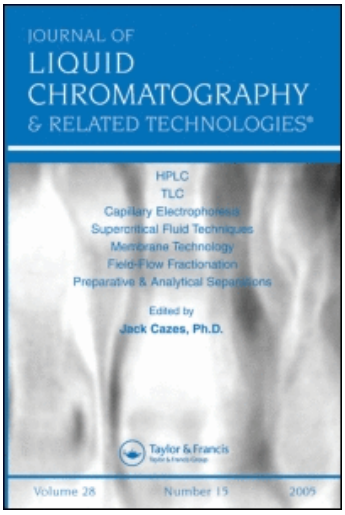

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THE SEPARATION OF POSITIONAL ISOMERS BY CAPILLARY ELECTROCHROMATOGRAPHY

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THE SEPARATION OF POSITIONAL ISOMERS BY CAPILLARY ELECTROCHROMATOGRAPHY

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ABSTRACT

The feasibility for separation of positional isomers using capillary electrochromatography (CEC) was investigated. Two groups of neutral isomers, trifluoroacetophenone and bromobenzonitrile, were separated by CEC. Effects of selected parameters, such as mobile phase pH, ionic strength, temperature, organic modifier, buffer type, diluent type, voltage, and column length, were studied using bromobenzonitrile isomers to elucidate the separation mechanisms. In parallel, the separation of the isomers was also performed by high-performance liquid chromatography (HPLC) to serve as a comparison. It was found that the retention time behavior as a function of acetonitrile concentration in CEC was similar to that observed in HPLC. The effect of mobile phase pH has a greater impact with CEC than with HPLC due to the change in electroosmotic flow. The effect of temperature with liquid chromatography showed a linear Van't Hoff plot, while deviations

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from linearity were noted with CEC. The optimized CEC method was validated in terms of linearity, limit of detection, and injection precision. The experimental results indicate that the separation of positional isomers by CEC is feasible and is an appropriate alternative technique to reverse phase HPLC.

INTRODUCTION

Capillary electrochromatography (CEC) is one of the newest techniques in the separation sciences. It is essentially a combination of high-performance liquid chromatography (HPLC) reverse-phase chromatography and capillary electrophoresis. Like HPLC, the capillary columns are packed with reverse stationary phase material. However, the driving force of the mobile phase is the electroosmotic flow (EOF) as in capillary electrophoresis. Therefore, solutes are separated based upon both their electrophoretic mobility and their partitioning between the stationary and the mobile phase. One of the advantages of the CEC technique is the unique feature of a flat flow profile in the capillary column. This property is beneficial since it does not directly contribute to the dispersion of solutes. This is in contrast to the parabolic flow profile driven by pressure in a HPLC separation system. Compared to HPLC, band broadening of solutes in CEC is significantly reduced, resulting in very high separation efficiency and resolution.

In 1974, Pretorius et al. (1) introduced the concept of capillary electrochromatography. This idea did not draw much attention until Jorgenson, Lukacs, Knox, and Grant demonstrated the technique's value, both theoretically and experimentally, in 1981 and 1987 (2,3). The initial studies on CEC were performed by using a capillary with internal diameter (ID) of 150 or 170 μm, fabricated in laboratories, with 5 or 10 μm octadecylsilica (ODS) particles (2–4). The performance of packed capillaries depended on the scientist's packing skill and experience. Variations in the efficiency of the same types of packed capillaries were observed (5–8).

Recently, several research groups have made contributions to the further development of this separation technique and several excellent review papers have been published (9–11). Currently, commercial CEC columns packed with a variety of stationary phases and a capillary electrophoresis instrument with updated CEC mode are available. With this improved instrumentation, external pressure can be applied on the inlet and outlet vials to prevent bubble formation in the capillary. These advances have made the CEC technique practical (12). However, separation of positional isomers by CEC in the pharmaceutical area is still a challenge, and many mechanistic and practical aspects need to be investigated.

In this paper, we demonstrate the separation of two groups of neutral positional isomers, trifluoromethylacetophenone and bromobenzonitrile. There are three positional isomers for each compound. The structures are shown in Figure 1. Analytical methods were required to separate these isomers and determine the purity of the desired isomer.

During our study, both CEC and conventional liquid chromatography (LC) techniques were evaluated using bromobenzonitrile isomers. Various operating parameters, such as mobile phase pH, ionic strength, temperature, organic modifier, buffer type, diluent type, voltage, and column length, were studied to understand the separation mechanisms. The advantages and disadvantages of each methodology were compared. The feasibility of the CEC method is demonstrated through the validation of its performance in terms of linearity, sensitivity, accuracy, recovery, ruggedness, and precision.

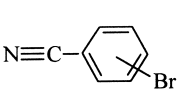
EXPERIMENTAL

Instrumentation

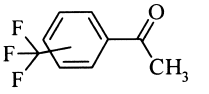
Unless otherwise specified, all CEC separations were performed on a Hewlett-Packard HP^{3D}CE instrument in the CEC mode using a Hewlett-Packard CEC-Hypersil C^{18} capillary column [100 μm ID, 40 cm long (effective length 32 cm)] with 3-μm particle size (Hewlett-Packard, Wilmington, DE, USA). A diode array detector (Hewlett-Packard) was used with monitoring at 210 nm.

The LC separations were performed on a Shimadzu SCL-10A liquid chromatograph with a Shimadzu SPD-10AV (UV)-Visible detector. The detector was operated at a wavelength of 210 nm. A Hypersil C^{18} column (25 cm length, 4.6 mm ID, 3 μm particle size; Keystone Scientific, Bellefonte, PA, USA) was used. The injection volume was set to 10 μL. Unless otherwise noted, the flow rate used in HPLC analysis was 0.8 mL/min. To maintain a constant column temperature during the runs, a Cera column cooler 250 was used.

A data system from PE Nelson (Cupertino, CA, USA) was used to collect and analyze the data.



Bromobenzonitrile



Trifluoroacetophenone

Figure 1. Chemical structure of trifluroacetophenone and bromobenzonitrile.

Reagents

Thiourea, 2-bromobenzonitrile, 3-bromobenzonitrile, and 4-bromobenzonitrile were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). 2-Trifluoromethylacetophenone, 3-trifluoromethylacetophenone, and 4-trifluoromethylacetophenone were obtained from May Bridge (Trevilett, Tintagel, Cornwall, UK). Tris(hydroxymethyl)aminomethane (TRIS) was purchased from Bio-Rad Laboratories (Richmond, CA, USA). Morpholinoethanesulfonic acid hydrate (MES) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium phosphate dibasic was purchased from J. T. Baker (Phillipsburg, NJ, USA).

Glacial acetic acid and HCl were obtained from Fisher Chemical (Fair Lawn, NJ, USA). Sodium hydroxide (50% NaOH) was purchased from EM Science (Gibbstown, NJ, USA). Acetonitrile (ACN) and methanol (MeOH) were obtained from Fisher Chemical. All water was purified with a Milli-Q system (Millipore, Bedford, MA, USA).

Solution Preparation

A 25 m*M* TRIS aqueous solution was prepared, and the pH was adjusted to 7.9 with HCl. To the resulting solution, the appropriate amount of ACN was added, resulting in solutions with compositions ranging from 80:20 (TRIS/ACN) to 30:70 (TRIS/ACN). The buffers for the pH effect study were prepared by using a 25 m*M* TRIS aqueous solution and altering the pH to the desired range (4.0–8.4) using acetic acid. Then the appropriate amounts of ACN were added to form 60:40 TRIS/ACN solutions. A stock solution of 120 m*M* TRIS was prepared, and the pH was adjusted to 8.0 using acetic acid for the ionic strength study. The solution was diluted to the desired concentration with deionized water. The pH measurement for each solution showed that values were in the range of 8.0 ± 0.05 units.

Each resulting buffer solution was then mixed with ACN to form 60:40 buffer/ACN solution. The samples were dissolved in 60:40 TRIS (25 m*M*, pH 7.9)/ACN. The target concentration of samples for CEC was 4 g/L and for LC was 0.4 g/L. All buffer and sample solutions were mixed and then filtered with 0.45-μm nylon membrane syringeless filters (Whatman, Clifton, NJ, USA).

Capillary Column Preparation

The capillary columns were preconditioned before each run by a high pressure flush (10 bar, 5 min) of the mobile phase, while applying a voltage step of 5

kV per 15 min starting at 5 kV and ending at 25 kV. Unless otherwise specified, all runs were performed using 30 kV applied voltage at 20°C, while applying pressure (10 bar) at both ends of the capillary column. All sample solutions were injected using the high flushing mode (25 kV, 3 s, 10 bar).

Calculations

The retention factor κ*(*κ* for CEC)* for the two bands (formerly referred to as the capacity factor *k′*) was determined as $\kappa = (t_R - t_0)/t_0$, where t_R and t_0 are retention times of retained and unretained compounds, respectively. In reverse-phase LC, t_0 was determined by injecting a concentrated solution of sodium nitrate (13) (detection at 210 nm). In CEC, the retention time of thiourea (12) (detection at 210 nm) was used for the measurement of EOF as well as for t_0.

RESULTS AND DISCUSSION

The bromobenzonitrile isomers were selected to demonstrate the systematic method development and the investigation of separation mechanisms. For comparative purposes, the bromobenzonitrile isomers were also injected on a conventional (HPLC) system with a Hypersil C^{18} column (25 cm × 4.6 mm ID, 3-μm particle size) side by side.

Effect of Column

The initial separation attempt using CEC was with a 25-cm Hypersil C^{18} 3-μm column. However, the base-line separation was not achieved for all three peaks after optimizing method parameters.

Because CEC relies on an electrically driven flow and not a pressure-driven flow, a longer column can be used without exceeding the "maximum column pressure," which is a common problem in LC. By using a longer (40 cm) capillary column, a base-line separation of the three bromobenzonitrile isomers in CEC was achieved.

All columns were packed with Hypersil C^{18} material; the particle size was 3 μm. The ID was 4.6 mm and the length was 25 cm for the LC column. The ID was 75 μm and the lengths were 25 and 40 cm, respectively, for the CEC columns. The mobile phase was 60:40 of 25 m*M* TRIS buffer (pH = 7.9)/ACN.

As is presented in Table 1, it is clear that the CEC technique is superior to conventional LC in terms of separation efficiency. The CEC column provided plate numbers about 6–8 times higher than the LC column and improved resolution.

Table 1. Comparison of Chromatographic Parameters of the Isomers on LC and CEC with Different Columns[a]

Column	N_1	N_2	N_3	$Rs_{1\text{-}2}$	$Rs_{2\text{-}3}$
LC (25 cm × 4.6 mm)	7947	8477	5478	3.2	0.7
CEC (25 cm × 75 µm)	55400	16005	30610	2.0	1.1
CEC (40 cm × 75 µm)	64616	34573	82456	3.8	1.5

[a]The subscripts 1, 2,and 3 represent 2-, 4-, and 3-bromobenzonitrile isomers, respectively. The subscripts 1-2 and 2-3 represent the resolution between 2- and 4- and 4- and 3-bromobenzonitrile peaks, respectively.

Effect of Organic Modifiers

ACN and MeOH were initially used as organic solvents in the study. Based on our preliminary experiments, we found that the capillary column equilibrated more rapidly using ACN/buffer mobile phase than with MeOH/buffer mobile phase. The base line of the electrochromatograms observed from MeOH/buffer system sometimes showed a long-term perturbation. This unpredictable base-line perturbation phenomenon has been described in the literature (18,19). Therefore, the ACN-based mobile phase was chosen for further studies.

The effects of varying the percentage of ACN in the mobile phases were studied for both CEC and HPLC modes. The mobile phase compositions ranged from 30:70 to 80:20 ACN/TRIS (25 m*M*, pH 7.9). The retention time (RT) data are plotted as log (RT) versus %ACN for bromobenzonitrile isomers, as shown in Figure 2a and b. Linear plots with correlation coefficients (R^2) greater than 0.99 for all three isomers were obtained for both CEC and HPLC. The resolution between the three isomers decreased as the %ACN increased for both CEC and HPLC.

This behavior is typical of reverse-phase HPLC retention mechanisms (13). However, the absolute resolution varied greatly between HPLC and CEC. No resolution was achieved between 3-bromobenzonitrile and 4-bromobenzonitrile by HPLC when the ACN ratio (v/v) in the mobile phase was higher than 40%. In comparison, partial resolution was achieved between 3-bromobenzonitrile and 4-bromobenzonitrile by CEC, even when the ACN ratio (v/v) in the mobile phase was higher than 60%. The primary reason for this increase in resolution is the increase in efficiency for the separation on CEC.

Effects of pH_{app}

The effect of the apparent pH (pH_{app}) on the separation of the isomers by CEC was studied by varying the pH_{app} of buffer (TRISacetate 25 m*M*) over a range from 4.0 to 8.4.

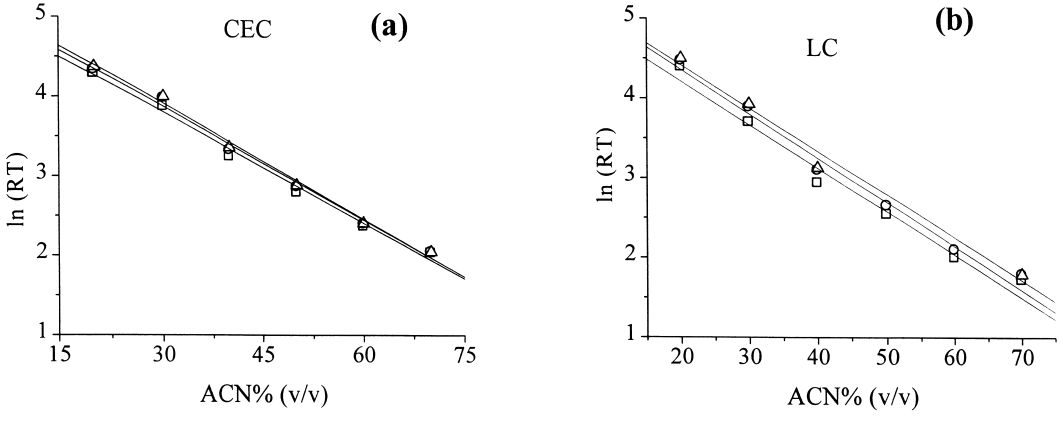


Figure 2. a) Effect of acetonitrile concentration on retention time (RT) of bromobenzonitrile isomers in CEC mode. Capillary column: Hewlett-Packard CEC-Hypersil C^{18} capillary column: 100 µm internal diameter, 40 cm in total length with 3-µm particle size. Mobile phase: 25 m*M* TRIS buffer (pH = 7.9) with acetonitrile. The concentration of acetonitrile was varied. Applied voltage: 30 kV. Capillary temperature: 20°C. Applied pressure on capillary: 10 bar. Injected mode: high flushing mode (25 kV, 3 s, 10 bar). Detection: UV at 210 nm. △, 3-bromobenzonitrile; ○, 4-bromobenzonitrile; □, 2-bromobenzonitrile. b) Effect of acetonitrile concentration on retention time (RT) of bromobenzonitrile isomers in HPLC mode. Column: Keystone Hypersil C^{18} column: 25 cm length, 4.6 mm ID, 3-µm particle size. Mobile phase: 25 m*M* TRIS buffer (pH = 7.9)/ACN. The concentration of acetonitrile was varied. Flow rate: 0.8 mL/min. Column temperature: 20°C. Injection volume: 10 µL. Detection: UV at 210 nm. △, 3-bromobenzonitrile; ○, 4-bromobenzonitrile; □, 2- bromobenzonitrile.

Upon examining the pH effect, a significant dependence of EOF velocity on pH_{app} was observed. The EOF was measured as the mobility of thiourea as shown in Figure 3a. The EOF increased very slowly as the pH_{app} of the mobile phase was increased from pH_{app} 4 to 7. However, a large increase in EOF was observed from pH_{app} 7 to 8.4. It has been documented that this behavior is due to the changes occurring on the silica surfaces of the capillary wall and packing material. At low pH_{app}, more silanol groups are fully protonated. Therefore, the EOF is low. Conversely, at higher pH_{app}, the silanol groups are mostly deprotonated, and EOF is increased (12,14). It is known that the EOF becomes significant above pH 4 for a fused silica capillary under aqueous medium (15). However, in our case, this region was between 7 to 8.4 due to the addition of organic solvent (16,17).

Figure 3a also demonstrates the retention behavior of the bromobenzonitrile isomers as a function of pH_{app} in CEC. The retention times of three isomers decrease inversely with the increase in the EOF. Because the bromobenzonitrile isomers are neutral compounds, they migrate toward the detector only by the EOF. The effects of pH on the separation of isomers by liquid chromatography were also studied to compare with effects seen on CEC. No significant effect was observed, which is expected, since the isomers are neutral compounds (Fig. 3b).

In comparison, it is clear that pH has a stronger effect on neutral isomer separation in CEC than in HPLC. This difference is due to different driving forces. For the LC mode, the driving force is pressure, which is independent of the pH of the mobile phase. For CEC, the only driving force for the neutral compounds is the EOF, which is directly dependent on the pH of the electrolyte.

Effects of Temperature

The influence of temperature on the separation of the bromobenzonitrile isomers was studied for both CEC and HPLC. In the CEC study, temperatures were varied from 10 to 50°C at 5°C degree intervals. In the HPLC study, temperatures were varied from 5 to 45°C at 5°C intervals.

The experimental results demonstrated an improvement in separation resulting from decreased column temperature. However, peak broadening increased as the temperature changed from 50 to 10°C. The temperature of 20°C seemed to be a good compromise in this case. As expected, the Van't Hoff plots of the κ versus T^{-1} was linear ($R^2 > 0.99$) for the LC mode (Fig. 4a), which reflected that the separation process is enthalpically driven. In the CEC mode, the κ used in HPLC is no longer valid for describing the retention characteristic with an electrically driven flow.

Because the bromobenzonitrile isomers are neutral compounds, resulting in no electrophoretic mobility, their electrochromatographic retention factor, κ^*,

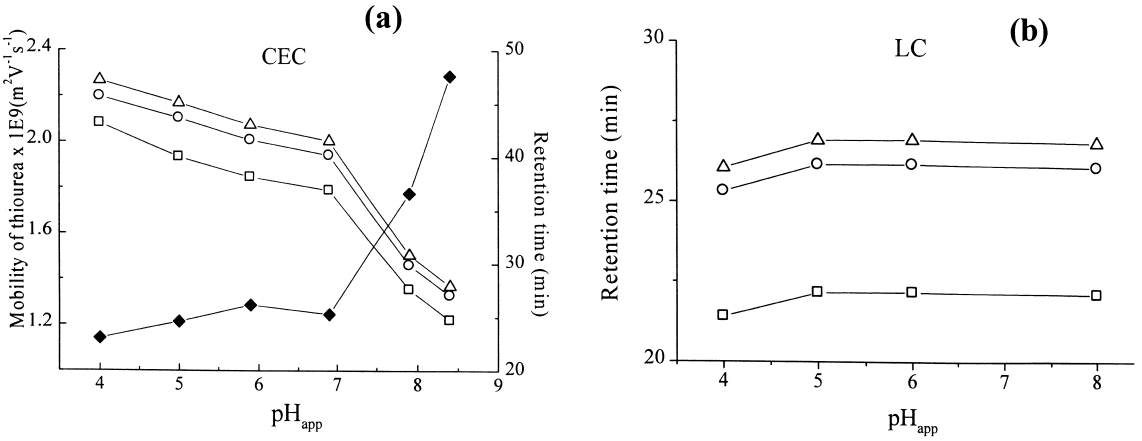


Figure 3. a) Effect of pH_{app} on EOF and retention time of bromobenzonitrile in CEC mode. Mobile phases: 25 m*M* TRIS acetate buffer at various pH values mixed with ACN at a ratio of 60:40. Other conditions are the same as in the legend to Figure 2a. △, 3-bromobenzonitrile; ○, 4-bromobenzonitrile; □, 2-bromobenzonitrile; ◆ mobility of thiourea (EOF). b) Effect of pH_{app} on retention time of bromobenzonitrile in HPLC mode. Mobile phases: 25 m*M* TRIS acetate buffer at various pH values mixed with ACN at a ratio of 60:40. Other conditions were the same as in the legend to Figure 2b. △, 3-bromobenzonitrile; ○, 4-bromobenzonitrile; □, 2-bromobenzonitrile.

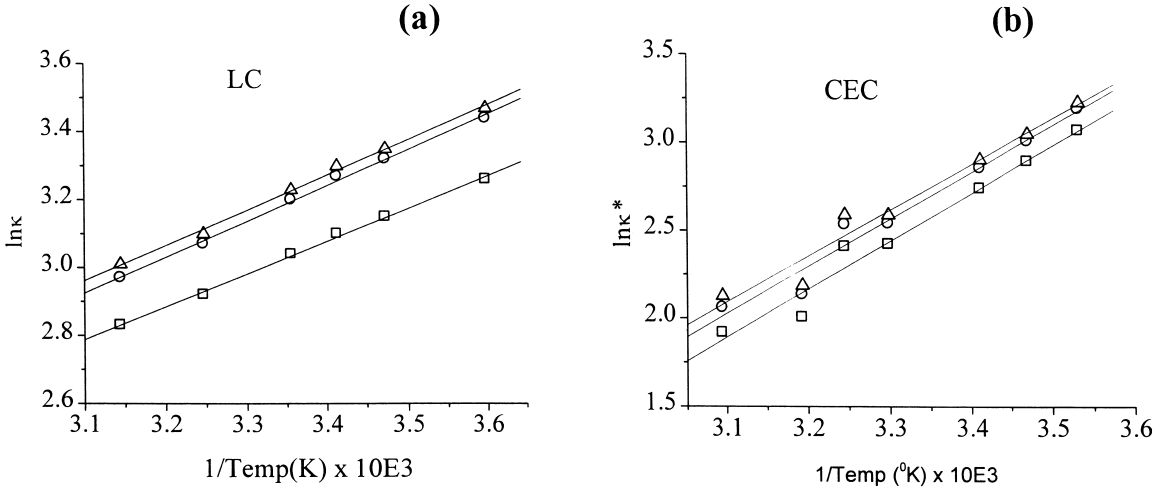


Figure 4. a) Effect of temperature on retention factor (ln κ) in HPLC mode. Conditions are the same as in the legend to Figure 2b except the temperatures were varied and the mobile phase was fixed at a 60:40 ratio (TRIS buffer/ACN). △, 3-bromobenzonitrile; ○, 4-bromobenzonitrile; □, 2- bromobenzonitrile. b) Effect of temperature on retention factor (ln κ*) in CEC mode. Conditions were the same as in the legend to Figure 2a except the temperatures were varied, and the mobile phase was fixed at a 60:40 ratio (TRIS buffer/ACN). △, 3-bromobenzonitrile; ○, 4-bromobenzonitrile; □, 2-bromobenzonitrile.

should reflect a purely chromatographic process (7). However, the plots ln κ^* versus T^{-1} on CEC showed deviations from linearity (Fig. 4b). These deviations may be attributed to the contribution of Joule heating arising during the EOF generation, especially in the higher temperature region.

Effects of Flow

In the LC mode, the solutes are driven by mobile phase flow. The retention time of the solutes should decrease as the flow rate increases. In the CEC mode, the neutral species are driven by EOF. It is expected that the EOF increases as applied voltage increases when all other operating conditions in the studies are held constant; hence, the solutes should be less retained on the column. Our investigation results confirmed these expectations as shown in Figure 5a and b. It is noticeable that the trend of "flows" versus retention times of the two techniques was very similar.

Effects of Buffer Ionic Strength

As shown in Figure 6a, the retention time of bromobenzonitrile isomers increased linearly with increasing ionic strength. This behavior is due to a reduction of the EOF as the zeta potential decreases as ionic strength is increased. Additionally, resolution between these isomers was fairly constant from 24 to 60 m*M* since these compounds are neutral. There was no significant effect of ionic strength on LC (Fig. 6b) since the studied compounds were neutral.

Effects of Different Buffers

The trend in retention time behavior observed using MES/ACN buffer was similar to the trends seen in the TRIS/ACN studies. In comparison with the TRIS/ACN study, the retention times of bromobenzonitrile isomers were longer when the MES/ACN was used at the same percentage of buffer. However, no significant differences in resolution of isomers were observed.

A 60:40 phosphate (20 m*M*, pH 8)/ACN buffer system was also tried for CEC. However, a large current was generated during the initial trial, and further studies were not attempted.

Sample Diluent Effects on CEC

Euerby et al. (18) investigated the effect of sample diluent on CEC performance. They reported that the sample solvent dramatically affected the peak effi-

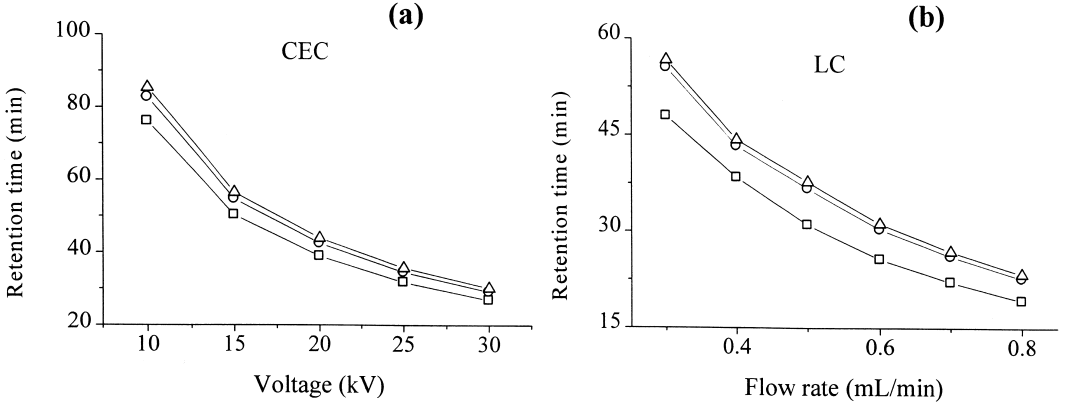


Figure 5. a) Effect of applied voltage on retention time in CEC mode. Conditions are the same as in the legend to Figure 2a except the voltages were varied and the mobile phase was fixed at a 60:40 ratio (TRIS buffer/ACN). △, 3-bromobenzonitrile; ○, 4-bromobenzonitrile; □, 2-bromobenzonitrile. b) Effect of flow rate on retention factor (ln κ) in HPLC mode. Conditions were the same as in the legend to Figure 2b except the flow rates were varied and the mobile phase was fixed at a 60:40 ratio (TRIS buffer/ACN). △, 3-bromobenzonitrile; ○, 4-bromobenzonitrile; □, 2-bromobenzonitrile.

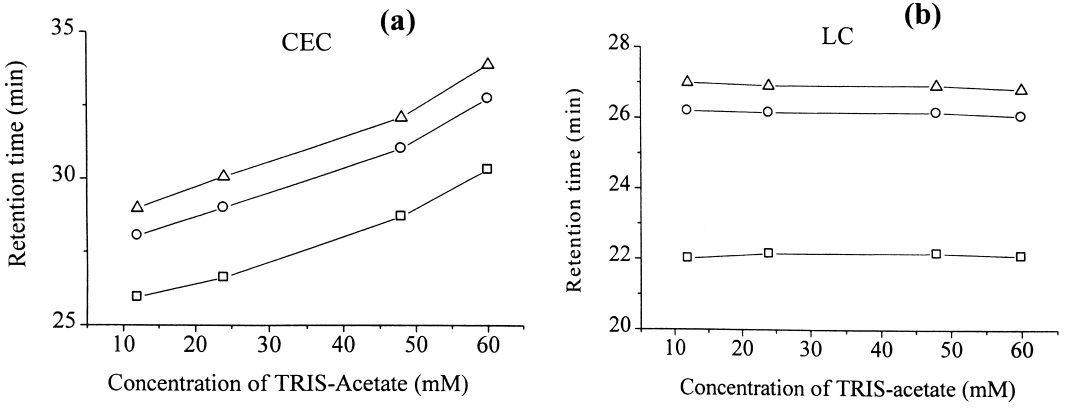


Figure 6. a) Effect of buffer concentrations on the retention time in CEC mode. Conditions are the same as in the legend to Figure 2a except the buffer concentrations were varied. △, 3-bromobenzonitrile; ○, 4-bromobenzonitrile; □, 2-bromobenzonitrile. b) Effect of buffer concentrations on the retention time in HPLC mode. Conditions were same as in the legend of Figure 2b except the buffer concentrations were varied. △, 3-bromobenzonitrile; ○, 4-bromobenzonitrile; □, 2-bromobenzonitrile.

ciency and symmetry in CEC. The peak efficiency was inversely proportional to the percentage of ACN, which ranged from 25 to 75% in sample diluent using the electrokinetic injection. Our study concurred with their results.

Additionally, we used electrokinetic injection combined with pressure to investigate the effects of the diluent type on peak shape and resolution of the bromobenzonitrile isomers. The diluents studied were 60:40 water/ACN, 60:40 TRIS (25 m*M*, pH = 7.9)/ACN, and 100% ACN. There was less than a 1 min difference in retention time for all sample solutions.

As shown in Figure 7, there was only a slight difference in resolution of the isomers for the water/ACN and TRIS/ACN trials. However, the resolution between peaks was much less using pure acetonitrile as the diluent because the peaks were broader. This type of behavior has been seen commonly in reverse-phase LC due to mismatched solvent strength (13).

Optimized Separation

As shown in Figure 8a and b, after optimization of the method parameters, the base-line separations were achieved for both the bromobenzonitrile isomers and the trifluoroacetophenone isomers in the CEC system with a Hypersil C^{18} column (40 cm × 75 μm ID, 3-μm particle size).

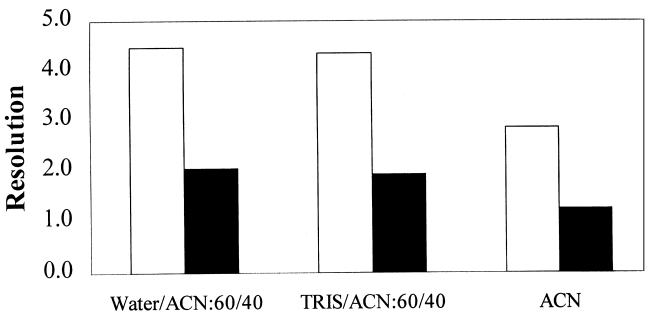


Figure 7. Effect of diluent types on resolutions. Conditions were the same as in the legend to Figure 2a except the mobile phase was fixed at 60:40 ratio (TRIS buffer/ACN). □, resolution between 2- and 4-bromobenzonitrile isomers; ■, resolution between 4- and 3-bromobenzonitrile isomers.

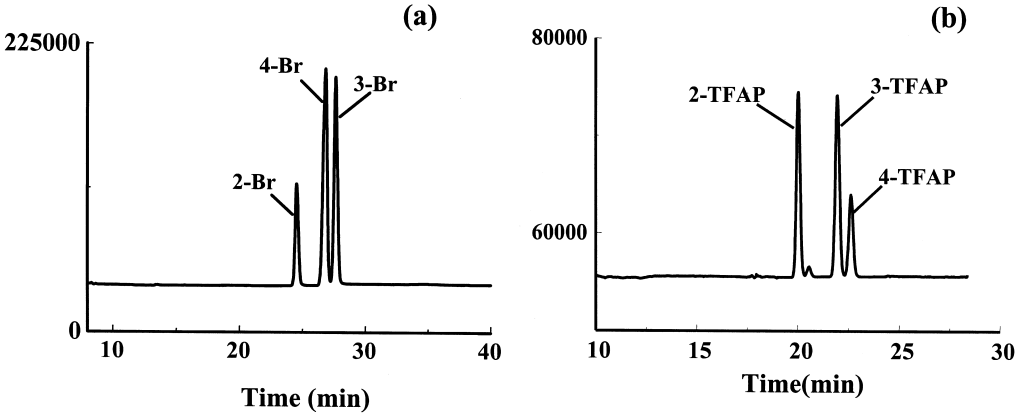


Figure 8. a) Optimized chromatograms of separations of trifluroacetophenone (TFAP) by CEC. Capillary column: Hewlett-Packard CEC-Hypersil C^{18} capillary column: 100 µm ID, 40 cm long (effective length 32 cm) with 3 µm-particle size. Mobile phase: 60:40 of 25 m*M* TRIS buffer (pH = 7.9)/ACN. Applied voltage: 30 kV. Capillary temperature: 20°C. Applied pressure on capillary: 10 bar. Injected mode: high flushing mode (25 kV, 3 s, 10 bar). Detection: UV at 210 nm. b) Optimized chromatograms of separations bromobenzonitrile by CEC. Experimental conditions are the same as Figure 8a.

Validation Studies

To demonstrate the feasibility of the CEC method, certain analytical criteria were tested. Serial dilutions of a 4-bromobenzonitrile solution were prepared between 0.05 and 120% of the target concentration (4 mg/mL) in 60:40 (H_2O/ACN). The UV detector response at 210 nm was linear ($R^2 > 0.99$). The signal/noise ratio of 9:1 was observed for a peak corresponding to 0.05% of the target concentration. The limit of detection was determined to be 0.1%. A 4-bromobenzonitrile solution was spiked with 0.1% (w/w) of the target concentration of minor isomers. The spiked solutions were injected five times consecutively.

The percent relative standard deviation was 1.4% based on the main component's area counts and 0.1% based on area percent. The average recovery was greater then 90%. The same solution was diluted 10-fold with 60:40 (H_2O/ACN), then injected on the HPLC system. The area percent results confirmed the accuracy of the CEC results.

CONCLUSIONS

Separation of neutral positional isomers by CEC is feasible. Reproducible CEC can be performed using a reverse-phase capillary column on a pressurized CE system. Many of the separation mechanism aspects of CEC are similar to reverse-phase HPLC, except that the flow profile is generated by the EOF. Therefore, many of the experimental parameters that control CEC performance can be predicted from a fundamental knowledge of both HPLC and CE. CEC is superior to LC in terms of separation efficiency and is superior to CE in terms of selectivity for neutral compounds and the detection limit. It can be used as an alternative analytical technique in the pharmaceutical industry.

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