

Gradient Elution in Capillary Electrochromatography

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In analogy to pressure-driven gradient techniques in high-performance liquid chromatography, a system has been developed for delivering electroosmotically driven solvent gradients for capillary electrochromatography (CEC). Dynamic gradients with submicroliter per minute flow rates are generated by merging two electroosmotic flows that are regulated by computer-controlled voltages. These flows are delivered by two fused-silica capillary arms attached to a T-connector, where they mix and then flow into a capillary column that has been electrokinetically packed with 3- μm reversed-phase particles. The inlet of one capillary arm is placed in a solution reservoir containing one mobile phase, and the inlet of the other is placed in a second reservoir containing a second mobile phase. Two independent computer-controlled, programmable, high-voltage power supplies (0–50 kV)—one providing an increasing ramp and the other providing a decreasing ramp—are used to apply variable high-voltage potentials to the mobile phase reservoirs to regulate the electroosmotic flow in each arm. The ratio of the electroosmotic flow rates between the two arms is changed with time according to the computer-controlled voltages to deliver the required gradient profile to the separation column. Experiments were performed to confirm the composition of the mobile phase during a gradient run and to determine the change of the composition in response to the programmed voltage profile. To demonstrate the performance of electroosmotically driven gradient elution in CEC, a mixture of 16 polycyclic aromatic hydrocarbons was separated in less than 90 min. This gradient technique is expected to be well-suited for generating not only solvent gradients in CEC but also other types of gradients, such as pH and ionic strength gradients, in capillary electrokinetic separations and analyses.

Analytical techniques with miniaturized columns, especially capillary zone electrophoresis (CZE)^{1,2} and high-performance liquid chromatography using capillary columns (micro-HPLC),^{3,4}

have received considerable attention. CZE, in which a potential is applied to a buffer-filled capillary to generate electroosmotic flow (EOF), provides excellent efficiency in separating charged species via their different electrophoretic mobilities but is unable to resolve neutral components. Micro-HPLC, on the other hand, provides high selectivity in a wide range of applications because a variety of stationary phases are available. However, the mobile phase in micro-HPLC is driven through the column by applying high pressure that causes a parabolic velocity profile of the mobile phase and thus reduces the column efficiency.

The high efficiency of CZE and the high selectivity of micro-HPLC can be combined, and the end result is a hybrid technique referred to as capillary electrochromatography (CEC). Since the first demonstration of the feasibility of electrochromatography by Pretorius et al.⁵ in 1974, CEC in packed columns has been applied to analyze neutral compounds that could not be separated by CZE.^{1,6–16}

To realize the full potential of CEC, it is necessary to develop the capability of gradient elution, as in HPLC¹⁷ and micro-HPLC,^{18,19} for successfully separating a wide variety of complex samples. So far, there appears to be no report on solvent gradient

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elution in CEC using direct, electroosmotically driven flow in packed capillary columns in an automated manner. Nevertheless, some related work should be noted. Behnke and Bayer²⁰ applied an electric potential in addition to pressure to a separation column in which a solvent gradient was delivered by a pressure-driven gradient system. Enhanced column efficiency and resolution were demonstrated when the electric potential was used in the separation column.

In addition, several approaches for generating pH gradients in isotachopheresis and CZE have been reported. Boček and co-workers^{21–23} used a system with two buffer chambers, each with its own electrode, to cause the migration of two different ionic species into the capillary during separation. In their approach, the two buffer chambers were separated from the capillary by semipermeable membranes. Šustáček et al.²⁴ used a syringe-type doser to pump the modifying electrolyte into the background electrolyte chamber to form pH gradients. Tsuda²⁵ proposed an apparatus that consisted of a programmed solvent delivery system and a split injector to generate pH gradients. Chang and Yeung²⁶ used a HPLC gradient system to generate pH gradients and flow gradients in CZE. Balchunas and Sepaniak²⁷ developed a stepwise gradient in MECC by manually pipetting aliquots of a gradient solvent containing 2-propanol into the inlet reservoir of the capillary.

In this paper, we report the development of an electrokinetic pumping system for performing solvent gradient elution in capillary electrokinetic separations. We demonstrate the performance of gradient elution in CEC by resolving a mixture of 16 polycyclic aromatic hydrocarbons (PAHs) in a single run within 90 min. This technique is capable of generating a dynamic gradient with submicroliter per minute flow rates. It should be well-suited for generating not only solvent gradients in CEC but also other types of gradients, such as pH and ionic strength gradients, in electrokinetic separations using capillaries or narrow channels in planar substrates.

EXPERIMENTAL SECTION

A schematic diagram of the solvent gradient CEC system and LIF detector is shown in Figure 1. The major components of this analytical system are described below.

Solvent Gradient Delivery System. The solvent gradient CEC system is composed of two mobile phase reservoirs, two 0–50-kV high-voltage power supplies (Glassman High Voltage, Inc., White House Station, NJ), two fused-silica capillaries (50- μm i.d., 365- μm o.d., 26-cm length), a home-made T-connector ($\sim 365\text{-}\mu\text{m}$ i.d.), and a packed separation column (described below). The T-connector was constructed by drilling a hole ($\sim 365\text{-}\mu\text{m}$) through the wall of a 2-cm length of poly(tetrafluoroethylene) tubing (365- μm i.d.). After a 50- μm -i.d. capillary was inserted into the hole, a drop of UV-cured optical adhesive (Norland Products, Inc., New Brunswick, NJ) was used to join the capillary to the

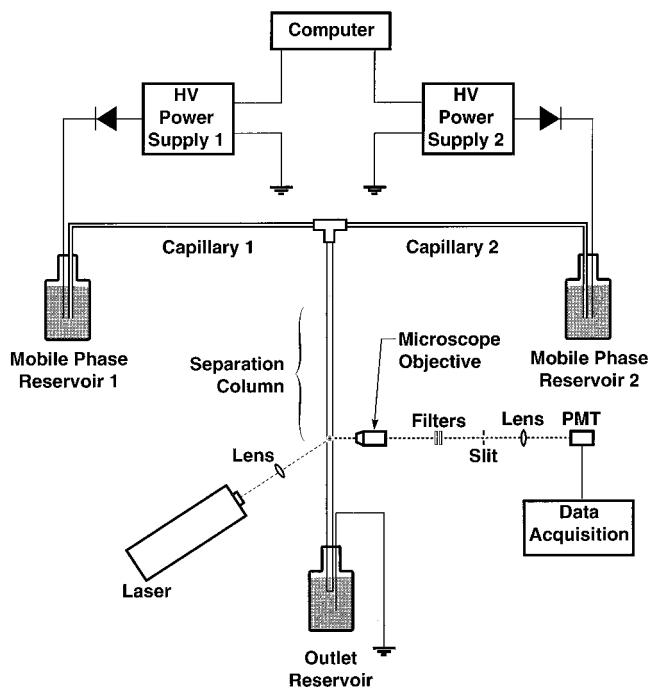


Figure 1. Schematic of the solvent gradient elution CEC apparatus.

connector. Because significant pressure is not created in CEC, this T-connector was used successfully in the solvent gradient system without any leakage problems. A program written with commercially available software (Labview for Windows, National Instruments, Austin, TX) was used with a digital-to-analog converter to control the voltages from the two power supplies.

Samples were introduced electrokinetically into the separation column by disconnecting it from the T-connector and placing its inlet into the sample vial. An application of 5 kV for 5 s caused the migration of a few nanoliters of the sample into the column. The column was then reconnected to the T-connector and the gradient elution initiated. A fan was used to assist the heat dissipation of the capillaries during the separation.

Preparation of the Packed Capillary Columns. Capillary columns with 75- μm i.d. were produced using an electrokinetic packing technique developed previously by Yan.²⁸ The procedure for fabricating the capillary columns has been described previously.^{14,16}

Detection Apparatus. The confocal design UV-LIF apparatus has been described previously.^{14,29} The UV laser radiation (257 nm) from an intracavity doubled argon ion laser (Coherent, Inc., Santa Clara, CA) was focused onto the detection window of the separation column. Fluorescence from the PAHs was collected perpendicular to the excitation beam by using a high numerical aperture (NA) microscope objective (Nikon, Melville, NY) having 40 \times magnification, a NA of 0.85, and a 0.37-mm working distance. The background fluorescence and scattered light from the mobile phase were minimized by using a set of filters (a 280-nm longpass in combination with two 600-nm shortpass filters), and the background from the capillary wall was spatially discriminated through a variable slit (Newport Corp., Fountain Valley, CA). The photocurrent from the photomultiplier tube (PMT) (Products for Research, Danvers, MA) was amplified with a lock-in amplifier (Stanford Research Systems, Sunnyvale, CA) while the output of

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the excitation laser was mechanically chopped. With sufficiently high signal levels, monitoring the PMT output with a picoammeter (Model 485, Keithly Instrument Inc., Cleveland, OH) was satisfactory. The output of the lock-in amplifier (or picoammeter) was displayed and stored by a commercial software package (Lab Calc, Galactic Industries Corp., Salem, NH) run on a personal computer.

Materials and Reagents. The fused-silica capillary columns were purchased from Polymicro Technologies, Inc. (Phoenix, AZ). The 3- μm ODS and the 1- μm silica particles were obtained from SynChrom, Inc. (Lafayette, IN) and Phase Separations, Inc. (Norwalk, NJ), respectively. The 5- μm silica gel for making the frits was provided by Waters Corp. (Milford, MA). The priority pollutant PAH mixture (standard reference material, SRM 1647c) was a gift from the National Institute of Standards and Technology. Sodium tetraborate, acetonitrile (HPLC grade), methanol, and individual PAHs were purchased from Aldrich Chemical Co. (Milwaukee, WI). The mobile phase was prepared by mixing the appropriate percentage of acetonitrile with a 4 mM sodium tetraborate solution and was degassed by ultrasonication before use. Water was purified with an Ultra-Pure water system from Millipore (Milford, MA). Stock solutions (1–10 mM) of the individual PAHs were first made in acetonitrile and then diluted to the desired levels in the mobile phase.

RESULTS AND DISCUSSION

Evaluation of the Solvent Delivery System. The theory of gradient elution is well understood in HPLC¹⁷ and is readily adapted to CEC. The mobile phase gradients in CEC can be described as a time function of the concentration, c , of the more efficient eluting component b in the mobile phase at the inlet of the separation column. This function may be expressed as

$$c = c_1 + (c_2 - c_1)f(t) \quad (1)$$

where c_1 is the concentration of b in solution 1, c_2 is the concentration of b in solution 2, and $f(t)$ ranges from 0 to 1. By choosing various combinations of c_1 and c_2 , different gradient programs can be used to achieve the same solvent gradient.

Simple gradients may differ from one another in three respects: (1) the shape of the gradient (linear, concave, or convex); (2) the slope and the curvature of the gradient; and (3) the initial and final concentrations of the more efficient component b in the mobile phase.

The quality and performance of the solvent gradient delivery system can be evaluated according to the following criteria: (1) accuracy of the gradient formation, i.e., agreement between the actual and the intended gradient profiles; (2) speed of response of the composition of the mobile phase to a change in the applied voltage program; and (3) reproducibility of the gradient profile in repeated runs.

For the evaluation of the solvent gradient delivery system, an open tubular capillary (50- μm i.d., 26-cm length) was used to replace the packed column. One reservoir (mobile phase reservoir 1) was driven by the first high-voltage power supply (HV₁) and was filled with 55% acetonitrile (c_1) in 4 mM sodium tetraborate buffer. The other reservoir (mobile phase reservoir 2) was driven by the second high-voltage power supply (HV₂) and was filled with 80% acetonitrile (c_2) in 4 mM sodium tetraborate buffer. Fluoranthene, added at a concentration of $\sim 10^{-7}$ M to mobile phase reservoir 2, served as a fluorescent tracer to indicate the amount of the solution from that reservoir

entering the mobile phase. Changes in fluorescence quenching caused by different mobile phase compositions during the gradient run were found to be insignificant over the composition range used in this study. The window for the LIF detection was located at about 2 cm after the T-connector, near the inlet of the separation column.

A gradient profile with the following parameters was selected for this demonstration: (1) initial concentration of acetonitrile in the mobile phase $c_1 = 55\%$ and final concentration $c_2 = 80\%$; (2) completion of the gradient in 20 min; and (3) 1-min holdup time before gradient elution and 5-min holdup time after the gradient elution. A reversed gradient was then used to reequilibrate the system (see Figure 2). The gradient profile (up to 26 min) can be described by the following time function of the acetonitrile concentration c :

$$\begin{aligned} c &= 55 & t < 1 \\ c &= 55 + 1.25(t - 1) & 1 \leq t < 21 \\ c &= 80 & 21 \leq t < 26 \end{aligned}$$

where c is the percentage (v/v) of acetonitrile (b) in the mobile phase, t is time, and the slope 1.25 was obtained by dividing the change in concentration by the selected duration of the gradient, i.e., $(80 - 55)/20$.

At the beginning of the gradient run, each arm was filled with its respective buffer, and the "separation column" (actually, an open column in this test) was filled with the 55% acetonitrile buffer. Both arms and the separation column are identical in this example (50- μm i.d., 26-cm length), and initial voltage settings of 20 kV in mobile phase reservoir 1 and 10 kV in mobile phase reservoir 2 were used. This is expected to result in the separation column being exclusively fed from reservoir 1, maintaining the composition of the separation column at 55% acetonitrile. This is because the voltage at the T-connector should be 10 kV. Holding mobile phase reservoir 2 at 10 kV should result in no voltage drop and, therefore, no electric field to drive the flow from this reservoir. In practice, at the beginning of the run, the starting voltage in reservoir 2 was held at a level slightly higher than 10 kV (at 10.5 kV) to assure that there was no reverse flow during the initial 1-min holdup period.

If the conductivities of both buffers were the same, the voltage at the T-connector would remain constant throughout the gradient run if the sum of HV₁ and HV₂ was maintained at 30.5 kV. Since the conductivities of the two buffers are not the same, the voltage at the T-connector (and therefore the voltage across the separation column) varies slightly. After the 1-min holdup time, gradient elution started by simultaneously decreasing HV₁ and increasing HV₂ in a linear fashion (see Figure 2). The gradient elution proceeded with a slope of 0.475 kV/min in each arm according to the preset program and completed in 20 min when HV₂ (with a positive slope) reached 20 kV and HV₁ (with a negative slope) reached 10.5 kV. The potential across capillary arm 1 from HV₁ should be ~ 0.5 kV at this time, and, therefore, the mobile phase containing 80% acetonitrile driven by HV₂ should be dominant.

Shown in Figure 2 are the voltage program and the experimental (actual) profile of the gradient. Deviations of the actual gradient profile from the ideal values were insignificant under the experimental conditions. The electroosmotic flow rate in the gradient system, measured using the baseline disturbance caused by an injection of a buffer mixture with a slightly different acetonitrile concentration, was ~ 0.6 mm/s, corresponding to a volumetric flow rate of 70 nL/min. The gradient delay time originating

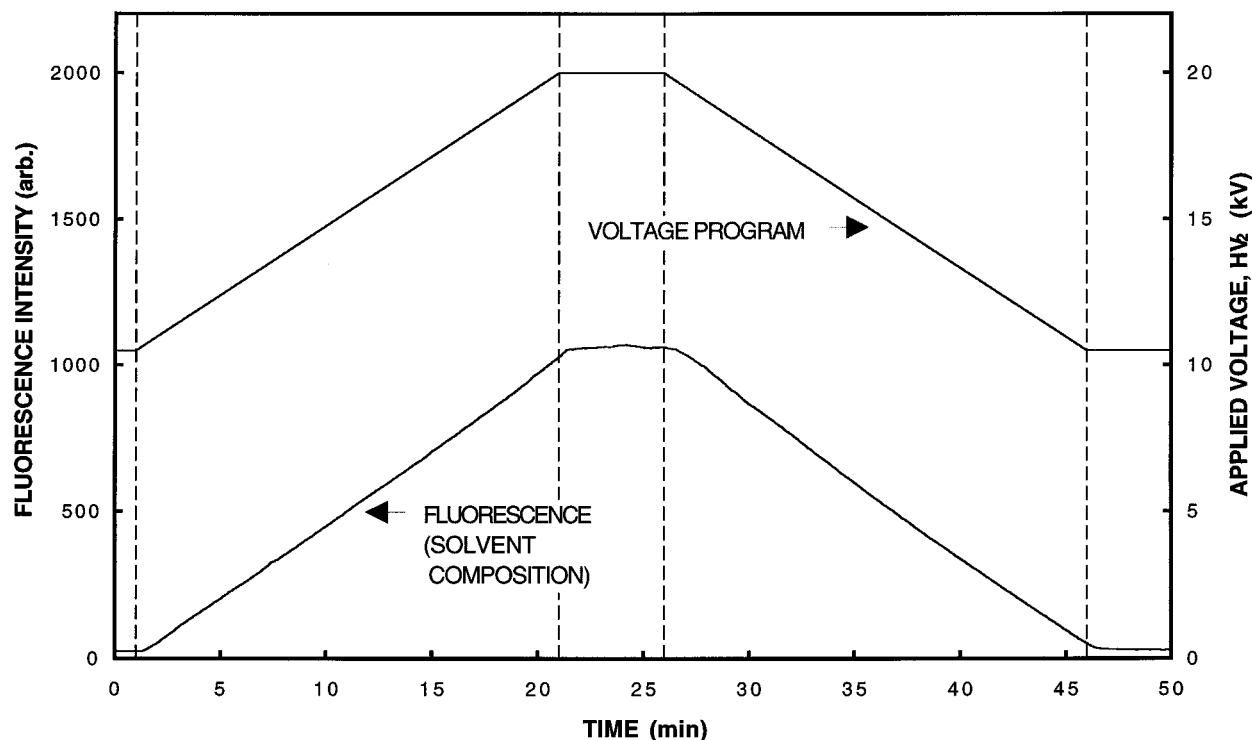


Figure 2. Time profiles for the voltage program and the solvent composition. The top curve is the voltage program for power supply 2 (HV₂). The bottom curve is the fluorescence response from fluoranthene (about 10^{-7} M) added to the 80% acetonitrile mobile phase.

from the dead volume of the T-connector and the capillary tubing between the T-connector and the detection window is 33 s; this time corresponds to a volume of ~ 40 nL. Because the volume of the capillary between the connector and the detector is also ~ 40 nL, the dead volume of the T-connector is negligible.

As an indication of the response time of the gradient system, the time between the beginning of the linear portion of the voltage program and the onset of the increase in measured fluorescence was measured. From three consecutive runs, the relative standard deviation (RSD) of this time interval was found to be $< 1\%$.

It is more difficult to evaluate precisely the solvent delivery system when a packed separation column is used. First, finding a fluorescent tracer that is truly unretained presents a challenge. Second, the dynamic change of the mobile phase composition during the gradient elution causes complications in retention and possible quenching of the tracer. Because of these complications in the case of a packed column, an actual separation is a better test for the gradient system.

Detection System Evaluation. Laser-induced fluorescence (LIF) detection allows sensitive detection in liquid chromatography and capillary electrokinetic separations. Under isocratic conditions (e.g., 80% acetonitrile/4 mM sodium tetraborate), we have achieved detection limits between 10^{-17} and 10^{-20} mol, with a linear response spanning 4 decades in concentration.¹⁴ However, as in gradient HPLC, a problem in gradient CEC is baseline drift, which may originate from fluorescence of impurities in the mobile phase and changes in the refractive index of the mobile phase during gradient elution. In our case, we sometimes observed the baseline drifting up when the acetonitrile content was increased. We should also point out that, for some species, the sensitivity of LIF detection depends on the composition of the mobile phase because of quenching effects. For example, a change from 80% acetonitrile to 55% in the aqueous buffer causes approximately a 2-fold decrease in fluorescent intensity of benzo[*k*]fluoranthene. There-

fore, it is essential for quantitative gradient analysis that the mobile phase program is reproducible, and calibration should be carried out under the same operating conditions as those used for the analysis of unknown samples.

Application. Many of the PAHs are suspected carcinogens, mutagens, or teratogens and can be found as pollutants in complex sample matrices, such as in airborne particulates, water, soil, and tissue. Isocratic separations of such complex samples are often inadequate to resolve the components. In our previous work,¹⁴ capillary electrochromatography was utilized to analyze 16 PAHs identified by the U.S. Environmental Protection Agency (EPA) as priority pollutants. Although good column efficiencies were obtained, we were unable to resolve all 16 PAHs under isocratic conditions in a single run in a reasonable time. Acenaphthene and fluorene coeluted when an isocratic mobile phase of 80% acetonitrile in 4 mM sodium tetraborate buffer was employed. Shown in Figure 3 is a series of electrochromatograms of the first four of the 16 PAHs obtained under isocratic conditions by using different mobile phase compositions. It is clear that the decrease of acetonitrile in the mobile phase dramatically increased the resolution and resulted in separation of acenaphthene and fluorene when the concentration of acetonitrile in the mobile phase was lowered to 60%. The increase in resolution was achieved, however, at the expense of long retention times. From an attempt to perform an isocratic separation of the 16 PAHs at 55% acetonitrile, in which the first 12 peaks of the NIST standard eluted in 4 h, we estimated that a complete separation of all 16 PAHs would take ~ 20 h under these experimental conditions.

A linear gradient elution in CEC was used to separate the 16 PAHs. A capillary column (75- μm i.d., 26-cm length) packed with 3- μm ODS particles was coupled to the T-connector. The length of the capillary (50- μm i.d.) in the solvent gradient system was chosen in such a way that the capillary filled with mobile phase has the same electrical resistance as that measured in the

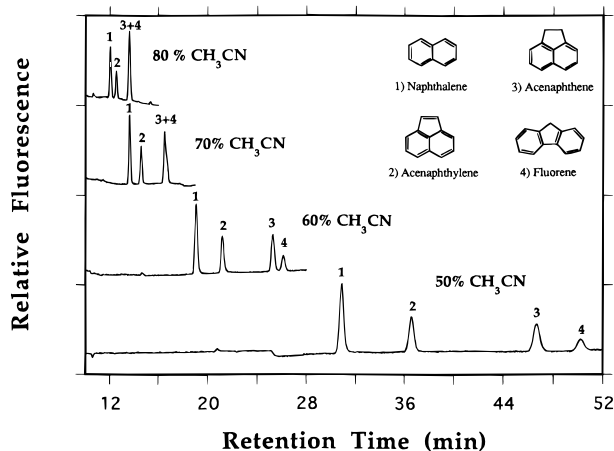


Figure 3. Electrochromatograms showing the separation of four PAHs under different isocratic mobile phase compositions. The column dimensions were 75- μ m i.d. \times 33-cm packed length. The applied voltage was 15 kV. Injection was performed electrokinetically at 5 kV for 5 s. The varying concentrations of acetonitrile were in a 4 mM sodium tetraborate mobile phase.

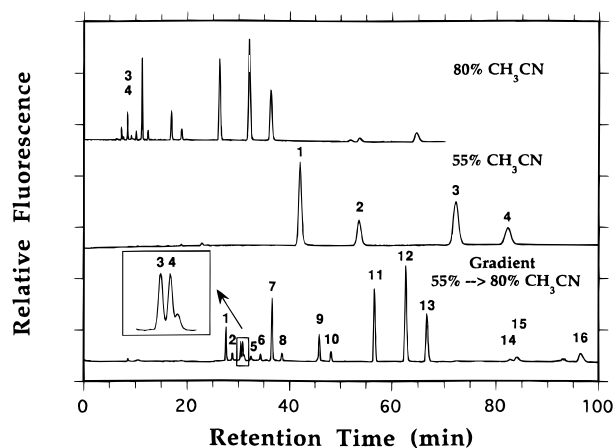


Figure 4. Electrochromatograms showing the comparison of isocratic and gradient elution for the separation of the 16 PAHs listed in Table 1. Peaks 3 and 4 correspond to compounds 3 and 4 in Figure 3. The column dimensions were 75- μ m i.d. \times 26-cm packed length. The applied voltage for the isocratic separations was 20 kV. The injection was performed electrokinetically at 5 kV for 5 s. See text for other details.

separation column when filled with the same mobile phase. The gradient profile was similar to that described in Figure 2, except that the initial voltages were 15 (for HV₁) and 30 kV (for HV₂).

Figure 4 demonstrates the performance of gradient elution in capillary electrochromatography. The top two electrochromatograms are isocratic separations. The bottom one shows the separation of the 16 PAHs using the gradient program described above. The 16 PAHs were resolved within 90 min. Using higher electric fields (higher voltages or a shorter column) should allow faster separations.

The reproducibility in terms of the retention times in gradient elution requires precise control of the variation of the mobile phase composition with time (gradient profile) and of electroosmotic flow rate. Table 1 lists the RSDs of the retention times for the 16 PAHs obtained from four consecutive runs in gradient CEC. These values ranged between 2.8% and 8.1%. The reproducibility of the retention times of the last three peaks was worse than the others. With automation and better temperature control in the future, we anticipate better reproducibility than was observed in this pre-

Table 1. Reproducibility of Retention Time in Gradient CEC

PAH	RSD (%)
1. naphthalene	4.6
2. acenaphthylene	5.0
3. acenaphthalene	4.8
4. fluorene	4.9
5. phenanthrene	4.9
6. anthracene	4.9
7. fluoranthene	4.7
8. pyrene	4.6
9. benz[<i>a</i>]anthracene	3.9
10. chrysene	3.2
11. benzo[<i>b</i>]fluoranthene	2.8
12. benzo[<i>k</i>]fluoranthene	3.8
13. benzo[<i>a</i>]pyrene	4.0
14. dibenz[<i>a,h</i>]anthracene	7.0
15. benzo[<i>ghi</i>]perylene	8.1
16. indeno[1,2,3- <i>cd</i>]pyrene	7.4

liminary study. We occasionally observed ghost peaks in the gradient elution process. We speculate that these peaks were caused by accumulation of adsorbed impurities in the column, which were then eluted when the mobile phase composition changed during the gradient.

CONCLUSIONS

It has been widely recognized that miniaturization of separation columns in HPLC and CEC offers several advantages, including improved efficiency and mass detection sensitivity, low solvent consumption, small sample quantity, and easier coupling to detectors such as mass spectrometers and flame-based detectors. However, to deliver a nanoliter per minute gradient flow into a capillary column (e.g., 10–100- μ m i.d.) packed with micrometer-size particles is a delicate problem.

An electrokinetically driven gradient system in capillary electrochromatography (CEC) offers a solution. The gradient apparatus presented here is capable of delivering extremely low flow rates without flow pulsation and solvent compressibility problems that are commonly encountered in gradient HPLC. With the present solvent gradient system, we expect that the applicability of CEC can be expanded to many separations and analyses that could not be achieved otherwise. This simple, cost-effective device can be easily coupled to other types of electrokinetic separation techniques in a capillary or narrow channel format, such as capillary zone electrophoresis, isotachopheresis, and isoelectric focusing. The gradient system can be readily automated and modified to generate multisolvent gradients not only for mobile phase composition purposes but also for other types of gradients.

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