.*** Peptide mapping of (A) $\beta$-lactoglobulin and (B) hGH by CEC on a BS-C23 column. Capillary column (packed with 5 μm end-capped BS-23): total length, 31.2 cm; effective length, 9.8 cm; 75 μm I.D. CEC conditons: mobile phase, Tris-HCl 75 mM, pH 2.6/acetonitrile (80/20, v/v %); injection, electrokinetic for 10 s at −5 kV; voltage, −15 kV with a ramp of 0.5 min; pressure, $6.9 \times 10^5$ Pa; temperature, 25°C; UV detection, 200 nm. $\beta$-Lactoglobulin 10 μg/μL, hGH 2 μg/μL. (From Ref.[32] with permission.)

were dominant and, therefore, the elution order of proteins was similar to that obtained in RP chromatography. It was recalled that the plots of migration factors of proteins and peptides against the acetonitrile contents exhibited different trends. According to the authors, this was most likely due to the greater chromatographic retention and slower electrophoretic mobility of proteins than that of peptides in the counterdirectional CEC system. Furthermore, the authors evaluated the significance of temperature as a variable parameter controlling the retention behavior of both peptides and proteins.[36] Without compromising the resolution, a two-fold increase in the speed of separation was attained by increasing the temperature from 25 to 55°C (Figure 13). The separation time could also be reduced through an increase in temperature for the separation of a tryptic digest of cytochrome *c*. This is one of the first examples of employing a monolith in CEC for the

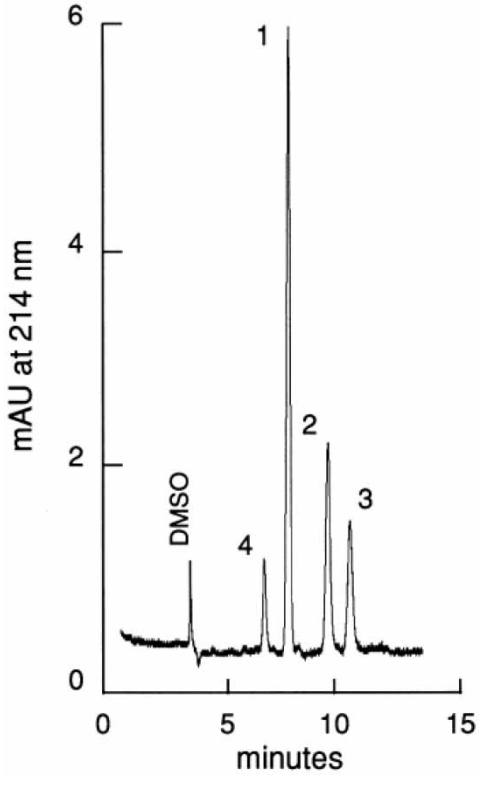


***Figure 12.*** Separation of acidic and basic polypeptides. Capillary column (porous styrenic monolith with quaternary ammonium and octyl functions): total length, 31 cm; effective length, 21 cm; 75 μm I.D. CEC conditions: mobile phase, 50 mM NaCl in 5 mM phosphate buffer with 25% acetonitrile, pH 3.0; voltage, −15 kV; injection, electrokinetic for 2 s at 5 kV. Peaks: 1, angiotensin II; 2, angiotensin I; 3, [$Sar^1$, $Ala^8$]-angiotensin II; 4, insulin; 4 mg/ml of each in buffer. (From Ref.[34] with permission.)

separation of more complex samples, especially in the context of proteomics. Scherer et al. developed and characterized new strong anion-exchange materials with additional hydrophobic moieties (SAX/$C_{18}$ mixed-mode stationary phase), of which the synthesis was based on polymer encapsulation of porous silica (Figure 14).[37] The authors reported the studies on the separation of small peptides (2-4 amino acids) on Spherisorb octadecyl silane (ODS) phase at acidic pH, and on the mixed-mode phase at weakly basic pH.[38] The elution order of model peptides were the same as those obtained on the reversed phase at 20% acetonitrile content, but acidic pH. However, the retention factors on the SAX/$C_{18}$ mixed-mode stationary phase were significantly higher than on the Spherisorb ODS stationary phase. This was in accordance with the higher carbon content of the SAX/$C_{18}$ stationary phase. When the organic solvent content in the mobile phase was increased, the separation efficiencies were lower: the efficiencies at 70% acetonitrile content decreased to about 50% of the efficiencies for certain peptides at 20% acetonitrile content. Although resolution was lower, the selectivities were different and separation time was shorter because the EOF was higher at the higher acetonitrile content.



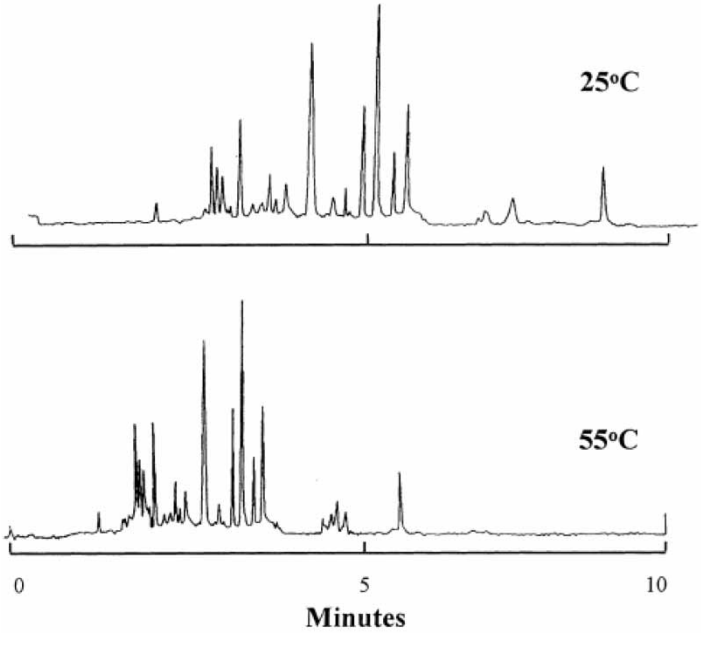


***Figure 13.*** Separation of tryptic digest of cytochrome *c* obtained by isocratic elution at 25°C and 55°C, respectively. Capillary column (styrenic monolith having quaternary ammonium functions): total length, 40 cm; effective length, 30 cm; 75 μm I.D. CEC conditions: mobile phase, 40% acetonitrile in 50 mM phosphate buffer, pH 2.5; voltage, −30 kV; UV detection, 214 nm. (From Ref.[36] with permission.)

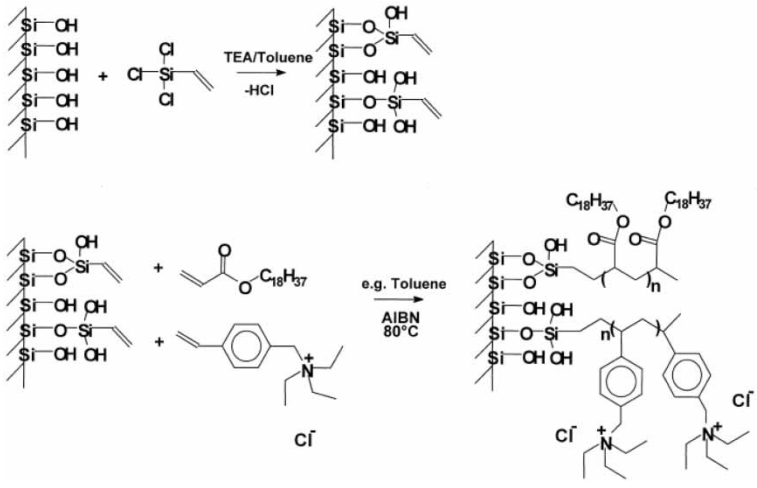


***Figure 14.*** Synthesis scheme for SAX/$C_{18}$ mixed-mode phases. (From Ref.[37] with permission.)

Very recently, Ye et al. proposed a silica-based monolithic column, chemically modified with 3-aminopropyltrimethoxysilane (APS), prepared by a sol-gel process for pressurized CEC.[39] APS produced a polar stationary phase with a weak anion-exchange site, which generated anodic EOF under

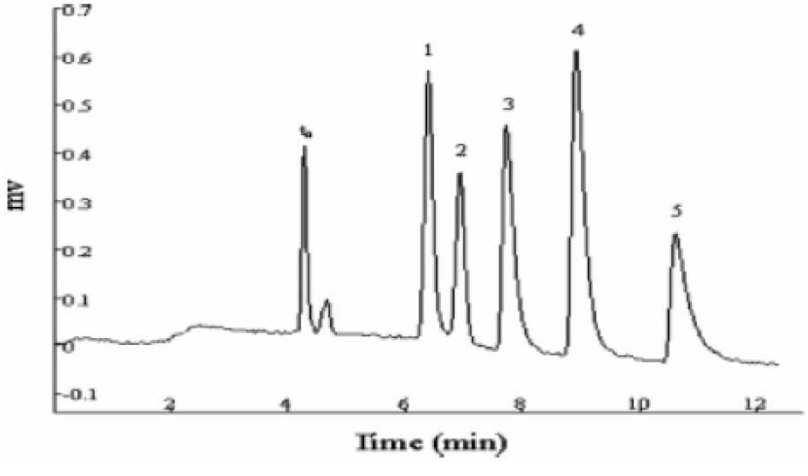


***Figure 15.*** Separation of nucleotides. Capillary column: total length, 55 cm; effective length, 25 cm; 75 μm I.D. CEC conditions: mobile phase, 40 mM TEAP buffer containing 60% acetonitrile (pH 3.5); voltage, 15 kV; UV detection, 254 nm; pressure, 100 psi; flow, 0.02 mL/min. Peaks: 1, 5′-UMP; 2, 5′-AMP; 3, 5′-IMP; 4, 5′-CMP; 5, 5′-GMP. (From Ref.[39] with permission.)

acidic conditions. The anion analytes such as the nucleotide were separated by the mixed-mode mechanism, which comprised hydrophilic interaction, weak anion-exchange, and electrophoresis (Figure 15).

## CONCLUSIONS

Several attractive approaches for the CEC of charged biomolecules with mixed-mode retention mechanism have been made and, at the same time, most of them provided significant progress in the design of the mixed-mode stationary phase suited for CEC.

Unfortunately, up to date, CEC with mixed-mode stationary phase has not yet found wide acceptance as a practical tool for biological analysis in chromatographic community. However, one of the research trends of CEC is the development of novel type mixed-mode column, so further improvement in this area is promising. CEC with mixed-mode stationary phase may be on the board of separation tools for biological analysis, especially for peptide mapping that requires new analytical techniques permitting faster analysis with higher efficiency.



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