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Gradient elution electrochromatography with a flow-injection analysis interface

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Abstract

A flow-injection analysis–capillary electrochromatography interface is used for gradient elution capillary electrochromatography giving purely electroosmotic flow through the analytical column. Solvent gradients were generated with a micro-LC system connected to the interface. Injections were carried out on-line using an inert rotary LC valve controlled by an electric actuator. Gradient shape was measured from acetonitrile (5% acetone)–water (50:50, v/v) to (100:0) in open tubular experiments. When compared to conventional instrumentation, peak tailing and peak width increased slightly using the interface. A test mixture of nine solutes was evaluated in isocratic and gradient elution modes. Using the interface, a gradient of MeCN–water (60:40) to (90:10) provided baseline separation of all nine solutes in under 18 min with good band spacing. Reproducibility of retention times in eight replicate injections was found to be better than 2% R.S.D. for all solutes. This interface also allows use of autoinjectors and dramatically lessens movement of the packed column, improving column lifetime.

Keywords: Electrochromatography; Flow injection analysis; Electroosmotic flow; Gradient elution; Polynuclear aromatic hydrocarbons

1. Introduction

Capillary electrochromatography (CEC) is a developing separation technique combining high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) where electroosmotic flow (EOF) is used to transport mobile phase through a fused-silica capillary packed with chromatographic stationary phase. The flat flow profile of EOF creates a more uniform flow velocity across the diameter of the column, decreasing contributions to band broadening when compared to the parabolic nature of hydrodynamic (laminar) flow. Theoretical plates (N) achieved using CEC are routinely observed to be up to 100% greater than those for equivalent separations by traditional LC [1]. The possibility of high efficiency separations of both neutral and charged analytes has generated a great deal of interest and subsequent research as evidenced by the increasing number of manuscripts [1–7] and reviews [8–10] concerning CEC.

Traditional CEC instrumentation is identical to that commonly used in CE, consisting of a high voltage power supply, inlet and outlet solvent reservoirs, a fused-silica capillary, electrodes and a detection device (most commonly UV–Vis). Application of high voltage (0–30 kV) across the capillary suspended between solvent reservoirs necessitates special safety considerations to prevent electrical shock, requiring the unit to be electrically isolated during separation. Because liquid in each reservoir is subject to the same potential as in the capillary,

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solvent composition must remain static during analysis for most CE based separations. Tsuda experimented with pH gradients in CE using a solvent delivery system with a split injector so that the solvent reservoirs were consistently being refilled [11]. Tsuda's experiment yielded improved separations of charged species in CE. In spite of the few gradient elution schemes in CE, the separation of neutral compounds in CEC traditionally has been limited to isocratic elution. In order for CEC to be accepted as a versatile analytical separation method simple, reproducible, convenient gradient elutions are necessary.

This 'isocratic only' problem has been explored by several groups resulting in different methods for achieving gradient elutions. There are at least three general approaches to generating gradients in CEC. Each approach has advantages and disadvantages depending on the nature of the separation.

Euerby utilized a step gradient in a CEC separation of six diuretics [12]. This was achieved by turning off the voltage during the run, replacing the mobile phase with a stronger solvent, then re-applying the voltage. Euerby obtained a retention time R.S.D. of less than 1% for three injections using automated equipment. This method is relatively easy to reproduce and it works well for a limited number of samples. However, the capillary is moved frequently. This diminishes the lifetime of the column and the usefulness of this method for large numbers of samples.

A second method for generating gradients was developed by Yan and coworkers. This method used instrumentation allowing delivery of purely electroosmotic solvent gradients by merging two electroosmotic flows generated by separate power supplies [13]. Using voltage ramps from each power supply, gradients were produced and delivered to the separation capillary through a T-connector. A sample of 16 polynuclear aromatic hydrocarbons (PAHs) was separated in 90 min by this technique showing improved resolution over isocratic methods. Since the gradient which is generated is purely due to electroosmotic flow this system seems ideal. However, to make a sample injection the CEC column must be disconnected from the gradient system, making it impractical for routine separations.

A third method for generating gradients has been explored by several groups. These methods use HPLC pumps to deliver a gradient to the separation capillary. Behnke and Bayer reported an instrument design for gradient separations with pressurized CEC employing split flow from a conventional gradient LC system [14]. Injection was made using a rotary LC injector in line with the pump and connected to a splitter delivering a designated fraction of solvent flow to a custom built interface acting as the inlet reservoir. Solvent gradients using this design showed improved separation efficiency and resolution when compared to the same in isocratic mode. Taylor et al. have used a gradient system similar to that described by Behnke and Bayer for routine analysis of corticosteroids in equine biofluids [15]. Flow was split using a second capillary loop connected between the column inlet and outlet, maintaining a head pressure of ~10–15 bar at the column inlet. Using an HPLC autosampler, over 200 samples were analyzed with reproducible results. Taylor's system applied a positive potential to the capillary inlet so that the outlet could be coupled with electrospray ionization (ESI) MS [16]. The injector was grounded and protected with a long narrow bore loading capillary which provided electrical resistance. Eimer avoided using the loading capillary by grounding the system at the flow splitter and applying a negative potential to the capillary outlet [17]. It is important to note that all of the CEC gradient systems of this type generate some pressure driven flow within the column, compromising the electroosmotic flow profile and contributing to band broadening.

At the First International Symposium on Capillary Electrochromatography there were three presentations on forming solvent gradients in CEC (Van de Goor, Choudhary, and Dorsey) [18]. In each case, LC pumps are interfaced with CE instrumentation to generate a solvent gradient in which there is no significant hydrodynamic flow through the separation capillary. Huber et al. used a commercial CE interfaced with LC pumps to generate a solvent gradient for the separation of phenylthiohydantoin (PTH) amino acids [19].

This paper discusses the application of a flow-injection analysis (FIA) CEC interface used to generate solvent gradients for packed column separations. Solvent mixing is carried out using a micro-HPLC gradient system to produce reliable gradients

while the interface design ensures that flow through the column is purely electroosmotic in nature. Separations of PAHs in both isocratic and gradient mode are reported and performance between the two modes is compared.

2. Experimental

2.1. Reagents

HPLC grade acetonitrile was purchased through Fisher Scientific (Pittsburgh, PA, USA) and used as received. Deionized water (18 MΩ) was obtained from a Barnstead Nanopure filtration system (Dubuque, IA, USA). Acetone, benzene, toluene and liquefied phenol were obtained from Fisher Scientific and all PAH solutes were purchased from Chem Service (West Chester, PA, USA).

2.2. Column Preparation

Fused-silica capillaries used for both CE and CEC were 75 μm I.D. unless noted otherwise and obtained from Polymicro Technologies (Phoenix, AZ, USA). Packed columns were prepared with Shandon 5-μm ODS Hypersil reversed-phase stationary phase particles (Cheshire, UK) using a slurry packing procedure previously described [20].

2.3. Instrumentation

All separations were performed on a laboratory-built CE instrument. A Glassman High Voltage power supply (Whitehouse Station, NJ, USA) in reversed-polarity mode was used with a working range from 0 to −30 kV. UV detection was carried out using an Isco CV^4 capillary absorbance detector (Lincoln, NE, USA) at 254 nm. Solvent gradients were generated using a micro-LC gradient system from Micro-Tech Scientific (Sunnyvale, CA, USA). Injection was carried out using a Cheminert low pressure rotary injection valve with electric actuator from Valco (Houston, TX, USA). An injection loop was made from polyether ether ketone (PEEK) tubing (0.010 in. I.D.; 1 in.=2.54 cm) with a volume of approximately 10 μl.

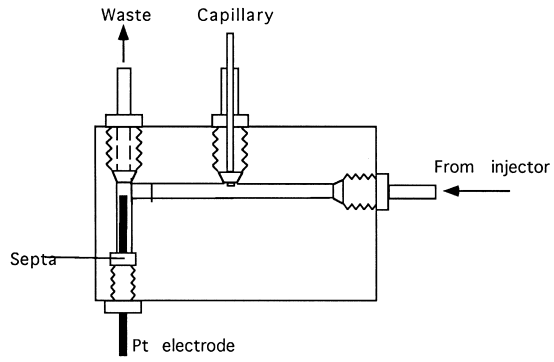

Fig. 1. Diagram of FIA CEC interface.

2.4. CEC–FIA Interface

Construction of the interface is nearly identical to that described elsewhere for CE FIA experiments [21]. The inert polymer Kel-F was substituted for the body of the interface due to its better suitability for machining compared to Plexiglas, as called for in the original paper. In Fig. 1 a schematic diagram of the interface is shown. Stainless steel tubing connected the gradient system to a piece of PEEK tubing through a zero-dead volume union. The PEEK section served as the solvent inlet to the injection port. All connections to the injector were made using PEEK fingertight nuts. A syringe containing sample was connected directly to the injector through a piece of PTFE tubing and used for filling the injection loop when in the load position. A 7-cm length of PEEK tubing (0.03 in. I.D.) connected the injector to the CEC FIA interface. All connections to the interface inlet, outlet, and waste were made using PEEK tubing with stainless steel fittings and ferrules. The Pt electrode was attached to the interface using a PTFE screw and septum. In reversed-polarity mode, high voltage was applied to the outlet reservoir with the electrode at the interface serving as ground.

3. Results and discussion

3.1. Interface grounding

The electrical grounding of all in-line instrumentation prior to the interface was initially a problem. Conductivity of the mobile phase creates a second

pathway for current flow back along the tubing connecting the capillary to the injector and pump, posing a hazard of instrument damage or injury due to high voltage. One design reported a stainless steel splitter used to deliver sub-microliter flow-rates was grounded to earth, preventing any damage to the pump electronics, although no mention was made of how this prevented shorting of the circuit path through the capillary [14]. In our own attempts using normal polarity mode, grounding of the injector caused a short resulting in greatly increased current flow through the grounded path and visible arcing at the electrode within the interface. This problem was overcome by changing the power supply to reversed-polarity mode and applying a negative potential at the capillary outlet as shown in Fig. 2 [21]. In this design, the electrode at the inlet serves as ground, isolating all tubing and instrumentation preceding the interface, preventing damage or injury as a result of the applied high potentials and insuring that the high voltage is applied entirely to the capillary resulting in maximum EOF.

3.2. Gradients

Open tubular experiments were used to determine the ability of the interface to produce solvent gradients. A fused-silica capillary was placed into the instrument and connected to the interface. Water and acetonitrile were prepared for use as mobile phases by sparging with helium. Approximately 5% acetone was added to the acetonitrile to allow UV detection of the gradient shape and slope. To insure that flow through the open capillary was due only to electroosmosis, a step gradient from 100% water to MeCN–water (50:50) was run through the interface with the power supply turned off. While collecting data before and for more than one hour after the step, no change in absorbance was observed, confirming that virtually no hydrodynamic flow was occurring in the direction of EOF. A similar experiment was used to determine the presence of hydrodynamic flow against EOF due to the Bernoulli effect. For an open tube, hydrodynamic flow was found to be ~10% of EOF, which was reduced to ~7% by fitting the capillary with a porous inlet frit. However, using packed capillaries no reverse hydrodynamic flow was observed for a measurement time in excess of 24 hours, indicating flow through the column was completely electroosmotic in nature. In Fig. 3 typical gradients from MeCN–water (50:50) to (100:0) over 20 min using a 60-cm capillary (30-cm to window) are shown. The first curve was produced by connecting the pump directly to the capillary inlet to determine the gradient shape produced by the pumps alone, undistorted by the interface. Although small deviations are present, the gradient shape was found to be relatively good. The second curve was generated using the interface with an applied potential of 15 kV. The gradient shape was found to track that of the first gradient well, showing that the interface was capable of delivering linear gradients. The dwell

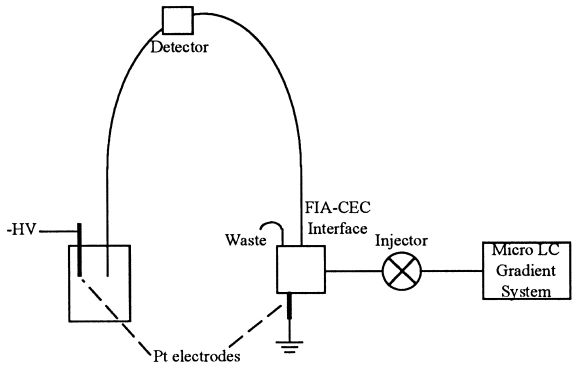

Fig. 2. Schematic diagram of instrument setup.

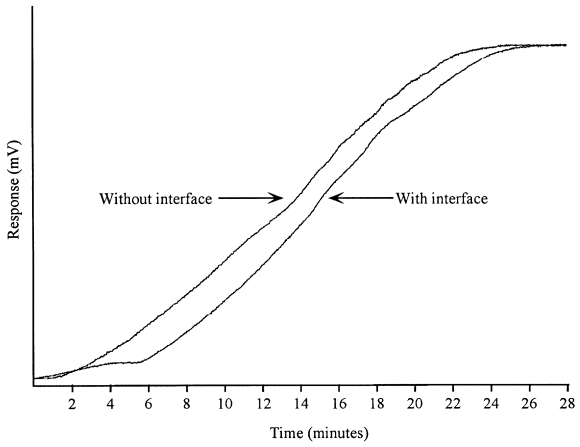

Fig. 3. Open-tubular gradients of MeCN–water (50:50) to (100:0). Gradient program length: 20 min. flow-rate: 200 μl/min. Applied potential: 15 kV.

volume (V_D) of the system was calculated to be ~250 μl, translating into a dwell time (t_D) of 1.25 min at a 200 μl/min flow-rate. The initial void time (t_o) using the interface is ~4.3 min with the beginning of the gradient observed at 5.6 min. Previous results from this lab have documented the change in magnitude of EOF with solvent composition for MeCN–water mixtures as shown in Fig. 4 where at solvent compositions above 80% organic flow increases almost three-fold over that of water [20]. It is probable that differences in gradient shape using the interface can be attributed to EOF changing with mobile phase composition, although these contributions were relatively minor under these conditions. However, significant changes in gradient slope were observed at high organic compositions when using a 20 kV potential with flow-rates of 200 μl/min or less.

In all experiments, MeCN–water mobile phases were used without supporting electrolyte. Currents were below the detectable range (≤1 μA) for the instrumentation described. Previous results have shown that currents for these mobile phases are in the low nanoampere (10^{-9} A) range for both open tubes and packed columns, minimizing any effects due to Joule heating [22]. Problematic bubble formation does not occur when unbuffered mobile phases are used with packed columns at ambient pressures.

3.3. Peak shape

Peak symmetry is affected when using the interface in comparison with traditional electrokinetic injection. Although the flat flow profile still exists, solute bands are distorted as they enter the capillary inlet due to the laminar flow profile of the liquid through the interface. Improper positioning of the capillary worsens the problem causing turbulent flow within the interface. A packed column was prepared and used in separations by CEC and using the FIA–CEC interface. In Fig. 5 an overlay of benzene peaks, scaled on the same time axis, is shown. Conditions between the two separations were identical, using the same column, mobile phase composition and applied potential. Although some tailing was observed with electrokinetic injection (B/A= 1.40), asymmetry increased by nearly 70% using the interface. Peak width was affected more, increasing

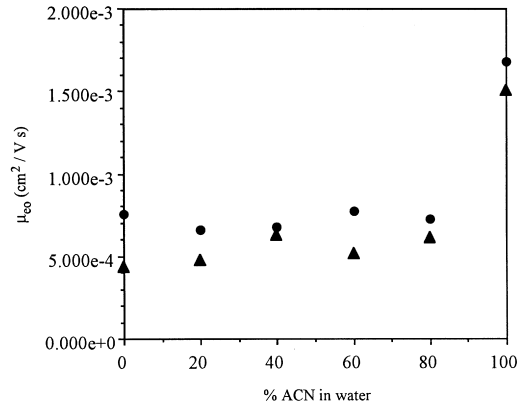

Fig. 4. Effect of increasing acetonitrile (ACN) concentration in water on μ_{eo}. (●) without 3% 1-propanol; (▲) with 3% 1-propanol. Each point is based on five replicate injections.

2.5 times over the CEC separation. Peak shape was found to improve with increasing capacity factor (k'), confirming that band broadening from the interface is an injection variance. This is a worst case example. For a gradient run, solute focusing would occur, and this increase in injection variance would be minimized or eliminated.

There are other factors which may contribute to peak broadening and asymmetry when an interface is used to generate a gradient. Although a 10 μl injection loop was used, the amount of sample introduced to the capillary is dependent upon the applied voltage and the flow-rate past the capillary. As sample concentration can influence peak shape

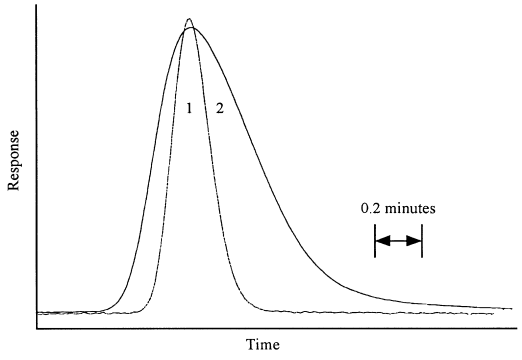

Fig. 5. Overlay of benzene peaks eluted using the same packed column under identical conditions. Mobile phase: MeCN–water (75:25) isocratic. Peak (1) Traditional CEC instrument, 15 kV; (2) FIA CEC interface, 15 kV, 200 μl/min. Column: 20 cm packed, 50 cm total.

and width, further studies must be performed to determine the amount of solute that is introduced to the separation capillary under varying conditions. Additionally, the exact bore size of the channels in the interface is not known. If the bore size varies from the tubing leading to it turbulent flow and sample dilution could occur causing wide asymmetrical peaks. Huber et al. avoided this problem by using a commercially manufactured microcross as an interface [19].

3.4. Gradient elution with packed columns

A 25-cm packed column (47 cm total) was prepared for use with the interface by flushing it with acetonitrile for 12 hours. Column performance was first evaluated using typical CEC instrumentation and when found to be functional, the column inlet was transferred from a mobile phase vial to the flow-injection interface. flow-rate from the pump through the interface was 200 μl/min and applied potential across the capillary was 15 kV. In isocratic mode, a test mixture of 9 solutes in MeCN–water (50:50) was separated in over 70 min with better than baseline separation for all solutes of interest at a mobile phase composition of MeCN–water (60:40). Upon changing the mobile phase to MeCN–water (90:10), the most retained peak eluted at ~10 min, however resolution worsened with significant peak overlap for earlier eluting species. In Fig. 6 the two isocratic separations of the test mixture and a separation using a solvent gradient of MeCN–water (60:40) to (90:10) programmed over 5 min are shown. In the gradient separation, baseline resolution was maintained for all major solutes and total analysis time was reduced to ~17 min. Small peaks occurring just after fluorene and anthracene were nearly baseline resolved in the gradient separation and attributed to solute impurities. Although analysis time was reduced significantly using this system, it is probable that the separation could be further improved while retaining baseline resolution. The gradient dwell time (t_D) observed in Fig. 3 may effectively cause early peaks to be eluted in isocratic mode. Decreasing the length of tubing between the pump and interface should reduce this effect to a minimum.

Repeated injections were made for the gradient separation to estimate reproducibility of retention times. Column re-equilibration occurred by running the system at the lower strength mobile phase for ~30 min between runs. Potential was increased to 20 kV during this period to increase the volume of mobile phase through the packed column and reduce

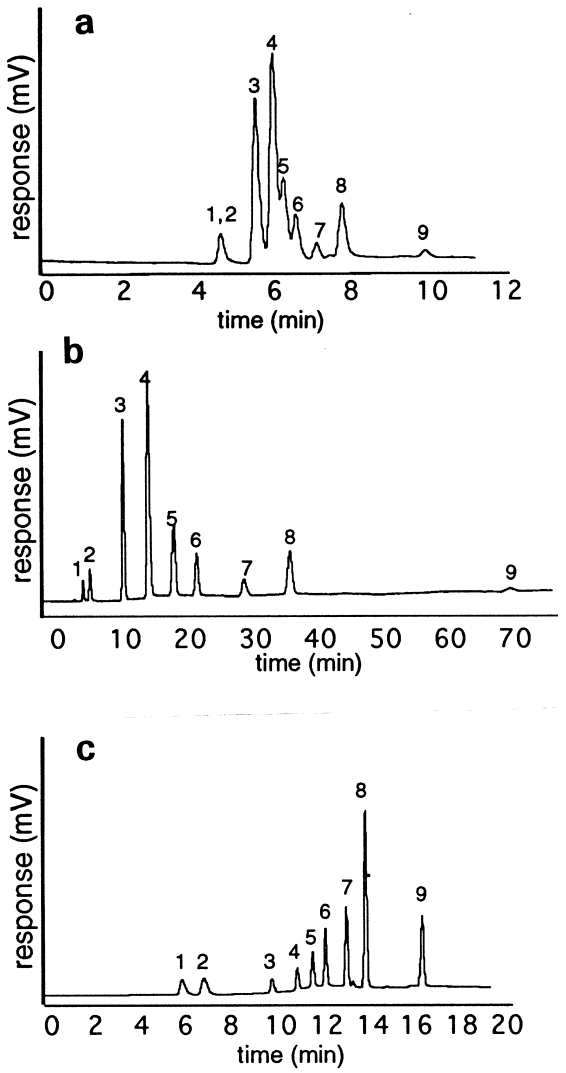

Fig. 6. Comparison of isocratic and gradient separation of test mixture. Gradient program length: 5 min. flow-rate: 200 μl/min. Potential: 15 kV. Capillary: same as in Fig. 5. (a) Acetonitrile–water (90:10) isocratic; (b) acetonitrile–water (60:40) isocratic; (c) acetonitrile–water (60:40) to (90:10) gradient. Peaks: (1) acetone; (2) phenol; (3) benzene; (4) toluene; (5) napthalene; (6) acenapthylene; (7) fluorene; (8) anthracene; (9) 1,2-benzanthracene.

equilibration time. Reequilibration time was not measured however, and likely occurs much faster than this [23]. For 8 replicate injections, retention time reproducibility was better than 2% R.S.D. for all solutes. By excluding both the first peak (acetone) and last peak (1,2-benzanthracene), R.S.D. improved to less than 1% for mobile phases containing no supporting electrolyte. The greater error in t_R for acetone is most likely an effect of injection variance due to the interface, affecting unretained and weakly retained species more than later eluting solutes. The last peak, 1,2-benzanthracene, elutes at a point where mobile phase velocity changes more rapidly over a short period of time, causing undetermined changes in EOF which could affect t_R reproducibility. Using a more shallow gradient at high organic mobile phase composition should slow the rate of velocity change resulting in more stable EOF and t_R, however, it would increase the run time. In addition, adding 3% 1-propanol to both A and B solvents as suggested by Cole and Dorsey would further reduce equilibration time for gradient separations [23]. Fig. 4 shows that the addition of 3% 1-propanol to MeCN–water mixtures decreases μ_{eo} values by ~30% while the dynamic velocity range from 0 to 100% MeCN remains nearly identical.

4. Conclusions

The FIA CEC interface has proven useful for generating solvent gradients in CEC from purely electroosmotic flow. Although promising, several concerns must be addressed to insure the reliability of this technique. Gradient shape is affected by both pump and capillary flow velocities and by changing mobile phase composition. Although not detrimental to these studies, peak tailing and band broadening effects due to the interface must be reduced in order to observe the full advantage of high efficiency separations by CEC.

In spite of these concerns, this technique was found to be reliable and relatively simple to operate, providing purely electroosmotic solvent gradients without the need for two separate power supplies. The feature of on-line injection appears to improve productivity of the fragile packed capillaries. Injection and changing solvent composition are both controlled remotely, reducing handling to a minimum and resulting in longer capillary lifetimes, all making the method more suitable for use in automated systems. It is expected that future developments improving on gradient elution in CEC will further the interest and acceptance of this promising technique.

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