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Separation of very hydrophobic compounds by hydrophobic interaction electrokinetic chromatography

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Abstract

Separations of very hydrophobic neutral analytes were achieved using hydrophobic interaction electrokinetic chromatography (HI-EKC). Alkyl aryl ketone homologues dodecanophenone (C_{18}), tetradecanophenone (C_{20}), hexadecanophenone (C_{22}) and octadecanophenone (C_{24}) were separated via hydrophobic interactions between free sodium dodecyl sulfate (SDS) monomers and the analytes. The first running buffer consisted of 50 m*M* SDS and 50% acetonitrile (pH 7.0). A complete reversal in the elution order of these analytes was obtained with the second running buffer, 20 m*M* cetyltrimethylammonium bromide (CTAB) and 50% acetonitrile (pH 2.8). With the second running buffer, electroosmotic flow was suppressed and the free CTAB monomers migrated toward the detector. Through hydrophobic interactions between the free CTAB monomers and the analytes, separations of these very hydrophobic alkyl aryl ketones were obtained in less than 10 min; analysis times were less than 5 min with the SDS-based separations.

1. Introduction

The introduction of capillary electrophoresis (CE) [1–3] in 1979 enabled the high-resolution separation of charged analytes with rapid analysis times. The separation power of CE was further enhanced with the development of micellar electrokinetic chromatography (MEKC) [4,5] in 1984. In this technique, the addition of micelles to the running buffer creates a pseudo-stationary phase by which neutral analytes can differentially partition. Since the advent of CE and MEKC, there has been considerable research in both the method development and application of these two powerful separation techniques.

CE has been used to successfully separate a diverse range of compounds such as drug-related impurities [6], warfarin enantiomers in human plasma [7], B vitamins [8], DNA fragments [9] and sulphonamides in pharmaceuticals [10]. A more complete review of CE-related applications can be found in the numerous review articles and books that have been published [11–14].

MEKC has been devoted primarily to the analysis of small molecules. The applications involving MEKC are considerably less than those accomplished with CE but are still numerous and span a broad range of analytes. To date MEKC has been utilized in the analysis of cardiac glycosides [15], mycotoxins [16], β-blockers [17],

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explosive residues in soils [18], herbicides [19], water- and fat-soluble vitamins [20–23], polymycins [24] and priority substituted phenols [25] just to mention a few. A recently published book provides a much more in depth review of the applications performed with MEKC [26].

It is easy to see the impact that CE and MEKC have had in separations. There are other modes of separation utilizing the basic principles of CE some of which include isoelectric focusing, capillary gel electrophoresis and isotachophoresis. These techniques are all employed for the analysis of ionic species. The separation of neutral compounds falls under the technique titled electrokinetic capillary chromatography (EKC). In EKC, additives to the running buffer, such as the micelles utilized in MEKC, provide the pseudophase by which the neutral molecules through their varying affinities for the pseudophase can be separated. Electrokinetic separations have been exclusively used for the analysis of small solutes.

Larger and more hydrophobic neutral analytes have been difficult to separate with EKC and more specifically MEKC. One of the primary problems with the analysis of very hydrophobic compounds by MEKC is that these very hydrophobic compounds tend to remain in the inner hydrophobic core of the micelle and eventually coelute with the micelle itself. One way to increase the affinity of the hydrophobic analytes for the aqueous mobile phase is the addition of organic modifiers to the running buffer. Although this method is effective in enhancing the resolution of more moderately hydrophobic analytes, the "cost" is often much longer analysis times and higher operating currents. Another limitation in dealing with very hydrophobic molecules is their minimal solubility in aqueous media. Sample matrixes often consist solely of organic solvent and introduction of the sample into a predominantly aqueous running buffer leads to the precipitation of the analyte in the capillary and of course this leads to capillary reconditioning procedures. As a result of a blocked capillary, organic solvents are often used to clear the capillary followed by purges with a base. This sort of treatment changes the chemistry on the surface of the capillary and often the result is a change in the electroosmotic velocity. This is an undesirable effect in terms of run-to-run reproducibility. Given these types of disadvantages associated with the existing EKC techniques, there is a strong need to develop new techniques which are specifically suited for the analysis of very hydrophobic neutral analytes.

In 1986 Walbroehl and Jorgenson [27] demonstrated the separation of aromatic ring compounds through proposed "solvophobic interactions" in systems consisting of tetrahexylammonium salts in water–acetonitrile mixtures. When the analytes are dissolved in this separation medium, they undergo a solvophobic interaction with the tetrahexylammonium ion (THA^+) and form positively charged species which can then migrate in an electric field. Although a solvophobic interaction mechanism was proposed, it was also hypothesized [26] that the π-electrons of the aromatic analytes may be attracted to the THA^+ ion in which case the separation mechanism would be based more on electrostatic interactions than true hydrophobic interactions. More recently Bullock [28] demonstrated the separation of Triton X-100 oligomers using hydrophobic interactions between free sodium dodecyl sulfate (SDS) monomers and the different poly(ethylene oxide) chains. The individual oligomers are separated based on differences in the length of these chains.

Based on the success of these studies and the need to develop a viable method by which very hydrophobic neutral analytes can be separated, we formally introduce a new mode of electrokinetic capillary chromatography: hydrophobic interaction electrokinetic capillary chromatography (HI-EKC). In this investigation we report the separation of some very hydrophobic alkyl aryl ketone homologues (C_{18}, C_{20}, C_{22}, C_{24}) using HI-EKC. Fast, high-resolution separations of these analytes, which differ by only two methylene groups, were achieved. The results of this report further support the need to develop a

gradient elution mode for MEKC similar to that found in high-performance liquid chromatography (HPLC).

2. Experimental

2.1. Apparatus

A Waters Quanta 4000 capillary electrophoresis system (Millipore, Waters Chromatography Division, Milford, MA, USA) equipped with fixed-wavelength UV detection at 254 or 214 nm was employed for all the separations performed in this study. HI-EKC was performed in either a 30 cm or 35 cm (injection to detection) × 50 μm I.D. × 370 μm O.D. fused-silica capillary tube (Polymicro Technologies, Tucson, AZ, USA). The total capillary length was 37.5 or 42.5 cm. Injections were made in the hydrostatic mode. The applied voltage ranged from 5 to 30 kV with operating currents less than 50 μA unless otherwise noted in the text. The data were collected at a rate of 20 points/s and analyzed with a Macintosh IIci computer (Apple, Cupertino, CA, USA) using a MacLab 4 channel ADC with the appropriate vendor software (ADInstruments, Milford, MA, USA). All experiments were done at ambient temperature (ca. 25°C).

2.2. Materials

The alkyl aryl ketone homologues (C_8–C_{24}) were purchased as a kit from Aldrich (Milwaukee, WI, USA). The alkylbenzenes (C_{13}, C_{14}, C_{16}) were purchased from Alltech (Deerfield, IL, USA). Anthracene, naphthalene, and phenanthrene were obtained from Aldrich. SDS was purchased from Sigma (St. Louis, MO, USA), while cetyltrimethylammonium bromide (CTAB) was obtained from Aldrich. Both surfactants were used as received. The concentration of SDS ranged from 2–50 m*M* for all the HI-EKC separations. Separations performed in utilizing CTAB were done at concentrations of 20 and 40 m*M*. Stock buffer solutions were prepared with $NaH_2PO_4 \cdot H_2O$ and sodium hydroxide to give a 100 m*M* phosphate buffer at pH 7.0 for the SDS-based separations. A phosphate buffer with pH 2.8 was used with the CTAB-based separations. A phosphate buffer concentration of 5 or 10 m*M* was used in all the experiments. Acetonitrile was obtained from Mallinckrodt (Paris, KY, USA). The non-micellar solutions were made by weighing appropriate amounts of SDS or CTAB and diluting with the stock buffer solution, in some cases 40 to 50% acetonitrile, and distilled water in a 100-ml volumetric flask to obtain the desired concentrations. All the running buffer solutions were filtered through 0.20-μm membrane filters obtained from Alltech and degassed before use. HPLC-grade distilled water used in the makeup of the micellar buffer solutions was obtained from J.T. Baker (Phillipsburg, NJ, USA). Sample solutions were made up of 100% acetonitrile with solute concentrations at or below 2.5 mg ml^{-1}.

2.3. Methods

The capillary was activated using a modification of a previously described procedure [29]. The capillary was first rinsed with 1 *M* potassium hydroxide for 20 min followed by subsequent rinses of 0.1 *M* KOH and distilled water for 20 min each. A final 20-min rinse was performed with the operating buffer before the capillary was used. Purges with the operating buffer were done after each run for 5 min using a vacuum of ca. 16.5 in.Hg (1 in.Hg = 3386.4 Pa) at the detector reservoir.

Electroosmotic velocities were measured where applicable using a method previously published [30].

3. Results and discussion

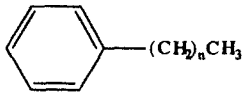
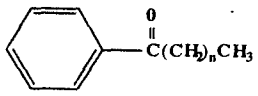
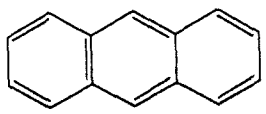
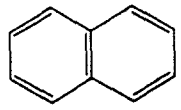
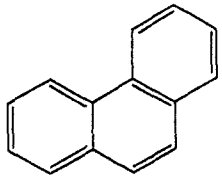
Hydrophobic interaction chromatography (HIC) has been utilized in many successful fractionations of both water-soluble proteins and membrane proteins [31]. In theses cases, gel beds consisting of uncharged amphiphilic agarose

derivatives were used although charged gels [32] can also be employed. Bonded phase HIC utilizes a hydrophilic polymeric layer that totally covers a silica or polymer support surface and into this layer are incorporated short alkyl or aryl chains in low density. The mild conditions of this type of HIC separate by selectivity based on the hydrophobicity of the surface amino acids and usually render proteins intact [33,34]. Unlike these forms of HIC, HI-EKC is performed in the free solution mode. All hydrophobic interactions take place between solubilized analytes and the hydrophobic portions of free (non-micellized) surfactant monomers in solution.

In this investigation, the surfactants SDS and CTAB were used with high amounts of acetonitrile such that micelles were not formed. Only the free surfactant monomers were present in the running buffer. Initial studies, however, focused on utilizing SDS at concentrations below the critical micelle concentration (CMC) in totally aqueous solutions. In these studies, very hydrophobic alkylbenzenes (C_{13}, C_{14}, C_{16}) were used as the probe analytes as well as the polyaromatic hydrocarbons anthracene, naphthalene and phenanthrene. Table 1 provides the structures, CMCs and aggregation numbers of these two surfactants as well as the general structure of the analytes studied, i.e., the alkyl aryl ketones, alkylbenzenes, and the three polyaromatic hydrocarbons.

The first attempts of achieving separations of very hydrophobic compounds by HI-EKC involved utilizing SDS at concentrations ranging from 2–5 m*M* with 10 m*M* phosphate buffer. Under these experimental conditions, no micelles were present. This was verified by performing runs with hydrophilic to moderately hydrophobic alkyl aryl ketones (C_8–C_{14}). If micelles had been present there would have been some retention of these analytes. This was not observed as all of the analytes eluted with the

Table 1
Structures, critical micelle concentrations and aggregation numbers of the surfactants; general structures of the analytes

Surfactants		
SDS $(CH_3(CH_2)_{11}0S0_3^-Na^+$	CMC = 0.008 *M*	Aggregation number 62
CTAB $(CH_3(CH_2)_{15}N^+(CH_3)_3Br^-)$	CMC = 0.0013 *M*	Aggregation number 78
Analytes		
Alkylbenzenes (n = 7, 8, 10)		
Alkyl aryl ketones (n = 10, 12, 14, 16)		
Polyaromatic hydrocarbons	 Anthracene	 Naphthalene
	 Phenanthrene	

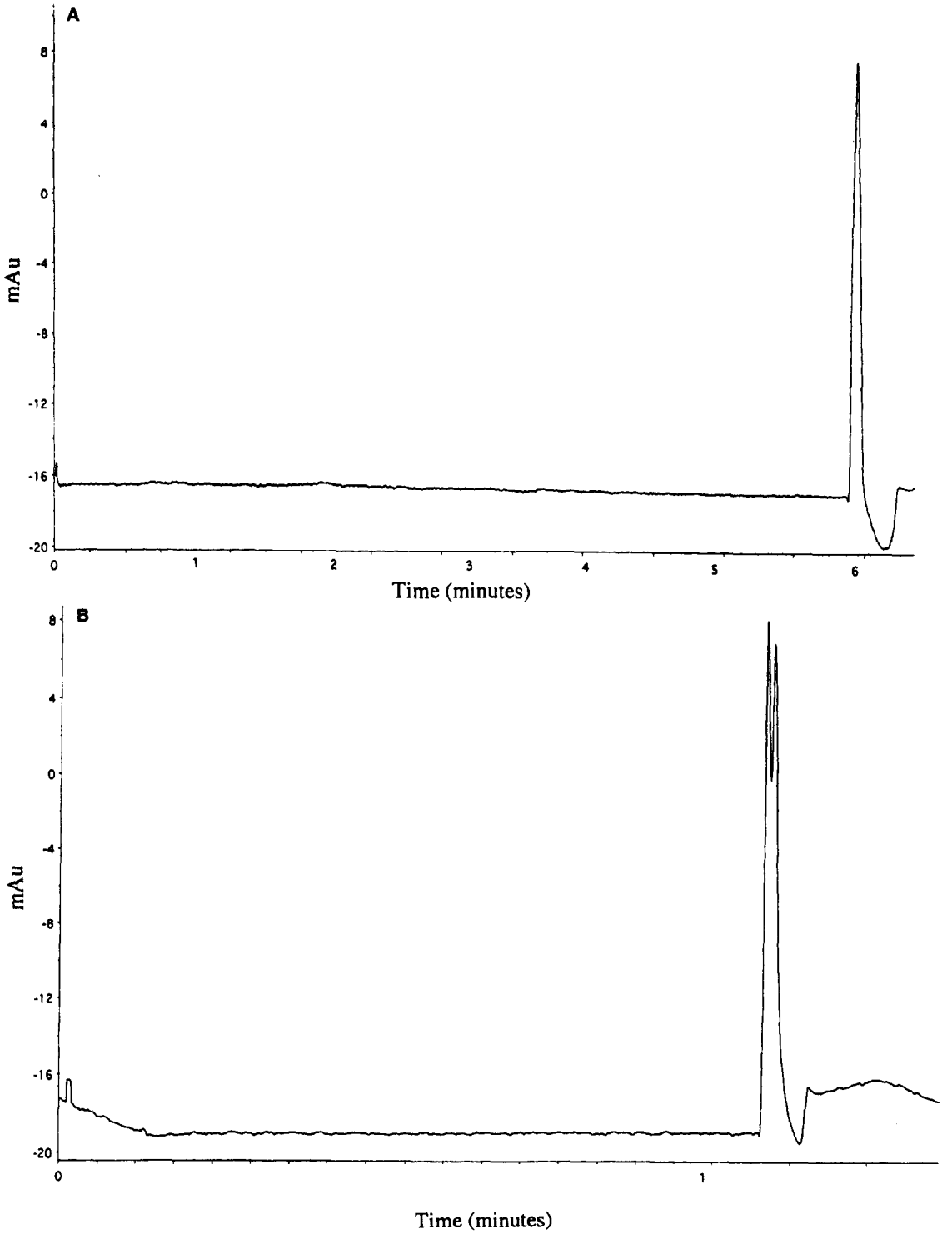


Fig. 1. Elution of very hydrophobic alkylbenzenes (*n*-heptyl-, *n*-octyl- and *n*-decylbenzene) with the electroosmotic flow marker under non-micellar electrokinetic conditions and an applied voltage (current) of (A) 5.0 kV (4.2 μA) and (B) 20.0 kV (27.8 μA). Running buffer: 3 m*M* SDS–10 m*M* phosphate buffer (pH 7.0). Other conditions as in the Experimental section.

electroosmotic flow marker (t_0). In order to test the theory of possible hydrophobic interactions between the free SDS monomers and the compounds of interest, separations of very hydrophobic alkylbenzenes were attempted. Since the alkylbenzenes chosen for this part of the investigation possessed long alkyl chains, it was hoped that these long chains would interact with the hydrophobic moiety of the SDS monomer and result in separation with the elution order following that of MEKC with the most hydrophobic analyte experiencing the greatest retention.

Fig. 1 shows the results obtained with a running buffer system containing 3 m*M* SDS. The operating voltage for this run was 5 kV (Fig. 1A). As can be seen all the alkylbenzenes elute with the electroosmotic flow marker. When the operating voltage was raised to 20 kV, a slight separation was observed as shown in Fig. 1B; reproducibility of this separation was verified with triplicate runs. When the operating voltage was raised to 30 kV no real improvement was observed in the resolution. Similar separations were tried with higher SDS concentrations (3.5, 4 and 5 m*M*) in an attempt to increase the number of free surfactant monomers available for hydrophobic interactions, but the results were similar to that attained with 3 m*M* SDS. The results indicated that there was an insufficient amount of free SDS monomers to achieve the amount of hydrophobic interactions necessary to obtain the desired separations.

Fig. 2 displays the results obtained for the three polyaromatic hydrocarbon compounds. As

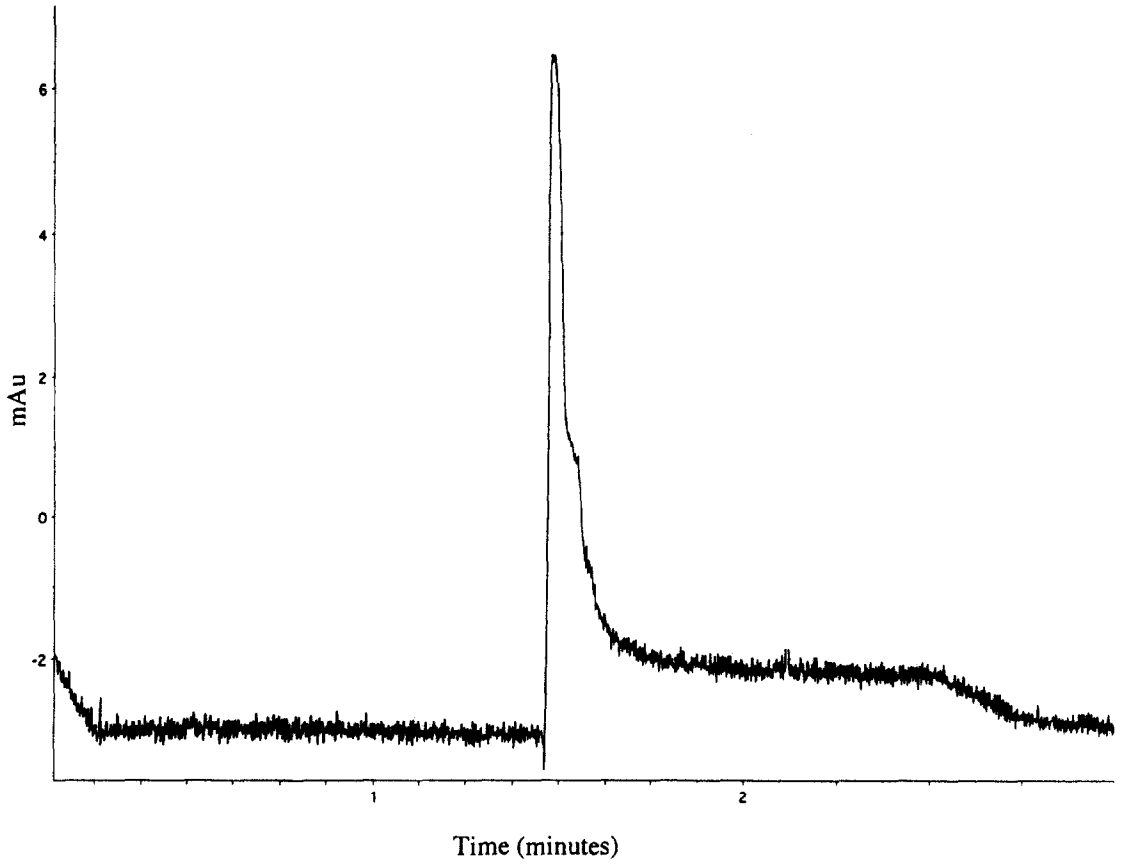


Fig. 2. Elution of three polyaromatic hydrocarbons (naphthalene, anthracene and phenanthrene) with the electroosmotic flow marker. Conditions as in Fig. 1B.

can be seen, all the analytes elute with the electroosmotic flow marker. The separation of these compounds was attempted in order to determine if a better separation could be obtained when the analyte structure contained more aromatic rings as opposed to the long alkyl chains of the alkylbenzenes. The results in Fig. 2 support the findings obtained with the alkylbenzene separations since even a change in solute structure did not produce the desired separation.

In an effort to increase the number of free SDS monomers, separations were attempted with running buffer solutions consisting of 50 m*M* SDS, 50% acetonitrile and 5 m*M* phosphate buffer. In order to ensure that no SDS micelles were present in the running buffer, separations of small neutral molecules were tried. As before, all the solutes eluted with the electroosmotic flow marker which indicated that no SDS micelles were present. Separations of C_{18} and C_{20} alkyl aryl ketones were then attempted (see Fig. 3) using applied voltages of 20, 25 and 30 kV; and hydrodynamic injection times of 5, 3 and 1 s,

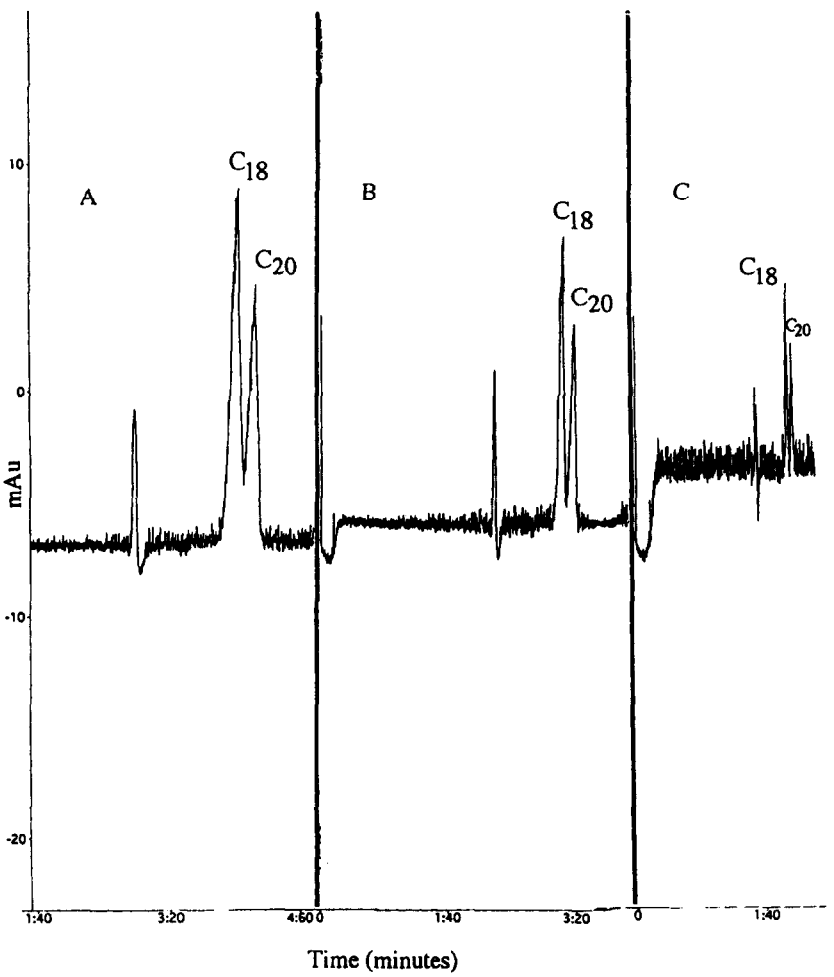


Fig. 3. Separation of alkyl aryl ketones, C_{18} and C_{20}, using a high concentration of *non*-micellized SDS (50 m*M* SDS–50% acetonitrile–5 m*M* phosphate buffer, adjusted to pH 7.0). Other conditions: (A) applied voltage: 20 kV, 5 s injection; (B) applied voltage: 25 kV, 3 s injection; (C) applied voltage: 30 kV, 1 s injection.

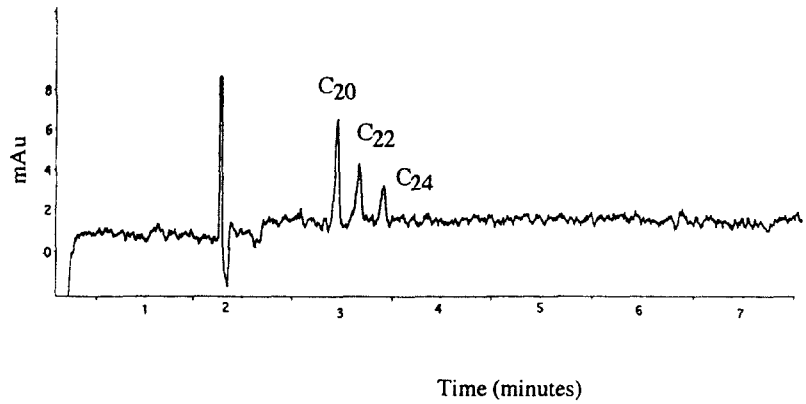


Fig. 4. Separation of C_{20}, C_{22}, C_{24} alkyl aryl ketone homologues using a high concentration of *non*-micellized SDS. Current = 106 μA, other conditions as in Fig. 3C.

respectively. As the operating voltage was increased from 20 to 30 kV with a simultaneous decrease in injection volume, complete resolution of these hydrophobic compounds was achieved in less than 2 min. The elution order followed that seen in MEKC as the less hydro-

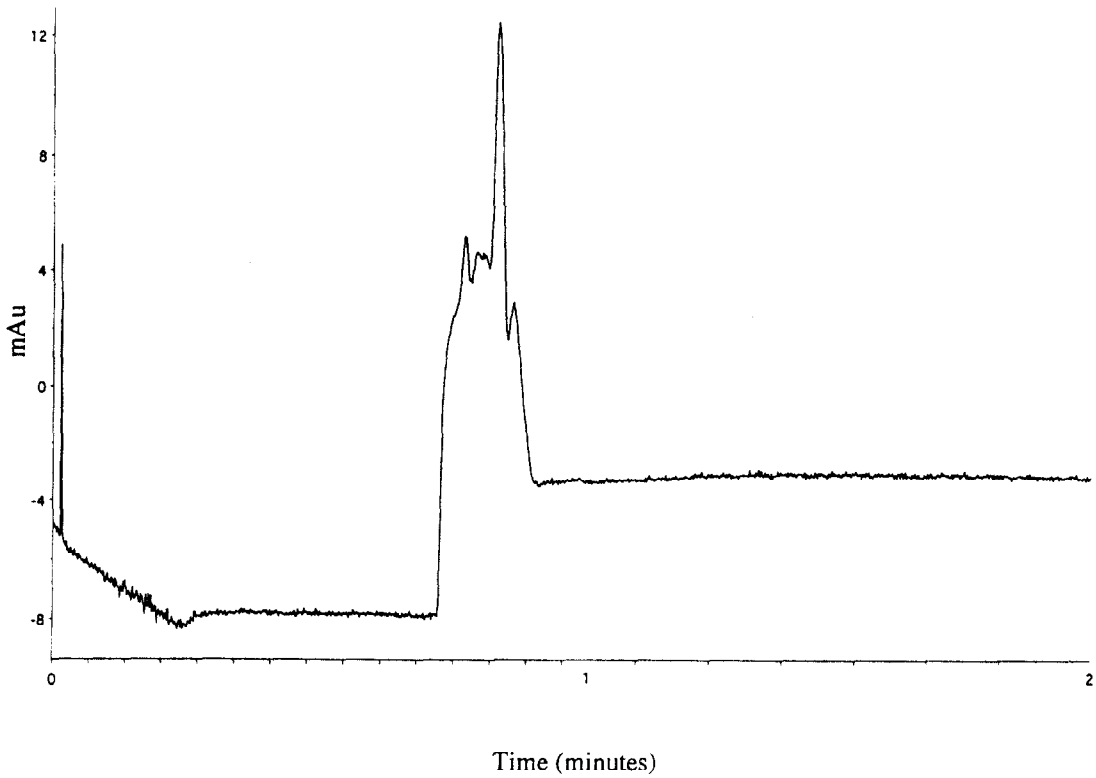


Fig. 5. Elution (but minimal separation) of C_{20}, C_{22}, C_{24} alkyl aryl ketone homologues using a micellar CTAB phase. Experimental conditions: 40 m*M* CTAB–40% acetonitrile–5 m*M* phosphate buffer (pH 7.0); applied voltage 30 kV; operating current < 60 μA.

phobic analyte experiences weaker interactions with the free SDS monomers and consequently elutes closer to the electroosmotic flow marker as opposed to the more hydrophobic analyte which experiences a stronger hydrophobic interaction and therefore increased retention. Fig. 4 shows the separation obtained of the three most hydrophobic alkyl aryl ketones (C_{20}, C_{22}, C_{24}) with this running buffer system. The analysis time for this separation is equally impressive. It is important to note that these homologues would have formed precipitates in a totally aqueous medium. The samples injected were dissolved in 100% acetonitrile and since the running buffer consisted of 50% acetonitrile, no precipitation took place in the capillary. Clearly this is an advantage as this had been a problem with MEKC separations where the amount of organic modifier in the running buffer is much less.

With the success of the SDS-based HI-EKC separations, the next logical approach was to attempt separations using a cationic surfactant instead of an anionic surfactant. A surfactant system consisting of 40 m*M* CTAB–40% acetonitrile was first tried. However, as seen in Fig. 5, all the alkyl aryl ketones elute in less than 1 min. This indicated the presence of CTAB micelles because these hydrophobic analytes would reside in the inner regions of the micelle and elute together. In addition, if CTAB micelles were present, one would expect the very quick traversion through the capillary, and indeed this was observed. We then tried a running buffer system consisting of 20 m*M* CTAB–50% acetonitrile. A separation of hydrophilic to moderately hydrophobic alkyl aryl ketone homologues with this system did not result in the elution of any of the analytes after a run time of 30 min, indicating that there were no CTAB

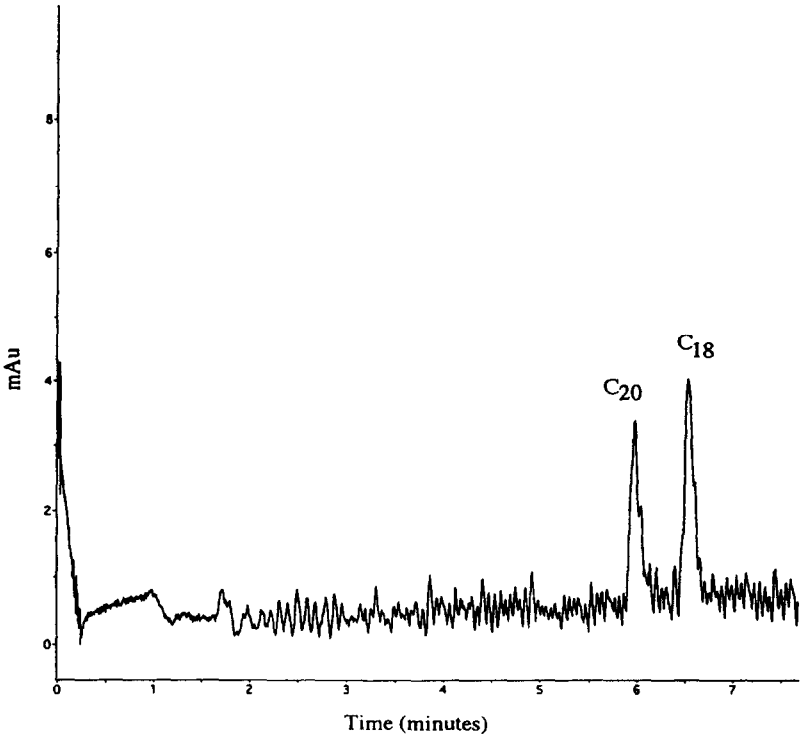


Fig. 6. Separation of alkyl aryl ketones, C_{18} and C_{20}, using a high concentration of *non*-micellized CTAB. Experimental conditions: 20 m*M* CTAB–50% acetonitrile–5 m*M* phosphate buffer (pH 2.8); applied voltage 30 kV; operating current < 40 μA; 1 s injection.

micelles present. Fig. 6 displays the separation of the C_{18} and C_{20} alkyl aryl ketones using the 20 m*M* CTAB–50% acetonitrile system. By comparison of these results with Fig. 3, it is easy to deduce the reversal in elution order of these two analytes. The buffer pH for the separations was 2.8. This was done to decrease the electroosmotic flow such that the positively charged CTAB monomers would serve as the mobile phase and the partially aqueous buffer would serve as the stationary phase. If the pH of the running buffer had been 7.0, both the CTAB monomers and buffer system would rapidly traverse the capillary before any significant hydrophobic interactions, i.e. separation could take place and consequently all the analytes would quickly exit the capillary. By operating at a low buffer pH with the CTAB system, any separations based on hydrophobic interactions result in a completely reversed elution order as the more hydrophobic analytes experience stronger hydrophobic interactions with the free CTAB monomers and travel toward the detector at a faster velocity. The analysis time was less than 7 min for this separation and operating currents were considerably lower than that observed with the SDS-based HI-EKC separations. In Fig. 7, separation of the three most hydrophobic alkyl aryl ketones (C_{20}, C_{22}, C_{24}) is shown. Once again, the reversal in elution order is easily seen in comparison to that observed in Fig. 4 with the SDS-based separations.

In addition, when HI-EKC separations are performed, the alkyl aryl ketone homologues

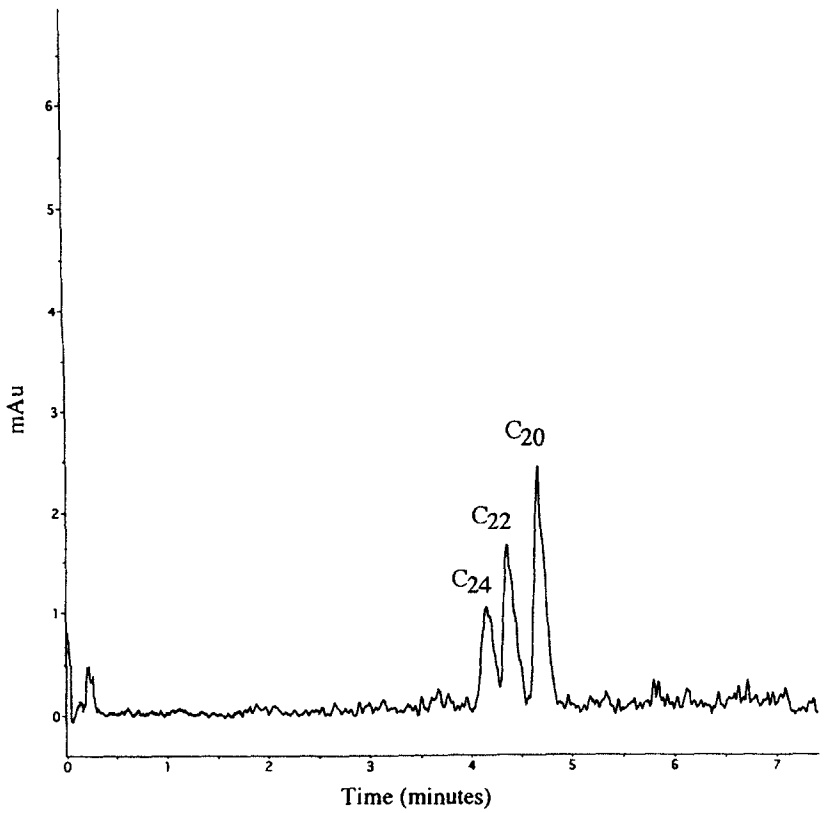


Fig. 7. Separation of C_{20}, C_{22}, C_{24} alkyl aryl ketone homologues using a high concentration of *non*-micellized CTAB. Conditions as in Fig. 6.

(C_{18}, C_{20}, C_{22}, C_{24}) could be used as retention index standards for the calculation of retention indices of very hydrophobic analytes.

4. Conclusions

Clearly, the combination of both the SDS- and CTAB-based HI-EKC methods represent a powerful new way to analyze very hydrophobic analytes. The rapid analysis times and high resolution seen in HI-EKC are very advantageous. If an organic solvent gradient system were available, one could easily run MEKC to separate the hydrophilic to moderately hydrophobic compounds in a sample and then increase the percentage of organic solvent to eliminate the micelles so that the very hydrophobic compounds could be separated as described here. Our approach may serve to bridge the gap between MEKC with predominately aqueous buffer systems and MEKC with non-aqueous buffers, the latter of which has yet to be explored.

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