Bonded Polymethacrylate Stationary Phases for Open Tubular Liquid Chromatographic and Electrochromatographic Separations

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Abstract: Using a new procedure developed in our laboratory for the preparation of thick polymethacrylate films bonded in fused silica capillaries, crosslinked polymeric stationary phases were synthesized in situ in 25-μm-i.d. capillaries. The effect of monomer and crosslinker concentrations on the resulting polymer film was studied by open tubular capillary electrokinetic chromatography (OTCEC) using *p*-hydroxy benzoates (parabens) as test solutes. Retention of analytes showed interesting trends versus monomer and crosslinker concentrations, exhibiting maxima at certain combinations rather than a continuous increase in capacity factor with increasing concentration. Because the electroosmotic flow can arise from both exposed silica and polymer surfaces, the flow velocity did not exhibit an obvious trend versus monomer and/or crosslinker concentration. Open tubular liquid chromatographic (OTLC) separations were also achieved using head pressure as the driving force for bulk flow, without employing a high-voltage power supply or micro LC pump. *J Micro Sep* **10:** 99–105, 1998

Key words: *open tubular liquid chromatography; electrokinetic chromatography; polymethacrylate; bonded stationary phases*

INTRODUCTION

According to chromatographic theory, the highest separation efficiency and speed can be obtained with small inner diameter (i.d.) open tubular columns, owing to their favorable flow characteristics [1]. For many years the full exploration of open tubular liquid chromatography (OTLC) and open tubular capillary electrokinetic chromatography (OTCEC) was hindered by two factors: (1) the lack of detectors and injectors which would not add significant band broadening to the miniaturized chromatographic system and (2) difficulties encountered with the immobilization of a uniform stationary phase layer on the inside surface of the capillary having sufficient sample capacity to avoid overloading.

Development of sensitive on-column detection techniques and novel injection schemes, especially techniques used in capillary electrophoresis, has resulted in open tubular liquid chromatography systems which provide for minimal external band broadening [2–5]. The challenge now is to fabricate suitable columns. Several methods have been utilized to realize a retentive layer: static coating procedures [5, 6], creation of a porous structure inside the capillary [7, 8], and chemical immobilization of a monolayer [9] or a polymeric stationary phase [2–5, 10–13] on the inner wall of the capillary.

Described herein is a new procedure developed in our laboratory [12] for the preparation of polymethacrylate films bonded inside narrow fused silica capillaries. This procedure relies on thermal initiation of polymerization, enabling the use of conventional polyimide jacketed capillaries in column preparation and affording greater control over the conditions for film synthesis. Previous studies on linear polymer films [12] showed that the electroosmotic flow velocity and capacity factor of solutes on the resulting columns are related to the initial concentration of monomer. Studies on crosslinked polymer films are presented in this article.

Open tubular capillary electrokinetic chromatography (OTCEC) was used to evaluate the chromatographic performance of these columns. OTCEC can easily accommodate very small sample volumes and very low flow rates, obviating the need

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for pumps and splitting devices. Two mechanisms act simultaneously in OTCEC: (1) partitioning between the stationary and mobile phases and (2) electrophoretic migration. However, since the test solutes (parabens) have essentially equivalent electrophoretic mobilities, partitioning is the principal and overwhelming separation mechanism under the experimental conditions used. In addition to the OTCEC work, reverse-phase separation of the parabens was also achieved using head pressure on the inlet vial as the driving force for bulk flow, without the use of a high-voltage power supply or a micro LC pump. These experiments provided data for the direct comparison of OTLC and OTCEC and demonstrated that LC separations in open tubular columns may be achieved using very simple instrumentation, eliminating the necessity of pumps and valves which are indispensable when conventional columns or packed capillaries are employed.

EXPERIMENTAL

Chemicals and materials. Polyimide jacketed fused silica capillaries (25 μm i.d.) were obtained from Polymicro Technologies (Phoenix, AZ). The anchoring reagent 3-(trimethoxysilyl)propyl methacrylate (γ-MPS), the monomer butyl methacrylate (BM), the crosslinker 1,4-butanediol dimethacrylate (BDM), and the initiator *tert*-butyl peroxide (BP) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Test solutes methyl *p*-hydroxy benzoate (MeP), ethyl *p*-hydroxy benzoate (EtP), *n*-propyl-*p*-hydroxy benzoate (PrP), *n*-butyl-*p*-hydroxy benzoate (BuP) were from Sigma Chemical Co. (St. Louis, MO). High-performance liquid chromatography (HPLC) grade toluene and acetonitrile were bought from Fisher Chemicals (Fair Lawn, NJ).

Apparatus. The equipment employed in column preparation was described in detail in a previous paper [12]. A Hitachi (Tokyo, Japan) L-6000 HPLC pump was used with a high-pressure rinsing device for reagent delivery. A homebuilt low-pressure capillary rinsing device containing a pressurized solvent reservoir and a capillary holder was also employed. The drying and conditioning of capillaries was performed using a Perkin Elmer (Norwalk, CT) model 8500 gas chromatograph. Two capillary electrophoresis (CE) systems were employed for column evaluation: an ATI-Unicam (now Thermo Separations Group, Franklin, MA) crystal CE system (model 300) with 4225 UV detector and a Beckman (Fullerton, CA) P/ACE System 2050 with UV detector.

Column preparation

Etching. Using the low-pressure capillary rinsing device at 750 kPa N_2 head pressure, each capillary (typically 2 m long) is etched with 0.5 M NaOH for 1 h, flushed with 0.03 M HCl followed by deionized water for at least 1 h each, and dried at 120°C under a stream of He gas for at least 10 h.

Silylation. Silylation was performed using the high-pressure rinsing device. The capillary was housed in a gas chromatography (GC) oven, and a HPLC pump was used to displace the silylating reagent (anchoring group). Steps in the process included silylation of the etched capillary by treatment with 50% (v/v) γ-MPS in dried toluene at 120°C for 1.0 h at 1000 kPa/m capillary length, washing of the capillary with toluene at room temperature for at least 1 h, and drying at 30°C under a stream of He gas for at least 4 h.

Preparation of the polymer film

- Monomer solutions were always prepared just prior to their use by adding appropriate amounts of monomer, crosslinker, and initiator to dried toluene (Table I).
- The capillary was filled with monomer solution and sealed at each end with a GC septum.
- The filled capillary was incubated in a GC oven at 120°C for 10.0 min, followed by rapid cooling. Capillaries were then examined for bubbles using a zoom stereomicroscope.
- Application of N_2 gas pressure at 750 kPa to one end of the coated capillary ensured that the lumen was unobstructed. This generally required less than 1 h.
- Curing of the capillary was carried out at 120°C under a flow of He gas for at least 2 h.

Table I. *Experimental conditions for column preparation.*[a]

Capillary number	Length (cm)	Monomer BM% (v/v)	Crosslinker BDM% (v/v)	Initiator BP% (v/v)
B-0-0[b]	100	0	0	0
B-10-0	100	10	0	0.5
B-10-2.5	100	10	2.5	0.5
B-10-5	100	10	5	0.5
B-10-7.5	100	10	7.5	0.5
B-10-10	65	10	10	0.5
B-0-5	100	0	5	0.5
B-2.5-5	100	2.5	5	0.5
B-5-5	100	5	5	0.5
B-7.5-5	100	7.5	5	0.5
B-12.5-5	100	12.5	5	0.5
B-15-15	95	15	15	0.5

[a]Incubation conditions: 120°C, 10.0 min. Inside diameter of uncoated capillary 25 μm.
[b]Capillary modified by emplacement of γ-MPS anchoring groups.

Equilibration of the column. Columns were treated as follows before use:

- Rinse with 60% acetonitrile/40% deionized water for about 30 min.
- Wash with deionized water for 10 min.
- Flush with the mobile phase for at least 40 min before use.

Open tubular capillary electrokinetic chromatography conditions. The columns with bonded stationary phases were evaluated by OTCEC. Some common experimental conditions are listed here; other conditions are described in the tables and figures. The mobile phase is a solution of 20% (v/v) acetonitrile/80% 10 mM phosphate buffer, pH 7. The paraben test mixture is comprised of 100 ppm each of MeP, EtP, PrP, and BuP in the mobile phase, with acetone as an unretained neutral marker. Injections were made electrokinetically at 5 kV for 3 s. On-column UV absorbance detection at 254 nm was used throughout.

RESULTS AND DISCUSSION

Preparation of OTCEC columns. Using the column preparation procedure described above, capillaries with bonded polymethacrylate films were produced. For monomer solutions without added crosslinking 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) [12]. Two series of crosslinked polymer films were studied: (1) a B-10-X series, with constant 10% (v/v) BM monomer and different concentrations (X) of BDM crosslinker, and (2) a B-X-5 series, with constant 5% (v/v) BDM crosslinker and different BM monomer concentrations (X). It was found that when the total concentration of monomer and crosslinker was below 20%, the success rate for column preparation was very near 100%. However, when the total concentration of monomer and crosslinker reached 20% or higher, the success rate for producing the columns was somewhat lower, since the resulting polymer solutions have higher viscosity. The success rate for B-10-10 was one of two and for B-15-15 was one of four.

The bonded stationary phases, as expected, exhibit high tolerance for organic solvents. The capillaries were rinsed with mobile phases containing up to 90% acetonitrile, yet no loss of stationary phase was observed. Columns can 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. For example, column B-15-15 was made and first tested in January 1996; further testing was conducted in March, October, and December of 1996. The chromatographic performance in each test was very similar. Studies on the column stability in different pH environments are currently underway in our laboratory.

Representative separations using the bonded polymethacrylate stationary phases are shown in Figure 1. For comparison, separations on a bare silica capillary and B-0-0 (which has only the anchoring group, no polymer film) are included. On the bare silica column, no separation of parabens was observed. The separation of acetone from the parabens indicates that, under the experimental conditions used, the parabens have finite electrophoretic mobilities. However, the mobilities of the parabens are so similar that they cannot be separated electrophoretically. The chromatogram obtained on the B-0-0 column shows higher capacity factors for parabens, and the flow velocity is decreased dramatically compared to the bare silica capillary. This evidence suggests that the monolayer of anchoring groups on the inner wall of the capillary acts as a stationary phase. More importantly, however, it serves as proof that the anchoring group

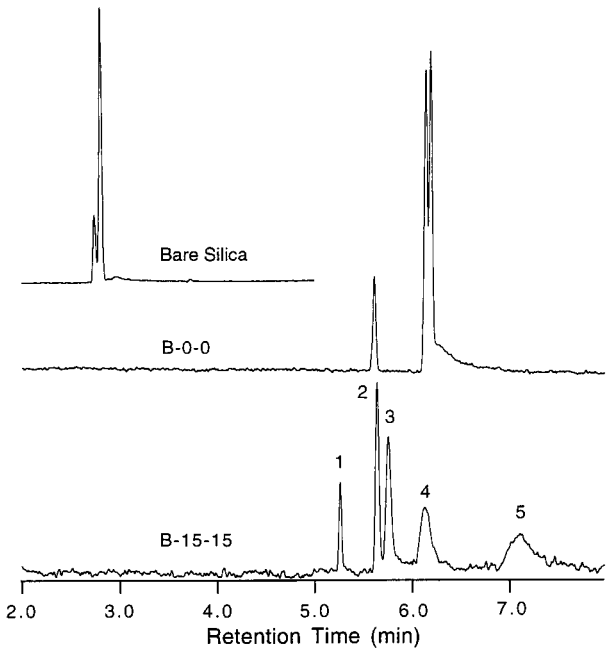

Figure 1. *Representative separation of the paraben mixture. Capillary: L_{TOTAL} = 60 cm, L_{DET} = 45 cm. Applied potential: 30 kV, positive polarity. Peaks: (1) acetone; (2) MeP; (3) EtP; (4) PrP; (5) BuP. Data were collected on an ATI CE system. Other conditions are listed in the text. The signal (Y) axis is compressed for the bare silica and B-0-0 capillaries.*

is in place and available for attachment of the polymer film. On column B-15-15, the four parabens are easily separated. Details on the chromatographic characteristics of the columns are discussed below.

Although there is sufficient evidence that there is a polymeric film bonded inside the capillary, the exact film thickness is unknown at present. Hydrodynamic methods based on the Poiseuille relation [2, 8], scanning electron microscopy [10], and mercury resistance methods [9] have been used to measure the inside diameter and film thickness of OTLC columns. Studies have shown that polymer films with similar chemistry to ours have a thickness of 0.3–2 μm [10]. Work is being carried out in our laboratory using both chromatographic methods and scanning electron microscopy to measure the film thickness and assess the uniformity of the film.

Effect of monomer and crosslinker concentrations on electroosmotic flow velocity. Reverse-phase OTCEC 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 the organic polymers themselves support a finite electroosmotic flow (EOF), which is at a lower magnitude than that for silica [14]. The EOF in our columns may arise from both sources. Using acetone in the test mixture as a marker for the flow rate, the electroosmotic flow velocities were calculated as follows:

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

where L_{DET} is the length from point of injection to point of detection, t_0 is the retention time of acetone. The results for the B-10-X columns are presented in Figure 2. In general, u_{eo} decreases with increasing crosslinker concentration, which suggests an increase in surface coverage by the polymethacrylate film. The flow velocity for B-10-7.5 was somewhat higher than expected. Two B-10-7.5 columns made separately produced the same result, and the standard deviation for the measurements was quite small. The large decrease in flow velocity from B-10-0 to B-10-2.5 suggested that the electroosmotic flows might arise predominantly from different sources in these two columns. It is suspected that the crosslinked polymer films provide for greater surface coverage than the linear films (as in B-10-0), reducing the fraction of electroosmotic flow which originates from the silica surface. The small error bars in Figure 2 indicate a high level of reproducibility in electroosmotic flow velocity.

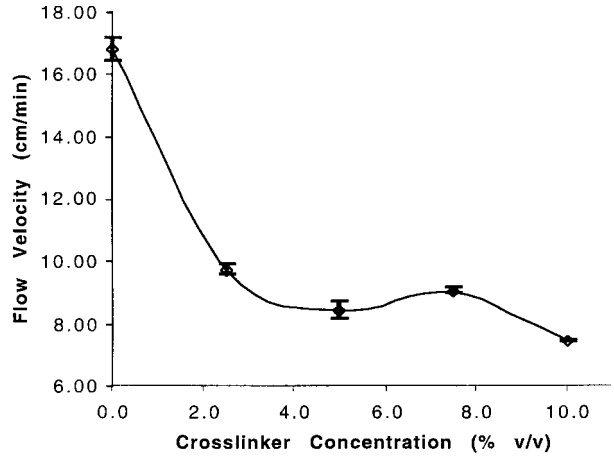

Figure 2. *Flow velocities for the B-10-X columns. Data points represent the mean of five measurements, error bars represent ±1 standard deviation. Capillary: L_{TOT} = 37 cm, L_{DET} = 30 cm. Potential applied: 30 kV constant, positive polarity. Measurements were performed using a Beckman CE system. Other experimental conditions are indicated in the text.*

The measured flow velocities in the B-X-5 columns are shown in Table II. No obvious trend was observed for these columns. Unlike the B-10-X columns, where the degree of crosslinking of the polymer film increased with crosslinker concentrations, the degree of crosslinking decreases in progression through the B-X-5 series while the concentration of monomer increases. These two factors may have different effects on the flow velocity; thus the total effect does not result in an obvious trend.

Effect of monomer and crosslinker concentrations on chromatographic performance parameters

Table II. *Electroosmotic flow velocities for B-X-5 columns.*[a]

Capillary number	Flow velocity (cm/min) (n = 5)	Standard deviation (n = 5)	% Relative standard deviation (n = 5)
B-0-0	8.37	0.48	5.7
B-0-5	3.01	0.32	10.6
B-2.5-5	9.31	0.31	3.3
B-5-5	8.61	0.19	2.2
B-7.5-5	3.37	0.14	4.2
B-10-5	8.08	0.03	0.37
B-12.5-5	9.31	0.04	0.43

[a]Capillary lengths: L_{TOT} = 60 cm, L_{DET} = 45 cm. Measurements performed on ATI CE system. Potential applied: 30 kV constant, positive polarity. Other experimental conditions are listed in the text.

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. Use of this chromatographic formula assumes a net electrophoretic migration of zero for each analyte. The mobilities of the species studied, while not exactly zero, are small enough to make this assumption reasonable. The capacity factors showed an interesting trend vs. crosslinker concentration in the B-10-X series columns (Figure 3). Values of k' initially increased with increasing crosslinker concentration, reaching a maximum at B-10-5, and then decreased. For B-X-5 columns, two maxima in k' were observed (Figure 4). At first glance, column B-5-5 appears to be an outlier; however, three B-5-5 columns made separately all yielded very similar test results. One might expect a continuous increase in k' with increasing monomer or crosslinker concentration. At this point, the source of the unusual trend in the k' data is not clear, though a similar trend (with a maximum at a given monomer concentration) is observed with linear polymer films [12].

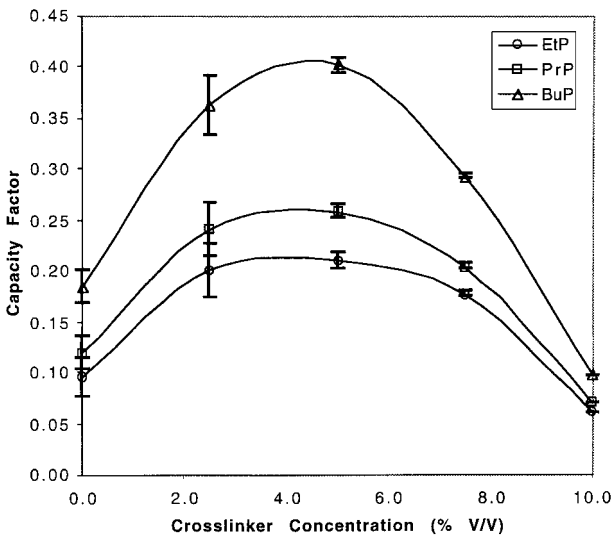

Figure 3. *Capacity factors for the B-10-X columns using parabens as test solutes. Data points represent the mean of five measurements, error bars represent ±1 standard deviation. Other experimental conditions are as indicated in Figure 1.*

These results indicated that simply varying the initial monomer concentration does not alter the selectivity of the stationary phase to a large degree. It is expected, though, that variation of alkyl chain length on the methacrylate monomer will enable the fine tuning of selectivity for this type of stationary phase. In addition, incorporation of pendant ionic groups in the polymer matrix or use of molecular imprinting techniques for stationary phase synthesis will provide unique selectivity for ion-exchange, affinity, and chiral separations. Research in this direction is currently underway in our laboratory.

Efficiency. The flow profile in OTCEC is plug-like because electroosmosis is the motive force for bulk transport, and thus high efficiencies are expected. Figure 5 indicates typical efficiencies obtained with B-10-X columns. The efficiencies of B-X-5 columns are shown in Figure 6. Data were calculated based on peak width at half height and retention time. Though 25-μm-i.d. capillaries are quite large for OTLC separations, efficiencies of up to 300,000 plates per meter were measured for the unretained acetone test probe. These efficiencies are comparable to those obtained with linear polymer films (270,000 plates per meter for a stationary phase produced from a 45% v/v monomer solution) [12]. Efficiencies for retained test solutes such as PrP are much lower than those for acetone. This is probably due to slow mass transfer between the mobile phase and the stationary phase, which leads to increased peak broadening for the retained analytes. The efficiencies shown here are by no means the highest achievable efficiencies for these columns.

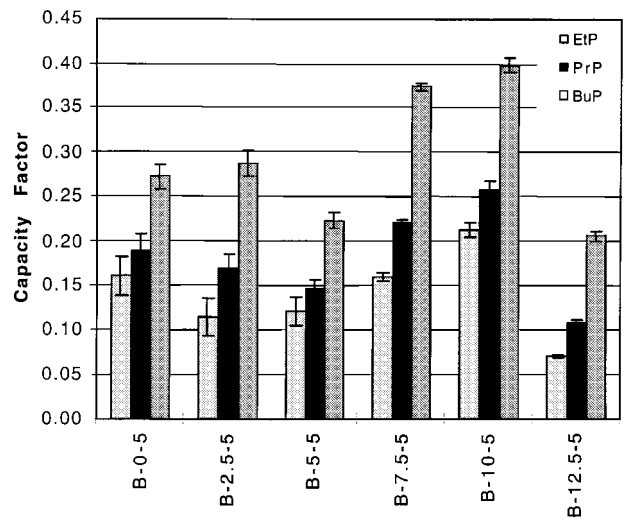

Figure 4. *Capacity factors for the B-X-5 columns. Data points represent the mean of five measurements, error bars represent ±1 standard deviation. Experimental conditions are as indicated in Table II.*

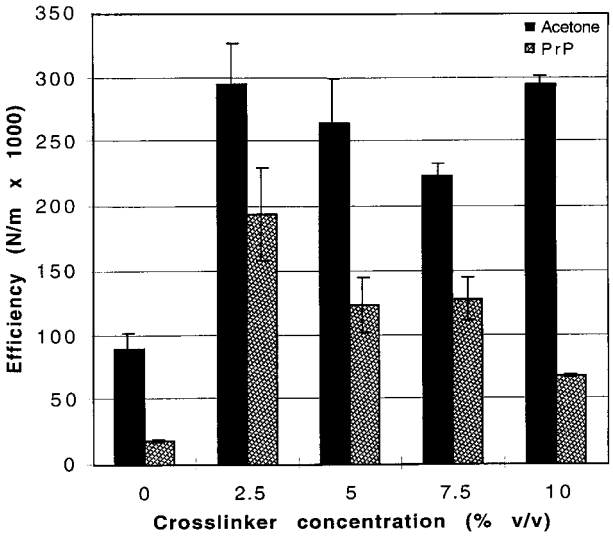

Figure 5. *Efficiency for the B-10-X columns. Calculations are based on peak width at half height. Experimental conditions are as in Figure 2.*

Because all columns were tested under the same conditions for the sake of comparison, little effort was made to optimize separation conditions. The capillary columns were necessarily overloaded for ease of detection.

Comparison of OTCEC and OTLC separations. Traditionally, a pump that can deliver constant flow is indispensable for any liquid chromatography separation. Even for OTLC studies, micro LC pumps or conventional LC pumps with flow splitting [3, 4], or home-made He gas pressurized solvent reservoirs with static splitting devices [5, 7, 8] are generally employed. We utilized the commercial ATI CE system, which is capable of applying variable head pressure (either positive or negative) to the inlet vial, to achieve pressure-driven OTLC separations without any modification to the instrument. A sample chromatogram is shown in Figure 7(A). Nearly complete separation of the paraben mixture was achieved by this simple method. This instrument is also capable of programming the pressure applied which can be utilized to improve separation. Another advantage of using a CE instrument for OTLC separation lies in the fact that no injection valve is needed. In addition, due to the low flow resistance of the open tubular column, only very low pressures are required to obtain the desired flow rate.

OTCEC is predicted to have high efficiency due to its flow profile, which is pluglike in comparison to the parabolic profile typical of OTLC. For comparison, separation of parabens at about the same bulk flow rate by these two techniques were carried out on the same column [Figures 7(A) and (B)]. It is easily seen that the early eluting peaks are much narrower in the OTCEC separation. Note that the paraben peaks were retained longer in the OTCEC chromatogram than in OTLC due to their finite negative electrophoretic mobilities. A more quantitative comparison of the efficiencies is given in Figure 8. Chromatograms C and D were obtained using

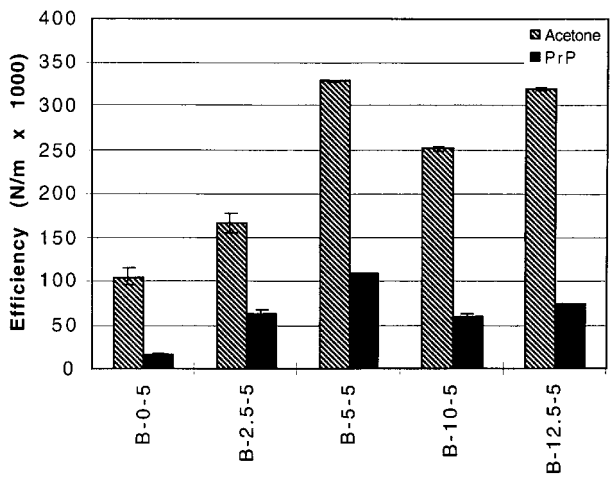

Figure 6. *Efficiency for the B-10-X columns. Calculations are based on peak width at half height. Experimental conditions are as in Table II.*

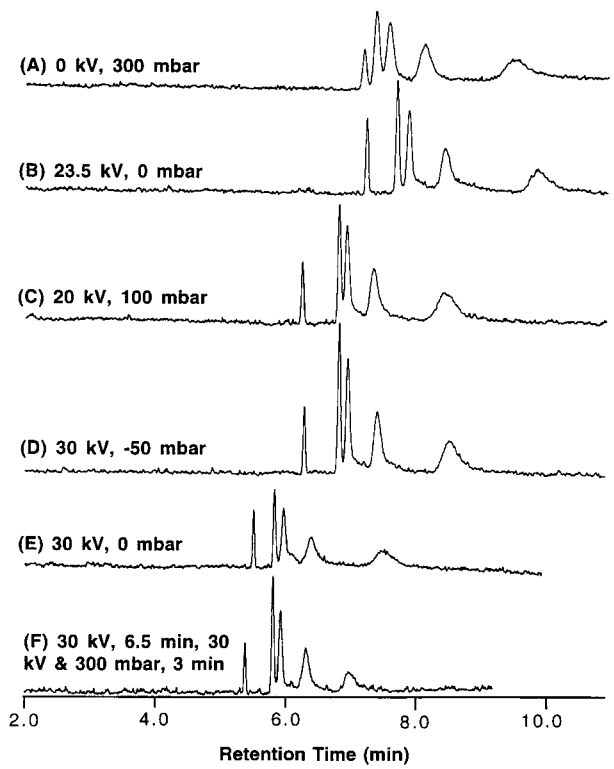

Figure 7. *Comparison of OTCEC and OTLC separations. Capillary: B-15-15, L_{TOTAL} = 60 cm, L_{DEC} = 45 cm. Other conditions are as in Figure 1.*

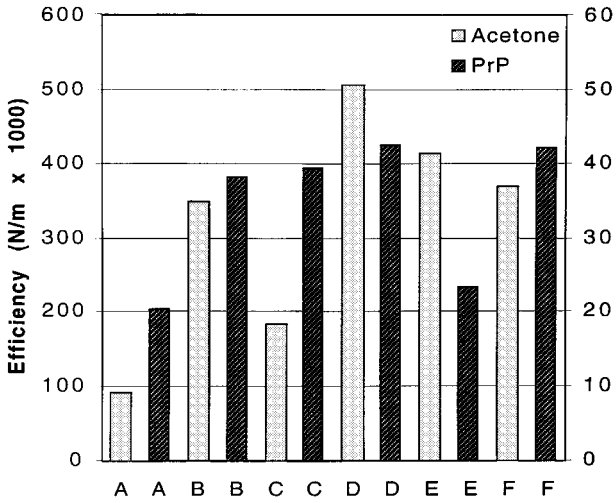

Figure 8. *Comparison of efficiencies for OTCEC and OTLC. Left axis: efficiencies for acetone, an unretained test probe. Right axis: efficiencies for PrP, a retained test probe. Experimental conditions are as displayed in Figure 7.*

combinations of voltage and pressure. They have almost identical flow rates, yet D demonstrates higher efficiency. This is most likely due to the fact that less pressure is applied in D, resulting in decreased flow velocity inhomogeneity. Chromatograms E and F demonstrate that the programmed combination of voltage and pressure can be manipulated to speed up late eluting peaks and achieve higher efficiencies. One can see from Figure 8 that efficiency for PrP is higher in F than in E.

CONCLUSIONS AND FUTURE DIRECTIONS

We have successfully applied the procedure developed in our laboratory to synthesize an immobilized polymethacrylate stationary phase in conventional polyimide-clad capillaries. Both linear and crosslinked polymer films can be prepared inside 25-μm-i.d. capillaries. Appreciable efficiencies were obtained given the large inner diameter capillaries employed and long analyte diffusion times. The stationary phases produced were stable enough to permit the use of strong mobile phases, greatly expanding the range of utility of this technique. Both OTCEC and OTLC separations can be performed on these columns, which makes the open tubular columns more competitive with packed capillaries. In addition, the selectivity of the polymeric stationary phases can be adjusted by altering the monomer and crosslinker concentrations and chemistries and by incorporating other functional groups in the polymer matrix. Application of this in situ thermal polymerization technique to fabricate stationary phases capable of conducting ion-exchange, chiral, or affinity separations is currently underway in our laboratory.

Open tubular columns with bonded polymeric stationary phases prepared using the method described here may be employed in both OTCEC and OTLC separations. Both techniques are easily conducted on commercial CE instruments, which makes the application of these high efficiency columns more cost effective.

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