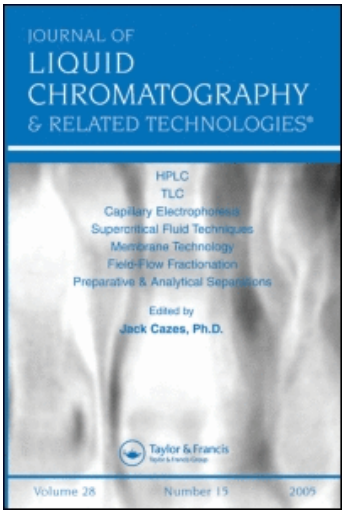

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Xin Xu[a]; Robert J. Hurtubise[a]
[a] Department of Chemistry, University of Wyoming, Laramie, WY, U.S.A.

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SEPARATION OF POLYCYCLIC AROMATIC HYDROCARBON METABOLITES BY γ-CYCLODEXTRIN-MODIFIED MICELLAR ELECTROKINETIC CHROMATOGRAPHY

Xin Xu, Robert J. Hurtubise*

Department of Chemistry
University of Wyoming
Laramie, WY 82071, USA

ABSTRACT

A γ-cyclodextrin (γ-CD) modified micellar electrokinetic capillary chromatographic (CD-MEKC) method has been developed to separate PAH metabolites. Baseline resolution of six acidic PAH metabolites was achieved within 14 min. The effects of several separation parameters were investigated in detail. These were the γ-CD concentration, sodium dodecyl sulfate (SDS) concentration, pH of the buffer, organic modifier, and urea concentration. The apparent capacity factors (k'_{app}) increased linearly with SDS concentration which was consistent with theory. Possible mechanisms that account for the differences in the apparent distribution coefficients (K_{app}) were proposed. The CD-MEKC method was compared with a capillary zone electrophoresis (CZE) method that we developed previously for the separation of the PAH metabolites.

INTRODUCTION

Micellar electrokinetic capillary chromatography (MEKC) has been proven to be a very useful method for the separation of neutral compounds.[1,2] Analytes are separated on the basis of the differential partition between the

micellar (pseudo-stationary) phase and the aqueous (mobile) phase. One of the limitations of conventional MEKC is the difficulty in separating extremely hydrophobic species such as polycyclic aromatic hydrocarbons (PAHs). This is due to the fact that highly hydrophobic solutes all tend to be completely associated with micelle phase and coelute with it.[3] Several approaches have been reported for the improvement of separation of hydrophobic compounds. For example, the addition of cyclodextrin,[3-5] organic solvents,[6,7] and urea[8] to a micelle solution, and the use of bile salts,[9-12] such as sodium cholate and sodium deoxycholate, instead of a surfactant such as SDS have proven to be useful.

Polycyclic aromatic hydrocarbons are a significant class of pollutants that are found in automotive exhaust, tobacco smoke, charcoal-broiled foods, and other materials. The control of the level of PAHs is receiving more and more attention because of their potential carcinogenicity. Some of the PAH metabolites, such as 1-hydroxypyrene (1-OH-Py), 3-hydroxybenz[a]anthracene (3-OH-B[a]A), and 3-hydroxybenzo[a]pyrene (3-OH-B[a]P) have been used as biomarkers for environmental and occupational PAH exposures.[13] Although a number of analytical approaches, such as high performance liquid chromatography (HPLC)[14,15] and gas chromatography (GC)[16] have been used to isolate and separated PAH metabolites, none of these methods match the resolving power of capillary electrophoresis (CE). Until now, very little work has been done in the separation of PAH metabolites with CE. However, cyclodextrin (CD)-modified micellar electrokinetic capillary chromatography (CD-MEKC) was recently used for the separation of PAH metabolites.[17,18] In both of these approaches, high concentrations of γ-CD were used. This would increase the cost of the method. We previously reported a capillary zone electrophoresis (CZE) method for the separation of nine PAH metabolites including tetrols, benzo[a]pyrene diols, and hydroxyl aromatics without a CD and micelle.[19] In this study, we investigated the influence of various buffer components (e.g. SDS and γ-CD) and buffer additives (e.g. organic solvent and urea) on the separation of PAH metabolites by γ-CD-modified MEKC to improve this approach for the separation of the metabolites. The interactions between PAH metabolites and the micelle, in a γ-CD system were also investigated.

EXPERIMENTAL

Apparatus

A P/ACE 5000 (Beckman, Fullerton, CA) instrument equipped with a UV detector, and a fused-silica capillary with a 75 μm i.d. (375 μm o.d.) and a 57 cm total length (50 cm from inlet to detector) were used. Electropherograms were recorded and analyzed on a IBM 350-P90 computer using Beckman's System Gold electrophoresis software (Beckman). The buffer pH was determined with an Orion research digital ionalyzer/501 equipped with a Orion 910200 combination electrode (Orion Research Inc., Cambridge, MA).

For the MEKC experiments, the electrophoresis system was operated in the conventional mode with the cathode at the detector end. Hydrodynamic injection was used and set for 2 s. The temperature of the capillary was maintained at 25°C by means of a liquid coolant in the capillary cartridge. All experiments were performed at a constant voltage of 20 kV with ultraviolet absorbance detector set at 254 nm.

Reagents

The benzo[a]pyrene metabolites and the benz[a]anthracene metabolite were purchased from the National Cancer Institute (NCI) repository at Midwest Research Institute (Kansas City, MO), and they were used without further purification. The 1-OH-Py sample was obtained from Aldrich (Milwaukee, WI). Figure 1 shows the structures of the nine PAH metabolites that were studied in this work. Sodium tetraborate (99.999%), urea (99%), and sudan III (certified, dye content 89%) were purchased from Aldrich. Sodium dihydrogen phosphate monohydrate (A.C.S. reagent) was purchased from Spectrum Quality Products, Inc. (New Brunswick, NJ). The γ-cyclodextrin was a gift from Cerestar USA, Inc. (Hammond, IN). Sodium dodecyl sulfate (SDS) was purchased from Sigma (St. Louis, MO). Methanol (MeOH), acetonitrile (ACN) and water were HPLC grade and were purchased from J. T. Baker Inc. (Phillipsburg, NJ).

Samples and Solutions

Stock solutions containing 60 μg/mL of PAH metabolites were prepared in MeOH/water (7:3, v:v). Working solutions for MEKC separation were prepared by diluting these stock solutions. All samples were stored in the dark at 4°C. The glassware was wrapped in aluminum foil to protect sample solutions from light.

The running buffer consisted of 7.5 mM $Na_2B_4O_7$ and various amounts of SDS and γ-CD. The pH of buffer solutions ranged from 8.44 to 10.05. In the experiments with organic solvents, buffer electrolytes contained either 10% (v/v) or 15% (v/v) ACN or MeOH. In the experiments with urea, the urea concentrations were varied from 1 mM to 3 mM. Before use, all buffer solutions were either filtered through a 0.45 μm Supor Acrodisc filter (for aqueous buffers) or a 0.45 μm GHP Acrodisc filter (for buffers with organic solvents) (Gelman, Ann Arbor, MI) and then sonicated 5 min to degas.

RESULTS AND DISCUSSION

Influence of γ-CD Concentration on the Separation

To study the influence of γ-CD concentration on the separation of PAH metabolites, the experiments were carried out with a buffer of 7.5 mM $Na_2B_4O_7$,

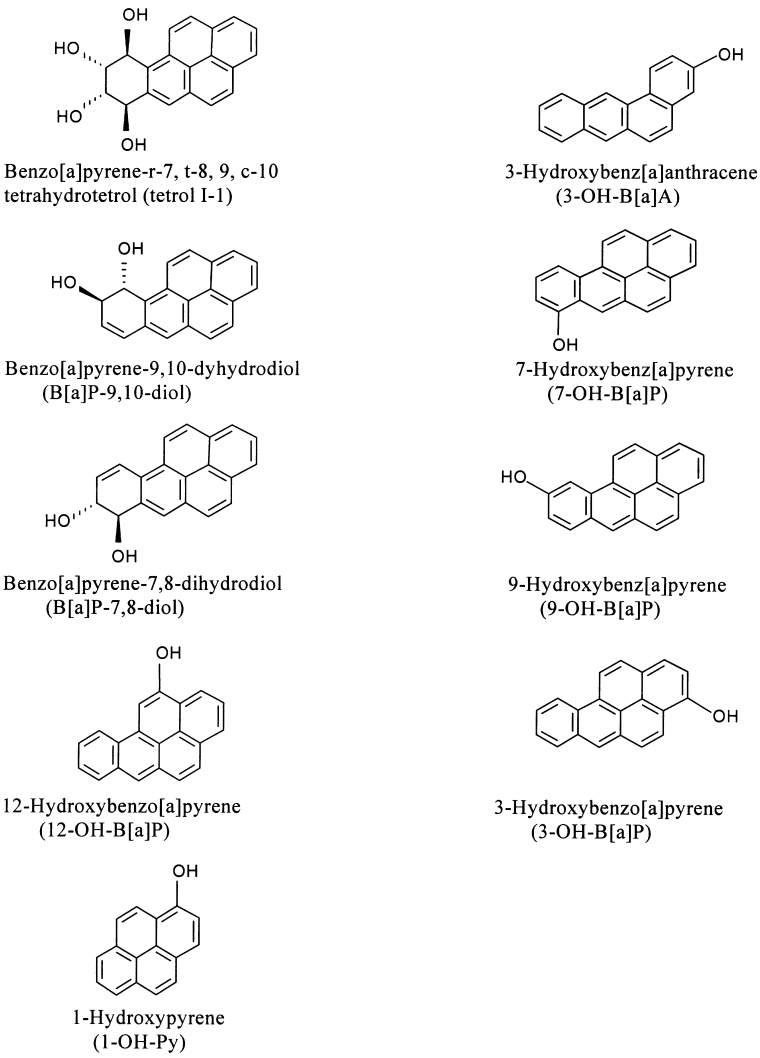

Figure 1. Structures of the polycyclic aromatic hydrocarbon metabolites investigated.

25 mM SDS and 0 to 40 mM γ-CD at pH 9.0. Figure 2 illustrates the dependence of the migration time (t_R) on γ-CD concentration. When there is no γ-CD in the running buffer, 7 out of 9 metabolites migrated together. Tetrol I-1 and B[a]P-9,10-diol didn't migrate with the other 7 compounds. The similar hydrophobicity of the PAH metabolites causes them to be totally incorporated

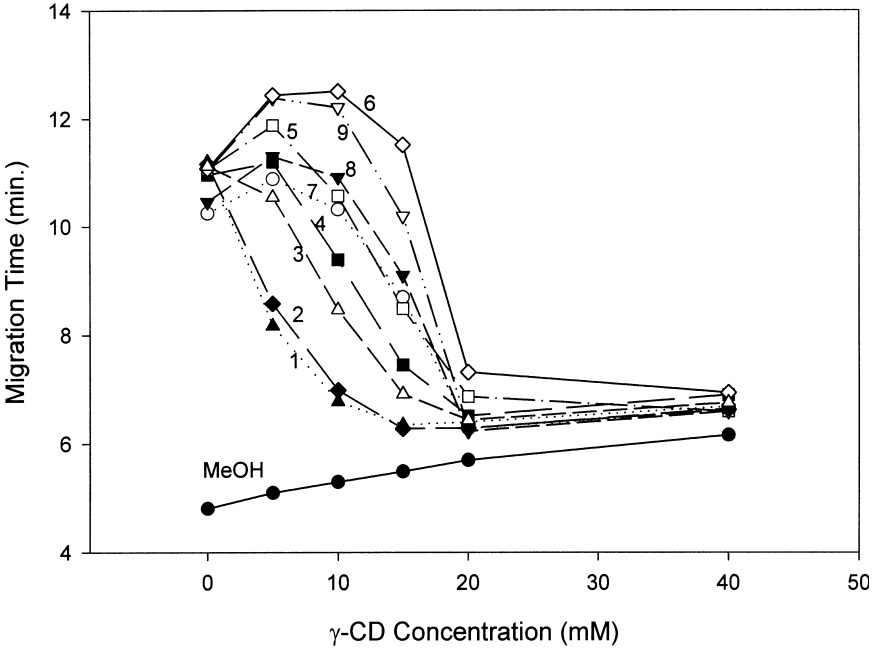

Figure 2. Effects of γ-CD concentration on the migration times of nine PAH metabolites. Buffer: 7.5 mM $Na_2B_4O_7$, 25 mM SDS, plus γ-CD, pH 9.00; voltage: 20 kV; temperature: 25°C. Compound identification: (1) 7-Hydroxybenzo[a]pyrene; (2) 3-Hydroxybenzo[a]-pyrene; (3) 9-Hydroxy-benzo[a]pyrene; (4) 1-Hydroxypyrene; (5) 3-Hydroxybenz[a]anthracene; (6) 12-Hydroxybenzo[a]pyrene; (7) Benzo[a]pyrene-r-7,t-8,9,c-10-tetrahydrotetrol (I-1); (8) Benzo[a]pyrene-trans-9,10-dihydrodiol; (9) Benzo[a]pyrene-trans-7,8-dihydrodiol.

into the micelle phase and co-migrate with no γ-CD present. When γ-CD was added to the micelle buffer, inclusion complexes between γ-CD and PAH metabolites formed, provided the size and shape of the metabolites conformed to the interior dimensions of the γ-CD cavity. Therefore, hydrophobic PAH metabolites would partition between the micelle and the γ-CD, resulting in a better separation.

As indicated in Figure 2, the higher the γ-CD concentration, the shorter the migration time, except in the γ-CD concentration range from 0 to 5 mM for compounds 4-9. This could be due to the competitive equilibria that exists between γ-CD and SDS for the analytes. That is, when the γ-CD concentration was increased from 0 to 5 mM, due to the competitive equilibria, SDS inter-

acted more strongly with the PAH metabolites. The longest migration times appeared at 5 mM γ-CD for compounds 4, 5, 7, 8, and 9 (in case of compound 6, the longest migration times appeared at 10 mM γ-CD). Figure 2 shows that all nine metabolites could possibly be separated with 10 mM γ-CD in the present buffer system. However, experiments showed that the separation of the three neutral compounds (tetrol I-1, B[a]P-9,10-diol and B[a]P-7,8-diol) was poor because of the low signal responses and the broad peaks for two of the compounds. Therefore, the γ-CD modified MEKC system was most efficient in separating the six more acidic metabolites (compounds 1-6).

Influence of SDS Concentration and pH on the Separation

Figure 3 shows the influence of SDS concentration on the migration times of six acidic PAH metabolites with 10 mM γ-CD in the buffer. The SDS concentration was varied from 17 mM to 40 mM. The migration times of all six

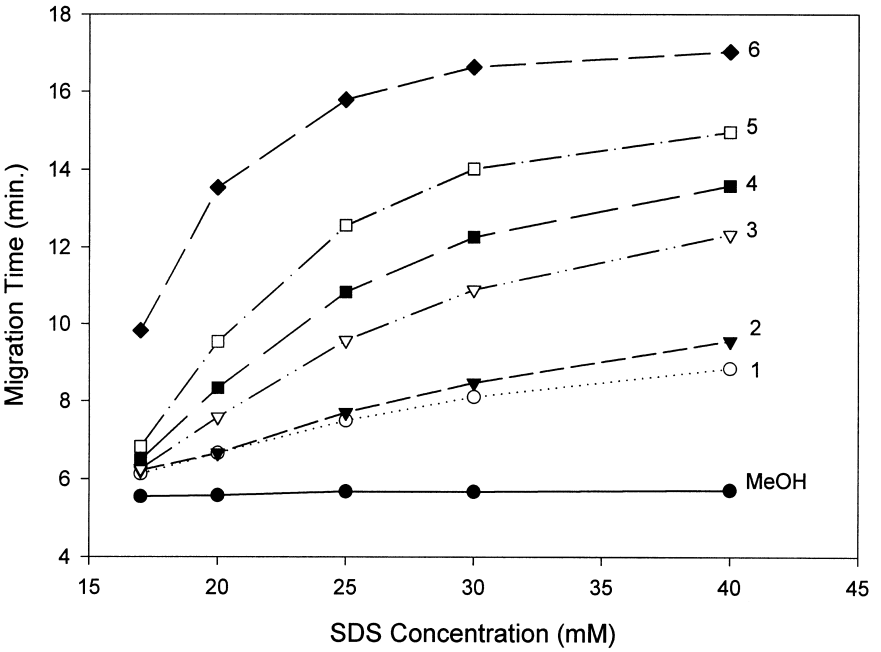

Figure 3. Effects of SDS concentration on the migration times of six acidic PAH metabolites. Buffer: 7.5 mM $Na_2B_4O_7$, 10 mM γ-CD, plus SDS, pH 9.00; voltage: 20 kV; temperature: 25°C. Compound identification is the same as for Figure 2.

compounds increased with increasing SDS concentration and the resolution of 7-OH-B[a]P and 3-OH-B[a]P was improved with higher SDS concentrations so that the two compounds could be separated. The best separation, but the longest analysis time (17 min), was obtained with 40 mM SDS. Considering the fact that with 25 mM SDS and 5 mM γ-CD in the buffer at pH 9.0, 7-OH-B[a]P and 3-OH-B[a]P could be baseline separated (result not shown), it was decided to use 25 mM SDS in future experiments.

The effects of buffer pH was also investigated. With 25 mM SDS and either 5 mM or 10 mM γ-CD in the buffer, the pH was changed from 8.5 to 10.0 with a interval of 0.5 pH units. It turned out that there was little effect on the separation in the pH range of 8.5 to 9.5. In a previous paper, we reported that the pK_a values of these acidic PAH metabolites are in the range of 8.81 to 9.30.[20] Therefore, in the above pH range, the difference in the degree of dissociation of the compounds would not change much, leading to somewhat similar migration characteristics. Although near-baseline resolutions of all six acidic compounds were obtained in the pH range 8.5 to 9.5, at pH 10.0, complete baseline resolutions of the six acidic metabolites were achieved. Therefore, the optimum separation pH was 10.0.

Influence of Organic Solvent and Urea on the Separation

In a previous report by us, organic solvents were the key factor in the separation of PAH metabolites with CZE.[19] In this work, MeOH and ACN were used as buffer modifiers in a buffer with γ-CD. First of all, the addition of either MeOH or ACN did not improve the peak shape of the three neutral compounds. The peaks were still broad with low signal to noise ratios.

Secondly, the migration times for all PAH metabolites increased significantly with organic modifier. Also, all peaks broadened with increasing amounts of organic solvent. In general, there was no improvement in the resolution for the separation of either the mixture with nine metabolites or the six acidic compounds mixture. We also examined the effects of urea in the buffer on the resolution. However, peak splitting appeared for 3-OH-B[a]P with 1 M urea in the buffer. The analysis time was greatly increased with the addition of urea, and no improvement was observed in the resolution.

After an investigation of all the conditions and modifiers discussed in this section and the previous two sections, the best buffer system for the separation of the six acidic metabolites was 7.5 mM $Na_2B_4O_7$ buffer, with 25 mM SDS and 5 mM γ-CD, at pH 10.0. Figure 4 presents the separation results.

In an effort to separate the three neutral compounds, several experiments showed that with 10 mM NaH_2PO_4-6 mM $Na_2B_4O_7$ and 10 mM SDS at pH 8.2,

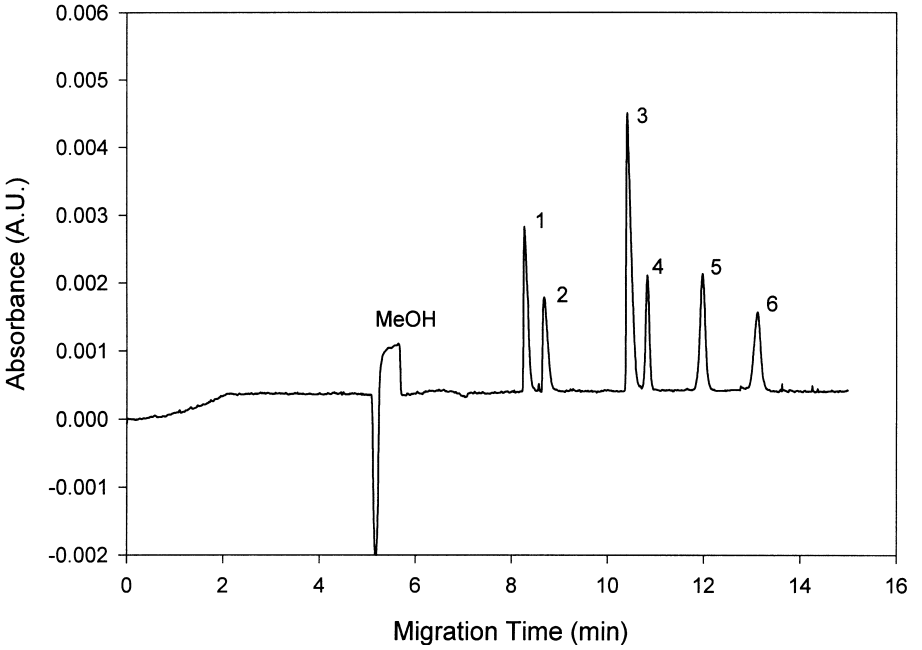

Figure 4. Electropherogram of a mixture of six acidic PAH metabolites. Buffer:7.5 mM $Na_2B_4O_7$, 25 mM SDS, 5 mM γ-CD, pH 10.00; voltage: 20 kV; temperature: 25°C. Peak identification is the same as for Figure 2.

the three neutral compounds could be baseline separated within 11 min (results not shown). However, this buffer system was not as effective in separating the six acidic metabolites as the buffer system discussed in the previous paragraph.

In earlier work, we developed a CZE method to separate nine PAH metabolites.[19] Separation of the nine PAH metabolites was achieved within 24 min with a CAPS buffer with 40% (v/v) MeOH, although a resolution of 0.8 was obtained for tetrol I-1 and B[a]P-9,10-diol. In terms of the six acidic metabolites, in the previous work[19] the best system was 100 mM CAPS with 45% (v/v) ACN. The theoretical plate values for the six acidic metabolites in that system ranged from 1.44×10^5 to 2.08×10^5. The resolution ranged from 1.5 to 11.2. In the present CD-MEKC system (Figure 4), the theoretical plate values ranged from 3.05×10^5 to 8.02×10^5. The resolution values ranged from 1.9 to 7.4. Therefore, in terms of separation efficiency, the present CD-MEKC system is somewhat advantageous in the separation of the six acidic metabolites. Another

advantage of the present buffer system is the low background absorbance. The value of the baseline absorbance of the present CD-MEKC system was approximately 0.3 x 10^{-3} A.U. Whereas, the value of the baseline absorbance of the previous CZE system with organic modifiers was approximately 1.5 x 10^{-3} A.U. The low background absorbance provided higher signal-to-noise ratio and better sensitivity. To develop a better understanding of the CD-MEKC system, one can also explore the interactions between the PAH metabolites and the micelle phase, as well as the γ-CD (see next section).

Investigation of the PAH Metabolites-Micelle Interactions

In order to explore the interaction mechanism between PAH metabolites and the micellar phase, the apparent capacity factors (k'_{app}) were determined using the following equation[3]

$$k'_{app} = \frac{t_R - t_0}{t_0(1 - t_R / t_{mc})} \quad (1)$$

where t_R, t_0 and t_{mc} are the migration times of the solute, the electroosmotic flow (EOF) marker (methanol), and the micelle marker (sudan III), respectively. The buffers contained 10 mM γ-CD at pH 9.0. The SDS concentration was varied from 17 mM to 40 mM. Figure 5 shows the dependence of k'_{app} on the concentration of SDS of five weakly acidic PAH metabolites (compounds 1-5). The slopes of the five straight lines (from line 1 to line 5) are 0.038, 0.053, 0.16, 0.28, and 0.67, respectively. The following equation gives the relationship between k'_{app} and several parameters:[3]

$$k'_{app} = \frac{K_{app}}{C_{CD}\overline{V}_{CD}}(C_{surf} - CMC)\overline{V}_{mc} \quad (2)$$

where C_{surf} is the surfactant concentration, K_{app} is the apparent distribution coefficient of the solute between the micelle phase and γ-CD, CMC is the surfactant's critical micelle concentration, C_{CD} is the concentration of γ-CD, and $\overline{V}_{CD}$ and $\overline{V}_{mc}$ are the partial specific volumes of the micelle and γ-CD, respectively. The slope of the straight line in Figure 5 is equal to $\frac{K_{app}\overline{V}_{mc}}{C_{CD}\overline{V}_{CD}}$.[3] If it is assumed that the partial specific volumes of γ-CD and SDS are 0.671 ml g^{-1} and 0.856 ml g^{-1}, respectively,[3] the apparent distribution coefficients (K_{app}) of the five acidic compounds can be calculated from the slopes of the lines in Figure 5. They are as follows: 3-OH-B[a]A: 5.3; 1-OH-Py: 2.2; 9-OH-B[a]P: 1.3; 3-OH-B[a]P: 0.42; 7-OH-B[a]P: 0.30. The relationship of k'_{app} vs. C_{surf} of

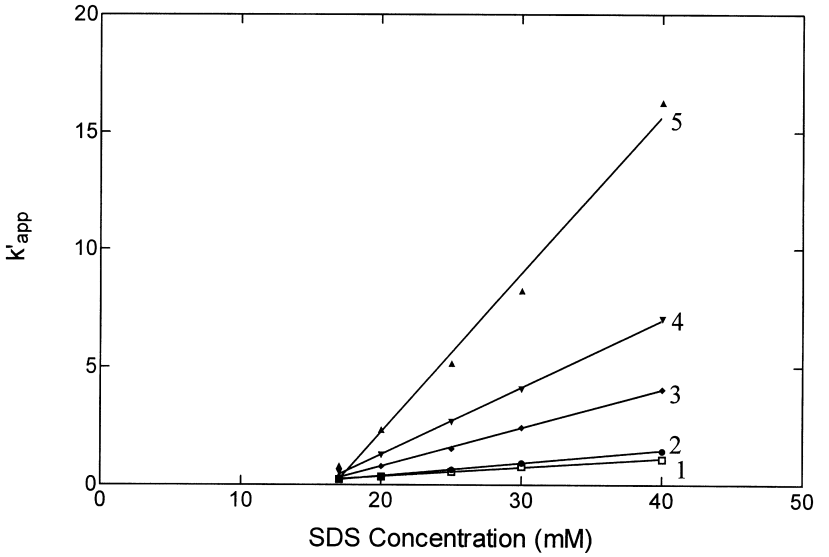

Figure 5. Dependence of the apparent capacity factors (k'_{app}) on the concentration of SDS. Compound identification and operating conditions are the same as for Figure 3.

12-OH-B[a]P was not included in Figure 5 because the migration times of 12-OH-B[a]P were very close to t_{mc} for all of the SDS concentrations studied. With the concentrations of SDS changed from 17 mM to 40 mM, the k'_{app} values of 12-OH-B[a]P ranged from 4.3 to 225. Also, the linear relationship between k'_{app} and C_{surf} of 12-OH-B[a]P ($r^2 = 0.92$) was not as good as those for the other five compounds ($r^2 \geq 0.99$). Nevertheless, the K_{app} value of 12-OH-B[a]P was calculated to be 74.3.

The K_{app} values give a measure of the relative affinities of the PAH metabolites for the micelle phase and for γ-CD. With a K_{app} value of 74.3, 12-OH-B[a]P had the strongest affinity for the SDS micelle phase. The metabolites, 3-OH-B[a]A and 1-OH-Py, with K_{app} values much greater than 1, also have a strong affinity for the micelle phase. With a K_{app} value a little greater than 1, 9-OH-B[a]P tends to be included into γ-CD somewhat more compared to compounds 4, 5, and 6. Because 3-OH-B[a]P and 7-OH-B[a]P have K_{app} values much less than 1, they have the strongest affinity for γ-CD.

The differences in the interactions of the metabolites with SDS and γ-CD are due to the differences in their sizes and their hydrophobicities. Generally,

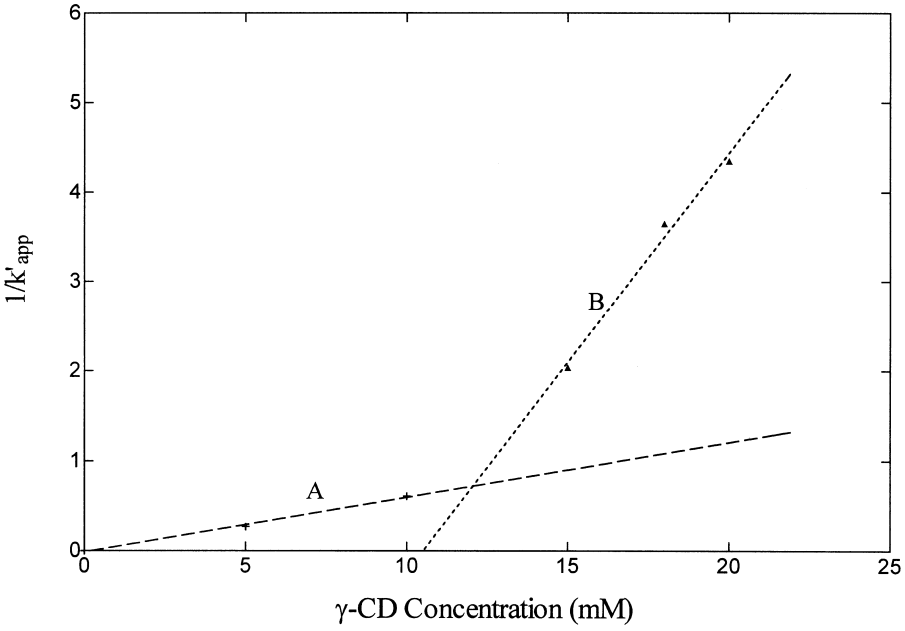

Figure 6. Dependence of the reciprocals of the apparent capacity factors ($1/k'_{app}$) of 9-OH-B[a]P on the concentration of γ-CD. Operating conditions are the same as for Figure 2.

separation is achieved because of distribution differences of the solutes between γ-CD and the micelle.[21] The quantity of solute in a micelle is dependent largely on its hydrophobicity. However, the amount of solute in the γ-CD is dependent on its capability to fit into the γ-CD cavity and its hydrophobicity. Based on the K_{app} values, a very large fraction of 12-OH-B[a]P would be in the micelle. A smaller fraction of 3-OH-B[a]A and 1-OH-Py would be in the micelle compared to 12-OH-B[a]P. However, because 9-OH-B[a]P, 3-OH-B[a]P, and 7-OH-B[a]P have significantly smaller K_{app} values, they would spend a greater proportion of their time in γ-CD. In fact, 7-OH-B[a]P with a K_{app} of 0.29 would spend the greatest amount of time in γ-CD. This is born out by the data in Figure 4 which shows that 7-OH-B[a]P has the shortest migration time.

By rearranging equation 2, one can see that there is a linear relationship between the reciprocal of k'_{app} and C_{CD}:

$$\frac{1}{k'_{app}} = \frac{\overline{V}_{CD}}{K_{app}\overline{V}_{mc}(C_{surf} - CMC)} \bullet C_{CD} \qquad (3)$$

In a buffer system consisting of 7.5 mM $Na_2B_4O_7$, 25 mM SDS (pH 9.0), and a γ-CD concentration from 0 to 20 mM, the values of the reciprocal of k'_{app} for 9-OH-B[a]P were obtained and plotted against C_{CD} (Figure 6). Figure 6 indicates that the partition mechanism for the solute changed at a γ-CD concentrations of about 12 mM because of the break in the plot. The reason why the partition mechanism was altered is not clear at this stage. However, a possible explanation is a change in the stoichiometric ratio of the inclusion complex between γ-CD and 9-OH-B[a]P from 1:1 to 1:2.[3] The slope of the plot of $1/ k'_{app}$ against C_{CD} is proportional to $1/K_{app}$. The larger the slope, the smaller the K_{app} value. The slope of line B in Figure 6 is about 8 times that of line A. That means, at concentrations of γ-CD higher than 12 mM, K_{app} decreased 8-fold compared to a C_{CD} concentration less than 12 mM . Therefore, 9-OH-B[a]P is more easily incorporated into the γ-CD cavity above approximately 12 mM γ-CD. Similar relationships of $1/k'_{app}$ vs. C_{CD} were also obtained for compounds 1, 2, 4 and 5.

CONCLUSION

The γ-CD-modified MEKC method was very effective in separating six acidic PAH metabolites, which are important in cancer research. The separation conditions can be readily optimized by changing the concentrations of γ-CD and SDS. Buffer modified with organic solvents and urea had little effect on the separation.

The concentration of γ-CD used in the present study was much lower than in the similar work reported earlier.[17,18] Considering the high cost of γ-CD, our γ-CD-MEKC method would be advantageous because of the smaller amount of γ-CD used. For the separation of the six acidic metabolites, the CD-MEKC system that was developed in this work is somewhat better than a previous CZE method[19] in terms of separation efficiency and sensitivity.

The K_{app} values obtained for the acidic PAH metabolites can be used to qualitatively explain the mechanism of interactions of the metabolites with micelle and γ-CD based on the differences in hydrophobicity and the size of different metabolites.

ACKNOWLEDGMENT

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