Review

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Polar stationary phases for capillary electrochromatography

This review article summarizes the variety of polar stationary phases that have been employed for capillary electrochromatographic separations. Compared with reversed-phase stationary phases, the polar alternatives provide a completely different retention selectivity towards polar and charged analytes. Different types of polar stationary phases are reviewed, including the possible retention mechanisms. Electrochromatographic separations of polar solutes, peptides, and basic pharmaceuticals on polar stationary phases are presented.

Keywords: Capillary electrochromatography / Monoliths / Particulate packing materials / Polar stationary phases / Review
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Abbreviations: HI, hydrophilic interaction; **HILIC**, hydrophilic interaction chromatography; **ODS**, octadecylated silica; **TEAP**, triethylamine phosphate

1 Introduction

Capillary electrochromatography (CEC) is an analytical separation technique that strives to combine the best features of HPLC and capillary electrophoresis (CE). Its definite advantage is that it combines the differential electrophoretic mobility of CZE with the partitioning mechanism of HPLC to increase the CEC separation selectivity. As with HPLC, retention is defined by solute-stationary phases interactions; however, in CEC the stationary phases play a dual role and provide the basis for electroosmotic pumping the mobile phase (bulk flow) through the capillary column in addition to chromatographic separation.

Since its introduction, CEC has experienced open-tubular, packed-column, and monolithic (or continuous-rod) column configurations in its developmental stages. In packed columns, traditional HPLC particulates specially designed for CEC are packed into capillaries, which are kept in place by the assistance of both inlet and outlet frits. Although the packed column was once the most favored format, the fabrication of mechanically stable retaining frits is not an easy task. As an alternative to packed columns, open-tubular columns are adopted in open-tubular CEC and thus eliminate the tedious procedures required to prepare the packed CEC columns. Open-tubular CEC represents a conceptually simple column design where stationary phases are covalently attached, coated, or adsorbed onto the capillary wall. However, low phase ratios, hence lower sample loading capacities are the main obstacles for this format of columns. Monolithic column is a relatively new column design where the particles or monoliths are consolidated

into a whole inside the capillary by the *in situ* polymerization of the monomers in the presence of porogens under thermal or UV conditions.

The stationary phase has always been the 'heart' of CEC. Early attentions have been focused mainly on CEC separations of neutral compounds using capillaries packed with reversed-phase packing materials (virtually exclusively octadecyl silica gel) [1, 2]. Under the reversed-phase CEC, the hydrophobicities of the stationary phases determine the selectivity of the separation, and retention can easily be regulated by adjusting either the composition of the mobile phase or the hydrophobicities of the surface of the stationary phase. Since CEC have obtained relatively satisfactory results for separations of nonpolar and neutral analytes, their separations should not be a major target for CEC in the future [3]. With the demands of proteomic and zeomic (integrated biopathway systems analysis) studies, the complexities of biomolecules, particularly biopolymers, such as peptides and proteins, present a challenge for CEC. Furthermore, separation of strongly basic analytes has provided a challenge in chromatography for many years. Traditionally the bonded phases used in CEC possess a high density of silanol groups for the purpose of generation of EOF, and their applications in the analysis of strongly basic analytes inevitably result in extensive secondary interactions, peak tailing, and increased retention.

An alternative to reversed-phase sorbents is the use of polar stationary phases, which exhibit a different retention mechanism. It is an effective solution to the separation of polar and charged analytes. The polar stationary phase was first used in HPLC as in the normal-phase mode with a less polar mobile phase as opposed to the reversed-phase chromatography introduced at a time later. Although reversed-phase chromatography has been proliferated rapidly since the advent of C_{18}-silica stationary phase, polar stationary phase has always been the next in popularity and used in the reversed-phase as well as the normal-phase mode in HPLC [4]. Polar stationary phases are by definition including polar sorbents (mainly refers to silica gel, and zirconia and alumina substrates also included), silica gel with chemically bonded polar phases, and polymers bearing polar functional groups. As complementary to reversed-phase stationary phases, polar stationary phases proved to be very useful in some applications. According to the principle of 'like dissolves like', polar stationary phases have good separation abilities toward polar analytes, such as positional isomers or enantiomers that differ only slightly in their structures for which the more common reversed-phase LC mode is not suitable. Secondly, polar stationary phases are often used in normal-phase mode with the combination of a less polar mobile phase. This separation mode allows the effective analysis of such organic compounds with rather poor solubility in polar solvents. Further applications of polar stationary phases in hydrophilic interaction chromatography (HILIC) allow the use of aqueous mobile phases in the normal-phase mode, which is extremely useful for the analysis of polar solutes in biological samples. As a recently developed microseparation technique, CEC has commons to HPLC. Therefore, it is reasonable to expect that the useful attributes of polar stationary phases be also maintained in CEC. The first application of polar stationary phases in CEC was silica gel and cellulose-based packing materials, pioneered by Maruska *et al.* [5, 6] in the normal-phase mode.

Preparation of stationary phases in CEC is a very active research area. Several reviews [7–10] have appeared in recent years concerning CEC stationary phases. These articles mainly focused on either the applications of CEC with reversed-phase stationary phases or enantiomeric separations. The developments of special column configurations are also reviewed [11]. Here, we will give an overview of CEC with polar stationary phases. The retention mechanisms and the applications are also included.

2 Basic concepts of CEC

In CEC, the propulsion of mobile phase through the capillary column is upon the influence of the electroosmotic flow (electric pump). Electroosmotic flow (EOF) is an electrokinetic effect originating from the electrophoretic movement of the diffuse layer of the electric double layer that is formed at the liquid-solid interface. In CE, EOF is generated only at the electric double layer formed at the inner capillary wall. In CEC with the packed or monolithic columns, however, properties of the solid materials inside the capillary, and not properties of the inner surface of capillary wall, determine the electroosmotic velocity. The linear flow rate (v_{eo}) is proportional to the ζ-potential, which characterizes the electric double layer, and also depends on the mobile phase properties (dielectric constant ε, viscosity η, and ionic strength I) as well as the applied field strength (E) as follows:

$$v_{eo} = -\frac{\varepsilon}{\eta}\zeta E = \mu_{eo} E \quad (1)$$

In CEC, the EOF is measured by the velocity of a neutral and nonretained solute as EOF marker. Thiourea is generally chosen as the EOF marker for reversed-phase packing materials since it has the least interaction with the nonpolar stationary phase. While for polar stationary

phases, especially when used in normal-phase mode, an apolar solute, such as toluene, is used as the EOF marker [12].

In addition to being transported by the EOF, charged species also have their electrophoretic migration with the direction determined by the positive or negative charges they bear (electrophoretic process). The magnitude of the electrophoretic (v_{ep}) velocity is given by the equation:

$$v_{ep} = \frac{ze}{6\pi\eta r}E = \mu_{ep}E \quad (2)$$

where z is the charge number, e is the charge of an electron in Coulomb, r is the ion radius, and μ_{ep} is the electrophoretic mobility. It should be noted that for the uncharged compounds the electrophoretic mobility approaches zero.

Therefore, the apparent linear flow velocity of a solute (v_{app}) is the sum of the EOF (constant for all species) and its electrophoretic mobility (varied for different species):

$$v_{app} = v_{eo} + \text{tf} = ''\text{ps}_\text{h}\text{eni}''\text{v}_{ep} \quad (3)$$

$$\mu_{app} = \mu_{eo} + \mu_{ep} \quad (4)$$

The above equations illustrate that the driving force for migration of solutes through the column can be either EOF or electrophoretic migration or a combination of both upon the applied electric field. However, the interactions with the stationary phase in the CEC column retard the movement of solutes. Rathore and Horváth [13] defined the electrochromatographic retention factor (k^*) as the ratio of separative and nonseparative virtual migration distances for the characterization of the whole migration processes. In their definition, both the contributions from chromatographic retention and electrophoretic migration process to the retention factors in CEC were described in a unified equation. The retention factor in HPLC can be easily calculated according to the migration time of an analyte and the void time, which is readily available from a chromatogram. Similarly, this definition of retention factor (k^*) was applied to CEC according to some authors [14–16]:

$$k^* = (t_r - t_o)/t_o \quad (5)$$

where t_r is defined as the migration time of an analyte and t_o as that for a neutral and unretained marker. The electrochromatographic retention factor (k^*) has been used to describe the migration processes of charged solutes in both reversed-phase CEC and ion-exchange CEC. Following this definition, the electrochromatographic retention factor for a charged analyte can be given by the equation according to Wu *et al.* [14]:

$$k^* = \frac{k' - \mu_{ep}/\mu_{eo}}{1 + \mu_{ep}/\mu_{eo}} \quad (6)$$

where k' is the retention factor defined by a pure chromatographic process, and μ_{ep} and μ_{ep} are the electrophoretic mobility of the analyte and EOF, respectively.

In a CEC system where pressurized flow is included, the electrochromatographic retention factor was proposed by Ye *et al.* [17] to take the form as follows:

$$k^* = \frac{k'(\mu_{eo} + u_p/E - \mu_{ep})}{\mu_{eo} + u_p/E + \mu_{ep}} \quad (7)$$

In this equation, two parameters, the electric field (E) and pressurized flow velocity (u_{ep}) were introduced. It was seen that in a pressurized CEC system k^* is dependent on either the applied electric field or applied pressure. Therefore, by manipulation of the applied pressure or voltage, unique control of selectivities of the charged analytes can be obtained.

3 Packed CEC columns with polar stationary phases

3.1 Porous organic beads

Whitaker and Sepaniak [18] reported in 1994 the nonaqueous CEC separation of large polycyclic aromatic hydrocarbons (PAHs) and fullerene mixtures with a reversed-phase packing (octadecyl silica, ODS) under even unbuffered nonaqueous mobile phases. The application of polar stationary phases in combination with nonaqueous and aqueous mobile phases in CEC was first reported by Maruška and Pyell [5, 6]. Although it is well-known that the octadecylated packing material was typically used in reversed-phase CEC, the octadecylated cellulose beads exhibited a kind of mixed-mode retention mechanism. The lipophilic property was considered from the hydrophobicities of the octadecyl ligands while the hydrophilic property from the hydroxyl groups consisted of both the unconverted hydroxyl groups of the cellulose matrix and the hydroxyl groups produced by the reaction of the octadecanol anchoring to the epoxy-activated cellulose. The PAHs and five benzoates were eluted in a sequence corresponding to their decreased polarity, which corroborated the apolar nature of the octadecylated cellulose packing material under the aqueous mobile phase while the polar nature of the matrix was demonstrated by the separation of polar aromatic compounds with the nonaqueous mobile phase. The less polar compounds, namely benzene, phenol, and resorcinol, were eluted first, whereas aminophenol and 1,2-dia-

minobenzene, being the most polar compounds, were eluted last. Further evidence of the amphiphilicities of this material was also given by the change of the elution pattern of phenolic compounds under an aqueous mobile phase containing varying acetonitrile concentrations. The elution order at low acetonitrile concentration is resorcinol and phenol, while it is phenol and resorcinol under high acetonitrile concentrations. This change of the elution sequence corresponds to changing the separation mechanism from reversed-phase to normal-phase mode. The utility of this amphiphilic stationary phase-packed capillary is that it can provide the opportunity to compare the performance of the same capillary in both reversed and normal-phase modes of CEC. However, the application of this stationary phase is limited by its relative low column efficiency not exceeding 21 000 plates/m with nonaqueous mobile phases.

Although polymeric beads have found increasing applications in HPLC for their favorable attribute of higher chemical stability over a wide pH range [19, 20], their applications as CEC stationary phases were rarely reported [21–23]. Horváth *et al.* [23] introduced polar vicinal diol groups by hydrolyzing epoxy groups on the poly(glycidyl methacrylate-*co*-divinylbenzene) beads, and its further reaction with epichlorohydrin yielded more hydroxyl groups in the polymeric beads. The styrene-based polymer is hydrophobic in nature. The built-in reactive glycidyl groups provide an effective approach for the hydrophilization of organic beads thus imparting the porous beads with hydrophilic properties.

3.2 Bare silicas

Although materials, such as alumina and zirconia, continue to receive attentions as chromatographic substrates for bonded phases, silica gel is still by far the most commonly used support material. Silica gel itself as a typical polar stationary phase is particularly suitable for chromatography of polar substances. It is generally accepted that the existing surface siloxane groups (Si-O-Si) constitute weak hydrophobic sites while the Si-OH groups (silanols) are the surface-active sites on silica gel for hydrophilic interactions. The vicinal silanols can form "bound silanol" *via* hydrogen bonds. Silanols are also present in the form of hydrated hydroxyl groups. They all serve as attachment points for the covalent silyl ether bonds that anchor bonded phases to the silica support.

Bare silica used as stationary phase in CEC separation was first reported by Maruška *et al.* [5, 6] in the normal-phase mode with methanol-ethanol-hexane as the less polar mobile phase. The separation of polar compounds was typically in the normal-phase mode. As the polarities of mobile phases decreased, increased retention accompanied with a better resolution of the polar solutes was observed. The elution order of the polar phenolic compounds and diaminobenzene according to their increasing polarities also demonstrated the separation is under normal-phase mode.

Wei *et al.* [24] studied the effect of mobile phase composition on CEC separation on bare silica of primary (aniline), secondary (ephedrine), and tertiary amines (codeine, cocaine, thebaine), and quaternary ammonium compounds (berberine, jatrorrhizine). Even on a bare silica phase, the retention mechanism seems to be multifunctional with contributions of reversed-phase, ion-exchange, and normal-phase chromatography, and electrophoresis. The retention factors of the unprotonated tertiary amines (pK_a 6–8) decreased with increased content of organic modifier in the mobile phase with main contribution by the reversed-phase mechanism, while the comparatively small and linear ephedrine eluted later by the electrostatic interaction. At acetonitrile (ACN) contents $>$ 80%, the elution pattern turned from reversed-phase to normal-phase. The best separation was obtained with 10 mM Tris buffer (pH 8.3) containing 80% ACN, though the last peak, codein, was tailing; a typical electrochromatogram for the separation of the above basic compounds on bare silica is shown in Fig. 1.

McKeown *et al.* [25] compared three unbound Hypersil silica stationary phases of different purity when separating a mixture of diverse basic drugs. The purity of the packing material affects its acidity and the range of the pK_a values of the silanol groups. The ion-exchange activity, as well as the EOF, differed substantially between the materials at low pH, while it was rather similar at high pH, when all available silanol groups are dissociated. Also, the proportion of acidic *versus* nonacidic silanol groups changes as a function of the metal content. Despite of similar ion-exchange activity at pH 7.8, the resolution and the elution order of the tested analytes changed between the columns as illustrated in electrochromatograms shown in Fig. 2. The chromatographic performance depended on the proportion and the type of silanol group and the best separation, for both neutral and basic analytes, was on the material with the highest proportion of acidic silanol groups.

Gillott *et al.* [26] also used a bare silica column for the separation of basic compounds by adding amines into the mobile phase to reduce peak tailing. Comparing triethanolamine (TEOA) and triethylamine (TEA) as additives, they found TEOA had the more desirable effects on separation to improve peak symmetry, while a high EOF was still maintained for its lesser degree of masking for silanol groups on the inner surface. Ye *et al.* [27] also

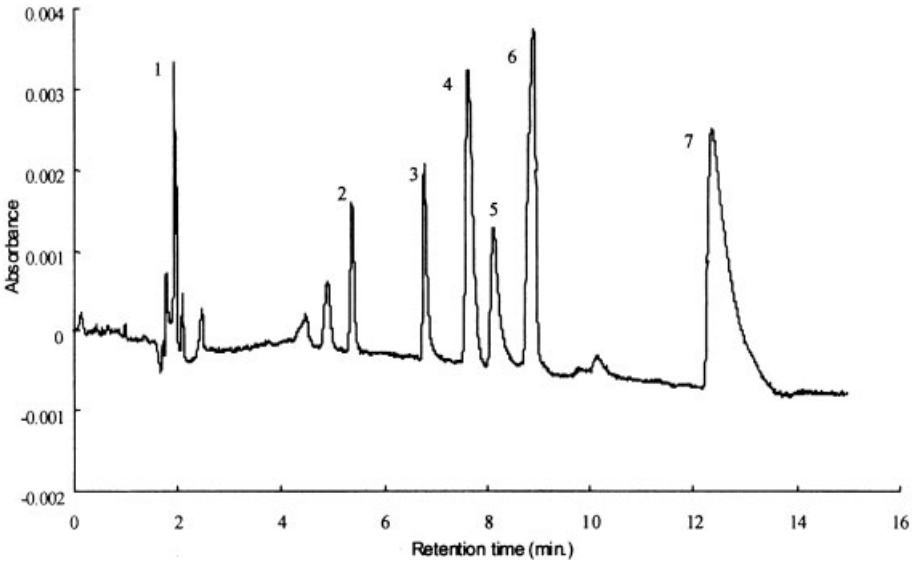


Figure 1. Separation of seven basic drugs on silica stationary phase. Conditions: packing material, 3 μm silica; mobile phase, ACN/10 mM Tris buffer (pH 8.29) (80/20). Solutes: (1) aniline; (2) cocaine hydrochloride; (3) berberine hydrochloride; (4) thebaine; (5) jatrorrhizine hydrochloride; (6) ephridine hydrochloride; (7) codeine phosphate. Reprinted from [24], with permission.

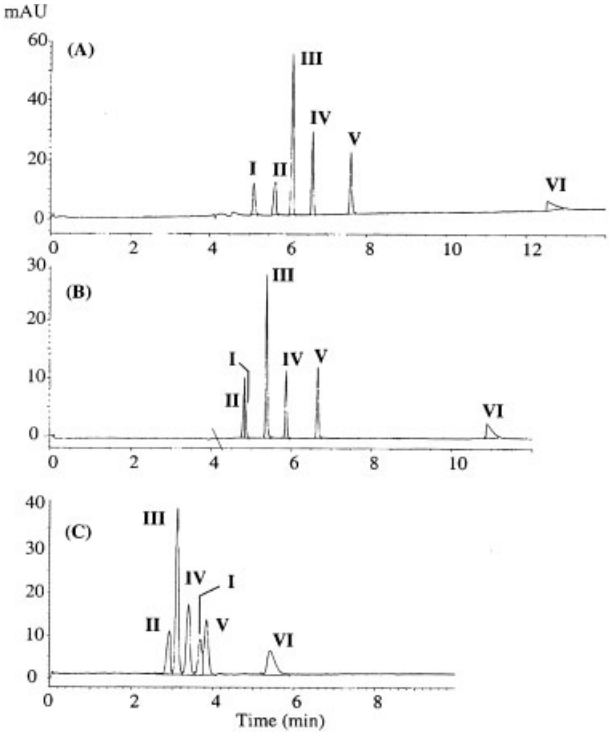


Figure 2. Comparison of CEC separation of a basic test mixture using bare-silica stationary phases of different purity: (A) Hypersil Silica; (B) Hypersil BDS silica; and (C) Hypurity silica. Conditions: mobile phase, 50 mM Tris (pH 7.8)/H_2O/CH_3CN (20/20/60); voltage, 20 kV; temperature, 20°C; injection, 5 kV for 3 s; detection, UV at 210 nm. Solutes: (I) AZ compound A; (II), benzylamine; (III) nortriptyline; (IV) diphenhydramine; (V) AZ compound B; (VI) procainamide. The EOF was marked by biphenyl and ranged between 1.03 mm/s and 1.04 mm/s for all three phases. Reprinted from [25], with permission.

regulated the retention properties of basic compounds on a bare silica column by dynamically modifying it with cationic surfactant.

Lai *et al.* [28] optimized the mobile phase compositions for the normal-phase separation of mixtures containing theophylline, caffeine, and related drugs on bare silica columns. The tertiary mobile phase consisting of isopropanol/hexane/Tris gave an excellent resolution toward these basic drugs and proved to be superior to both the binary and quaternary mobile phases. Column efficiencies were up to 63 000 plates/m and the limits of detection were in the low μg/mL rang.

3.3 Fluorinated and cyanopropylated silicas

Chaiyasut *et al.* [29] studied the separation behaviors of some bases under the pressurized-flow CEC mode using a capillary column packed with fluorinated-bonded silica. Due to the unique properties common to fluorinated packing materials, the retentions of fluorinated bases were stronger than those of nonfluorinated ones. It was unexpected that even the applied voltage can alter the nature of stationary phase. The reduction of the charge density at the surface of stationary phase upon the application of positive voltage was evidenced by the nonlinearity of EOF in the range of positive voltages. In addition, the adoption of either negative or positive voltage deteriorated the separation of neutral analytes and caused the change of elution orders of anionic solutes.

Wei *et al.* [30] studied the dual mechanism of normal-phase and reversed-phase mode on the cyanopropylated silica packed column when separating thiourea, benzyl

alcohol, and naphthalene. Under low acetonitrile concentrations, the solutes are eluted in a typical reversed-phase manner, while under high acetonitrile concentrations the solutes are eluted according to their polarities in the sequence of naphthalene, benzyl alcohol, and thiourea.

3.4 Hydrophilic interaction particulate materials

The term "hydrophilic interaction chromatography" (HILIC) was proposed in 1990 by Alpert [31], and has been applied for the separation of polar substances in HPLC [31–34]. Compounds are separated in HILIC by using a mostly organic mobile phase and a neutral hydrophilic stationary phase. This CEC system causes the elution of solutes in order of increasing hydrophilicity, *i.e.*, the inverse of reversed-phase HPLC. This mode is well-suited for the analysis of polar compounds and has been used successfully for the separation of biopolymers, such as polypeptides, carbohydrates, oligonucleotides, and proteins. In essence, HILIC is a kind of normal-phase liquid chromatography (LC) in which polar sorbents and apolar mobile phases are used, but it is unique regarding the presence of water in the mobile phase. It is crucial to establish a stagnant, aqueous-enriched layer on the surface of the stationary phase into which analytes can selectively partition. In HILIC, a high organic modifier concentration is used to promote hydrophilic interaction between the polar solutes and the hydrophilic stationary phase. The idea of using HILIC materials as CEC stationary phases was recently pioneered by Ye *et al.* [12]. A hydrophilic, strong cation-exchange packing material called poly(2-sulfoethyl aspartamide)-silica (Polysulfoethyl A) was packed into a capillary for hydrophilic-interaction CEC (HI-CEC). This packing material has been found to be very hydrophilic, particularly in comparison with other silica and nonsilica-based matrices. Figure 3 shows a typical separation of polar compounds by HI-CEC with a column of PolySulfoethyl A and a mostly organic mobile phase. As a version of normal-phase CEC, the elution order of solutes should be the opposite of that observed in reversed-phase CEC. Because reversed-phase CEC is difficult to implement under low acetonitrile concentrations, thus reversed-phase HPLC was chosen to investigate the retention of polar compounds. As expected, the elution order of the analytes on HI-CEC was approximately the opposite of that obtained in reversed-phase HPLC (Fig. 4). Toluene as the most hydrophobic of the ten solutes was eluted first in HI-CEC while eluted last in reversed-phase HPLC. Even the extremely hydrophilic solutes of formamide and thiourea were well-resolved in HI-CEC. The column efficiencies for the tested solutes varied from 79 000 to 111 000 plates/m,

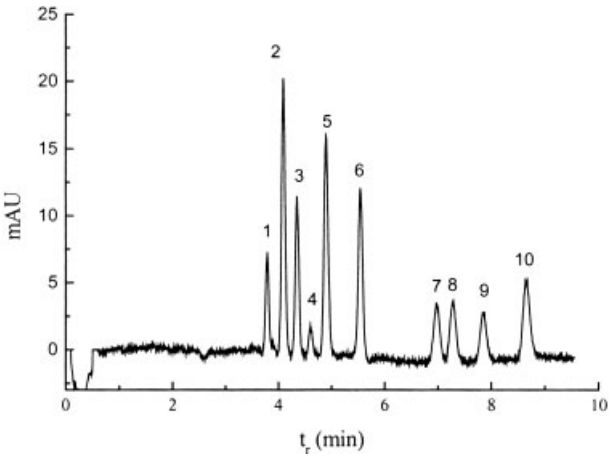


Figure 3. Separation of polar compounds by HI-CEC. Conditions: packing material, 5 μm PolySulfoethyl A; applied voltage, 10 kV; injection, 5 kV for 5 s; mobile phase, 2 mM triethylamine phosphate (TEAP; pH 6.5) containing 80% acetonitrile. Solutes: (1) toluene; (2) *p*-cresol; (3) benzyl alcohol; (4) acetone; (5) aniline; (6) dimethylformamide; (7) thymine; (8) dihydrouracil; (9) formamide; (10) thiourea. Reprinted from [12], with permission.

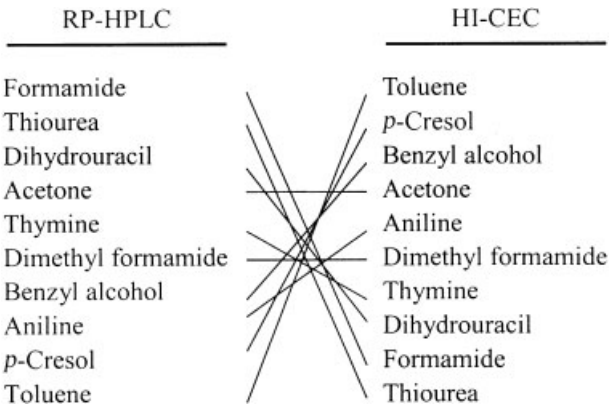


Figure 4. Elution order of polar solutes in reversed-phase HPLC and HI-CEC. Reversed-phase HPLC: stationary phase, 5 μm Hypersil ODS2; mobile phase, linear gradient from water to 100% acetonitrile in 30 min. HI-CEC conditions as in Fig. 3. Tie lines connect the same solutes. Reprinted from [12], with permission.

which was much better than the reported efficiency obtained in normal-phase CEC with nonaqueous mobile phases [5, 6].

The concentration of acetonitrile is an important parameter in adjusting the retention of solutes in HI-CEC. Nine polar solutes including three basic solvents were under investigation by varying the acetonitrile concentration from 40% to 80%. Figure 5 shows the relationship between the retention factors of the selected solutes and the acetonitrile concentration in CEC. An increase in the

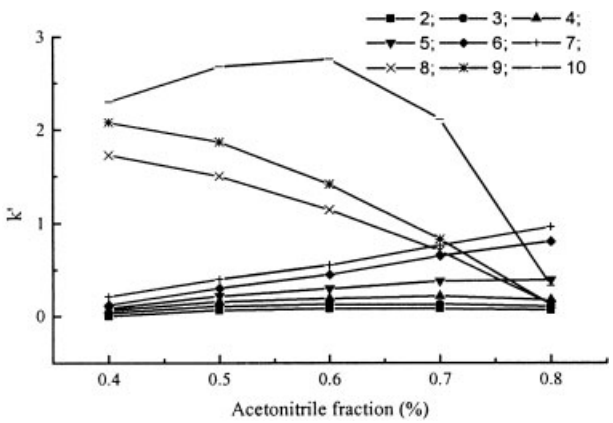


Figure 5. Effect of acetonitrile concentration on the retention factor of polar solvents. Conditions: packing material, 5 μm PolySulfoethyl A; mobile phases, 20 mM TEAP (pH 6.5) with acetonitrile content varying from 40% to 80%. Solutes: (2) ethyl acetate; (3) butanone; (4) acetone; (5) dimethylformamide; (6) dimethylsulfoxide; (7) formamide; (8) quinoline; (9) aniline; (10) pyridine. Reprinted from [12], with permission.

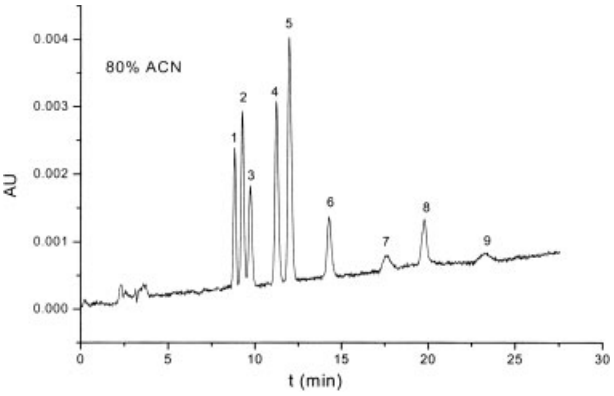


Figure 6. Separation of peptides by HI-CEC. Conditions: packing material, 5 μm PolySulfoethyl A; voltage, 5 kV; injection, 5 kV × 5 s; mobile phase, 100 mM TEAP buffer (pH 2.8) containing 80% acetonitrile. Solutes: (1) Ala-Ile; (2) Gly-Leu; (3) Gly-Phe; (4) Gly-Met; (5) Gly-Val; (6) Gly-Tyr; (7) Gly-Thr; (8) Gly-Ser; (9) Gly-Asp. Reprinted from [35], with permission.

acetonitrile concentration from 40% to 70% resulted in the increase of the retention of all the neutral solutes, a fact that is consistent with the behavior under normal-phase mode. The retention factors of strongly polar solutes, such as dimethylsulfoxide, formamide and dimethylformamide, increased with increasing acetonitrile concentration under investigation. The retention factors of the less polar solutes, such as ethyl acetate, methyl ethyl ketone and acetone, reach a maximum at 70% acetonitrile concentration. The deviation from the

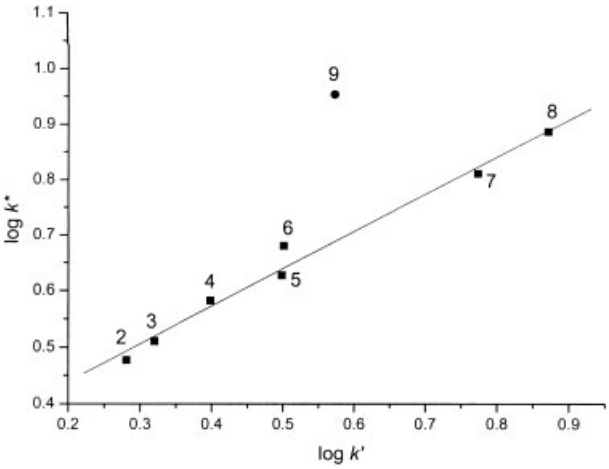


Figure 7. Plot of k^* of peptides in HI-CEC *vs.* k' of peptides in μ-HPLC. μ-HPLC: packing material, 5 μm PolyHydroxyEthyl A; mobile phase, 100 mM TEAP buffer (pH 2.8) containing 80% acetonitrile. HI-CEC conditions and solutes as in Fig. 6. Reprinted from [35], with permission.

expected tendency may be caused by their increased solubility in the mobile phase, while the retention of basic solutes was more complex because of the mixed-mode mechanisms by the contributions of hydrophilic interaction, ion exchange, and electrophoresis to the migration process. Under low acetonitrile concentrations, the basic solutes were less partitioned into the mobile phase and electrostatic interactions were relatively strong. Therefore, it was an ion-exchange mechanism mainly responsible for the retention of basic solutes.

Fu *et al.* [35] have shown the efficient separation of small peptides in HI-CEC. Figure 6 demonstrates the separation of nine peptides under 80% acetonitrile concentration in CEC using a capillary packed with PolySulfoethyl A. As the acetonitrile concentration increases, the retention of the peptides increases. Except for Gly-Asp, a good linear relationship (r = 0.9915) between the log k' in μ-HPLC with neutral hydrophilic stationary phase and log k^* in HI-CEC was observed (Fig. 7). This result suggested that the retention of the solutes is governed mainly by hydrophilic interaction, since the retention increases as the polarities of solutes increased. The acidic peptide Gly-Asp is eluted before Gly-Thr and Gly-Ser in μ-HPLC, whereas in HI-CEC, because of the involvement of electrophoretic migration in the retention process, it is eluted after.

As mentioned above, the electrostatic interactions will inevitably take place between the positively charged peptides and the negatively charged packing surface. The ionic strength of the mobile phase, therefore, should have a strong influence on the separation of peptides in

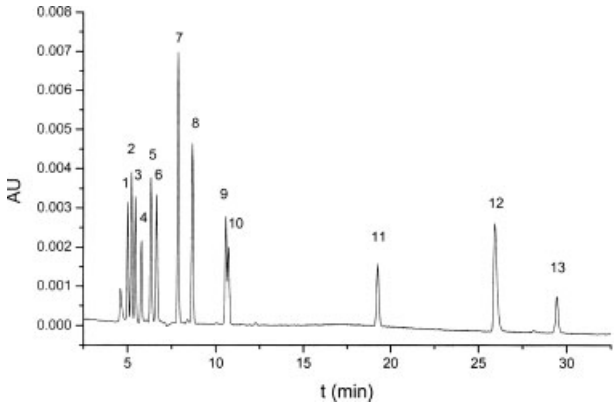


Figure 8. HI-CEC separation of standard basic drugs. Conditions: packing material, 5 μm PolySulfoethyl A; mobile phase, 100 mM TEAP buffer (pH 2.8) containing 80% acetonitrile; voltage, 10 kV; injection, 0.5 psi for 90 s; temperature, 25°C; detection, UV at 214 nm. Solutes: (1) amobarbital; (2) phenobarbital; (3) barbital; (4) caffeine; (5) sulfanilamide; (6) theophylline; (7) 2, 4-dimethylquinoline; (8) propranolol; (9) metoprolol; (10) gramine; (11) atenolol; (12) nicotine; (13) quinoline. Reprinted from [36], with permission.

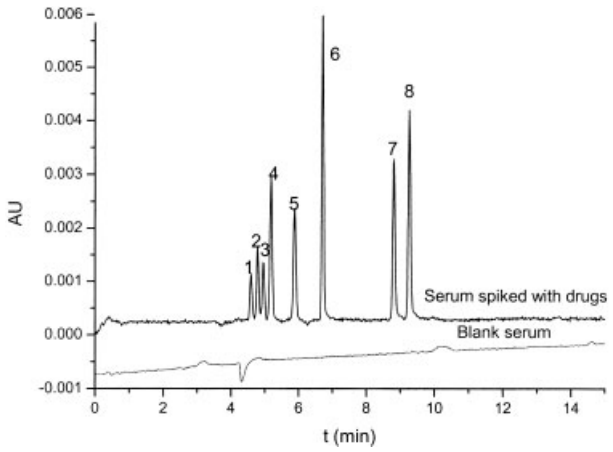


Figure 9. HI-CEC separation of basic drugs spiked in human serum. Concentration of each basic drug is at 40 μg/mL. Conditions as in Fig. 8. Solutes: (1) amobarbital; (2) phenobarbital; (3) barbital; (4) caffeine; (5) sulfanilamide; (6) theophylline; (7) 2, 4-dimethylquinoline; (8) propranolol. Reprinted from [36], with permission.

HI-CEC. However, as the PolySulfoethyl A particles have different hydrophilicities from Spherisorb-SCX, and the involvement of the electrophoretic migration also contributed to the migration process, a linear relationship between the logarithm of retention factors of solutes and the logarithm of ionic strength observed in ion-exchange CEC was not obtained.

The separation of basic compounds was always a challenge in CEC. Because of the applicability to the separation of polar compounds, Fu *et al.* [36] extended HI-CEC to the separation of basic pharmaceuticals. Figure 8 demonstrates a typical separation of 13 standard basic drugs under HI-CEC with the optimized conditions. Symmetrical peaks were observed. Further application of HI-CEC in the determination of basic drugs spiked in human serum was also demonstrated. Figure 9 illustrates the separation of seven basic drugs extracted from spiked human serum. The linearities of amobarbital, phenobarbital, and barbital were found in the range of 5–160 μg/mL, and the detection limits of them were below 5 μg/mL.

A mathematical model has been proposed by Ye *et al.* [17] to quantitatively describe the relationship between the electrochromatographic retention factor (k^*) of charged analytes and the applied voltage and pressure. Separation of peptides under the pressurized flow HI-CEC with a PolyHydroxyl A packed column was applied to validate the model.

3.5 Chemically bonded types of chiral stationary phases with cellulose derivatives

Enantioseparation is a very important aspect of CEC research. It is expected that enantioseparation would not be less important as in HPLC since the high efficiency associated with CEC would not compensate for any short fall in selectivity. As in HPLC, different retentions towards the enantiomers by the specially designed chiral stationary phases (CSPs) are responsible for the enantioseparations. Among the CSPs used in CEC, polysaccharides, such as cellulose and amylose, are some of the most readily available optically active polymers and their derivative-based CSPs have shown the powerful chiral recognition ability for a variety of racemic compounds [37, 38]. To date, the polysaccharide derivative-coated type CSPs on the silica gel are most widely used both in HPLC and CEC [39–44]. The main drawback of the coated phases is the solubility of the chiral selector in a number of solvents, which resulted in the limitation of the mobile phases employed for chiral separations. In order to overcome this problem, the bonded-type CSPs with polysaccharide derivatives chemically immobilized onto the silica gel have been developed for CEC separation of enantiomers [45, 46].

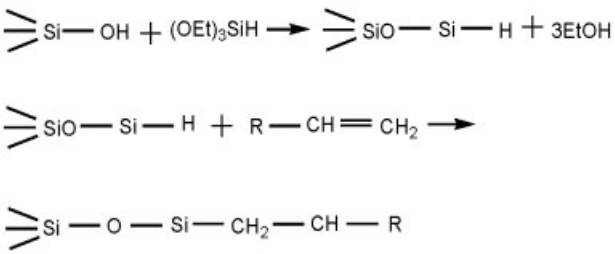
A polysaccharide derivative, cellulose trisphenylcarbamate, was chemically bonded onto the surface of silica gel *via* a spacer for nano-LC and CEC separation of enantiomers [47]. Enantiomers of warfarin and praziquantel were baseline-resolved with column efficiencies of 82 300 and 38 800 plates/m, respectively. Enantiosep-

arations of *trans*-stilbene oxide and praziquantel were also achieved in aqueous CEC with 111 100 and 107 400 plates/m, respectively.

Chen *et al.* [48] prepared positively charged CSPs by chemically immobilizing cellulose derivatives onto diethylenetriaminopropylated silica (DEAPS) with tolylene-2, 4-diisocyanate (TDI) as a spacer reagent. The anodic electroosmotic mobility was observed in both nonaqueous and aqueous mobile phases due to the presence of positively charged amines on the surface of the prepared CSPs. Separation of enantiomers in CEC was performed with the nonaqueous mobile phases of pure ethanol or a mixture of hexane-alcohol and the aqueous phases of acetonitrile-water or 95% ethanol. Fast separation of enantiomers was achieved on the newly prepared CSPs.

4 Polar stationary phases in open-tubular CEC

The use of open-tubular columns is probably the simplest way to implement CEC. In open-tubular columns, the inner wall of a capillary column is modified with the stationary phase. However, open-tubular columns often suffer from low surface coverage, which leads to a low phase ratio, and hence low sample loadability. Pesek *et al.* [49–53] developed a method to prepare the etched capillary to address this problem. Starting with a 20 μm ID capillary and etching with caustic reagent, a variety of surface structures ranging from ripples to coral-like rods can be produced. After etching, the stationary phase is affixed at the surface *via* silanization/hydrosilation reactions. This is accomplished by reacting the etched capillary wall with triethoxysilane, forming a silica hydride surface, which is then reacted with an olefin to form a variety of stationary phases. In this way, a much more stable Si-C bond against hydrolysis under acidic aqueous environment is formed:



In this way, Pesek *et al.* [49, 50] have prepared CEC columns with diol-bonded stationary phases. In contrast to the suffering from insufficient retention, traditional diol-bonded open-tubular CEC columns the former showed improved separation of basic proteins. The results suggested that the etching process in the case of hydride-based capillaries could greatly increase the roughness of capillary inner wall, and thus led to a great increase of the inner wall surface area and amount of surface silanols. A much better reproducibility of migration times for protein separation on the hydride-based capillaries than on capillaries treated with the traditional process was obtained. The authors [51, 53–54] also developed cholesteryl-modified capillaries on a silica hydride surface for CEC, and entirely different selectivities as compared to ODS phases in reversed-phase HPLC and CEC were observed. The intermediate hydrophobic/hydrophilic properties of the bonded cholesterol material allows this stationary phase to be used for both reversed-phase and normal-phase separations. The chemically etched capillaries have been applied for the preparation of CEC columns with 4-cyano-4'-pentoxybiphenyl- and cholesterol-10-undecenoate-bonded liquid crystal stationary phases [51]. The performance of the prepared columns was evaluated by electrochromatographic separation of mixtures of peptides, proteins, pyrimidine/purine bases and a nucleoside, benzodiazepines, the synthetic and metabolic compounds of serotonin, and other small molecules. The efficient separation of these samples was ascribed to the high resolving power of the liquid crystal properties in their native states.

5 Monolithic CEC columns with polar stationary phases

One of the most competing column technologies spurred by the technical difficulties associated with particulate-packed columns in CEC is the monolithic format. In this format, an integrated stationary phase is thus formed in the capillary column by the *in situ* polymerization of monomers or consolidation of packed materials. Currently, monolithic stationary phases prepared for CEC are classified into inorganic monoliths mainly referring to silica-based and organic monoliths.

5.1 Silica monoliths

The silica-sol-gel monoliths present an inorganic alternative for fritless packed columns. Although Fields [55] already prepared the silica xerogel as a monolithic column support and demonstrated the feasibility in HPLC, no further data were reported in the CEC mode. Tanaka *et al.* [56–58] pioneered the preparation of monolithic silica inside a capillary column using a sol-gel-transition process. A typical procedure can be described as follows: silanes, such as tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS) undergo hydrolytic polymerization

catalyzed by aqueous acetic acid in the presence of polyethylene glycol (PEG). The sol was converted to monolithic silica having network structures attached to the tube wall in a fused-silica capillary in order to prevent shrinkage of the skeletons. After the formation of the silica network, ammonia was introduced allowing the formation and tailoring of the mesopores. Therefore, monolithic silica with independently controlled macropores and mesopores can be formed. Monolithic silica prepared in this way has large through-pore/skeleton size ratios and higher porosities allowing good permeability for rapid separation without compromising column efficiency. The monolithic silica, similar to the silica particles developed for packed column CEC, is a valuable polar stationary phase; the separation of polar compounds on such a stationary phase is still under investigation (Zou and Xie, in preparation).

The monolithic silica is a good chromatographic support for bonded phases due to its high permeability and low backpressure. Chemical modification of this material now mainly concentrated on derivatization in the reversed-phase mode with ODS [59, 60]. Recently, Allen *et al.* [61] introduced two synthetic routes for the introduction of polar funcitionalities on the monoliths, one is to produce surface-bound cyano groups (CN-monolith), and the other is to bond the surface with mixed ligands of a cyano group as the top layer and a hydroxyl group as the sub-layer (CN-OH-monolith). Because the CN-OH-monolith yields a stronger polarity and provides a higher "polar" phase ratio, thus stronger retention and better selectivity towards polar compounds can be expected. The CN-OH monolithic column proved to be very useful for normal-phase CEC of a wide range of polar compounds including phenols, chlorophenols, nucleic acid bases, nucleosides, mono- and oligosaccharides. In the analysis of *para*-nitrophenyl derivatives of mono- and oligosaccharides, as the number of glucose residues increased, more hydroxyl groups are available for the interaction with the polar monolith, and in turn stronger retentions were observed. Column efficiencies for the oligosaccharides derivatives with a degree of polymerization up to 5 reached 111 000 plates/m at a flow velocity of 1.0 mm/s. Normal-phase separation of the oligomers in the elution sequence of increased glucose units is shown in Fig. 10.

A photopolymerization approach was introduced by Dulay *et al.* [62] to prepare the sol-gel monoliths. A parent monolith was prepared from a solution of methacryloxypropyltrimethoxysilane hydrolyzed in the presence of hydrochloric acid, and followed by functionalizing the monolith with the silanizing reagent bearing amino- and fluorine-groups. Despite the hydrophobic nature of parent monolith, the derivatization altered the surface polarity and yielded better chromatographic properties. Higher stability at pH values above 4 and column efficiencies up to 180 000 plates/m were achieved, and the enhanced separation of biologically and pharmaceutically important compounds, such as nucleosides, positively charged peptides, and taxol derivatives, were also obtained.

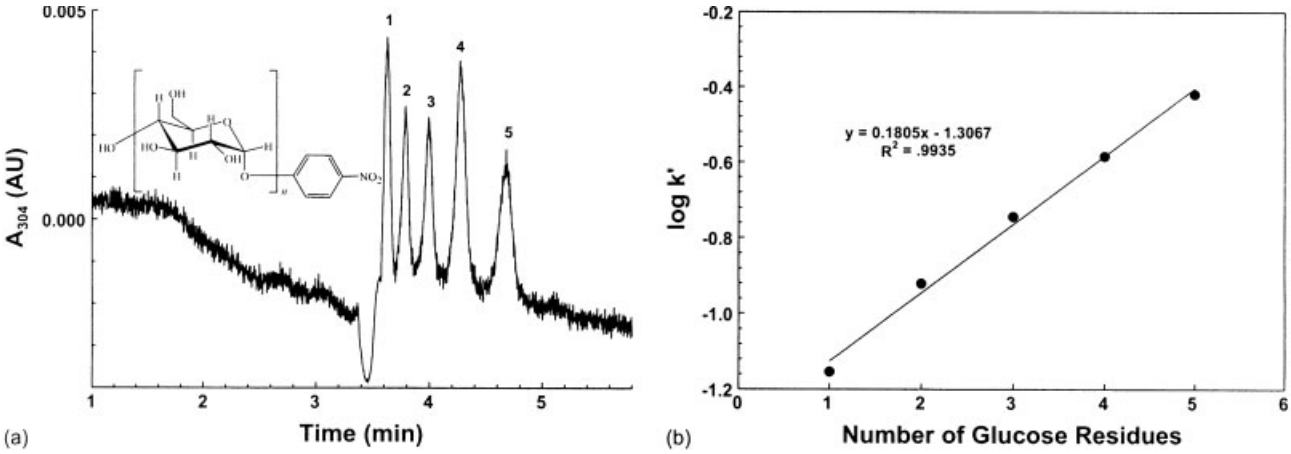


Figure 10. (a) Separation of *p*-nitrophenyl-α-glucose (pNP-αGlc) and *para*-nitrophenyl (pNP) maltooligosaccharides and (b) log *k' vs.* number of glucose residues. Conditions: capillary column, 27 cm (effective length 20 cm) × 100 μm ID with CN-OH-monolithic rod; mobile phase, 5 mM NH_4Ac (pH 4.5) at 80% v/v acetonitrile; voltage, 20 kV; column temperature, 20°C. Solutes: (1) pNP-αGlc; (2) pNP-α-D-maltoside; (3) pNP-α-D-maltotrioside; (4) pNP-α-D-maltotetraoside; (5) pNP-α-D-maltopentaoside. Solute concentration, 2.8×10^{-4} M. Reprinted from [62], with permission.

5.2 Monoliths based on polyacrylamides

An approach towards the preparation of highly cross-linked acrylamide polymer-based monolithic CEC column was reported by Hjertén *et al.* [63]. The initial purpose was to prepare a monolithic column involving a multiple-step process for reversed-phase CEC. However, the incorporation of hydrophilic monomers into the matrix can be finished in one step. The typical polymerization mixture consisted of an aqueous solution of acrylamide monomers and the cross-linking agent piperazine diacrylamide. Ammonium sulfate was included as the pore-generating agent. Following this procedure, acrylamide-based monoliths were prepared by Maruška *et al.* [64], which demonstrated normal-phase separation mechanism for capillary LC. In explaining the mechanism for the separation of polar aromatic compounds, it was ascribed by the adsorption of aromatic compounds on the polar sites of the polyacrylamide-based matrix.

In a modified procedure, Hoegger and Freitag [65–67] performed a thorough investigation of the conditions for the synthesis of acrylamide-based monolithic CEC columns and the retention behaviors of aromatic compounds. The pore size of the polymer can be modulated through the salt concentration. Although functional monomers of *N*,*N*-dimethylacrylamide, methacrylamide, 2-hydroxymethacrylate, butylacrylate, and hexylacrylate differed in polarities, the elution orders of aromatic compounds on the prepared monolithic columns from these monomers are the same, but distinctly different from that obtained on a commercial column packed with ODS silica particles (Fig. 11). In assessing the retention mechanism, it was assumed that the carbonyl and hydroxyl groups of the monomers as well as the hydrophobic polymer backbone are the main sites of interactions with solutes.

Novotny and Palm [68] substantially simplified the incorporation of highly hydrophobic ligands into the acrylamide-based matrices with C_4-, C_6-, or C_{12}-alkyl acrylate as monomers, respectively. Columns with high efficiencies were only obtained when the polymerization reaction was performed in the presence of poly(ethylene oxide) in the polymerization solution. In addition to preparing monolithic columns for reversed-phase CEC, Novotny and co-workers [69–71] also incorporated polar monomers, such as 2-cyanoethyl acrylate and 3-amino-1-propanol vinyl ether, into the acrylamide-based monoliths as polar stationary phases for CEC. In the analysis of bile acids, the free and glycine-conjugated bile acids were separated by using a hydrophobic C_{12} stationary phase

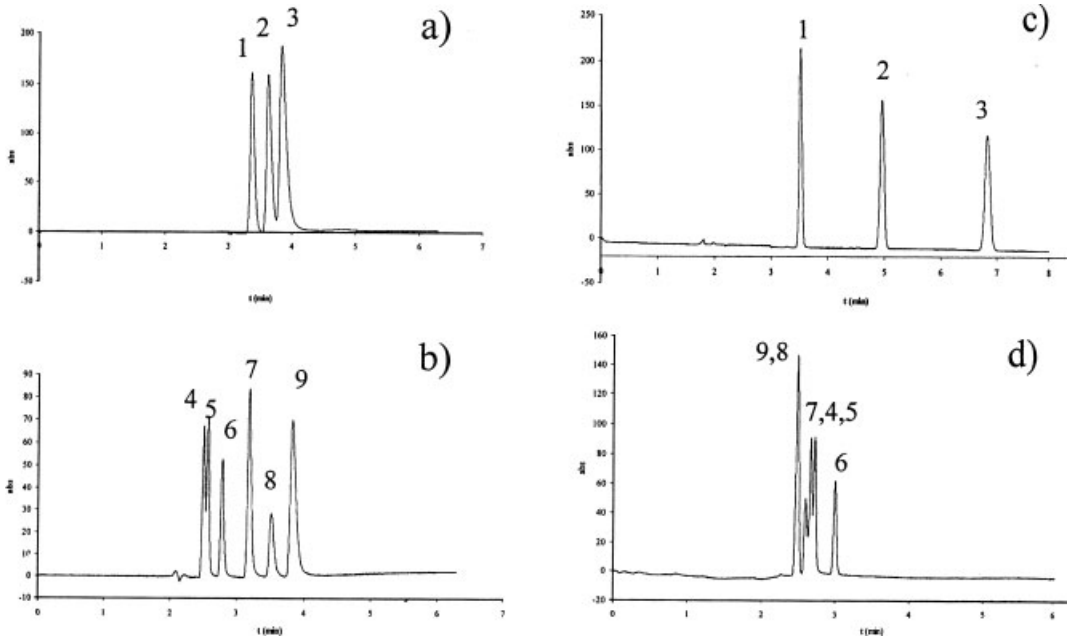


Figure 11. Comparison of the elution order of aromatic compounds in separations on a poly(dimethylacrylamide-*co*-piperazine diacrylamide-*co*-vinylsulfonic acid) column (a, b) *vs.* separations on a commercial column packed with ODS silica particles (c, d). Conditions: mobile phase, (a, b) methanol-acetonitrile (6/4), (c, d) acetonitrile-5 mM phosphate, pH 7 (7/3). Solutes: (1) naphthalene; (2) phenanthrene; (3) pyrene; (4) hydroquinone monomethyl ether; (5) phenol; (6) 2-naphthol; (7) catechol; (8) hydroquinone; (9) resorcinol. Reprinted from [66], with permission.

with positive-ion detection mode, and a mixture of glycine- and taurine-conjugated components by using a polar amine stationary phase with negative-ion detection mode. The former exhibits reversed-phase elution patterns, while the latter is apparently of normal-phase mode. Que and Novotny [69, 70] also extended their polar columns to the separation of complex mixtures of glycans. The separation mechanism was predominantly based on the hydrophilic partition involving hydrogen-bonding and dipole-dipole interactions between the hydroxyl groups of the saccharide solutes and the polar stationary phases. The hydrophilic column allowed ideal separation of the glycan mixtures in coupling with MS for detection and identification of the individually separated analytes proved to be a very powerful technique for carbohydrate analysis.

5.3 Monoliths based on polymethacrylates

Alternatives to the acrylamide-based monoliths are methacrylate-based monoliths prepared from a typical mixture consisting of butylmethacrylate (for hydrophobic retention), ethylene dimethacrylate (a cross-linker), and 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS), which provides a charge carrier for EOF. Mixture of 1-propanol and butanediol is often used as porogenic solvents. Although this type of monolith is mainly designed for reversed-phase CEC [72, 73], the incorporation of polar monomers into the monoliths was also reported. Lämmerhofer *et al.* [74] prepared poly(2-hydroxyethyl methacrylate-*co*-ethylene dimethacrylate-*co*-2-dimethylaminoethylacrylate) monolith in the presence of a binary porogenic mixture of dodecanol and cyclohexanol. The polar functionalities of the hydrophilic monolith can be used for the separation of neutral and even basic compounds in the normal-phase CEC mode (Fig. 12). Substituted phenols were separated according to their hydrophilicities: 4-iodoanisole, which does not contain hydroxyl groups, was eluted first, followed by vanillin and phenol with their single phenolic hydroxyl group, while 4-methylcatechol with two phenolic hydroxyl functionalities is the most polar, hence eluting last in the mixture. The column efficiencies in the nonaqueous mobile phase even exceeded 80 000 plates/m. It should be noted that the abilities of columns for normal-phase separation of analytes are preserved even in aqueous mobile phase. The symmetrical peaks and efficient separation achieved for basic compounds in an aqueous mobile phase with pH 10.7 demonstrated the usefulness of this monolith for an anion-exchange/normal-phase mixed-mode separation of analytes.

Lämmerhofer *et al.* [75, 76] examined the effect on enantiomeric separations when polar monomers were incorporated in the methacrylate-based chiral monolith.

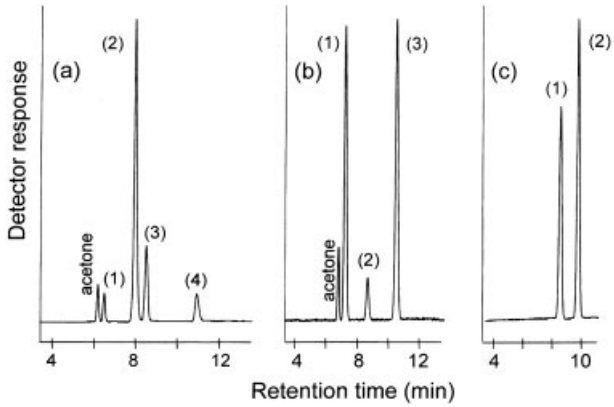


Figure 12. Separation of (a) phenolic compounds, (b) xanthines, and (c) *N*-ethylaniline and aminopyridine in normal-phase CEC using a strong anion-exchange monolithic column. Conditions: on column alkylated monolith prepared from mixtures consisting of 8% 2-dimethylaminoethyl methacrylate, 24% 2-hydroxyethyl methacrylate, 8% ethylene dimethacrylate, 20% cyclohexanol, 40% 1-dodecanol; UV-initiated polymerization at room temperature for 16 h; $d_{p,\,mode}$ = 1423 nm. Column dimensions: 100 μm ID, 33.5 cm total length, 25 cm effective length. Mobile phase: 0.4 M acetic acid and 4 mM triethylamine in acetonitrile-methanol (a) (60/40), and (b) (80/20); voltage, −25 kV; injection, −5 kV for 5 s; temperature, (a) 50°C and (b) 25°C; UV detection at 250 nm. Solutes: (a) 4-iodoanisole (1), vanillin (2), phenol (3), and 4-methylcatechol (4); (b) caffeine (1), theobromine (2), and theophylline (3); (c) *N*-ethylaniline (1), and 2-aminopyridine (2). Reprinted from [74], with permission.

The substitution of glycidyl methacrylate (GMA) for butyl methacrylate, and the subsequent hydrolysis of the epoxide groups into vicinal diol moieties, considerably improved the column efficiency. Simply replacing GMA with the more polar monomer 2-hydroxyethyl methacrylate leads to the increase of the separation efficiency of these CEC columns by a factor of 8 from 500 to 4000 plates/m, and simultaneously the enantioselectivity for the separation of racemic *N*-3,5-dinitrobenzoylleucine (DNB-Leu) is increased from an α-factor of 1.62–3.36. Explaining the effect of the polar groups on the separation, they attributed the improved chiral separation to the significant reduction of nonspecific interactions, as well as the absence of lateral epoxypropyl functionalities with uncontrolled stereochemistry at the central carbon atom.

Zhang *et al.* [77] prepared a cationic acrylic monolith by using a mixture of propanol and formamide as progenic solvents. The epoxide groups at the surface of the poly(glycidylmethacrylate-*co*-ethylene dimethacrylate-*co*-methyl methacylate) were functionalized with *N*-ethylbutylamine to introduce hydroxyl and tertiary amino functionalities with ethyl- and butyl-chains. Although the

separation of proteins and peptides was mainly contributed by the interaction with short alkyl chains, the existence of abundant hydroxyl groups on the monolith was also considered as interaction sites for separation of polar compounds under nonaqueous mobile phases.

6 Polar stationary phases for microfluidic CEC devices

A recent progress of CEC column technology is its application to microfluidic devices. It enables the separation in well-controlled microfabricated channels in a miniaturized format. As such devices offer several advantages, including speed of analysis, low sample consumption, ability to multiplex and compatibility with integration, leading towards the development of micrototal analysis systems (μTAS), they have found various potential applications and gained undeniable success. Up to now, all column configurations, open channel [78, 79], packed [80], and monolithic [81] structures have been fabricated in microchips. The fabrication of acrylamide-based monoliths in microfabricated channels was developed by Hjertén *et al.* [81]. The channels of the microchip were completely filled with polymerization solution, followed by reversely replacing about 2 cm of the solution with PEG solution to fabricate the UV detection window. A sample of uracil, phenol, and benzyl alcohol was separated by electrochromatography in less than 20 s with a separation efficiency of ~350 000 plates/m. The electrochromatographic separations of both low-molecular-weight (alkyl phenones, antidepressant drugs) and high-molecular-weight substances (standard proteins) were demonstrated. A comparison between the monolithic CEC microchips and a monolithic capillary column prepared with the same monomers showed similar separation efficiencies.

Svec *et al.* [82] has adopted a UV-initiated polymerization procedure to prepare monoliths in the channels of microfluidic devices. Using a mask, the polymerization may be strictly confined to the areas exposed to irradiation while no polymerization is observed in the dark areas. Therefore, by using different monomers, including the polar monomers of glycidyl methacrylate and 2-hydroxyethyl methacrylate, and the hydrophobic monomer of butyl methacrylate, monoliths possessing different surface chemistries can be fabricated in the microchannels.

7 Conclusions

Silica-based reversed-phase packing materials have been widely used in CEC for the analysis of neutral and nonpolar analytes. With the demands of proteomics studies and related biopathway system analysis, the separation of charged and polar analytes should be emphasized in CEC research in the future. In HPLC, the use of polar stationary phases provides different separation selectivity complementary to reversed-phase and ion-exchange stationary phases, and a great number of polar stationary phases have been developed. However, the transferring of polar stationary phases from HPLC to CEC is still very limited, thus specially designed polar packing materials for CEC should be developed.

A monolithic column with stationary phases inside the capillary being consolidated into integrity is a competitive CEC column format, which requires less efforts than the slurry-packed columns. The direct way to prepare the polar monoliths is by including polar monomers in the polymerization mixture, which can be further converted to a monolith. A major advantage of this approach is the availability of a wide variety of polar monomers and the simplicity of the column preparation procedure. However, the conversion to the polar functionalities *via* the building-in of active sites in the monolith with reactive monomers also provides an alternative. This method is promising because of the matured column technology and readily available chemical derivatization protocols. Biomolecules, including metabolites, peptides, proteins, and carbohydrates, are polar analytes present in complex biological systems; much effort is needed to develop polar stationary phases with novel properties for the highly efficient separation of these biomolecules by CEC.

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