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Capillary electrochromatography of methylated benzo[a]pyrene isomers
II. Effect of stationary phase tuning

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Abstract

For Part II of our ongoing study, we present a strategy for stationary phase optimization for the capillary electrochromatographic (CEC) separation of the 12 methylated benzo[a]pyrene (MBAP) isomers. Utilizing the optimum mobile phase conditions from Part I of our study as a guide, seven commercially available stationary phases have been evaluated for their ability to separate highly hydrophobic MBAP isomers. Ranging in design from high-performance liquid chromatography (HPLC) to CEC application, each phase was slurry packed in house and tested for CEC suitability and performance. Several stationary phase parameters were investigated for their effects on MBAP separation including bonding type (monomeric or polymeric, % carbon loading, surface coverage), pore size, particle size, and type of alkyl substituent. In this manner, the present state of commercially available packings has been assessed in our laboratory. Utilizing the optimum polymeric C_{18}-5 μm-100 Å-PAH stationary phase, the effects of CEC packed bed length and capillary inside diameter (I.D.) were also evaluated. A 50 μm I.D. capillary, 25 cm packed bed length and 75% (v/v) acetonitrile, 12.5 m*M* Tris, pH 8.0, 20 °C at 30 kV, provided resolution of 11 out of 12 MBAP isomers thus showing the effectiveness of CEC for analysis of structurally similar methylated polyaromatic hydrocarbons.

Keywords: Stationary phase optimization; Stationary phases, electrochromatography; Electrochromatography; Methylbenzo[a]pyrene isomers

1. Introduction

In recent years one of the most exciting developments in the field of capillary scale separation has been the advent of capillary electrochromatography (CEC). The use of both open and packed capillary columns is feasible in CEC, in which the mobile phase is electroosmotically driven through the column at high field strengths with instrumentation similar to that used for capillary zone electrophoresis (CZE). As a consequence, CEC has provided excellent separation efficiency and high peak capacity, which has opened the door to the separation of compounds that differ minimally in their structure.

CEC is rapidly growing in popularity as a separation technique capable of analysing structurally

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similar positional and geometrical isomers of polycyclic aromatic hydrocarbons (PAHs) [1–4]. However, application of CEC for developing selective separations of polychlorinated biphenyls (PCBs), tetrachlorodibenzodioxins (TCDDs) and various industrial chemicals will require development of CEC compatible stationary phases for improved separation performance. In addition, much of the efficacy of CEC both for understanding fundamental principles of solute shape recognition and in the development of the aforementioned application lies in the optimization of mobile phase and/or stationary phase conditions that can be useful to researchers both in academia and in industry.

The present study extends our earlier work (Part I, Ref. [5]) by examining in detail the effect of varying stationary phase properties on improving the very difficult separation of 12 methylated benzo[a]pyrene (MBAP) isomers. In this regard, seven stationary phases commonly used for either CEC or reversed-phase high-performance liquid chromatography (HPLC) were obtained from a commercial source. Each phase was then slurry packed into fused-silica capillary in our laboratory. These stationary phases were tested for their ability to separate 12 MBAP isomers under the optimum mobile phase conditions described in Part I of our study. In addition, various stationary phase parameters, such as the type of bonding chemistry (monomeric or polymeric, % carbon loading, surface coverage), pore size, particle size, ligand chain length, and column dimensions (packed bed length, inner diameter) were studied to optimize the CEC of MBAP isomers.

2. Experimental

2.1. Reagents and chemicals

These are essentially the same as in Ref. [5]. The monomeric and polymeric stationary phases were purchased from Column Engineering (Ontario, CA, USA).

2.2. CEC instrumentation

CEC instrumentation was as described in Part I [5].

2.3. Column preparation

Capillary columns were packed according to the method described in a previous paper [5].

2.4. Buffer and sample preparation

Buffer and sample preparation were as described in Part I [5].

3. Results and discussion

A total of seven stationary phases were evaluated in this study (Table 1). For the monomeric phases, we investigated the effect of particle size and bonded group (C_8, C_{18} and phenyl groups). For the polymeric phases, both particle and pore size were studied using C_{18} bonded group. In addition, the % carbon loading and surface coverage specifications of each phase are also summarized in Table 1. The stationary phase that provided the optimum MBAP separation was utilized for further study to investigate the effects of varying the volume fraction of acetonitrile (ACN) in small increments. Finally, the packed bed length and the inside diameter of the capillary were evaluated.

3.1. CEC separation of standard test mixture

All seven packed CEC columns were initially tested by running a neutral test mixture. This was conducted in order to establish correct operation of the column. A brief summary of the chromatographic performance of the seven stationary phases for neutral test mixture separation is provided in Tables 2 and 3. Thiourea was chosen as a model analyte marking the dead time of the column. When the runs were performed under identical mobile phase conditions of 80% (v/v) ACN–20% (v/v) 12.5 m*M* Tris, pH 8.0 buffer, highly efficient separations are achieved as qualitatively illustrated in Figs. 1 and 2, while chromatographic data is reported in Tables 2 and 3.

3.1.1. Comparison of monomeric phases

A comparison of four monomeric stationary phases using a mixture of five neutral test solutes is

Table 1
Commercial stationary phases

Stationary phase	Particle size (μm)	Pore size (Å)	% Carbon loading[a]	Surface coverage (μmol/m^2)	Endcapping
Reliasil Monomeric-CEC-C_{18} (M-CEC-C_{18}-3 μm-100 Å)	3	100	14.0	2.5	No
Reliasil Monomeric-C_{18} (M-C_{18}-5 μm-100 Å)	5	100	13.9	2.5	Yes
Reliasil Monomeric-C_8 (M-C_8-5 μm-100 Å)	5	100	8.0	2.5	Yes
Reliasil Monomeric-Phenyl (M-phenyl-5 μm-100 Å)	5	100	5.3	2.6	Yes
Reliasil Polymeric-C_{18} (P-C_{18}-3 μm-100 Å)	3	100	15.9	2.8	Yes
Reliasil Polymeric C_{18}-PAH (P-C_{18}-5 μm-100 Å-PAH)	5	100	16.1	2.8	Yes
Reliasil Polymeric-C_{18} (P-C_{18}-5 μm-300 Å)	5	300	6.7	2.7	Yes

[a] % Carbon loading and surface coverage data provided by Column Engineering.

shown in Fig. 1 and Table 2. There are several trends observable in terms of analysis time, efficiency, resolution, selectivity and the elution window. First, when increasing the particle size from 3 to 5 μm (M-CEC-C_{18}-3 μm-100 Å to M-C_{18}-5 μm-100 Å), while maintaining the same pore size and ligand properties (Fig. 1a,b), both the analysis time and width of the elution window are doubled. Since the % carbon loading and surface coverage are essentially the same for both of these phases (Table 1), the shorter analysis time of the M-CEC-C_{18}-3 μm-100 Å phase can be attributed to a faster electroosmotic flow (EOF) resulting from non-endcapped silanols that are responsible for generating EOF. Second, comparing monomeric phases of the same particle and pore size as well as similar surface coverage (Fig. 1b–d), the fastest separation is achieved with monomeric phenyl phase (M-phenyl-5 μm-100 Å) with the lowest % carbon loading which consequently generates the highest EOF. Generally, the retention time of all neutral solutes decreases as the alkyl substituent on the packing surface is decreased due to a concomitant decrease in % carbon loading (Table 1), which is expected. This is a similar trend as observed in HPLC, for which Sander and Wise reported that retention is observed to increase with an increase in % carbon loading [6]. Furthermore, under the experimental conditions, columns packed with phenyl substituent provided higher peak efficiency (sharper peaks) because of decreased retention time for all four test solutes as compared to C_8 and C_{18} substituent of the same particle size (Fig. 1d vs. b,c).

Two critical pairs of analytes (dimethylpthalate/diethylpthalate (DMP/DEP) and biphenyl/*o*-terphenyl (BP/TP)) are used to compare *N*, α and *Rs* trends (Table 2). Noticably, higher plate numbers (*N*) are observed (~221 000 plates/m) with 3-μm smaller particles containing C_{18} phase compared to 5-μm C_{18} phase particles. However, this is offset by lower *Rs* and α values of critical pairs of DMP/DEP and BP/TP using the former phase. The highest efficiency achievable with smaller particle size can be attributed to reduced eddy diffusion and resistance to mass transfer [7]. The shorter ligand M-C_8-5 μm-100 Å phase demonstrates good separation, however, its *Rs* and *N* values are significantly lower than M-CEC-C_{18}-3 μm-100 Å or M-C_{18}-5 μm-100 Å phases most probably due to lower carbon content (Table 1). Finally, M-phenyl-5 μm-100 Å phase shows the narrowest elution window represented by significantly lower k' and *Rs* values, however, higher plate numbers (~180 800 plates/m) and reasonable α values are still observed. Overall, when comparing the electropherograms in Fig. 1 and the data in Table 2, it was concluded that the M-CEC-C_{18}-3 μm-100 Å phase provided the best separation of test solutes as it provided the highest peak efficiencies with

Table 2
Comparison of capacity factor (k') selectivity factor (α), resolution (Rs), and efficiency for four different monomeric CEC columns[a]

	k'	α	Rs	N
M-CEC-C_{18}-3 μm-100 Å				
Thiourea (t_0 = 3.18 min)	–	–	–	–
Dimethylphthalate	0.15	–	–	50 100
Diethylphthalate	0.23	1.53	4.01	47 100
Biphenyl	0.51	–	–	55 300
o-Terphenyl	0.77	1.51	9.11	45 200
M-C_{18}-5 μm-100 Å				
Thiourea (t_0 = 3.54 min)	–	–	–	–
Dimethylphthalate	0.27	–	–	28 500
Diethylphthalate	0.47	1.74	5.78	22 400
Biphenyl	1.26	–	–	33 300
o-Terphenyl	2.28	1.81	15.90	28 000
M-C_8-5 μm-100 Å				
Thiourea (t_0 = 3.56 min)	–	–	–	–
Dimethylphthalate	0.24	–	–	7900
Diethylphthalate	0.38	1.58	2.45	8700
Biphenyl	0.70	–	–	14 600
o-Terphenyl	1.09	1.56	6.68	20 200
M-phenyl-5 μm-100 Å				
Thiourea (t_0 = 2.68 min)	–	–	–	–
Dimethylphthalate	0.063	–	–	37 100
Diethylphthalate	0.078	1.24	0.71	30 000
Biphenyl	0.11	–	–	45 200
o-Terphenyl	0.15	1.36	1.90	44 000

[a] Columns: 25.0 cm packed (37.4 cm total)×75 μm I.D.; electrolyte: Tris–HCl (pH 8)–ACN (20:80) (ionic strength 12.5 m*M*); applied voltage: +30 kV; UV detection: 254 nm; electrokinetic injection: 5 s (+5 kV) sample+3 s (+5 kV) running buffer.

reasonable resolution. Therefore, this phase was also expected to provide the optimum separation of MBAP isomers. However, this expectation was based upon less hydrophobic solutes. For more hydrophobic solutes that differ minimally in their structure (e.g. MBAP isomers), one would expect that the phase with the highest Rs and α values (with reasonable N) would be most suitable. Furthermore, the separation of 12 solutes (MBAPs) instead of five (test solutes) should require a wider elution window. From this point of view, it was expected that the M-C_{18}-5 μm-100 Å would provide the optimum separation of MBAP isomers.

Table 3
Comparison of capacity factor (K) selectivity factor (α), resolution (Rs), and efficiency for three different polymeric CEC columns[a]

	k'	α	Rs	N
P-C_{18}-3 μm-100 Å				
Thiourea (t_0 = 4.04 min)	–	–	–	–
Dimethylphthalate	0.28	–	–	51 100
Diethylphthalate	0.49	1.75	8.58	50 000
Biphenyl	1.28		–	55 000
o-Terphenyl	2.29	1.79	20.85	51 300
P-C_{18}-5 μm-100 Å-PAH				
Thiourea (t_0 = 3.24 min)	–	–	–	–
Dimethylphthalate	0.25	–	–	30 300
Diethylphthalate	0.43	1.72	5.11	18 200
Biphenyl	1.28	–	–	33 600
o-Terphenyl	2.15	1.70	13.86	27 800
P-C_{18}-5 μm-300 Å				
Thiourea (t_0 = 6.89 min)	–	–	–	–
Dimethylphthalate	0.08	–	–	2700
Diethylphthalate	0.14	1.82	0.75	3500
Biphenyl	0.33	–	–	5100
o-Terphenyl	0.60	1.79	2.91	3700

[a] Conditions as in Table 2.

3.1.2. Comparison of polymeric phases

Beside monomeric phases, three polymeric stationary phases were also evaluated for the separation of the neutral test mixture. A CEC comparison under identical operating conditions for the test mixture on three different packing materials is shown in Fig. 2. Interestingly, the α values of the critical pairs exhibited by the three phases are very similar, as demonstrated in Table 3. The neutral test analytes were retained for a shorter time on the P-C_{18}-5 μm-100 Å-PAH phase (Fig. 2b) as compared to the P-C_{18}-3 μm-100 Å phase (Fig. 2a). The retention data are not consistent with the data on % carbon loading which seems to be slightly higher for the former PAH phase (Table 1). However, the polymeric C_{18} phase with the lowest carbon content and largest pore size of 300 Å (P-C_{18}-5 μm-300 Å) provided the lowest k', Rs and N values (Fig. 2c and Table 3). In addition, the test solutes eluted in a much narrower elution window on the P-C_{18}-5 μm-300 Å phase than on the P-C_{18}-(3 μm or 5 μm)-100 Å phase. Since the surface area (2.7–2.8 μmol/m^2) is nearly the same for these polymeric phases, the

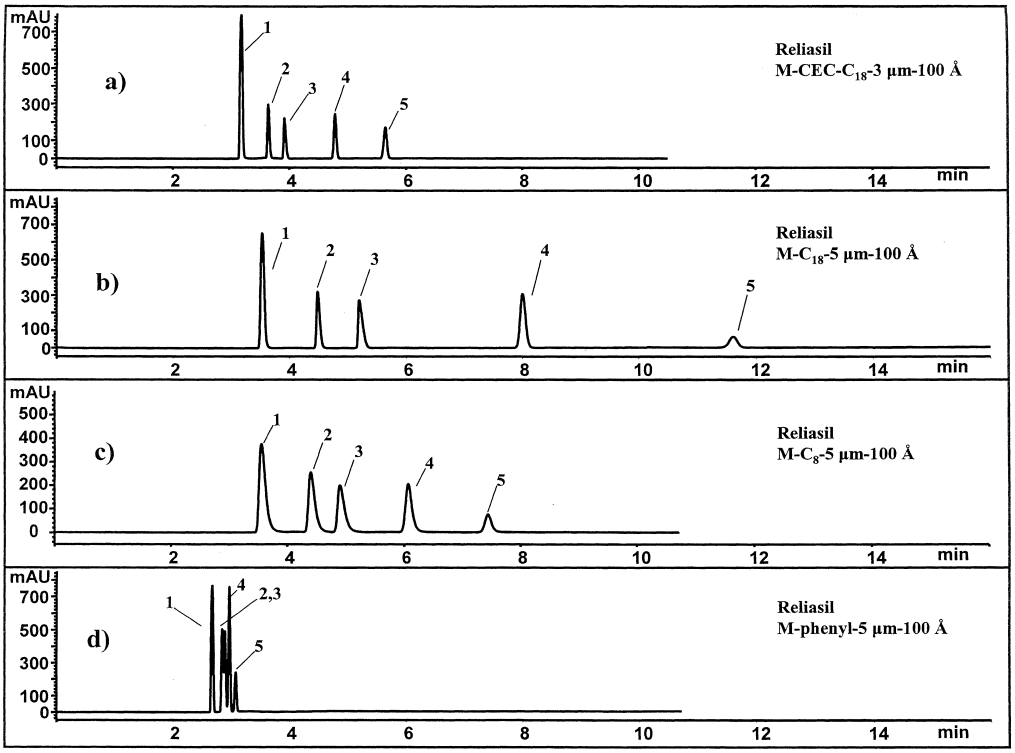

Fig. 1. Electrochromatograms showing the CEC separation of the test mixture on four different monomeric reversed-phases under identical mobile phase conditions. Conditions: 80% (v/v) ACN–20% (v/v) 12.5 m*M* Tris; pH 8.0; 20 °C; 30 kV.

poor chromatographic performance of the P-C_{18}-5 μm-300 Å phase was attributed to very low % carbon loading and weaker EOF under conditions of 80% (v/v) ACN. This is due in part to poor wetting, therefore requiring a higher concentration of ACN to properly wet the stationary phase and maintain stable CEC current. To test this theory, the test mixture was run at higher volume fraction, i.e. 90% (v/v) ACN. As shown in Fig. 2c inset at 90% (v/v) ACN, not only the peak shape but also the efficiency of the test solutes were dramatically improved. Although several reports have shown improved *N* with macroporous particles [8], in most studies it was demonstrated that this effect was not significant until pore size was above 500 [9] and even 1000 Å [10].

Although the two best polymeric 100-Å stationary phases of 3- or 5-μm particle diameter (P-C_{18}-3 μm-100 Å or P-C_{18}-5 μm-100 Å-PAH) provided equally good CEC separations of the neutral test mixture (Fig. 2a,b), the test solutes were retained for a shorter time on P-C_{18}-5 μm-100 Å-PAH phase (Fig. 2b) compared to P-C_{18}-3 μm-100 Å phase (Fig. 2a). This lower retention of test analytes on the former stationary phase is accompanied by lower *Rs* and *N* values (Table 3).

In general, the differences between the CEC separations of the neutral test mixture on both monomeric and polymeric phases are not overwhelming. However, due to relatively greater surface coverage of the polymeric phases as compared to the monomeric phases (Table 1), a slower EOF is achieved with the polymeric phases (Table 3), with the exception of one polymeric PAH phase (P-C_{18}-5 μm-100 Å-PAH), which does not follow the trend and exhibited a dead time closer to that of the monomeric phases (Table 2). It is interesting to note that the two smaller (3-μm particle diameter) monomeric and polymeric phases exhibited almost identi-

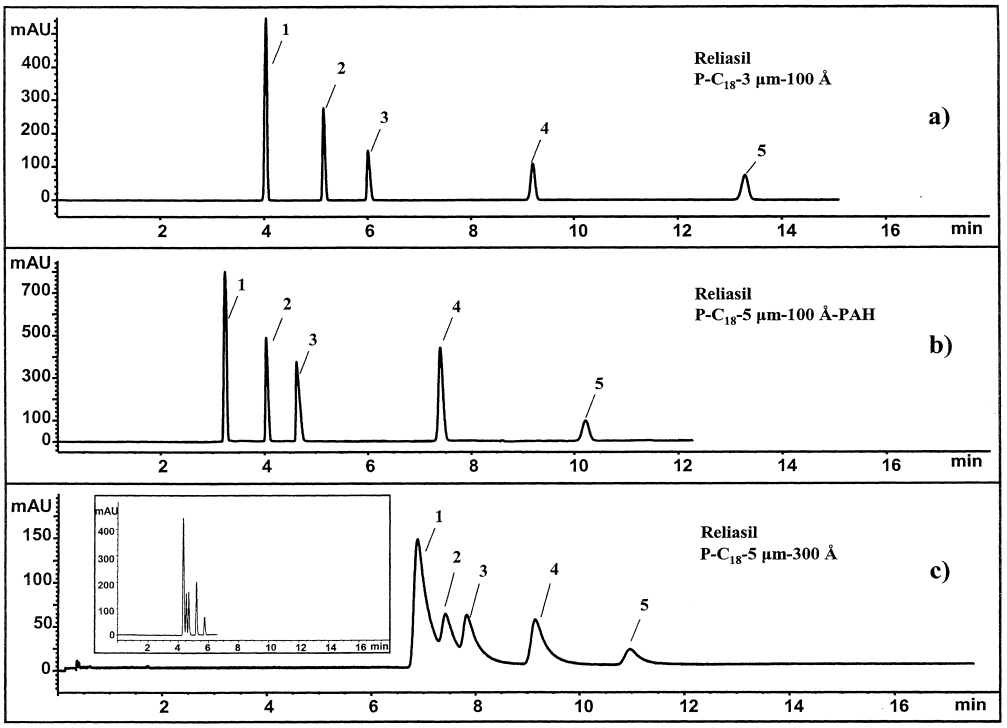

Fig. 2. Electrochromatograms showing the CEC separation of the test mixture on three different polymeric reversed phases under identical mobile phase conditions. Conditions are the same as in Fig. 1, except (c) inset was 90% (v/v) ACN–10% (v/v) 12.5 m*M* Tris.

cal *N* values. However, when increasing the particle size from 3 to 5 μm on both monomeric and polymeric phases, opposite trends in *Rs*, analysis time and elution range were observed. For example, for the polymeric phases, the *Rs* values between critical solute pairs (DMP/DEP and BP/TP) are significantly lower using 5-μm particle diameter compared to 3-μm particle diameter. This is the opposite trend as compared to monomeric phases where a smaller particle size provided lower *Rs* between the same test solute pairs (Table 2).

3.2. CEC separation of MBAP isomers

Following the test mixture separation, each of the seven stationary phases were tested for their ability to separate 12 MBAP isomers. Optimum mobile phase conditions from Part I of our study indicated that 75% (v/v) ACN–25% (v/v) 12.5 m*M* Tris, pH 8.0, provided the best chromatographic separation of MBAP isomers. In Part I of our study, the temperature and voltage were also optimized, where it was concluded that 20 °C and 30 kV afforded the best resolution (data not shown). Therefore, all of these parameters were maintained for Part II, with the exception of ACN content, which was varied in small increments for all monomeric and polymeric phases in order to optimize the separation. In this manner, we were able to assess the suitability of each stationary phase for CEC application with regards to separating capability of MBAP isomers, and the ability to maintain stable current and EOF over a wide range of ACN fractions. Furthermore, it was found that some stationary phases were not able to maintain stable current and EOF when typically <80% (v/v) ACN was used in the mobile phase. Therefore, we have compared the optimum operating CEC conditions for separation of MBAP isomers in Figs. 3 and 4.

3.2.1. Comparison of monomeric phases

The four monomeric phases were evaluated for

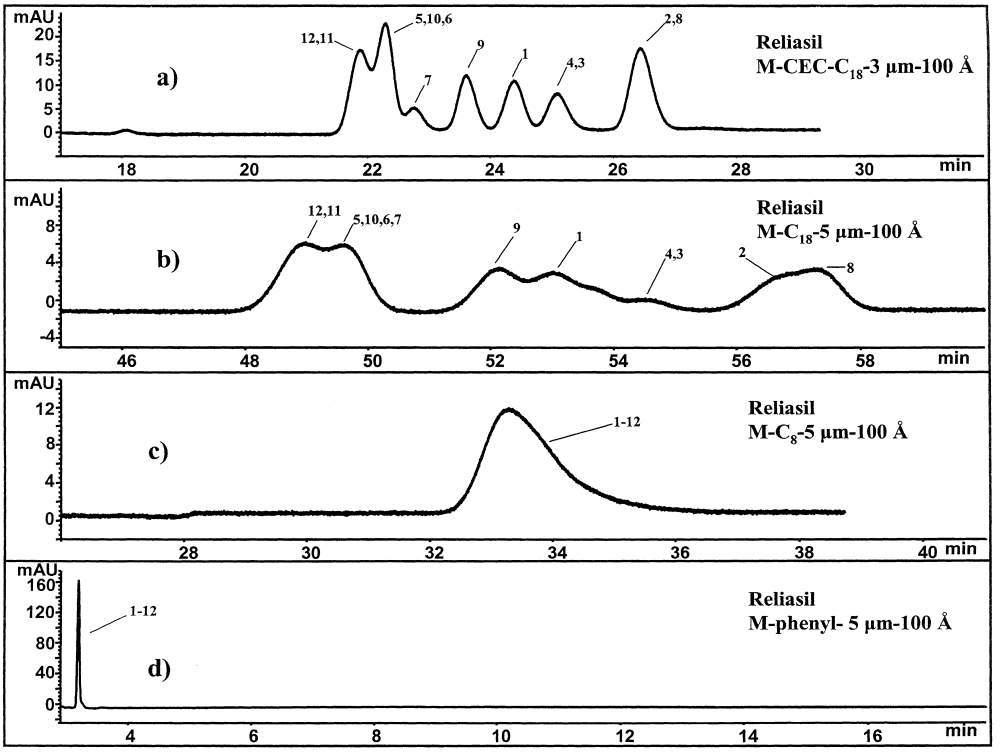

Fig. 3. Electrochromatograms showing the CEC separation of 12 MBAP isomers on four different monomeric reversed phases. Conditions are the same as in Fig. 1, except for 75% (v/v) ACN in (a), (b), and (c) and 80% (v/v) ACN in (d).

MBAP isomer separation as shown in Fig. 3a–d. Reliasil M-CEC-C_{18}-3 μm-100 Å phase is designed specifically for CEC with uncapped residual silanols (Table 1). This monomeric stationary phase was able to maintain the most stable and robust current over the widest range of ACN as compared to the other monomeric phases investigated in this study. In general, CEC phases have been shown to provide faster separations [11], which is consistent with our results. Hence, shorter retention with higher *N* of MBAP isomers was observed using the M-CEC-C_{18}-3 μm-100 Å phase (Fig. 3a) even though the surface coverage and % carbon loading of this phase are essentially the same as M-C_{18}-5 μm-100 Å phase (Table 1). This suggests that faster EOF of the CEC phase is predominantly responsible for improved chromatographic performance. The effect of increasing the particle size from 3 to 5 μm (while maintaining same pore size of 100 Å and surface coverage of 2.5 μmol/m^2 as well as similar carbon content) provided no improvement in *Rs* and α values of neighboring peaks of MBAP isomers (Fig. 3b) and is counter to expectations. This is because the data shown in Table 2 indicated that both *Rs* and α values of critical test solute pairs of similar hydrophobicity are higher for M-C_{18}-5 μm-100 Å phase than for M-CEC-C_{18}-3 μm-100 Å phase. As shown in Fig. 3b, MBAP isomers are barely resolved at optimum mobile phase conditions. Raising the volume fraction of ACN increased *N* values but was offset by a loss in *Rs* values and coelution of several early eluting MBAP isomers (data not shown).

The separating capability of M-C_8-5 μm-100 Å phase for MBAP isomers was very poor, as was expected for a shorter alkyl chain length with a low carbon content (Table 1). This phase exhibited weaker molecular interactions between the C_8 ligands and MBAPs, as was evident by one peak representing the coelution of all 12 isomers in Fig. 3c. Previously, C_8 phase has shown some utility for CEC separations of substituted barbiturates [12], but in general has been reported to have inferior resolv-

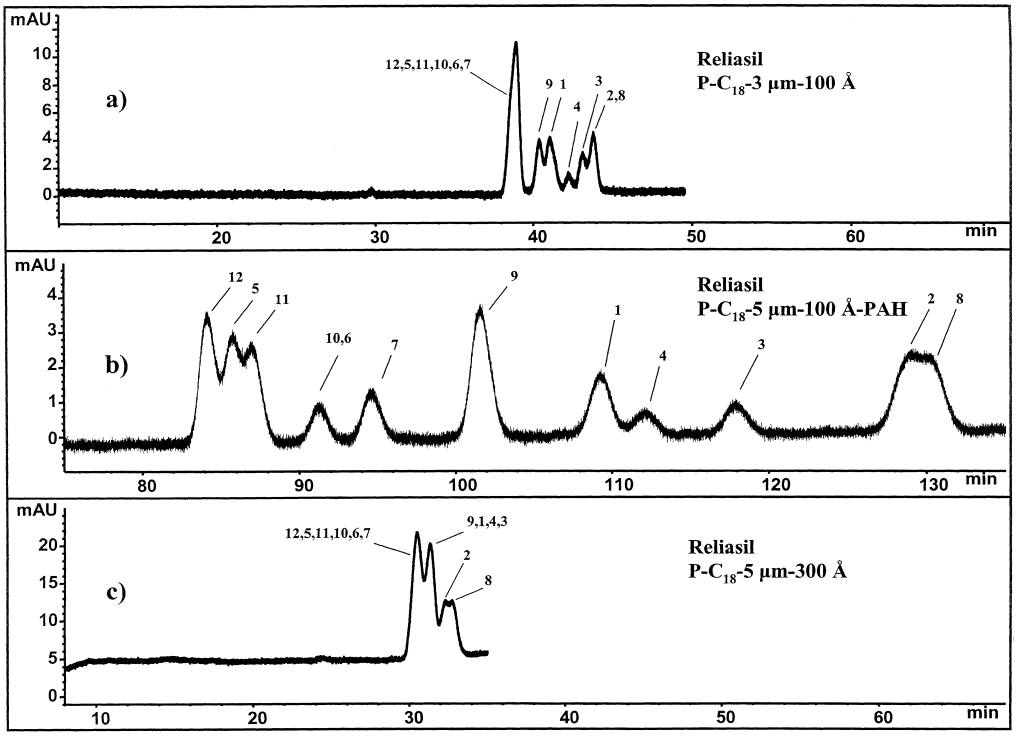

Fig. 4. Electrochromatograms showing the optimized CEC separation of 12 MBAP isomers on three different polymeric reversed phases. (a) 80% (v/v) ACN–20% (v/v) 12.5 m*M* Tris, pH 8.0; (b) 75% (v/v) ACN–25% (v/v) 12.5 m*M* Tris, pH 8.0; and (c) 80% (v/v) ACN–20% (v/v) 12.5 m*M* Tris, pH 8.0.

ing capability as compared to C_{18} [13,14]. Since no selectivity of MBAP isomers was observed in the range of ACN fractions studied, no further investigation was conducted for this phase.

Phenyl stationary phase was studied with the hopes of seeing separation due to π–π interaction between the phenyl ring and the MBAP aromatic rings. In the literature, the π–π retention mechanism was reported to be partly responsible for separation of a series of smaller ring benzodiazepines by Cahours et al. [15]. To our surprise, however, this phase exhibited no selectivity towards MBAP isomers. As shown in Fig. 3d, not only did all isomers coelute as a single band, but they also exhibited extremely short retention time. Similar to C_8 phase, this is due to the % carbon loading which is overall lowest among all stationary phases studied (Table 1). After seeing no selectivity using either C_8 or phenyl phases, no further study of these phases was pursued.

3.2.2. Comparison of polymeric phases

The three polymeric stationary phases were next studied under optimum mobile phase environment (Fig. 4a–c). Polymeric phases are known for enhanced shape recognition, thus being well suited to PAH separations. Sander and Wise attribute this to the fact that polymeric C_{18} phases typically have phase densities (i.e. carbon loading) nearly twice that of monomeric C_{18} phases [6]. However, in this study, the surface coverage (obtained from the manufacturer) shows that the carbon content of the two polymeric phases of similar alkyl chain and pore size is only slightly higher compared to the monomeric phases (Table 1). First, a small particle size polymeric phase was investigated using P-C_{18}-3 µm-100 Å material. Optimum separation for this phase was achieved at 80% (v/v) ACN. A comparison of the performance of polymeric stationary phases of different particle sizes but constant pore size for

MBAP isomers is illustrated in Fig. 4a,b. In contrast to the monomeric phases, the results of the polymeric phases with the same surface coverage show that the latter phases with a larger particle size (e.g. P-C_{18}-5 μm-100 Å-PAH with 16.1% carbon content) have significantly higher selectivity with a much wider elution window compared to the phase with a smaller particle size (e.g. P-C_{18}-3 μm-100 Å phase with 15.9% carbon content) (Table 1). For example, compared to the separation of only six MBAP isomers on P-C_{18}-3 μm-100 Å phase (Fig. 4a), the use of large particle size, P-C_{18}-5 μm-100 Å-PAH phase shows dramatic selectivity for ten MBAP isomers (Fig. 4b).

The effect of varying pore size was also studied utilizing a P-C_{18}-5 μm-300 Å phase. For HPLC [6] and CEC [16], increasing the pore size on polymeric C_{18} phases has been established to have pronounced effects on selectivity and efficiency, respectively. In our CEC application of MBAP isomers, stable current was only obtained when ACN fractions in the mobile phase were ≥80% (v/v). A larger pore size of 300 Å provided poor resolution of MBAP isomers with a very narrow elution window (Fig. 4c) due in part to significantly lower % carbon loading (Table 1). Furthermore, no appreciable gain in selectivity or efficiency of MBAPs was offered by varying the ACN content using this stationary phase (data not shown).

3.2.3. Effect of type of stationary phase on the electroosmotic flow (EOF)

One of the most influential properties of the column packing material in CEC is the ability to support EOF [17]. The effect of varying the ACN volume fraction on the electroosmotic mobility (μ_{eo}) for MBAP separation using six of the seven stationary phases is shown in Fig. 5, while maintaining all other optimized separation conditions. It was observed that some stationary phases were more capable of maintaining stable EOF and CEC current over a wider ACN (v/v) range than others. We found that three monomeric phases provided stable EOF and CEC current over the range of 75–95% (v/v) ACN. However, the phenyl monomeric stationary phase was omitted from the plot as it provided extremely short retention and coelution of MBAPs,

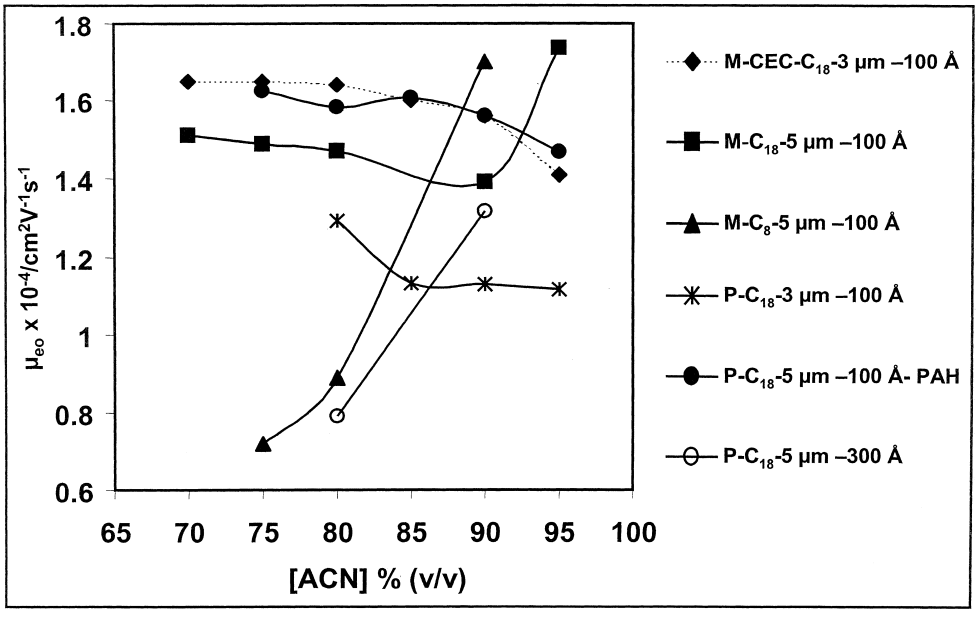

Fig. 5. The effect of ACN volume fraction on the electroosmotic mobility (μ_{eo}) for separation of 12 MBAP isomers on monomeric and polymeric stationary phases. All other conditions are the same as in Fig. 1.

and was only run at one volume fraction of ACN (80% (v/v) ACN; $\mu_{eo}=1.95\times10^{-4}/cm^2\ V^{-1}\ s^{-1}$).

Two of the monomeric phases, the M-CEC-C_{18}-3 μm-100 Å and M-C_{18}-5 μm-100 Å, provided very strong and stable μ_{eo} in the range of 70–90% (v/v) ACN. However, opposite trends in μ_{eo} were observed when increasing from 90 to 95% (v/v) ACN. For example, the larger M-C_{18}-5 μm-100 Å phase showed a large increase in μ_{eo}, which is in accordance with the greater dielectric constant to viscosity (ε_D/η) ratio reported for ACN [18]. Conversely, the μ_{eo} of the M-CEC-C_{18}-3 μm-100 Å phase decreased, possibly due to unfavorable interaction of ACN with exposed silanol groups responsible for promoting the EOF. However, when comparing both aforementioned monomeric phases over the same range of ACN, the effect of increasing the particle size from 3 to 5 μm lowered the μ_{eo}. This is contrary to other investigators who report either no change or a decrease in μ_{eo} with decreasing particle size [19,20]. This variation in μ_{eo} trend is most likely attributed to the differences in surface chemistries of the packings, i.e. endcapping, thus providing differences in the zeta potential. For the shorter ligand M-C_8-5 μm-100 Å phase, a significant increase in μ_{eo} was noticed with increase in ACN from 80 to 90% (v/v) which seems to correlate well with the lower carbon content of this phase (Table 1).

Polymeric stationary phases exhibited significant differences in μ_{eo} values. The P-C_{18}-5 μ-100 Å-PAH phase demonstrated the highest mobility values compared to the other polymeric phases tested. Upon decreasing the particle size of polymeric phase from 5 to 3 μm, a large drop in μ_{eo} was observed, which is in accordance with other reports [20]. For the largest 300 Å phase (P-C_{18}-5 μm-300 Å), very low μ_{eo} was observed at 80% (v/v) ACN which was largely responsible for peak tailing of test solutes observed in Fig. 2c. However, peak tailing was not as significant for the MBAPs as shown in Fig. 4c. Furthermore, a significant increase in μ_{eo} was observed upon raising the ACN fraction from 80 to 90% (v/v), similar to the monomeric C_8 phase.

A comparison of the EOF profiles between the best monomeric CEC phase (i.e. M-CEC-C_{18}-3 μm-100 Å) and the best polymeric phase (i.e. P-C_{18}-5 μm-100 Å-PAH) for MBAP separation shows that several similarities are apparent. Interestingly, the polymeric PAH phase showed an almost identical μ_{eo} profile to the monomeric CEC phase. This is surprising since the two phases possess different surface chemistry. Specifically, the polymeric PAH phase has relatively higher % carbon loading and surface coverage with endcapped silanols (Table 1), while the monomeric phase is non-endcapped. Therefore, it was expected that the monomeric CEC phase should have exhibited a higher μ_{eo} than the polymeric PAH phase. Nevertheless, both of these phases also exhibited the overall flattest μ_{eo} profile as compared to other phases tested. In addition, a similar drop in μ_{eo} for the two phases was observed in the range of 90–95% (v/v) ACN.

3.2.4. Comparison of optimum monomeric and polymeric stationary phases

The capacity factor (k') provides an accurate representation of analysis time and the differences in k' value can provide the width of the migration window. A comparison of k' values for MBAP isomers at varying ACN volume fraction under identical conditions is shown for the best monomeric and polymeric phases in Fig. 6a,b, respectively. For the monomeric CEC phase, at 70% (v/v) ACN, the width of the elution window, $\Delta k'$, was 2.08, which decreased to 0.25 at 95% (v/v) ACN. For the polymeric PAH phase, $\Delta k'=14.31$ at 75% (v/v) ACN, which then decreased to 2.29 at 95% (v/v) ACN. Comparison at optimum 75% (v/v) ACN for MBAP separation revealed that $\Delta k'$ for monomeric phase was ~1.43 as compared to 14.31 for polymeric phase. Therefore, a much wider elution window (~10-fold) is observed with the polymeric phase which is consistent with relatively higher carbon content and surface area of this phase resulting in better separation of the 12 MBAP isomers. Interestingly, when going from 75 to 95% (v/v) ACN, an overall 83% decrease in $\Delta k'$ for the monomeric phase was observed, which was very similar to a 84% decrease in $\Delta k'$ for the polymeric phase. Overall, the observed trends in retention factors for the MBAPs using either monomeric or polymeric phases are similar to other CEC work with neutral solutes, indicating partitioning as the main retention mechanism [21].

Efficiency (N), resolution (Rs), and selectivity (α) for certain critical pairs of MBAP isomers are

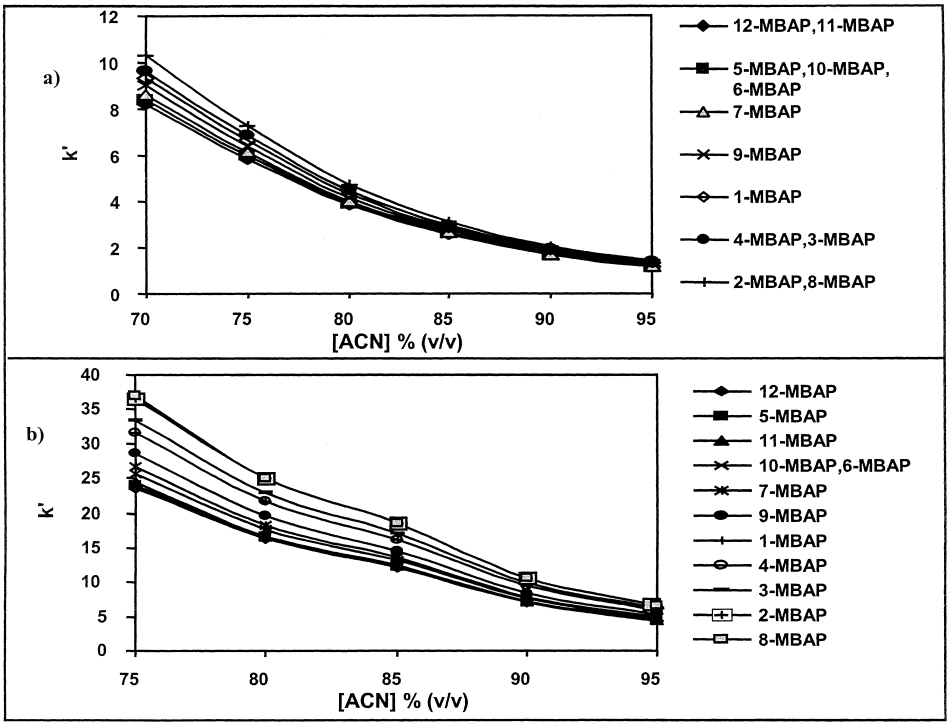

Fig. 6. The effect of ACN volume fraction on the capacity factor (k') for the separation of 12 MBAP isomers on: (a) monomeric CEC C_{18}-3 μm-100 Å stationary phase; (b) polymeric C_{18}-5 μm-100 Å-PAH stationary phase. All other conditions are the same as in Fig. 1.

compared for the two optimum phases in Fig. 7a–c, respectively. The top panel (Fig. 7a), compares *N* values of 9- and 1-MBAP, which were both clearly resolved on both phases. Generally, it is apparent at the lower range of ACN fraction (i.e. 75–85%, v/v) that similar efficiency for both MBAP isomers is achievable using either the 3-μm monomeric CEC or 5-μm polymeric PAH phase. However, at the higher range of ACN (85–95%, v/v), efficiency was higher with the monomeric CEC phase. This is surprising since CEC columns packed with smaller particles should deliver higher efficiency than larger particles irrespective of the concentration of organic solvents. Therefore, it appears that in the case of MBAP, a smaller monomeric 3-μm particle size only contributes to higher efficiency at larger ACN fraction, and that changing the ACN fraction on 5-μm polymeric phase had little to no effect on *N* values of 9- and 1-MBAP isomers.

The *Rs* and α values using monomeric and polymeric phases are compared for separation of three critical pairs: 12/5 MBAP, 7/9 MBAP, and 9/1 MBAP isomers (Fig. 7b,c). For the earlier eluting 12/5 MBAP isomers, similar *Rs* and α values are observed using either monomeric or polymeric phases. For the moderate to later eluting isomers (i.e. 7/9 and 9/1 MBAP), *Rs* and α values are considerably higher on the polymeric phase. Previously, it has been shown that in HPLC, higher *Rs* and α values are achieved with phases of greater phase density such as polymeric phases [6,22], which is consistent with our CEC results.

Finally, for the best stationary phase (i.e. P-C_{18}-5 μm-100 Å-PAH), the effect of ACN volume fraction on the MBAP separation was studied over the range of 75–95% (v/v) (Fig. 8). At a lower ACN fraction (e.g. 75%, v/v), a wider elution window is achievable at the expense of lower peak efficiency. At higher ACN fraction (e.g. 95%, v/v), the resolution of several isomers (e.g. 5/11 MBAP and 10/7 MBAP) deteriorates, however, nine peaks are still clearly resolved with high *N* in under 30 min. The

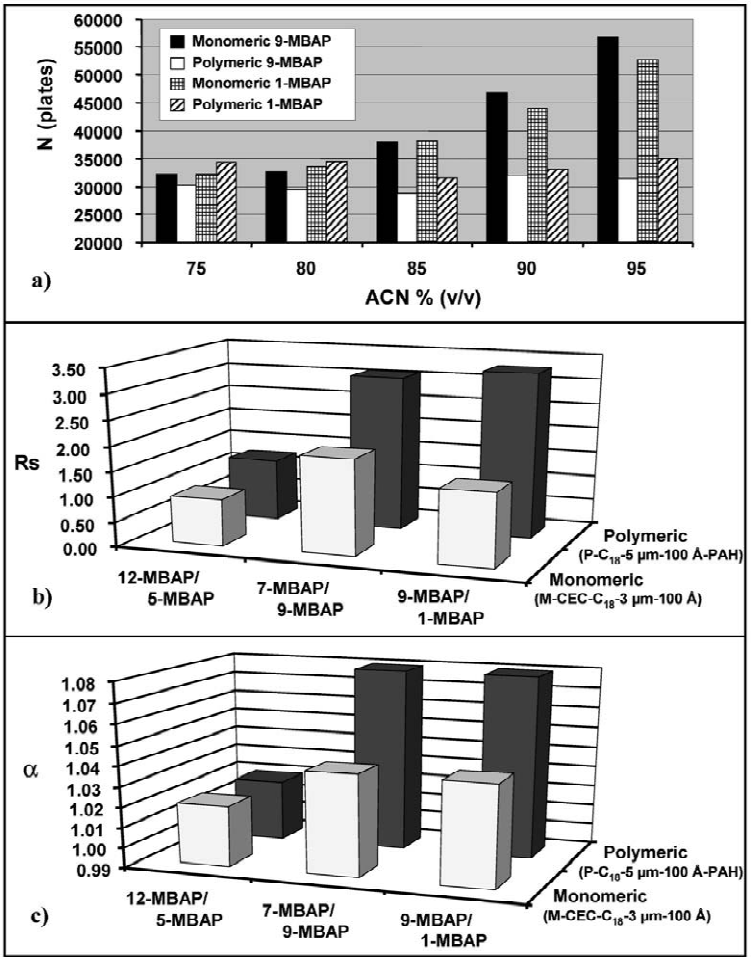

Fig. 7. Bar plots showing comparison of (a) efficiency for 9-MBAP and 1-MBAP, (b) resolution, and (c) selectivity between 12-MBAP/5-MBAP, 7-MBAP/9-MBAP, and 9-MBAP/1-MBAP isomers at various ACN fractions on optimum monomeric and polymeric stationary phases. Conditions are the same as in Fig. 1 except [ACN] was varied.

inset plots (Fig. 8a) demonstrate the effect of ACN (v/v) on linear velocity (V) which generally decreases with increasing ACN (v/v), with the exception of 85% (v/v) ACN. Although this trend of μ_{eo} is not similar to other reports in the literature [23], it can be partially explained by differences in the surface charge of the packing materials (Table 1), hence the complexity of the relationship. Insert (b) of Fig. 8 shows the correlation of $\log k'$ versus length to breadth (L/B) ratio. The trend is similar to that obtained in reversed-phase HPLC [14]. However, the correlation between $\log k'$ and L/B ratio is slightly lower for CEC (correlation coefficient is 0.56 for CEC compared to 0.81 for HPLC) [6].

3.3. Effect of capillary length and capillary internal diameter (I.D.)

Since the polymeric P-C_{18}-5 μm-100 Å-PAH stationary phase provided the best separation selectivity of MBAP isomers, this phase was also utilized to study the effects of capillary length and capillary internal diameter (I.D.) under optimum mobile phase conditions. The effects of varying the packed bed

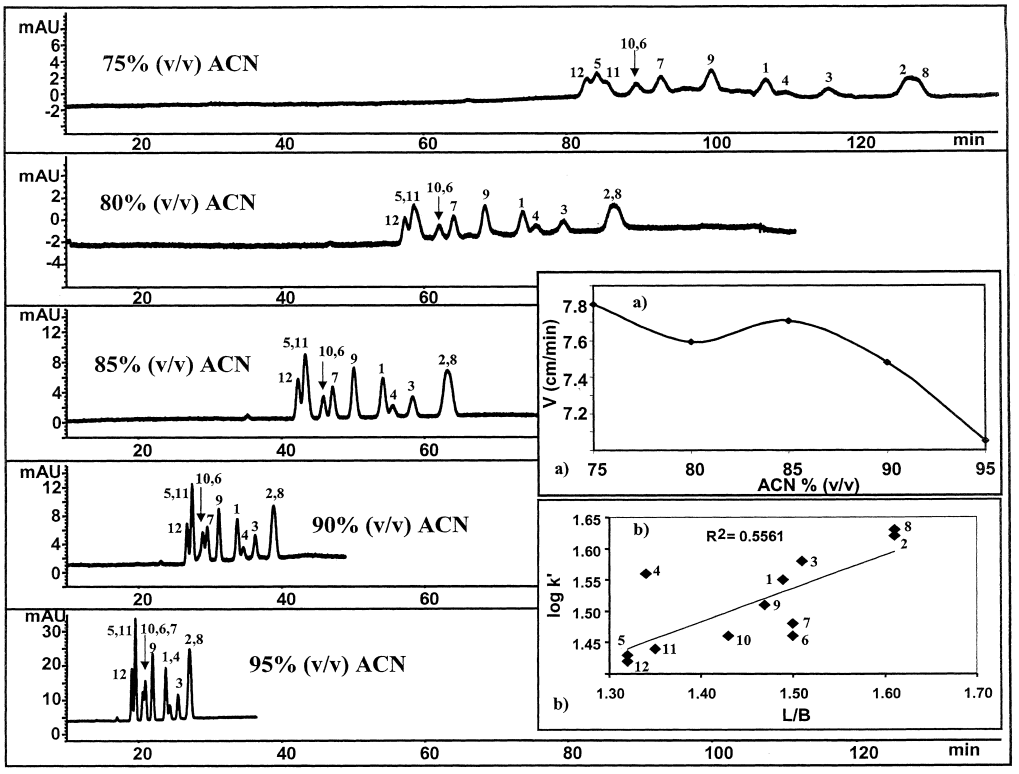

Fig. 8. Electrochromatograms showing the effect of ACN volume fraction on the separation of 12 MBAP isomers. Inset (a) shows effect of [ACN] on linear velocity and inset (b) shows correlation of $\log k'$ to length/breadth ratio using a polymeric C_{18}-5 μm-100 Å-PAH stationary phase. Conditions are the same as in Fig. 1 except [ACN] was varied.

length on separation of MBAP isomers are shown in Fig. 9a–c. It was important for this study to maintain constant field strength when varying the packed bed length, so as not to influence the separation. Initially, a packed bed length of 18.0 cm was studied. This length provided poor resolution and shorter retention time, as shown in Fig. 9a, due to a higher μ_{eo} indicated in the inset plot. At the same field strength, a 25.0-cm packed bed length provided dramatically better peak resolution of MBAPs, however, analysis time was increased nearly four times (Fig. 9b) as a result of decreased μ_{eo}. Further increase in the packed bed length to 40.0 cm at constant field strength was able to almost baseline resolve 11 of the 12 isomers, with a similar μ_{eo} compared to 25.0 cm. The total analysis time of MBAP isomers for this packed bed length was ~360 min, which greatly lessened the detectability, and thus was not deemed feasible for analysis. Therefore, it was concluded that a 25.0-cm packed segment provided the best overall performance for MBAP separation.

Next, the effect of varying the capillary I.D. was investigated. First, a 50-μm I.D. capillary with a packed bed length of 25.0 cm was run at optimum mobile phase conditions. This smaller diameter provided a superior MBAP separation at a reasonable retention time, clearly resolving 11 out of the 12 isomers as shown in Fig. 10a. The improved peak shape recognizing capability of the polymeric phase was best demonstrated by resolution of the first three and last two eluting isomers, which was only previously accomplished by increasing the packed bed length to 40.0 cm (Fig. 9c). Increasing the capillary I.D. to 100 μm did not improve the separation, as shown by a loss in resolution between the first three and last two coeluting MBAP isomers (Fig. 10b). The effect of varying the capillary I.D. showed no significant variation on the μ_{eo}, concluded by the

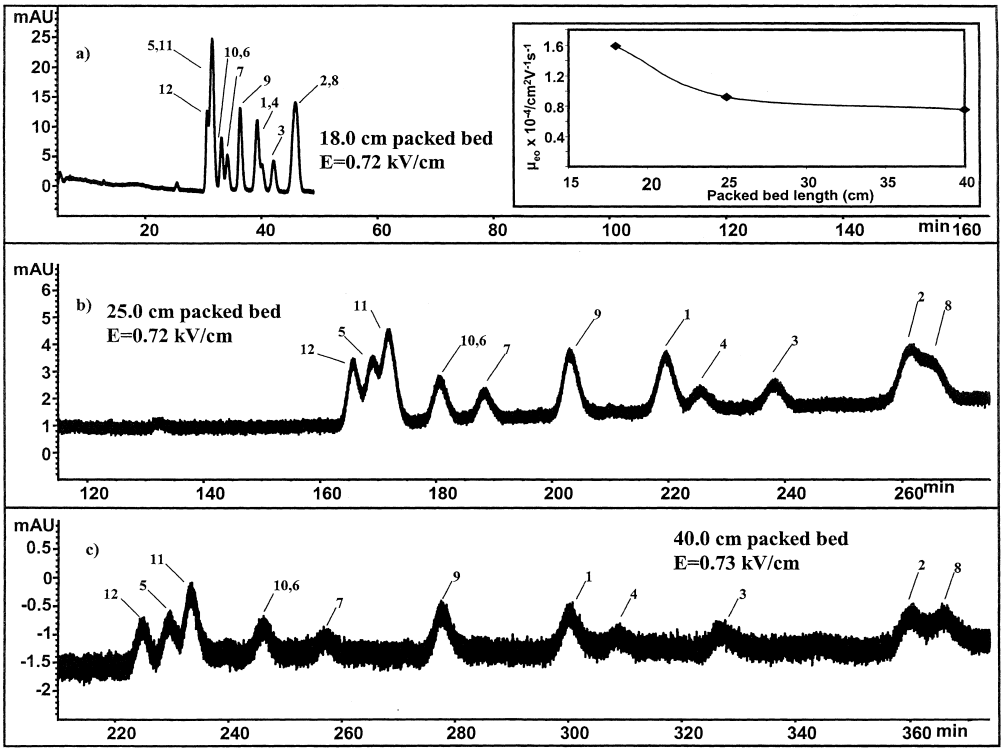

Fig. 9. Electrochromatograms showing the effect of packed bed length on the separation of 12 MBAP isomers. Conditions are the same as in Fig. 1 except BGE content was 75% (v/v) ACN–25% (v/v) 12.5 m*M* Tris and packed bed length was (a) 18 cm, (b) 25 cm, and (c) 40 cm. Voltage for constant field strength (*E*) of 0.72 kV/cm was 13, 18, and 29 kV for (a), (b) and (c), respectively.

same dead time of thiourea and retention time of MBAPs at all diameters.

4. Conclusions

The challenging separation of 12 MBAP isomers is extremely difficult to achieve due to the structural similarity and highly hydrophobic nature of these isomers. However, this report has demonstrated that CEC is a capable and powerful technique for resolution of these carcinogenic methylated-BAPs. Comparison between the optimum CEC monomeric and polymeric-PAH phase for MBAP separation showed a strikingly similar EOF profile for both phases, however, a 10-fold wider k' window, and better *Rs* and α values were achievable on the polymeric phase due in part to relatively higher % carbon loading and surface coverage which is similar to HPLC. After evaluation of seven stationary phases and studying various phase parameters, it was concluded that a polymeric P-C_{18}-5 μm-100 Å-PAH phase provided the best separation. Optimum separation conditions were: 25-cm packed bed length, 50-μm capillary I.D., 75% (v/v) ACN–25% (v/v) 12.5 m*M* Tris, pH 8.0, at 20 °C and 30 kV. The resolution of 11 out of 12 MBAP isomers has been accomplished in ~2 h using these conditions (Fig. 10a). The partial separation of 5-MBAP/11-MBAP and 2-MBAP/8-MBAP pairs of isomers could be further improved by exploring specifically designed CEC-compatible stationary phases and alternative column technologies. Although better separations over HPLC were not achieved by the best mobile conditions optimized for CEC (Part I, Ref. [5]), our studies on stationary phase tuning (Part II) clearly indicate that CEC offers higher peak capacity and higher selectivity than HPLC for separation of MBAP isomers. However, for CEC to become a mainstay there is an urgent need to develop more

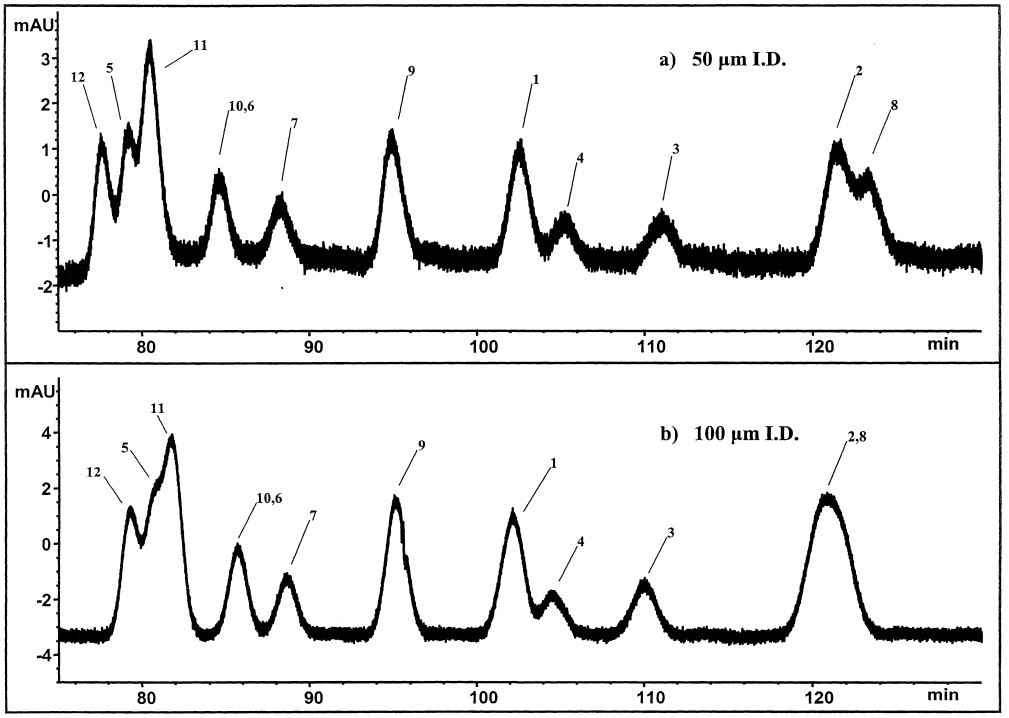

Fig. 10. Electrochromatograms showing the effect of capillary internal diameter (I.D.) on the separation of 12 MBAP isomers. Conditions are the same as in Fig. 1 except BGE content was 75% (v/v) ACN–25% (v/v) 12.5 m*M* Tris–HCl and capillary I.D. was (a) 50 μm, and (b) 100 μm.

stationary phases designed specifically for CEC with the ability to promote a robust and durable EOF over a wide range of buffer conditions. In conclusion, our two-part study has presented a novel and complete CEC method for the separation of these MBAP isomers. Even though the data is preliminary, we can state with confidence that CEC has the potential to establish itself as a powerful tool for separation of MBAP isomers and can be further applied for analysis of MBAP in environmental samples.

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