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Some aspects of chromatographic behavior in capillary electrochromatography

Yukui Zhang, Wei Shi, Lihua Zhang, Hanfa Zou*

National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116011, China

Abstract

Retention behavior and column efficiency in capillary electrochromatography (CEC) were compared with those in micro-high-performance liquid chromatography (μ-HPLC). Using a unified microcolumn separation apparatus, capacity factors of 27 neutral solutes in pressurized electrochromatography (PEC), CEC and μ-HPLC were investigated and no significant differences were found among these three modes. By linear solvation energy relationship analysis, the same linear equations were obtained in CEC, PEC and μ-HPLC. Systematic investigation of the retention behaviors under mobile phases with four different kinds of organic solvents showed that some of the retention rules in HPLC can be applied in CEC for neutral solutes. Gradient elution of ketones and aldehydes is discussed. © 1998 Elsevier Science B.V.

Keywords: Electrochromatography; Retention behavior; Column efficiency; Linear solvation energy relationships; Aromatic compounds

1. Introduction

Since the use of an electric field was introduced into chromatography by Pretorious et al. [1], capillary electrochromatography (CEC) has attracted more and more attention [2]. According to the types of packing material, CEC can be categorized as reversed-phase CEC [3,4], ion-exchange CEC [5], chiral CEC [6,7], etc. It has been proved theoretically that the theoretical plate number could be doubled by replacing pressurized flow with a plug, as in electroosmotic flow (EOF) [8,9] and, in practice, column efficiency as high as 300 000/m has been achieved in RP-CEC [10,11].

While according to the propelling mode, there is a variant of CEC, pressurized electrochromatography (PEC) [12–15], which can easily eliminate the bubbles that result from Joule heating. PEC can also be utilized to achieve gradient elution [16]. In this work, the term ''electrochromatography'' is used to refer to both CEC and PEC.

The retention behavior in CEC, PEC and micro-high-performance liquid chromatography (μ-HPLC) was investigated by several authors [14,15,17], who often came to different conclusions. In this paper, these three microseparation modes were performed on a unified apparatus, which made the experiments more convenient and reliable. By strictly controlling the experimental conditions, the capacity factors of 27 neutral solutes were measured and no significant differences were observed using these methods (at least under the experimental conditions used in this work). The same linear solvation energy relationship (LSER) equations were obtained in CEC, PEC an HPLC. The retention behavior was systematically investigated using mobile phases with four different

*Corresponding author.

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kinds of organic solvents, and all of these further proved that the same retention rules work, both in HPLC and in electrochromatography.

2. Experimental

2.1. Apparatus and procedures

For μ-HPLC and PEC, a BT3010 pump (Biotronik, Germany) was used under constant pressure, combined with a laboratory-made CE apparatus, to construct a CEC–PEC–μ-HPLC unified apparatus (Fig. 1), and a postinjection splitting technique was used. Capillaries (25 or 100 μm I.D.; 80 cm in length; Yong Nian Optic Fiber Plant, Hebei, China) were used as the splitting resistant column. A six-port injection valve (Elite, Dalian, China) was used with a 10-μl injection loop. For PEC, the applied pressure was varied between 10 and 120 bar, and the applied voltages were between 0 and 30 kV. The outlet of the column was dipped into the mobile phase in the cathode vial, and the anode was connected to the three-way tee at the inlet of the column. The UV wavelength used was 200 nm. Whenever the electric voltage was turned on/off, the column was equilibrated for more than 10 min before performing the next separation run. The procedure was further modified to perform CEC, as will be described in Section 3.1.2.

CEC experiments were also performed on P/ACE 5510 (Beckman, Fullerton, CA, USA) with a liquid cooling system controlling the column temperature at 20.0±0.1°C (Section 3.1 Section 3.3), and on a laboratory-made CE apparatus (Section 3.2), modified to perform gradient elution as described in Fig. 2, without a temperature controlling system. The UV detection wavelength used was 200 nm, and injections were performed by applying a voltage of 2 kV for 3 s, unless stated otherwise. All parts of the instruments shown in Figs. 1 and 2 were from Elite, unless otherwise stated.

A Waters 510 pump (Waters, Milford, MA, USA) was used to pack the capillary columns.

Slidewrite Plus 2.0 (Advanced Graphics Software, Netherlands) was used to do the single component regression analysis, and self-programmed software was used to do the multiple components linear regression, using a Legend personal computer (Pentium 100).

2.2. Reagent and materials

Spherisorb-ODS_2 (3 μm) was purchased from Phase Separations (Norwalk, NJ, USA). The fused-silica capillary used was 375 μm O.D.×75 μm I.D. (Yong Nian Optic Fiber Plant). Isopropanol, tetrahydrofuran (THF), thiourea, aromatic hydrocarbons, Trihydroxymethylaminomethane (Tris) (Shanghai

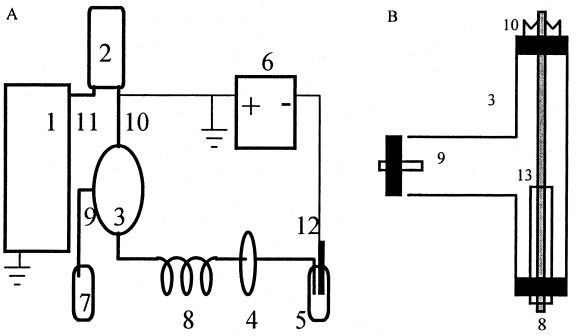

Fig. 1. Diagram of the CEC–PEC–μ-HPLC unified apparatus that was used. (A) Unified apparatus; (B) cross-sectional diagram of the inner structure of 3. (1) pump; (2) six-port injection valve; (3) three-way tee; (4) UV detector; (5) buffer vial; (6) high voltage supplier; (7) vial; (8) separation column; (9) splitting capillary; (10) stainless steel tube; (11) PEEK tube; (12) Pt electrode and (13) PTFE tube.

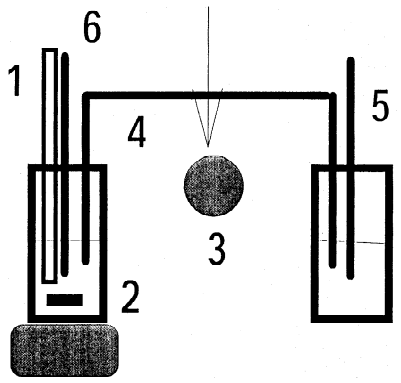

Fig. 2. Titration apparatus for gradient elution in CEC. (1) pipet or pump; (2) magnetic stirrer; (3) PMT; (4) capillary column; (5) cathode and (6) anode.

No. 1 Chemical Plant, China) were all of analytical grade. 2,4-Dinitrophenylhydrazine (DNPH), derivatized ketones and aldehydes were provided by the US Environmental Protection Agency. Acetonitrile (ACN) and methanol (Yuwang Chemical Plant, Shangdong, China) were of chromatographic grade. Double deionized water was used.

Tris was dissolved in water to give a buffer concentration of 40 mmol/l. The volumes of organic solvent and buffer were measured separately, with the v/v ratio varying from 6:4 to 9:1, then they were mixed together to give a mobile phase containing 4 mmol/l Tris, pH 9.2, without adjusting the pH value. The mobile phase was then degassed in an ultrasonic bath for 15 min. Samples were dissolved in the mobile phase at a concentration of ca. 60 mmol/l. The dead times were determined using thiourea.

2.3. Column preparation

The slurry packing method was used to pack the column, as stated in the literature [3,4,18]. The initial frit was sintered with water-wetted 5 μm silica gel particles at the end of the capillary. The slurry was composed of the stationary phases and ACN (ca. 0.01 g/ml), and was prepared using an ultrasonic bath and then packed into the capillary by the HPLC pump under a pressure of about 420 bar. After that, the injection- and detection-end frits were made by sintering the stationary phases, and the initial frit was cut off. The residue particles behind the detection-end frit were flushed out using the mobile phase, and the detection window was made by removing 1 mm of the polyimide coating using a blade. The detection window was about 2 mm from the detection-end frit. Before each run, a voltage of 5 kV was applied for 20 min to equilibrate the column.

3. Results and discussion

3.1. Comparison between μ-HPLC and electrochromatography

3.1.1. Column efficiency

The apparatus used to perform μ-HPLC experiments was described in Section 2.1. A typical chromatogram is shown in Fig. 3. The number of theoretical plates obtained for ethylbenzene was about 97 000/m. The relative standard deviations (R.S.D.s) of the retention times measured by seven consecutive injections were 1.69, 1.58 and 1.71% for thiourea, toluene and ethylbenzene, respectively, which showed the stability and reproducibility of the prepared capillary column. The CEC experiments were performed on P/ACE 5510, and the R.S.D.s of the retention times, measured by seven consecutive injections, were 0.18, 0.20 and 0.12% for thiourea, benzyl alcohol and benzene, respectively.

According to Van Deemter's equation, the relationship between total plate height (H_{tot1}) and the linear velocity, u, of the mobile phase in HPLC could be expressed as

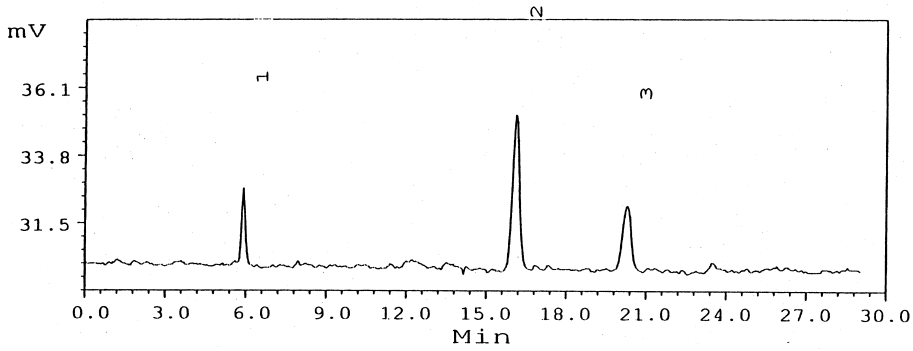

Fig. 3. Chromatogram of the separation of aromatic compounds by μ-HPLC. Experimental conditions: Instrument, unified apparatus; column, packed/total length = 20/27 cm with 3 μm Spherisorb-ODS; injection, 10 μl; pump pressure, 30 bar; isocratic elution with ACN–buffer (70:30, v/v) containing 4 m*M* Tris, pH 9.2; detection wavelength, 214 nm. Peaks: (1) thiourea, (2) toluene and (3) ethylbenzene.

$$H_{tot1} = H_{col1} + H_{ext1}$$
$$= A_1 + B_1/u + C_1 u + H_{ext1} \quad (1)$$

where H_{col1} is the contribution of the μ-HPLC column, H_{ext1} is the extra column contribution. A_1, B_1 and C_1 are the coefficients for eddy diffusion, longitudinal molecular diffusion, and resistance to mass transfer in the stationary phase and mobile phase, respectively. In μ-HPLC, the extra column contribution was mainly caused by introducing sample into the detection apparatus. With good splitting injection techniques as well as on-column detection, the extra column contributions caused by injection and detection could be minimized to the same magnitude as those in CEC. In CEC, the relationship between total plate height, H_{tot2}, and linear velocity, u, of the mobile phase could also be expressed in a similar way, as

$$H_{tot2} = H_{col2} + H_{ext2}$$
$$= A_2 + B_2/u + C_2 u + H_{joul} + H_{ext2}$$
$$= A_2 + B_2/u + C_2 u + H_{ext2} \quad (2)$$

where H_{col2} is the contribution of the CEC column, A_2, B_2 and C_2 are the coefficients for eddy diffusion, longitudinal molecular diffusion, and resistance to mass transfer in the stationary phase and mobile phase, respectively. H_{joul} is the Joule heating contribution, and H_{ext2} is the contribution made on injection and detection. In the experiment, samples were injected at 1 kV for 1 s, and the contribution of injection to H was approximately 0.004 μm [19]; with a detection window of 800 μm, the contribution of detection was about 0.3 μm [19], so H_{ext2} was about 0.3 μm. Because the electric current generated in the column was very small (around 1 μA) and an effective liquid cooling system (provided by Beckman P/ACE) was used, the Joule heating contribution could be calculated to be around 0.001 μm [20], which could be neglected, as shown in Eq. (2).

Relationships between plate height, H, and the linear velocity, u, of the eluent in μ-HPLC and CEC were investigated, and the regression results are shown in Fig. 4, according to Eqs. (1) and (2). The extracolumn effects in both modes were similar and the intercept of Eq. (2) was much smaller than that of Eq. (1), which meant that the contribution made by the eddy flow to plate height in μ-HPLC was much larger than that in CEC; the flow was parabolic in HPLC, while in CEC, the flow profile was plug-like. The values of parameters B_2 and B_1 were quite close, which indicated that the contribution made by longitudinal diffusion in both modes was quite similar. Therefore, in practice, higher column efficiency can be achieved in the CEC mode. Fig. 4 also suggested the possibility of high speed CEC separation, which will be discussed later in Section 3.3.

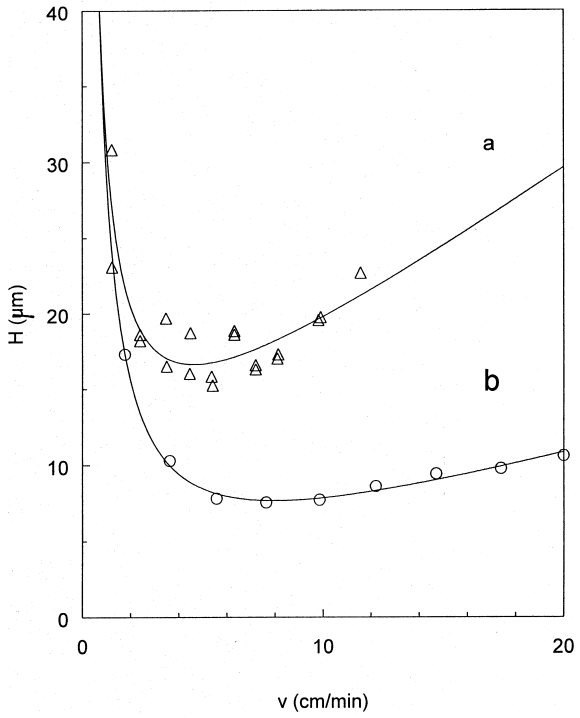

Fig. 4. Dependence of plate height on the linear velocity of the mobile phase. Column: Packed/total length = 20/27 cm × 75 μm I.D. with 3 μm Spherisorb-ODS_2. (a) Ethylbenzene in μ-HPLC; instrument, unified apparatus; detection window, 1 mm. Applied pressure, 10–120 bar. Regression equation: $H = 6.43 + 23.62/u + 1.10\ u$. (b) Propylbenzene in CEC; instrument, P/ACE 5510 system; injection, 1 kV, 1 s; detection window, 800 μm. Applied voltage, 3–30 kV. Regression equation: $H = 0.653 + 28.04/u + 0.441\ u$. For other experimental conditions, see Fig. 3 Section 2.

3.1.2. Retention behavior

The retention behavior in CEC, PEC and μ-HPLC has been investigated by several authors. The different retention behaviors between PEC and HPLC were observed previously by Tsuda [14], who con-

sidered that the surface of the stationary phase, when applying voltage, might become more polar due to polar adsorptive materials of charged species and/or the environment of the electric field. Eimer et al. [15] also found a 36–40% decrease in k' for more hydrophobic analytes in PEC compared to μ-HPLC, but found increased k' values for phenytoin in PEC compared to HPLC, which was supposed to be caused by an increase in its polar interactions with residual surface silanol groups, due to alteration of the stationary phase. However, this phenomenon was not observed by us in this work (see Table 1). The reason was probably that the neutral compounds tested by us were different from the charged species used by Tsuda [14] and Eimer et al. [15]. Charged compounds may be retained in the column and adsorbed to the surface of the support, thereby influencing the retention of neutral compounds in the next separation run.

Some researchers thought the Joule heat may contribute to the decrease in the k' value in CEC. Vissers et al. [17] have corrected the k' values on a packed column of 320 μm I.D. by theoretically derived equations and experimentally determined parameters, using the correlation of ln k' vs. $1/T$. The corrected results were generally about 1–6% higher than the uncorrected k' values. However, they observed that the retention in CEC for neutral compounds of 4-aminoacetophenone, *o*-nitrophenol, 2,6-dimethylphenol and naphthalene was about 20% slower than that in μ-HPLC, on the same 320 μm I.D. packed column. On the other hand, Yan et al. [21] found approximately the same retention in CEC and in HPLC for benzyl alcohol, benzaldehyde, benzene and naphthalene.

The reason for this incongruity may be due to the different test solutes used by these authors. Solutes with different molecular structures may show different variations in k' in electrochromatography and μ-HPLC. Therefore, more kinds of solutes must be measured to thoroughly investigate this phenomenon. At the same time, the disparity between the experimental conditions used, such as different column packing methods, different column temperatures, performance procedures, etc., also contributed significantly to these ambiguous results. Strictly controlled experiments to compare these methods, using a vast number of neutral solutes, are urgently needed to solve this problem, and one of our goals was directed towards this end. To obtain convincing results regarding the retention behaviors in CEC, PEC and μ-HPLC, several important points must be met, as discussed above. Firstly, the column used must be the same column, otherwise column-to-column reproducibility must be guaranteed and stated. A long equilibrating time was needed to make the column performance stable after mode change.

Table 1
Capacity factors of 27 solutes in μ-HPLC and PEC, determined using column I

Solute	μ-HPLC	PEC	Difference (%)
Benzene	0.73	0.74	1.4
Toluene	1.03	1.04	0.97
Ethylbenzene	1.38	1.38	0.0
Propylbezene	1.97	1.97	0.0
Butylbenzene	2.81	2.80	−0.36
Benzaldehyde	0.39	0.38	−2.6
Acetophenone	0.39	0.38	−2.6
Propylphenone	0.60	0.58	−3.3
Butylphenone	0.84	0.80	−4.8
Benzonitrile	0.39	0.38	−2.6
Phenyl methyl ether	0.64	0.61	−4.7
Phenyl ethyl ether	0.89	0.85	−4.5
Phenol	0.20	0.20	0.0
p-Methylphenol	0.26	0.26	0.0
Ethyl benzoate	0.82	0.79	−3.6
Benzyl alcohol	0.20	0.20	0.0
Ethylphenyl alcohol	0.25	0.25	0.0
Propylphenyl alcohol	0.36	0.36	0.0
Nitrobenzene	0.49	0.48	−2.0
p-Nitrotoluene	0.66	0.65	−1.5
Aniline	0.25	0.25	0.0
Bromobenzene	1.14	1.10	−0.88
Naphthalene	1.29	1.27	−1.6
Chlorobenzene	1.01	0.99	−2.0
p-Dichlorobenzene	1.47	1.44	−2.0
p-Dimethylbenzene	1.49	1.45	−2.7
Biphenyl	1.64	1.59	−3.0

The applied pressure was 50 bar for μ-HPLC;
The applied pressure and voltage were 50 bar and 10 kV for PEC. Operating current, 0.5 μA. Column I, 3 μm Spherisorb ODS_2, 75 μm I.D., packed/total length = 20/27 cm.
For PEC, a 75-μm I.D. capillary (15 cm long) was connected to the outlet of column I.
Mobile phase, 80% ACN, 4 m*M* Tris, pH 9.2; ambient temperature, 23.5±0.4°C.
Splitting tube, 80 cm×25 μm I.D.
For other conditions, see Section 2. $\Delta = (k_{PEC} - k_{\mu\text{-HPLC}})/k_{\mu\text{-HPLC}} \times 100\%$.

Secondly, the apparent column temperatures must be controlled to the same degree. Thirdly, the effects of Joule heating on the retention behavior in CEC and PEC must be corrected, unless the Joule heat can be proved to be negligible under the experimental conditions used. Finally, solutes with different functional groups must be investigated systematically.

We used a CEC–PEC–μ-HPLC unified apparatus to perform CEC, PEC and μ-HPLC together. This is described in Section 2.1 Fig. 1. When CEC was performed, the splitting tube (9) was removed and both pump 1 and the high voltage supplier (6) were switched on initially; after that, the rotary injection valve (2) was switched to the ''load'' position and a 10-μl sample was loaded by a syringe. The valve was then switched back to the ''injection'' position. In the meantime, the workstation was initialized to start data collection, which was similar to the injection and data collection process in μ-HPLC. The sample zone was moved quickly through the three-way tee (3) by a low pressure (10 bar) and was sucked by the inlet of the column (8) due to the electric field. After an interval of 4 s, residual sample was flushed completely out via the purge tube, the pump was stopped and the pressure was reduced to zero immediately, completing the injection procedure. The low pressure flush for 4 s had little effect on the flow velocity on the column and did not effect the injection volume. When in μ-HPLC mode, the voltages were switched off, and the splitting tube was fixed again. On injection, the splitting tube was connected to a trash bottle and, after 20 s, the splitting tube was reconnected to the mobile phase reservoir. In PEC, the splitting tube setting was the same as in μ-HPLC, and injection was performed using both pressure and voltage, as stated in Section 2.1.

The most exciting aspect of this unified apparatus was that the mode could be changed without the need to dismantle the column. With the inlet integrated into the splitter, the detection window and the detection-end frit fixed in a cartridge, the capillary column was protected and made more rugged. Furthermore, the electric field was not stopped throughout the CEC experiments, which should improve the reproducibility. Finally, it is worth mentioning that the buffer depletion effect was minimized during CEC experiments because the buffer in the ''micro reservoir'' surrounding the inlet of the column was replenished by the flow of mobile phase upon injection. The sample absorbed on the outside wall of the column inlet was flushed off and dispersion of the injection zone was reduced. In summary, this design will facilitate mode changes, improve the reproducibility of CEC experiments on laboratory-made machinery and make the electrochromatography experiments more comparable to those of μ-HPLC.

The experimental conditions were strictly controlled. The same column was used for the three microcolumn methods and the column was packed very compactly so that the column bed would not shrink when pressure and/or voltage were applied. This was important, as the retention time was not reproducible on a loosely packed column, which would lead to increased errors. Moreover, when pressure was applied, the packings would move with the pressurized flow. However, when voltage was applied, the packings would move in the opposite direction to the EOF (at high pH values). This difference would make the column behave differently when the different methods were used, which would lead to erroneous results, such as different k' values for the same solute. The same 400 ml mobile phase was used for all of the experiments with CEC, PEC and μ-HPLC. The temperature in the laboratory was controlled by an air conditioner, so that the temperature of the ambient air surrounding the column was kept constant during the experiments. The temperature was constantly measured by two thermometers, at intervals of about 10 min. One set of experiments was done at 23.5±0.4°C and the other was at 15.3±0.3°C.

Theoretically, in CEC, the temperature difference between the wall and the surrounding air, ΔT_{air}, was calculated by Eq. (3) [8]:

$$\Delta T_{air} = \frac{4EI}{\pi d_o^{0.3}} \quad (3)$$

where d_o = outer diameter of the tube, E = the field strength and I = current. In our experiments, the applied voltage was 20 kV, the total length of the column was 47 cm, I was 1 μA and d_o was 375 μm. The ambient temperature was 15.3°C, so the ΔT_{air} was approximately 0.6°C, which would lead to a

0.4% lower k' value, following the work of Vissers et al. [17]. Therefore, the thermal effect was negligible.

In our experiments, the dependence of the linear velocity, u, of EOF on the voltage was linear, with a regression coefficient of 0.999, as shown in Fig. 5. The common curve found when a u(EOF) vs. voltage plot of capillary zone electrophoresis (CZE) results is performed was not observed here because the thermal effect was so small. It can also be seen (Fig. 5) that the k' of benzene remained the same when the voltage was increased from 4 to 30 kV, therefore, the thermal effect on the retention was negligible, which was in agreement with that predicted by the theoretical calculations above.

For the first set of experiments, column I (total length, 27 cm) was used (in PEC mode, a 75-μm I.D. empty capillary, 15 cm long, was connected to the outlet of column I by a 380-μm I.D. PTFE tube to obtain better electro-contact) and ACN–buffer (80:20, v/v) was chosen as the mobile phase. The k' values of 27 solutes in PEC and in μ-HPLC were measured, as listed in Table 1. Every datum was the average of two experiments. The dead time changed from 2.93 min in μ-HPLC to 2.03 min in PEC. It can

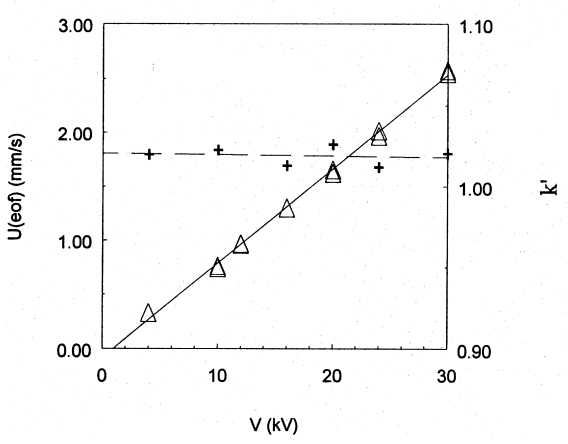

Fig. 5. Dependence of the linear EOF velocity u(eof) and the k' value of benzene on voltage (V) in CEC. Experimental conditions: instrument, unified apparatus; column, packed/total length, 15.0/47.0 cm with 3 μm Spherisorb-ODS_2; injection, 10 μl; pump pressure, 0 bar; isocratic elution with ACN–buffer (70:30, v/v), containing 4 mM Tris, pH 9.2. Operating current <1.0 μA. Ambient temperature, 15.3±0.3°C. (△) dependence of u(eof) on V, u(eof) $= -0.0865 + 0.0873$ V, $r = 0.9990$; (+) dependence of k' on V.

be seen from Table 1 that the k' values of the solutes in PEC were the same as those in μ-HPLC. For 41% of the solutes, the relative errors were within ±1.0%, and all of the relative errors were smaller than ±5.0%.

The elution strength was then decreased and ACN–buffer (70:30, v/v) was used as the mobile phase to increase the k' values. The reason for changing the conditions was to amplify any small variation in the retention behaviors in the three methods, if there were any. Column II (total length, 47.0 cm) was used for this experiment. In CEC, when the voltages were switched on, the column was pre-equilibrated for 30 min, then five injections of benzene were made to observe the condition of the column with R.S.D. of 1.9% for the k' values. When in μ-HPLC mode, pressure was applied and the column was equilibrated for 2 h, then benzene was injected to test the column, and the R.S.D. of the k' values for the last twelve injections reached 1.08%. In PEC, pressure of 50 bar and a voltage of 20 kV were applied together and the column was equilibrated for 30 min. The experimental results are listed in Table 2; every datum is the average of two experiments. We can see that the capacity factor values were the same for 27 solutes. This result was convincing, at least for simple neutral solutes under common simple mobile phases. The relative errors in the k' values between CEC an PEC were less than 3.0% for 96% of the solutes studied, and were less than ±1.0% for 48% of the solutes. The k' value of *p*-methylphenol in CEC was 5.4% lower than that in μ-HPLC, which may be due to experimental error. The dependence of k'(CEC) on k'(μ-HPLC) was studied (Fig. 6), and the result of linear regression was:

$$k'(\text{CEC}) = (0.009 \pm 0.006) + (0.984 \pm 0.003) \cdot k'(\mu\text{-HPLC}) \quad R = 0.9998 \tag{4}$$

with R being the correlation coefficient. From Eq. (4), we determined that the k' value in CEC was the same as that in μ-HPLC for neutral solutes ($k' = 0$–5), with ACN–buffer (70:30, v/v) as the mobile phase.

There was no surprise when the relative errors for the k' values between PEC and μ-HPLC were within ±3.5% for 92% of the solutes studied, and they were

Table 2
Capacity factors of 27 solutes, determined by μ-HPLC, CEC and PEC using column II

Solute	μ-HPLC	CEC	PEC	Difference 1 (%)	Difference 2 (%)
Benzene	1.037	1.014	1.036	−2.2	−0.10
Toluene	1.523	1.500	1.518	−1.5	−0.33
Ethylbenzene	2.135	2.091	2.134	−2.1	−2.1
Propylbezene	3.199	3.141	3.214	−1.8	−1.8
Butylbenzene	4.783	4.698	4.830	−1.8	0.98
Benzaldehyde	0.521	0.507	0.496	−2.7	−4.8
Acetophenone	0.539	0.543	0.517	0.74	−4.1
Propylphenone	0.860	0.852	0.853	0.93	−0.81
Butylphenone	1.200	1.222	1.212	1.8	1.0
Benzonitrile	0.548	0.549	0.556	0.18	1.5
Phenyl methyl ether	0.886	0.873	0.889	−1.3	0.34
Phenyl ethyl ether	1.279	1.250	1.282	−2.3	0.23
Phenol	0.276	0.274	0.267	−0.72	−3.3
p-Methylphenol	0.373	0.353	0.373	−5.4	0.00
Ethyl benzoate	1.186	1.194	1.112	0.67	−6.2
Benzyl alcohol	0.249	0.249	0.254	0.00	2.0
Ethylphenyl alcohol	0.325	0.324	0.328	−0.31	0.92
Propylphenyl alcohol	0.480	0.472	0.470	−1.7	−2.1
Nitrobenzene	0.690	0.692	0.687	0.29	−0.43
p-Nitrotoluene	0.996	0.986	0.974	−1.0	−2.2
Aniline	0.336	0.334	0.328	−0.60	−2.4
Bromobenzene	1.656	1.685	1.655	1.8	−0.06
Naphthalene	1.952	1.954	1.957	0.10	0.26
Chlorobenzene	1.468	1.494	1.466	1.8	−0.14
p-Dichlorobenzene	2.287	2.264	2.209	−1.0	−3.4
p-Dimethylbenzene	2.287	2.270	2.212	−0.74	−3.3
Biphenyl	2.670	2.623	2.581	−1.76	−3.3

Applied pressure, 50 bar for μ-HPLC.
Applied voltage, 20 kV. Operating current <1 μA for CEC.
Applied pressure and voltage, 50 bar, 20 kV. Operating current <1 μA for PEC.
Column, 375 μm O.D.×75 μm I.D., packed/total length=15.0/47.0 cm.
Ambient temperature, 15.3±0.3°C.
Splitting tube=80 cm×100 μm I.D.
For other conditions, see Section 2.
$\Delta 1=(k'_{CEC}-k'_{\mu\text{-HPLC}})/k'_{\mu\text{-HPLC}}\times 100\%$; $\Delta 2=(k'_{PEC}-k'_{\mu\text{-HPLC}})/k'_{\mu\text{-HPLC}}\times 100\%$.

within ±1.0% for 41% of the solutes. The k' value for ethyl benzoate in PEC was 6.2% lower than that in μ-HPLC, which may be due to experimental error. The results and discussion above may suggest that the retention rules in HPLC should also be applied in electrochromatography. Two examples of famous retention rules in HPLC were examined systematically in CEC, as stated below.

For one example, LSER methodology, which was first proposed by Kamlet et al. [22], was widely used in HPLC to predict the retention values and to investigate the retention mechanism [23]. It provided a quantitative equation between k' and the solvatochromic parameters $V_W/100$, π^*, β, α of the solutes as follows:

$$\log k' = \log k'_0 + mV_W/100 + s\pi^* + b\beta + a\alpha \qquad (5)$$

Here V_w, π^*, β and α stand for the Van der Waals volume, the dipolarity, the hydrogen-accepting basicity and the hydrogen-donating acidity of the solutes, respectively. The coefficients $\log k'_0$, m, s, b and a reflected the corresponding contribution of the mobile phases and the stationary phases to the retention of solutes. The solvatochromic parameters of the solutes investigated in this study are listed in Table 3, and were cited in ref. [24]. The k' values in

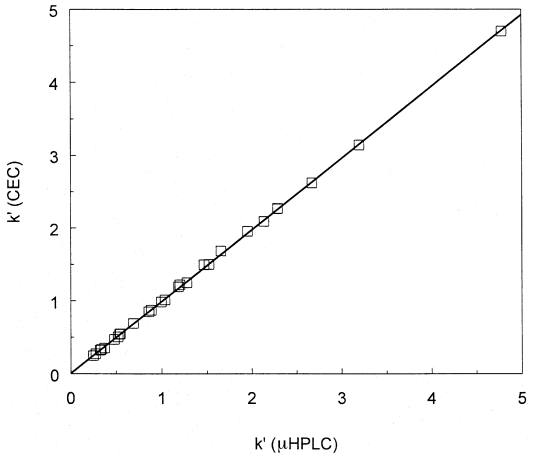

Fig. 6. Relationship between k' in CEC and in μ-HPLC. For experimental conditions, see Table 2.

Tables 1 and 2 were used for LSER analysis, and it was found that the LSER equations obtained in CEC, PEC and μ-HPLC were the same (Table 4). All had good linearity, therefore, the retention can be quantitatively correlated with the structure of solutes.

As another example, we investigated the dependence of k' on the concentration of organic modifiers in CEC. In RP-HPLC, it is well known that the effect of the concentration of organic modifier in a limited range can be determined using the following equation:

$$\log k' = \log k_w - SC \quad (6)$$

where C is the concentration of organic modifier, log k_w is the extrapolated logarithm value of the solutes with pure buffer as the mobile phase, and S is the slope of the equation. The retention capacity factors for each of 27 neutral solutes in CEC at methanol, ACN, THF and isopropanol concentrations ranging from 0.60 to 0.90 volume fractions were measured three times, and the arithmetic average was calculated. Linear regression analysis of the experimental data was carried out using Eq. (6), and the results obtained are listed in Table 5. It can be seen that in the limited range of organic modifier concentrations tested, all solutes used in CEC followed Eq. (6) very well, with correlation coefficients higher than 0.99, with the exception of bromobenzene (0.985) and naphthalene (0.982) in methanol–water. The values of S were positive for all solutes, which meant that the retention values of solutes in RP-CEC decreased with increasing concentrations of organic modifier. This meant that late-eluting peaks in RP-CEC could be eluted faster by using high concentrations of organic solvent, which was necessary when separating complex samples.

From the discussion above, it can be seen that some of the important retention rules in HPLC proved to be the same in CEC, which, combined with the identical k' values, further proves that the retention behaviors in electrochromatography are the same as those in HPLC.

Table 3
Solvatochromic parameters of 27 solutes [24]

Number	Solute	$V_w/100$	π^*	β	α
1	Benzene	0.491	0.59	0.10	0
2	Toluene	0.592	0.55	0.11	0
3	Ethylbenzene	0.668	0.53	0.12	0
4	Propylbenzene	0.769	0.51	0.12	0
5	Butylbenzene	0.867	0.49	0.12	0
6	Benzaldehyde	0.606	0.92	0.44	0
7	Acetophenone	0.690	0.90	0.49	0.04
8	Propylphenone	0.788	0.88	0.49	0
9	Butylphenone	0.886	0.86	0.49	0
10	Benzonitrile	0.590	0.90	0.37	0
11	Phenyl methyl ether	0.630	0.73	0.32	0
12	Phenyl ethyl ether	0.727	0.69	0.30	0
13	Phenol	0.563	0.72	0.33	0.61
14	*p*-Methylphenol	0.634	0.68	0.34	0.58
15	Ethyl benzoate	0.834	0.74	0.41	0
16	Benzyl alcohol	0.634	0.99	0.52	0.39
17	Ethylphenyl alcohol	0.732	0.97	0.55	0.33
18	Propylphenyl alcohol	0.830	0.95	0.55	0.33
19	Nitrobenzene	0.631	1.01	0.30	0
20	*p*-Nitrotoluene	0.729	0.97	0.31	0
21	Aniline	0.562	0.73	0.50	0.26
22	Bromobenzene	0.624	0.79	0.06	0
23	Naphthalene	0.753	0.70	0.15	0
24	Chlorobenzene	0.581	0.71	0.07	0
25	*p*-Dichlorobenzene	0.571	0.70	0.03	0
26	*p*-Dimethylbenzene	0.668	0.51	0.13	0
27	Biphenyl	0.920	1.18	0.20	0

3.2. Gradient elution by titration

As mentioned above, gradient elution was important in CEC to separate complex solutes. A simple gradient elution mode was developed by titration of the mobile phase. The gradient elution system is shown in Fig. 2. The composition of the

Table 4
Regression results between log k' and structure parameters for the solutes listed in Tables 1 and 2

Mode	log k'_0	m	s	b	a	S.D.	F	R	n
μ-HPLC (column I)	−0.476±0.061	1.38±0.080	−0.320±0.058	−1.14±0.069	−0.596±0.053	0.042	363.6	0.992	27
PEC (column I)	−0.467±0.060	1.36±0.079	−0.327±0.057	−1.15±0.068	−0.570±0.052	0.042	373.2	0.993	27
μ-HPLC (column II)	−0.362±0.065	1.53±0.086	−0.341±0.062	−1.28±0.073	−0.600±0.056	0.045	379.4	0.993	27
CEC (column II)	−0.370±0.064	1.52±0.084	−0.332±0.061	−1.28±0.072	−0.614±0.056	0.045	391.9	0.993	27
PEC (column II)	−0.363±0.064	1.53±0.084	−0.347±0.061	−1.29±0.072	−0.596±0.056	0.044	393.0	0.993	27

For experimental conditions, see Tables 1 and 2.
n is the number of test solutes; R is the correlation coefficient of linear regression; S.D.is the standard deviation of the y estimate; F is the statistical value of the F-test when $\alpha=0.01$, $F(0.01, 1, 21)=7.99$.

mobile phases could be changed by adding mobile phases into the inlet reservoir with pipet 1 or pump proportionally. The mobile phases were mixed quickly by stirring the mixture using a micromagnetic stirrer (2) immersed in the mobile phase. Nine DNPH-derivatized ketones and dehydes were sepa-

Table 5
Linear regression analysis of the experimentally measured data using Eq. (6) for the listed solutes in Table 3[a]

	MeOH–water			ACN–water			THF–water			Isopropanol–water		
Number	log k_w	S	R	log k_w	S	R	log k_w	S	R	log k_w	S	R
1	2.140±0.054	2.733±0.072	0.9990	1.598±0.036	2.271±0.047	0.9994	1.440±0.11	2.348±0.15	0.9941	1.210±0.030	1.934±0.040	0.9994
2	2.687±0.066	3.161±0.087	0.9989	1.904±0.040	2.475±0.053	0.9993	1.757±0.090	2.689±0.12	0.9971	1.474±0.037	2.134±0.049	0.9992
3	3.128±0.128	3.522±0.162	0.9979	2.211±0.047	2.704±0.062	0.9992	1.975±0.099	2.913±0.13	0.9970	1.664±0.032	2.298±0.042	0.9995
4	3.695±0.151	4.003±0.191	0.9977	2.548±0.050	2.944±0.066	0.9993	2.214±0.11	3.157±0.14	0.9968	[b]		
5	4.282±0.183	4.505±0.231	0.9973	2.881±0.056	3.179±0.074	0.9992	2.435±0.12	3.381±0.16	0.9966	[b]		
6	1.302±0.116	2.142±0.147	0.9953	1.101±0.076	1.961±0.101	0.9961	1.157±0.045	2.386±0.059	0.9991	0.3516±0.046	1.292±0.061	0.9967
7	1.564±0.082	2.399±0.108	0.9969	1.116±0.028	1.961±0.038	0.9994	0.9952±0.045	2.189±0.059	0.9989	0.4356±0.048	1.414±0.063	0.9970
8	2.103±0.120	2.802±0.158	0.9952	1.510±0.032	2.247±0.042	0.9995	1.389±0.048	2.540±0.064	0.9990	0.8277±0.031	1.748±0.041	0.9992
9	2.608±0.098	3.253±0.130	0.9976	1.806±0.030	2.440±0.040	0.9996	1.680±0.069	2.827±0.092	0.9984	1.131±0.037	2.018±0.048	0.9991
10	1.536±0.067	2.482±0.088	0.9981	1.285±0.042	2.204±0.055	0.9991	1.131±0.058	2.356±0.077	0.9984	0.4433±0.038	1.509±0.050	0.9983
11	2.084±0.064	2.710±0.084	0.9986	1.542±0.047	2.281±0.062	0.9989	1.392±0.062	2.442±0.082	0.9983	0.9376±0.034	1.701±0.045	0.9990
12	2.562±0.071	3.108±0.094	0.9986	1.853±0.041	2.497±0.054	0.9993	1.674±0.066	2.714±0.087	0.9985	1.271±0.034	2.004±0.045	0.9992
13	1.167±0.052	2.222±0.70	0.9985	0.8948±0.024	2.083±0.032	0.9998	1.349±0.075	2.810±0.10	0.9981	0.6350±0.043	2.044±0.057	0.9988
14	1.642±0.069	2.610±0.091	0.9982	1.054±0.022	2.112±0.030	0.9997	1.462±0.050	2.891±0.066	0.9992	0.7971±0.041	2.130±0.054	0.9990
15	2.617±0.099	3.239±0.131	0.9976	1.726±0.037	2.358±0.050	0.9993	1.553±0.083	2.693±0.11	0.9975	1.035±0.048	1.915±0.064	0.9983
16	1.164±0.050	2.256±0.066	0.9987	0.488±0.010	1.554±0.014	0.9999	0.7370±0.044	2.115±0.058	0.9989	0.3514±0.057	1.664±0.076	0.9969
17	1.561±0.052	2.583±0.068	0.9990	0.687±0.026	1.662±0.034	0.9994	0.9983±0.057	2.339±0.076	0.9984	0.5475±0.036	1.819±0.048	0.9990
18	2.026±0.064	2.951±0.085	0.9992	1.031±0.026	1.909±0.034	0.9995	1.330±0.066	2.627±0.088	0.9983	0.8089±0.036	2.022±0.048	0.9991
19	1.874±0.058	2.637±0.077	0.9987	1.523±0.086	2.417±0.113	0.9967	1.374±0.066	2.566±0.086	0.9983	0.7277±0.029	1.613±0.038	0.9992
20	2.377±0.073	3.042±0.097	0.9985	1.748±0.056	2.500±0.074	0.9987	1.620±0.085	2.790±0.11	0.9976	0.9993±0.030	1.827±0.039	0.9993
21	0.943±0.037	2.061±0.048	0.9992	0.808±0.035	1.837±0.046	0.9991	0.9869±0.057	2.272±0.076	0.9983	0.1026±0.056	1.234±0.074	0.9946
22	3.010±0.272	3.506±0.359	0.9846	1.936±0.023	2.456±0.031	0.9998	1.740±0.096	2.751±0.13	0.9968	1.464±0.038	2.107±0.050	0.9992
23	3.350±0.316	3.778±0.417	0.9822	2.127±0.043	2.642±0.057	0.9993	1.814±0.091	2.871±0.12	0.9974	1.603±0.038	2.163±0.050	0.9992
24	2.745±0.080	3.276±0.106	0.9984	1.886±0.040	2.461±0.052	0.9993	1.727±0.099	2.735±0.13	0.9966	1.399±0.037	2.085±0.049	0.9992
25	2.678±0.229	3.005±0.289	0.9909	2.218±0.043	2.680±0.056	0.9993	2.065±0.096	3.082±0.13	0.9975	[b]		
26	3.174±0.150	3.505±0.189	0.9971	2.210±0.042	2.675±0.056	0.9994	1.959±0.093	2.874±0.12	0.9972	[b]		
27	3.262±0.082	3.446±0.100	1.000	2.486±0.046	2.965±0.061	0.9994	1.901±0.063	2.954±0.083	0.9988	[b]		

[a]Experimental conditions: Instrument, Beckman P/ACE 5510; column, packed/total length, 20/27 cm, with 3 μm Spherisorb-ODS; mobile phases contained 4 mM Tris; detection wavelength, 200 nm; column temperature, 20.0±0.1°C.
[b]Data were not measured because the retentions were too strong.

rated quickly (not shown) using a mobile phase of ACN–buffer (60:40, v/v) from 0 to 12.83 min, and then titrating to 80:20 (v/v). The R.S.D. values of the retention times for acetaldehyde, propionone, propionaldehyde, benzaldehyde, butyraldehyde, *o*-tolualdehyde, valeraldehyde, 2,5-dimethylbenzaldehyde and heptaldehyde were 7.56, 6.85, 6.82, 3.30, 2.03, 2.22, 2.49, 3.23 and 3.74%, respectively, for five consecutive separations, which showed that the gradient elution separation was reproducible. Furthermore, separation of fifteen aromatic compounds was performed in RP-CEC with isocratic and gradient elution, and the electrochromatograms obtained are shown in Fig. 7. The column efficiency of the thirteenth peak by isocratic elution was 174 000/m and, in the case of gradient elution, the late peaks were sharpened greatly. Comparing the electrochromatogram obtained by isocratic elution with that by gradient elution, we could see that the separation time was shortened and the detection limit for late-eluting solutes was greatly improved.

3.3. Rapid separation by CEC

As shown in Fig. 4, with a linear velocity as high as 20 cm/min, the plate height in CEC was still quite small and rapid separation of samples could be achieved with high column efficiency. The separation of thiourea and toluene took 1.6 min on a 6.5-cm-long column packed with 3 μm Spherisorb C_{18} (Fig. 8), and the velocity was so high that the theoretical plate numbers for thiourea and toluene were $4.3 \cdot 10^4$/m and $6.8 \cdot 10^4$/m, respectively. Fur-

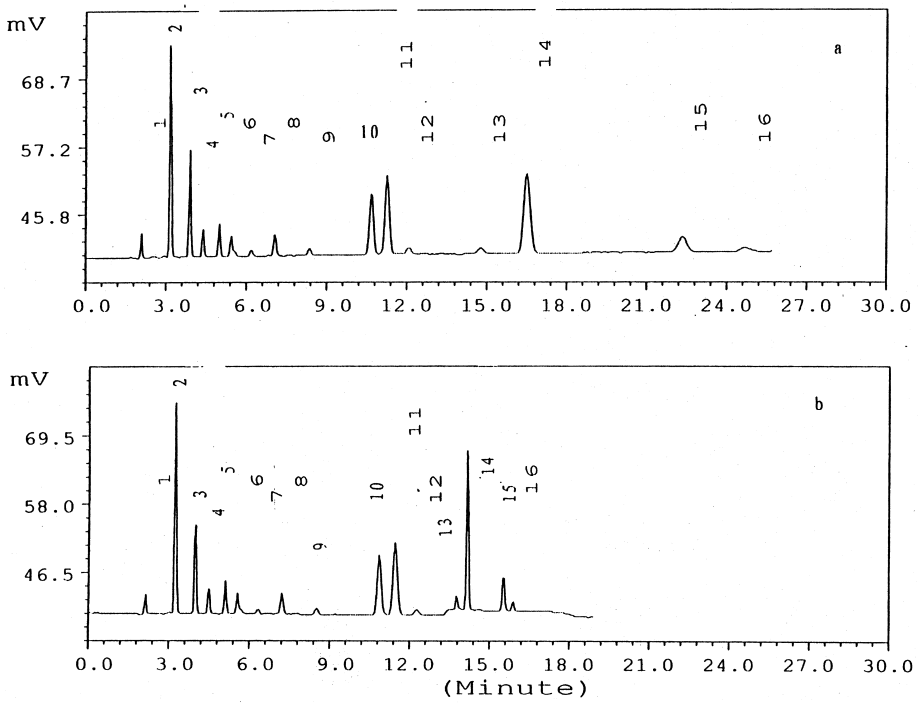

Fig. 7. Separation of aromatic compounds by CEC. Experimental conditions: Instrument, laboratory-made apparatus; column, packing/total length, 15.8/43.5 cm with 3 μm Spherisorb-ODS_2; detection wavelength, 200 nm; electrokinetic injection, 5 kV, 5 s; applied voltage, 20 kV; operating current, 1.0 μA. For other experimental conditions, see text. (a) Isocratic elution with ACN–buffer (60:40, v/v), containing 4 m*M* Tris, pH 9.2. (b) Gradient elution: Mobile phase, ACN–buffer (60:40, v/v), containing 4 m*M* Tris, pH 9.2, from 0 to 12.25 min, then titrated to a mobile phase of ACN–buffer (80:20, v/v), containing 4 m*M* Tris, pH 9.2. Peaks: (1) thiourea, (2) phenol, (3) phenylpropanol, (4) 2,3-dimethylphenol, (5) nitrobenzene, (6) 2,4-dinitrotoluene, (7) benzene, (8) ethyl benzoate, (9) toluene, (10) naphthalene, (11) ethylbenzene, (12) *p*-dichlorobenzene, (13) 1,2,3-trimethylbenzene, (14) *n*-propylbenzene, (15) 1,2,4,5-tetramethylbenzene and (16) *n*-butylbenzene.

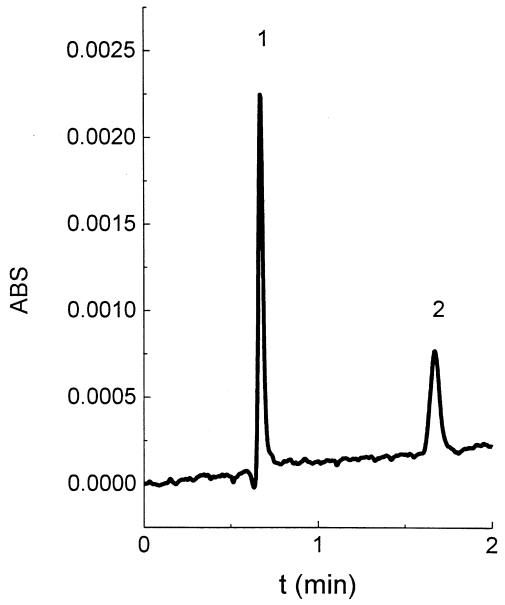

Fig. 8. Electrochromatogram showing the fast-speed separation of thiourea and toluene. Experimental conditions: Instrument, P/ACE 5510 system; column, packed/total length of 6.5/27 cm with 3 μm Spherisorb-ODS; applied voltage, 29 kV; electrokinetic injection 1 kV, 1 s; mobile phase, ACN–buffer (70:30, v/v), containing 4 m*M* Tris, pH 9.2. Operating current, 1.4 μA. For other experimental conditions, see Section 2. Peaks: (1) thiourea and (2) toluene.

ther work is being done in our laboratory using nonporous silica C_{18} as the stationary phase [25].

4. Conclusion

In this work, CEC, PEC and μ-HPLC were performed using one unified apparatus to compare the retention behaviors of neutral solutes with different structures. A unified apparatus was found to facilitate switching between the three methods and made the CEC experiments more reliable. Other experimental conditions, such as the columns used, mobile phases, temperature, etc., were strictly controlled to make the data more comparable. The results show that there were hardly any differences in the capacity factors for the 27 solutes tested using these three methods, which meant that the retention rules for HPLC could also be applied in CEC for further optimization work. The same LSER equations applied in electrochromatography and HPLC, and the same relationship between ln k' and organic modifier concentration was observed, which strengthened the hypothesis that, for neutral compounds, the retention behavior is the same in electrochromatography and in HPLC.

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