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Preparation and Evaluation of Bonded Linear Polymethacrylate Stationary Phases for Open Tubular Capillary Electrokinetic Chromatography

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A new procedure for the preparation of thick polymethacrylate films bonded in 25 μm i.d. fused-silica capillaries is developed. The etched silica surface is first modified with an unsaturated organosilane, which is later incorporated into the polymer film. The capillary is then filled with a monomer solution, and polymerization is initiated by incubation at elevated temperature. This thermoinitiation method enables the use of ordinary polyimide-jacketed capillaries in preparing the columns. The effect of monomer concentration on the resulting polymer film was studied by open tubular capillary electrokinetic chromatography using *p*-hydroxybenzoates (parabens) as test solutes. Good separations were achieved using short capillaries. Run-to-run retention time reproducibility was excellent, with RSDs of 2% ($n = 50$) being representative. For the linear polymer films produced, retention of analytes increased as the monomer concentration increased to a certain value, at which point the capacity factors level off with further increases in monomer concentration. The electroosmotic flow velocity decreases with increasing monomer concentration. The efficiency for an unretained test probe (acetone) reaches 270 000 plates/m.

Chromatographic theory predicts a significant improvement in efficiency when small inner diameter (i.d.) open capillaries are used for liquid chromatographic separations.[1] However, relatively thick films of stationary phase are required in order to combine this high efficiency with sufficient capacity for facile detection. These films are somewhat difficult to prepare when polymer solutions are used, such that coated films, rather than immobilized polymer layers of stationary phase, have received primary attention to date.

Much effort has been made to covalently bond a thick polymer film inside a fused-silica capillary, perhaps most notably in the work of Poppe's group.[2−7] In situ photopolymerization was used to incorporate thick polyacrylate films in 5−10 μm i.d. capillaries. Open tubular liquid chromatography (OT-LC) columns yielding good efficiency were produced; however, special capillaries with UV transparent coatings were used,[2−4] and there was a problem in stopping polymerization after UV irradiation. The relation between various experimental conditions and the resulting film thickness was also not clear. Etched borosilicate glass capillary columns (6−15 μm i.d.) with a bonded monolayer of an octadecyl stationary phase were also evaluated. A tedious nine-step etching procedure was employed before the actual bonding.[8] Precipitation coating followed by cross-linking has been successfully applied to fabrication of stable polymer films inside small-bore fused-silica capillaries.[9] To increase the inner surface area and facilitate the diffusion of the solutes in the stationary phase, the sol−gel technique was employed to fabricate a thin porous glass film onto the inner wall of OT-LC columns.[10,11]

Difficulty in preparing OT-LC columns with appropriate stationary phases, having sufficient retention and mass loadability characteristics, is not the only factor hindering the development of OT-LC. Because very small inner diameter columns must be used, sophisticated low flow rate pumps and/or flow splitting are often necessary to handle the very small sample volume and flow rate, and on-column UV or laser-induced fluorescence (LIF) detection is usually employed.

In the work described herein, thermal initiation of polymerization was employed in the preparation of polymethacrylate films bonded inside fused-silica capillaries. This enabled the use of readily available and inexpensive polyimide-jacketed capillaries in column preparation and afforded greater control over film synthesis conditions. The electroosmotic flow velocity and capacity factor of solutes on the resulting columns are related to the initial concentration of monomer. Limited by the sensitivity of the UV detector in our laboratory, the column preparation procedure was developed using 25 μm i.d. capillaries, which is quite large for OT-LC. Nonetheless, efficiencies of 270 000 plates/m were achieved for an unretained test probe (acetone) even though the experimental conditions were not fully optimized.

The chromatographic performance of these columns was evaluated by open tubular capillary electrokinetic chromatography (OT-CEC). OT-CEC can easily accommodate very small sample volumes and very low flow rates, obviating the need for pumps and splitting devices. Two mechanisms act simultaneously in OT-CEC: (1) partitioning between the stationary and mobile phases; (2) electrophoretic migration. However, since the test solutes (parabens) have essentially equivalent electrophoretic

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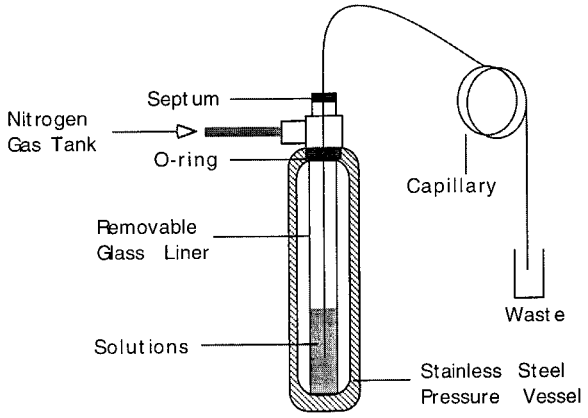

Figure 1. Low-pressure capillary rinsing device.

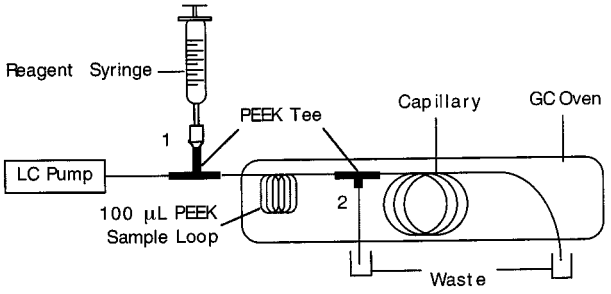

Figure 2. High-pressure capillary flushing device. Ports 1 and 2 were capped after filling the sample loop with reagents.

mobilities, partitioning is the principal and overwhelming separation mechanism under the experimental conditions used. Separations with selectivity comparable to reverse phase HPLC are achieved, while efficiencies rival capillary zone electrophoresis (CZE).

EXPERIMENTAL SECTION

Chemicals and Materials. Fused-silica (25 μm i.d.) capillaries were purchased from Polymicro Technologies (Phoenix, AZ). 3-(Trimethoxysilyl)propyl methacrylate (γ-MPS), butyl methacrylate (BM), 1,4-butanediol dimethacrylate (BDM), and *tert*-butyl peroxide (BP) were obtained from Aldrich Chemical Co. (Milwaukee, WI). HPLC grade toluene and acetonitrile were bought from Fisher Chemicals (Fair Lawn, NJ). Methyl *p*-hydroxybenzoate (MeP), ethyl *p*-hydroxybenzoate (EtP), *n*-propyl *p*-hydroxybenzoate (PrP), and *n*-butyl *p*-hydroxybenzoate (BuP) were from Sigma Chemical Co. (St. Louis, MO).

Apparatus. A Hitachi (Tokyo, Japan) L-6000 HPLC pump, with constant pressure and constant flow rate modes, was used for reagent delivery. A Perkin-Elmer (Norwalk, CT) Model 8500 GC, was used for drying and conditioning of capillaries. An ATI-Unicam (Madison, WI) Crystal CE system (Model 300) with 4225 UV detector and a Beckman (Fullerton, CA) P/ACE System 2050 with UV detector were employed for column evaluation. The low-pressure (Figure 1) and high-pressure (Figure 2) rinsing devices were designed in-house for manufacturing the capillary columns with bonded polymer films.

Column Preparation (1) Etching. Using the low-pressure rinsing device (Figure 1) at room temperature and 100 psi N_2 pressure, each capillary (25 μm i.d., typically L = 200 cm) was etched with 0.5 M NaOH for at least 1 h and flushed with 0.03 M HCl followed by deionized water for at least 1 h each. The etched capillary was then dried at 120 °C under a stream of He gas for at least 12 h.

Table 1. Experimental Conditions for the Syntheses of the Bonded Polymethacrylate Films[a]

capillary no.	% monomer BM (% v/v)
A-15-0	15
A-25-0	25
A-35-0	35
A-45-0	45
A-55-0	55

[a] The following conditions applied for all experiments: cross-linker BDM 0% (v/v); initiator BP, 0.5% (v/v); incubation temperature, 120 °C; incubation time, 10 min.

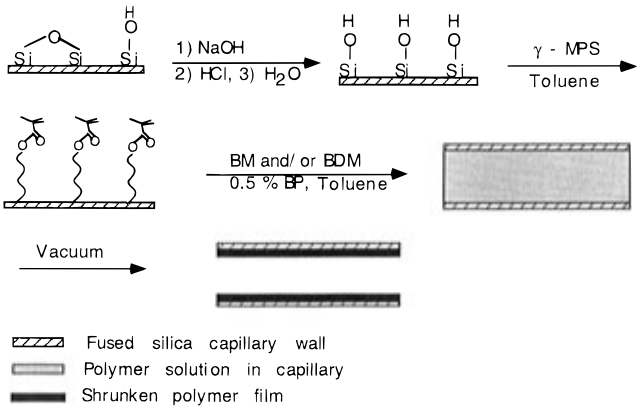

Figure 3. Schematic of column preparation procedure.

(2) Silylation. Using the high-pressure flushing device (Figure 2, LC pump operated in the constant-pressure mode), a solution of 50% (v/v) γ-MPS in dried toluene was pumped through the etched capillary at 120 °C for 1 h, followed by washing the capillary with dried toluene at room temperature for at least 1 h to leach out any unreacted reagents. The capillary was then dried at 30 °C under a stream of He gas for at least 4 h.

(3) Preparation of the Polymethacrylate Film. The monomer solutions were prepared just prior to use by adding appropriate amounts of monomer and initiator in dried toluene. Exact conditions are listed in Table 1. The silylated capillary was filled with monomer solution and sealed at both ends with a GC septum. Polymerization was initiated by placing the filled capillary in a GC oven maintained at 120 °C, and the reaction was allowed to proceed for exactly 10 min. At this point, the capillary was quickly cooled in a refrigerator. To evaporate the solvent and shrink the polymer film, 100 psi N_2 gas pressure was applied at one end of the filled capillary, and a vacuum pump was connected to the other end. The capillary was examined under a zoom stereomicroscope periodically until it was opened. The evaporation and shrinkage process usually required less than 2 h for the linear polymer films prepared. Finally, the resulting column was cured at 120 °C under a flow of He gas for at least 2 h. Figure 3 shows a schematic of the general procedure for column preparation.

(4) Equilibration of the Column. Each column containing a bonded polymethacrylate film was rinsed with 90% acetonitrile/10% deionized water for ~30 min, followed by flushing with the mobile phase for at least 30 min before use. The column was stored overnight filled with deionized water. After testing, columns may be stored either filled with deionized water or in a dry state. No particular care was taken in column storage, and there was no noticeable difference in column performance before and after storage.

Open Tubular Capillary Electrokinetic Chromatography Conditions. Unless otherwise indicated, the following OT-CEC experimental conditions were used. (1) Mobile phase: 20% (v/v) acetonitrile/80% 10 mM phosphate buffer, pH 7. (2) Test sample: 100 ppm each of MeP, EtP, PrP, and BuP in 50% (v/v) acetonitrile/50% 10 mM phosphate buffer, pH 7, with acetone as an unretained neutral marker. The sample mixture was prepared in a solvent stronger than the mobile phase because relatively high concentrations of analytes were required for facile UV detection. In spite of the fact that the sample solution was a stronger eluent than the mobile phase, only minimal zone defocusing was noted. (3) Injection: electrokinetic, 5 kV, 3 s, constant potential, positive polarity. (4) Detection: on column UV absorbance detection at 254 nm.

RESULTS AND DISCUSSION

Preparation of OT-CEC Columns. Using the column preparation procedure described above, capillaries with bonded polymethacrylate films were produced. For monomer solutions without added cross-linking agents, the resulting stationary phases were linear polymer chains attached to the capillary wall. Due to the relatively low viscosity of this type of polymer solution, no problems were encountered in establishing flow through these capillaries where the initial monomer concentration was as high as 55% (v/v). In monomer solutions containing cross-linkers, the success rate for producing the columns was somewhat lower, since the resulting polymer solutions have higher viscosity.[12] Results for the linear polymethacrylate stationary phases are presented here.

Effect of Monomer Concentration on Electroosmotic Flow. Reverse phase OT-CEC separations of the parabens were achieved using the capillaries prepared as described above, which suggested that although a layer of polymer film was bonded to the inner surface of the capillary, the polymer film coverage may still be sparse enough to expose a fraction of the capillary wall, supporting electroosmosis for bulk transport. It has been shown that organic polymers have a finite electroosmotic flow (EOF), which is at a lower magnitude than for silica.[13] The EOF in our columns may arise from both sources. However, the monomer concentration used to prepare the polymethacrylate film did have an effect on the electroosmotic flow velocity (u_{eo}) of the resulting column. The experimental results are shown in Figure 4. Electroosmotic flow velocities were calculated as follows:

$$u_{eo} = L_{DET}/t_0 \quad (1)$$

L_{DET} is the length from point of injection to point of detection; t_0 is the retention time of acetone, an unretained neutral marker of u_{eo}. As can be seen in Figure 4, u_{eo} decreases almost linearly with increasing monomer concentration for modified capillaries, which indicates an increase in surface coverage by the polymethacrylate film. A measurement of u_{eo} for a bare silica capillary is also included in this figure. It is notable that this experimental data point deviates considerably from the regressed intercept using data points for modified capillaries. The small error bars in Figure 4 indicate a high level of reproducibility in electroosmotic flow velocity.

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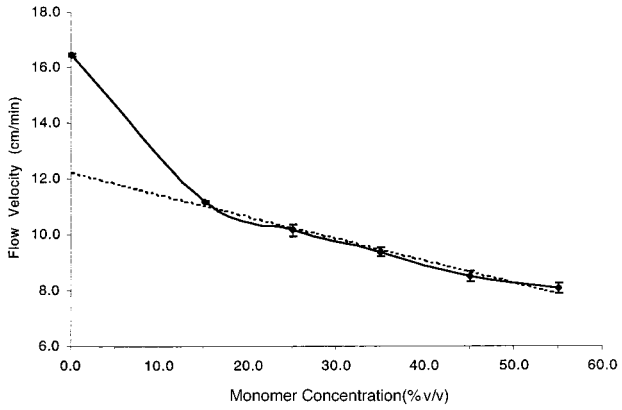

Figure 4. Effect of monomer concentration on electroosmotic flow velocity. Capillary: L_{tot} = 60 cm, L_{det} = 45 cm, and i.d. = 25 μm. Separation: ATI-Unicam Crystal CE system, 30 kV, constant E, and + polarity. Other conditions as listed in the Experimental Section. Calculations are for the acetone peak in the chromatograms for the paraben mixture sample. Data shown are the average of five runs, and the error bars represent ± standard deviation. The dashed line is the linear regression of flow velocities for the modified capillaries (r^2 = 0.984).

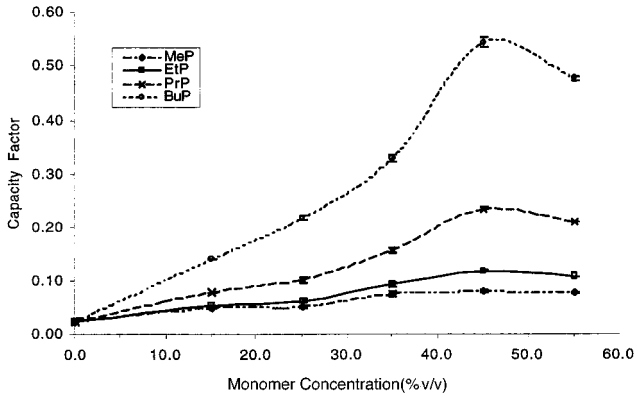

Figure 5. Effect of monomer concentration on analyte capacity factor. Experimental conditions are as described in Figure 4.

Effect of Monomer Concentration on Chromatographic Performance Parameters. (1) Capacity Factor. The observed electroosmotic flow indicated that the inner surface of the capillary was not completely covered by the polymer film. On the other hand, studies on analyte capacity factors showed that the surface-bound polymer layer was sufficient to support reverse phase partitioning behavior. The capacity factors (k') of paraben samples were calculated as follows:

$$k' = (t_R - t_0)/t_0 \quad (2)$$

where t_0 and t_R are the elution times of the unretained test probe and the paraben analytes, respectively. The capacity factors showed an interesting trend vs monomer concentration (Figure 5). Values of k' initially increased with increasing monomer concentration, reaching a maximum at 45% (v/v) monomer, and then slightly decreased. One might expect a continuous increase of k' with increasing monomer concentration. This was found to be true for monomer concentrations up to 45% (v/v); however, because all polymerization reactions were initiated at the same temperature (120 °C) and stopped at the same time (10 min), in some instances the reaction may not have progressed to the point that all of the monomer was reacted. Under the conditions used

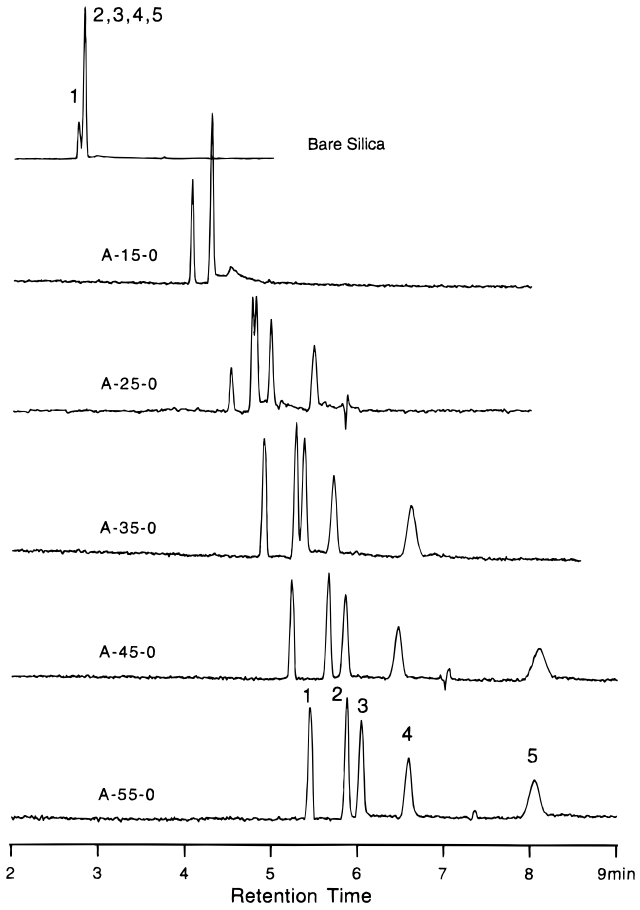

Figure 6. Effect of monomer concentration on overall separation performance. Data for the bare silica capillary is shown 8 times smaller than the others. Capillary: L_{tot} = 60 cm, L_{det} = 45 cm, and i.d. = 25 μm. Separation: ATI-Unicam CE, 30 kV, constant E, and + polarity. Key: (1) acetone, (2) MeP, (3) EtP, (4) PrP, and (5) BuP. Other conditions as listed in the Experimental Section.

in this experiment, the polymer chain resulting from 55% (v/v) monomer solution may be no longer than that resulting from 45% (v/v) solution. At this point, the source of the unusual trend in the k' data is not clear, though a similar trend is observed with cross-linked polymer films.[12]

From the capacity factor graph, one can also conclude that the selectivity of the polymer film also increases with increasing monomer concentration. This provides a mechanism of control over the selectivity of the stationary phase by simply varying the initial monomer concentration. We expect that the addition of different concentrations of cross-linkers, or variation of alkyl chain length on the methacrylate monomers, will enable us to fine tune the selectivity of this type of stationary phase. Research in this direction is currently underway in our laboratory.

The effect of monomer concentration on overall separation performance can be more easily seen in Figure 6. On the bare silica column, all parabens eluted together. The separation of acetone from the parabens indicated that the parabens had a finite electrophoretic mobility under these conditions. As the initial monomer concentrations increased, the resulting polymer chains were made longer, which led to improved separation of the paraben test solutes. The overall separation in OT-CEC is based on both partitioning between stationary and mobile phases and (for species with substantially differing, nonzero electrophoretic mobilities) electrophoresis.

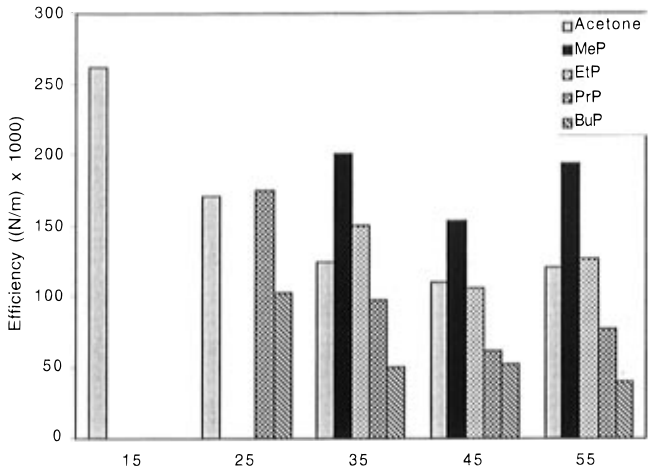

Figure 7. Effect of monomer concentration on column efficiency. Experimental conditions are as listed in Figure 4. Data for unresolved peaks are not included.

(2) Efficiency. As electroosmosis is the motive force for bulk transport in OT-CEC, high efficiencies are expected. Figure 7 indicates the typical efficiencies obtained with linear polymer film columns; efficiencies for unresolved peaks are not included. The efficiency for the unretained acetone test probe reached 270 000 plates/m. This is, however, not the highest achievable efficiency on this kind of column, as little effort was made to optimize separation conditions, and the capillary columns were necessarily overloaded for ease of detection.

One interesting experimental result was that in the cases where MeP and EtP were separated [monomer concentrations greater than 35% (v/v)], the efficiencies achieved for MeP and EtP were higher than or equal to that for acetone. This is quite surprising when one considers that the sample was dissolved in a solvent stronger than the mobile phase. This result, however, was consistent in all of the experiments conducted. Additionally, it was noted that the current was always lower after the injection than before the injection for the same applied potential. Because the sample was dissolved in a less conductive solution than the mobile phase, the introduction of the sample plug led to a potential gradient discontinuity in the column. As the parabens each have a finite electrophoretic mobility, the discontinuity resulted in some degree of sample stacking, which led to the higher efficiencies measured for the MeP and EtP peaks.

(3) Plate Height vs Flow Velocity. A plot of reduced plate height (h) vs reduced velocity (ν) is shown in Figure 8. Flow rates were adjusted by varying field strength. For all the experiments conducted, electroosmotic flow velocity was found to be linear with electric field strength. The experimental data were fitted by nonlinear regression using the following equations:

$$h = A + B/\nu + C\nu \quad (3)$$

$$h = H/d_c \quad (4)$$

$$\nu = (u_{eo}d_c) \times 10^{-4}/D_m \quad (5)$$

For these open tubular columns, the A term of eq 3 was set to zero. In the equations above, H is the plate height (nm), d_c is the diameter of the column (d_c = 25 μm), u_{eo} is the electroosmotic flow velocity (cm/s), and D_m is the diffusion constant of the sample

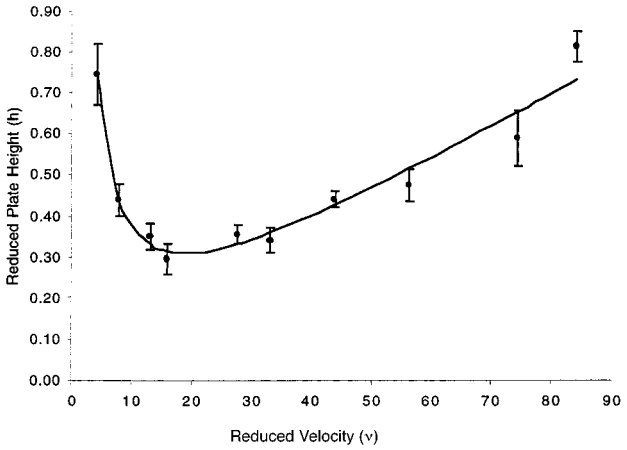

Figure 8. Effect of flow velocity on plate height. Calculations are for the acetone peak in the chromatograms for the paraben mixture sample. Capillary: A-45-0, L_{tot} = 27 cm, L_{det} = 20 cm, and i.d. = 25 μm. Separation: Beckman P/ACE System 2050. Data shown are the average of five runs, and the error bars represent ± standard deviation. Other experimental conditions as described in the text.

in mobile phase ($D_m = 10^{-5}$ cm^2/s in the calculations). Calculations were based on the acetone peak in the chromatograms for the paraben mixture. All data points (with their error bars) fitted well to the trend line except the last point (at 30 kV), which was above the line. This may be explained by Joule heating-induced zone spreading at high potential. It is anticipated that if care were taken to further optimize test conditions, plate heights lower than those shown in Figure 8 could be obtained.

Flow-Programmed OT-CEC. Separations can be improved by employing a gradient in any of several separation factors, (e.g., temperature/viscosity, solvent strength, flow velocity, etc.). In liquid chromatography, flow programming (though seldom applied due to excessive pressure concerns) is achieved by varying the pressure applied to the column, and precise control of the flow rates requires sophisticated pumps. In CEC, flow programming can be easily accomplished by changing the applied potential. The effect of flow programming on the resolution is demonstrated in Figure 9. At constant flow rate (constant potential, Figure 9A and B), a slower flow rate lead to better separation of early-eluting peaks (MeP, EtP), but the last eluting peak (BuP) was so broad that it was almost lost. When flow programming was applied (Figure 9C), not only were MeP and EtP baseline separated but also the peak shape of BuP was improved. Although flow programming cannot substitute for solvent gradient programming in all cases, it may prove useful in instances where only a shallow solvent composition gradient would be needed. The great simplicity of flow programming in OT-CEC is its most attractive feature.

Qualitative Reproducibility. Reproducibility of retention time for acetone was evaluated in 50 consecutive runs. The buffer solution was used without degassing and was replenished after the 18th and the 36th run. The flow rates measured are plotted in Figure 10. Most of the data points fell into one standard deviation range. The relative standard deviation was 2%, which was comparable to that achievable in CZE. This shows that good qualitative reproducibility can be achieved without need of extreme caution regarding eluent conditions.

CONCLUSIONS AND FUTURE DIRECTIONS

We have developed a simple method to synthesize an immobilized polymethacrylate stationary phase inside conventional

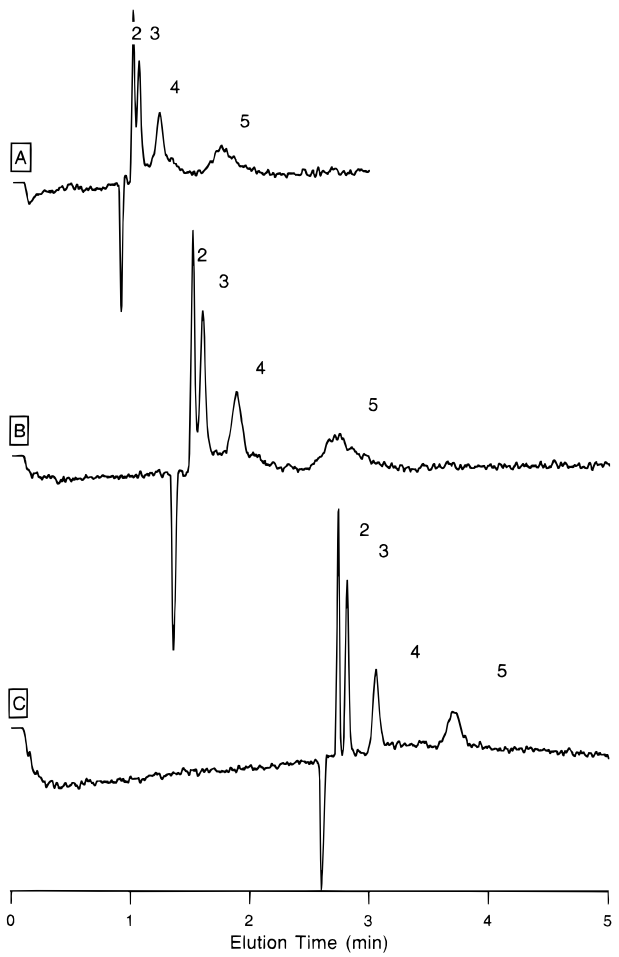

Figure 9. Flow-programmed separation of paraben mixture. Capillary: A-45-0, L_{tot} = 27 cm, L_{det} = 20 cm, and i.d. = 25 μm. Sample: paraben mixture solution without acetone. Separation: Beckman P/ACE system 2050. (A) 30 kV, constant *E*; (B) 20 kV, constant *E*; and (C) 3 kV, to 30 kV gradient in 3 min, hold at 30 kV for 3 min. Other experimental conditions as described in the text.

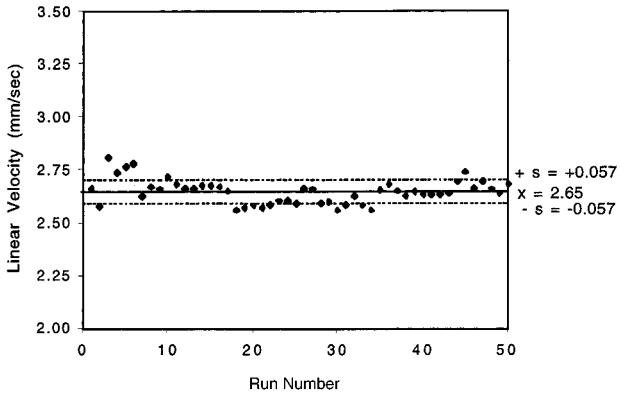

Figure 10. Qualitative reproducibility for modified column. Calculations based on acetone peak. Constant *E* = 30 kV. Other experimental conditions are listed in Figure 8.

polyimide-clad capillaries using a thermal initiator. This procedure has the following advantages: (1) No special outer coating of the capillary is required; (2) experimental conditions are simple and well controlled; (3) performance of the linear films can be directly related to experimental conditions; and (4) columns with bonded polymer films can be stored in either wet or dry conditions without noticeable effect on chromatographic performance.

The capillary columns produced by this procedure were tested using OT-CEC. The surface-bound polymer layer was shown to

be sufficient to support reverse phase partitioning behavior but was sparse enough to expose a fraction of the capillary wall, supporting electroosmosis for bulk transport. The selectivity of the resulting stationary phase was tunable by varying the initial monomer concentration. Appreciable efficiencies were obtained given the large inner diameter capillaries employed and long analyte diffusion times. Qualitative reproducibility comparable to that achievable in conventional CZE was demonstrated. The stationary phases produced were stable enough to permit the use of strong mobile phases, greatly expanding the range of utility of this technique.

The column preparation procedure has been successfully applied to synthesize cross-linked polymer films, and evaluation of these cross-linked stationary phases is underway in our laboratory. Preliminary results show that these cross-linked stationary phases have higher analyte retention ability under reverse phase separation conditions.

ACKNOWLEDGMENT

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