Hydrophilic Interaction Capillary Electrochromatography for the Separation of Polar Compounds

The authors used hydrophilic interaction capillary electrochromatography (CEC) to separate polar compounds. Their system had a capillary column packed with a hydrophilic, strong cation-exchange material of poly(2-sulfoethyl aspartamide)-silica and a hydrophobic mobile phase, which typically contained 80% acetonitrile. The elution order of polar compounds in hydrophilic interaction CEC is similar to that obtained by normal-phase chromatography, and the retention of solutes increases with their hydrophilicity. The authors obtained column efficiencies of 79,000–111,000 plates/m with separation of some model polar compounds under optimal conditions. The repeatability of their system was good with relative standard deviations of approximately 0.4% and less than 0.7% for void time and solute retention factors, respectively, in 10 consecutive runs.

Capillary electrophoresis (CEC) embodies many features of capillary zone electrophoresis (CZE) and high performance liquid chromatography (HPLC). The combination of CZE’s high peak efficiency and HPLC’s high selectivity appears to be the major advantage of this separation method. Most of the CEC work to date has concerned the separation of hydrophobic compounds using reversed-phase stationary phases (1–4). The separation of very polar compounds in reversed-phase CEC is difficult. Reversed-phase CEC separations can be hampered by the formation of bubbles caused by the stationary phase’s nonwettable characteristics when highly aqueous mobile phases are used to promote the retention of polar compounds (5). The formation of bubbles can be eliminated by pressurizing both sides of the capillary (6,7). However, this practice requires a more complex instrument and cannot be performed on most standard CZE instruments.

Seifar and co-workers (8) reported that adding sodium dodecyl sulfate (SDS) to the mobile phase was useful for stabilizing the current and the electroosmotic flow. Bailey and Yan (5) reported that they successfully separated high polarity explosives using CEC with nonporous C18-coated material and a mobile phase with a low concentration of organic modifier and SDS added to the mobile phase to increase the wettability of the particle surface. Zhang and colleagues (9) also reported that very polar compounds could be separated by mixed-packing CEC with a stationary phase that was a physical mixture of strong cation-exchange and C18 materials. The hydrophilicity of the packing increased greatly because of the presence of the sulfonic acid group on the surface of the strong cation-exchange material, and a mobile phase with a low concentration of organic modifier could be used in this system to avoid bubble formation. Polar ionic compounds can be separated by ion-exchange CEC with high efficiency (10–12), and hydrophobic neutral solutes can be separated in ion-exchange CEC after adding surfactant to the mobile phase to dynamically modify the packing surface (13,14).

A chromatographic technique called hydrophilic interaction chromatography has proven to be an efficient method for separat-
ing polar compounds, including amino acids, peptides, nucleic acids, and carbohydrates (15–17). In essence, hydrophilic interaction chromatography is a kind of normal-phase liquid chromatography (LC) in which polar solvents 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, as Alpert (15) described. In traditional normal-phase LC, nonaqueous mobile phases such as hexane or chloroform typically are used.

Normal-phase CEC with a nonaqueous mobile phase also has been performed; however, both the column efficiency and the electroosmotic flow were poor (18,19). Lai and Dabek-Zlotorynska (20) reported that column efficiency and electroosmotic flow could be improved by adding some amount of aqueous buffer to the mobile phase in normal-phase CEC with bare silica particles. They achieved an efficiency of 63,000 plates/m when separating some drugs.

Hydrophilic interaction chromatography may be more suitable than normal-phase LC for use with CEC because of the aqueous-rich mobile phases used. Recently, Que and co-workers (21) achieved apparent efficiencies as high as 610,000 plates/m for separations of bile acids and their conjugates on an amino phase with a partially aqueous mobile phase. The separation of solutes was based mainly on a normal-phase chromatography mechanism, which means that the CEC was performed in the hydrophilic interaction mode.

In our study, we packed a hydrophilic, strong cation-exchange packing material called poly(2-sulfoethyl aspartamide)-silica (PolySulfoethyl A [PolyLC Inc., Columbia, Maryland]) into a capillary column for hydrophilic interaction CEC. This packing has been characterized as very hydrophilic, particularly in comparison with other silica- and nonsilica-based matrices (15,22). Recently, Mant and colleagues (16) demonstrated that mixed-mode hydrophilic interaction–cation-exchange chromatography using PolySulfoethyl A as the stationary phase was an excellent technique for separation of peptides. In our study, we separated polar compounds in the hydrophilic interaction CEC mode with PolySulfoethyl A. We compared the selectivity for separation of polar solutes with that of reversed-phase HPLC. We also investigated the effects of ionic strength and acetonitrile concentration on separation.

Experimental

Instrumentation and materials: All CEC experiments were performed using a Beckman P/ACE 5510 instrument (Beckman Coulter, Fullerton, California). The HPLC experiments were performed using LC10AT pumps and an SPD-10A UV detector (both from Shimadzu, Kyoto, Japan) and a WDL-95 workstation (National Chromatographic R&A Center, Dalian, China). We used a Waters model 510 pump (Waters Corp., Milford, Massachusetts) to pack the capillary columns. We obtained 75- and 50-μm i.d. (365-μm o.d.) fused-silica capillary from the Yongnian Optic Fiber Plant (Hebei, China). The 5-μm d_p, 300-Å PolySulfoethyl A was a gift from PolyLC Inc. We obtained a 200 mm × 4.6 mm, 5-μm d_p, Hypersil ODS2 HPLC column (Thermo Hypersil Ltd., Runcorn, United Kingdom) from the Dalian Elite Corp. (Dalian, China) and used it for the reversed-phase chromatography experiments.

Chemicals and buffers: The acetonitrile we used was of chromatographic grade, and the other reagents we used were of analytical reagent grade. The ultrapure water we used for preparing solutions was produced by a Milli-Q water-purification system (Millipore Corp., Bedford, Massachusetts). We prepared a stock solution of triethylamine phosphate, 500 mM in phosphate, by adding triethylamine to a solution of phosphoric acid until we obtained a pH level of 6.5. The triethylamine phosphate stock solution was filtered through a 0.45-μm membrane. The mobile phases were prepared by adding the required volume of acetonitrile and triethylamine phosphate buffer to a volumetric flask, followed by adding water to 1 mL below the volume mark on the flask. Then, we placed the flask in a sonicator bath for 5 min. After the solution reached room temperature, we added water to the mark. Before starting the run, we degassed the mobile phase in an ultrasonic bath for 30 min.

Column preparation and separation conditions: The CEC columns were packed in-house by a slurry packing technique, as reported in the literature (15,23). All columns were 27 cm long with packed lengths of 20 cm. Before using a column in a CEC experiment, we flushed it with mobile phase for 30 min using a syringe. The column then was conditioned on the instrument with the mobile phase for at least 2 h. The applied voltage was first ramped from 0 to 10 kV in 10 min and then maintained at 10 kV. The temperature was maintained at 25 °C, and the detection wavelength was set at 200 nm. The separation voltage was set at 10 kV if not otherwise stated. All CEC experiments were performed in the isocratic elution mode.

The HPLC experiments were performed in gradient elution mode with a linear gradient from 100% water to 100% acetonitrile in 30 min. The flow velocity was set at 0.9 mL/min, and the detection wavelength was set at 200 nm.

Results and Discussion

The term hydrophilic interaction chromatography originally was coined to describe separations based upon solute hydrophilicity (15). As a variant of normal-phase LC, separation by hydrophilic interaction chromatography depends upon hydrophilic interactions between the solutes and a hydrophilic stationary phase in a manner similar to normal-phase chromatography; that is, solutes are eluted in order of increasing hydrophilicity. A characteristic of hydrophilic interaction chromatography separations is the presence of a high organic modifier concentration to promote hydrophilic interactions between the polar solute and the hydrophilic stationary phase. Therefore, we adopted a mobile phase containing 80% acetonitrile in the initial hydrophilic interaction CEC experiments. Although hydrophilic interaction chromatography can be applied to separate neutral solutes using a mobile phase without buffer solution (15), we added at least 2 mM triethylamine phosphate (pH 6.5) to the mobile phase to confer reproducible electroosmotic flow.

Figure 1 shows the separation of polar compounds by hydrophilic interaction CEC with a column of PolySulfoethyl A and a mostly organic mobile phase. As a version of normal-phase chromatography, the elution order of solutes should be the opposite of that obtained with reversed-phase chromatography. However, it was impossible to make a direct comparison with reversed-phase CEC. First, the reversed-phase CEC runs were interrupted by the formation of bubbles because of the nonwettable property of the packing surface at the low acetonitrile concentrations necessary to ensure retention of the more polar standard solutes. Second, because the standards differ greatly in hydrophobicity, we needed a linear gradient to elute them in a reasonable time in reversed-phase chromatography.

For those two reasons, we separated these solutes using reversed-phase HPLC. We adopted a linear gradient from water to 100% acetonitrile in 30 min, and the last peak, toluene, was eluted in 25 min.
Figure 2 compares the elution orders of the same solutes in reversed-phase HPLC and hydrophilic interaction CEC. As expected, the elution order in hydrophilic interaction CEC is approximately the opposite of that obtained in reversed-phase HPLC. Toluene is the most hydrophobic of the 10 solutes, and it is eluted last in reversed-phase HPLC. Conversely, it is eluted first in hydrophilic interaction CEC because of the minimal hydrophilic interaction with the polar stationary phase. We assumed that the interaction between toluene and the hydrophilic stationary phase was insignificant, so we selected toluene as the $t_r$ marker in hydrophilic interaction CEC, where $t_r$ is the retention time of unretained compounds. The last-eluted two solutes in hydrophilic interaction CEC — formamide and thiourea — are extremely hydrophilic. They are used in reversed-phase CEC and micellar electrokinetic capillary chromatography as $t_r$ markers because they are virtually unretained by the hydrophobic stationary phase (23,24). Baseline separation of these two solutes was not achieved, even when no organic modifier was in the mobile phase in reversed-phase HPLC. However, these two solutes can be separated with high resolution in hydrophilic interaction CEC, as shown in Figure 1. It is interesting that the elution order of formamide and thiourea is the same in both systems. Because the hydrophobic interactions of these two solutes are negligible with a hydrophobic stationary phase, the partial separation of the solutes by reversed-phase HPLC can be based upon the hydrophilic (silanophilic) interaction with the residual silanol groups on the C18 packing surface.

Alpert and co-workers (25) explained another separation of carbohydrates in hydrophilic interaction chromatography by the different contact region of the anomers with the hydrophobic stationary phase. Thiourea was retained more strongly than formamide in hydrophilic interaction CEC, and it could be explained by the fact that the contact region of thiourea is more favorable for interaction with the stationary-phase surface. They also reported that highly polar compounds that are not retained on reversed-phase stationary phases can be separated successfully by hydrophilic interaction chromatography (25). This finding supports our observations that hydrophilic interaction CEC is a complementary technique to reversed-phase CEC and that it has the potential to analyze highly polar compounds.

The column efficiencies for peaks in Figure 1 varied from 79,000 to 111,000 plates/m. Although these efficiencies are not as high as those of typical reversed-phase CEC separations performed with 5-μm particles, they are much better than those obtained in normal-phase CEC with nonaqueous mobile phases (18,19). The reported efficiency did not exceed 21,000 plates/m with cellulose-based packing and native silica gel as stationary phases in normal-phase CEC (18). Lai and colleagues (20) obtained relatively higher efficiency in normal-phase CEC with a 3-μm $d_p$ silica gel–packed column with a mobile phase that contained a small amount of aqueous buffer. The highest efficiency for the separation of theophylline, caffeine, and related drugs was 63,000 plates/m, which also is poorer than that in hydrophilic interaction CEC in our study. Que and co-workers (21) achieved apparent efficiencies of as much as 610,000 plates/m for separations of bile acids and their conjugates on an amino stationary phase in hydrophilic interaction CEC. These results also indicate that hydrophilic interaction chromatography is better suited to CEC than the conventional normal-phase chromatography mode because of the favorable aspects of a partially aqueous mobile phase.

The retention of neutral polar solutes on PolySulfoethyl A in the hydrophilic interaction chromatography mode is approximately 25% less than with PolyHydroxy-
ethyl A (PolyLC), which is a neutral polar stationary phase developed especially for hydrophilic interaction chromatography (15). However, PolySulfoethyl A has potential for use in CEC because of the high charge density on its packing surface, which causes strong electroosmotic flow. Figure 3 shows the fast separation of seven solutes with 25-kV voltage. All seven solutes were baseline separated in only 2.5 min due to the great linear velocity. The electroosmotic flow is much stronger than that in normal-phase CEC with a nonaqueous mobile phase. The linear velocity was only approximately 0.48 mm/s in normal-phase CEC at 30 kV voltage using a mobile phase of methanol–ethanol–hexane and a column with 34.1 cm total length and 30.1 cm packed length (16). By contrast, the linear velocity in hydrophilic interaction CEC in our study was 2.94 mm/s at 25 kV, which is more than fivefold that in normal-phase CEC.

Maruska and Pyll (19) reported that electroosmotic flow was substantially decreased with an increasing volume fraction of a nonpolar component (hexane) in the mobile phase in normal-phase CEC. However, an increase of the acetonitrile fraction (nonpolar component) in our present study results in an increase in electroosmotic flow, as shown in Figure 4. This tendency is similar to that of reversed-phase CEC with acetonitrile as the organic modifier, which may be the result of a decrease in the mobile phase’s viscosity (26).

Triethylamine phosphate buffer typically was used in hydrophilic interaction chromatography because of its good solubility in solvents with high organic modifier concentrations. However, triethylamine salts sometimes yield rising baselines and artifact peaks in HPLC gradient elution (15). Drifting baselines also are observed in hydrophilic interaction CEC, especially when changing mobile phases.

The baseline can be stabilized after a long period of conditioning with high voltage. In our work, we conditioned the column by applying a voltage of 5 kV for 20 min and then applying 10 kV for 30 min when changing the mobile phase. We investigated the repeatability of this system with a mobile phase that contained 80% acetonitrile and 5 mM triethylamine phosphate (pH 6.5). We selected six compounds — toluene, benzyl alcohol, dimethyl formamide, thymine, formamide, and thiourea — as the test solutes. We selected the apolar solute, toluene, as a t<sub>0</sub> marker. We obtained good repeatability for t<sub>0</sub> with a relative standard deviation (RSD) of 0.4% for 10 consecutive runs, which indicates that electroosmotic flow in this system is very reproducible. The RSD values for the retention factors of the five polar solutes were less than 0.7%, which also demonstrated the good repeatability of the retention of these solutes.

The concentration of acetonitrile is an important parameter in adjusting the retention of solutes in hydrophilic interaction chromatography. We investigated the acetonitrile concentration’s effect on the separation by varying the acetonitrile concentration from 40% to 80%. We selected nine polar solvents, including three basic solvents — quinoline, aniline, and pyridine — as the test solutes. We adopted a mobile phase with a relatively high buffer concentration — 20 mM triethylamine phosphate (pH 6.5) — to elute the basic solvents in reasonable time at low organic modifier concentration at which strong electrostatic attraction occurs between these solutes with the negatively charged packing surface.

The CEC experiments may be hampered by the formation of bubbles, which may occur more easily when Joule heating is strong. To decrease the Joule heating, we used a capillary with an inner diameter of 50 μm. We observed no bubble formation throughout our investigation.

Figure 5 shows the relationship between the retention factor of the selected solutes and the acetonitrile concentration. An increase of acetonitrile concentration from 40% to 70% results in an increase in the retention of all the neutral solvents, as expected in normal-phase but not in reversed-phase chromatography. These results demonstrate that hydrophilic interactions are promoted at high acetonitrile concentrations. The retention factors of the strong polar solvents — dimethyl sulfoxide, formamide, and dimethyl formamide — increase with increasing acetonitrile concentration at all the concentrations we investigated. However, the maximum retention factors for the three less polar solvents — ethyl acetate, methyl ethyl ketone, and acetone — were obtained at 70% acetonitrile concentration. The retention factors of these solutes were slightly decreased by further increasing acetonitrile concentration. This deviation from the expected tendency may be because the solubility of those solutes in the mobile phase increases and, consequently, the amount of them partitioning into the stationary phase decreases with increasing acetonitrile concentration.

The effect of acetonitrile on the retention of the basic solvents was more complex than that of neutral solvents because three mechanisms — hydrophilic interaction, ion exchange, and electrophoresis — may contribute to the migration process. Figure 5 shows that we obtained strong retention of three basic solvents with a mobile phase that contained 40% acetonitrile. The retention factors for the neutral solutes were less than 0.21, which means that their hydrophilic interactions with the stationary phase were very weak under this condition. The electrophoretic mechanism accelerates basic solutes to be eluted from the column because the electrophoretic mobility of these solutes has the same direction as that of the electroosmotic flow, and this acceleration results in weak retardation in the column. Therefore, the ion-exchange mechanism mainly was responsible for the strong retention.

![Figure 3](image_url)  
**Figure 3:** High-voltage hydrophilic interaction CEC separation of polar compounds. Mobile phase: 80% acetonitrile, 5 mM triethylamine phosphate (pH 6.5); separation voltage: 25 kV. See Figure 1 for other conditions. Peaks: 1 = toluene, 2 = benzyl alcohol, 3 = aniline, 4 = dimethyl formamide, 5 = thymine, 6 = formamide, 7 = thiourea.

![Figure 4](image_url)  
**Figure 4:** Effect of acetonitrile concentration on electroosmotic flow mobility. Mobile phase: 20 mM triethylamine phosphate (pH 6.5) and acetonitrile (fraction varying from 40% to 80%); column inner diameter: 50 μm. See Figure 1 for other conditions.
Alpert (15,27) reported that the retention of peptides and amino acids on PolySulfoethyl A in hydrophilic interaction chromatography increased with an increased acetonitrile concentration because hydrophilic interaction was promoted at high acetonitrile concentrations. In hydrophilic interaction CEC, the hydrophilic interaction between these basic solvents and the stationary phase also should increase with increasing acetonitrile concentration and result in strong retention. However, as Figure 5 shows, the retention of quinoline and aniline decreased steadily with increasing acetonitrile concentration, and the retention of pyridine first slightly increased and then dramatically decreased with increasing acetonitrile concentration. This tendency is similar to what occurs in ion-exchange chromatography with relatively hydrophobic materials as the stationary phases, in which the retention of ionic compounds decreases with increasing acetonitrile concentration because of the reduction of hydrophilic interaction (12,22).

In our study, the stationary phase was very hydrophilic, and the hydrophobicity of the basic solvents was weak; therefore, their hydrophobic interaction on the surface was negligible. Because ion-exchange is the dominant mechanism for the strong retention of basic solvent at low acetonitrile concentrations, a possible reason for the reduced retention of these solutes is that the electrostatic interactions are lower at high acetonitrile concentrations. Another possible reason is that the solubility of these three basic solvents will increase in the mobile phase, and, consequently, the amount of solutes retained on the stationary phase will decrease with an increasing acetonitrile fraction.

Figure 5 shows that the separation of quinoline and aniline was not achieved with a mobile phase that contained 80% acetonitrile. Although most baseline separations of these solutes were obtained with a mobile phase that contained 60% acetonitrile, the separation was relatively long; that is, the last peak was eluted at 14.5 min. Figure 5 shows that the optimal acetonitrile concentration for separation of these solutes should be 74%, so we separated the solutes at this concentration. Figure 6 shows the obtained chromatogram; we successfully separated 10 solutes in 10 min with column efficiencies varying from 77,000 to 145,000 plates/m.

We investigated ionic strength’s effect on the separation of the same nine polar solvents by varying the triethylamine phosphate concentration from 10 to 30 mM. Figure 7 shows the dependence of retention factors on the triethylamine phosphate concentration. Undoubtedly, the retention of basic solutes decreased quickly with increased ionic strength because the electrostatic interaction was reduced at high ionic strength. The retention factor of three basic solvents — quinoline, aniline, and pyridine — decreased by 39.1, 41.1, and 56.7%, respectively, when the triethylamine phosphate concentration increased from 10 to 40 mM.

Ye and colleagues (11,12) reported a similar tendency for ionic strength’s influence on the retention of ionic solutes in ion-exchange CEC. Neutral solutes are retained in this column by hydrophilic interaction because increases in salt concentration result in increases in solution hydrophilicity, so their retention should be decreased at high ionic strength. We found that the retention factors of the six polar neutral solutes decreased by 14.6–21.0% when the triethylamine phosphate concentration increased from 10 to 30 mM.

The increase of hydrophilicity in the mobile phase may not be the only reason for the decreased retention of neutral solutes. Strong Joule heating, caused by the use of high ionic strength mobile phase, also might be the reason for the decreased retention of solutes. Figure 8 shows a typical separation of the nine polar solvents by hydrophilic interaction CEC with mobile phase that contained 60% acetonitrile and 30 mM triethylamine phosphate buffer. Under this condition, the electrostatic interaction was negligible and the electrostatic interaction between ionic solutes and the stationary phase was reduced. Because increases in salt concentration result in increases in solution hydrophilicity, so their retention should be decreased at high ionic strength. The retention factor of three basic solvents — quinoline, aniline, and pyridine — decreased by 39.1, 41.1, and 56.7%, respectively, when the triethylamine phosphate concentration increased from 10 to 40 mM.
actions still are relatively strong, and the three basic solvents are eluted late. We achieved successful separation of these solutes in 13 min with column efficiencies greater than 100,000 plates/m, but the selectivity was different from that with 74% acetonitrile, at which the hydrophilic interaction of solutes with stationary phase was relatively strong, as shown in Figure 6.

Conclusion
We have demonstrated that hydrophilic interaction CEC has potential for the analysis of polar compounds. Compared with normal-phase CEC under nonaqueous mobile phases, hydrophilic interaction CEC is more suitable because of the aqueous mobile phase used and the higher efficiency and shorter separation times that can be obtained. Hydrophilic interaction CEC provides selectivity complementary to reversed-phase CEC because of the different separation mechanisms involved. Hydrophilic interaction CEC is a powerful technique for separating highly polar compounds that are unretained or weakly retained in the reversed-phase mode. Because hydrophilic interaction chromatography has proven to be an effective method for separating polar compounds such as amino acids, peptides, carbohydrates, nucleic acids, and carbohydrates (15–17), the application of hydrophilic interaction CEC to the analysis of these polar compounds must be studied.

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References
(6) N.W. Smith and M.C. Evans, Chromatographia 38, 649 (1994).