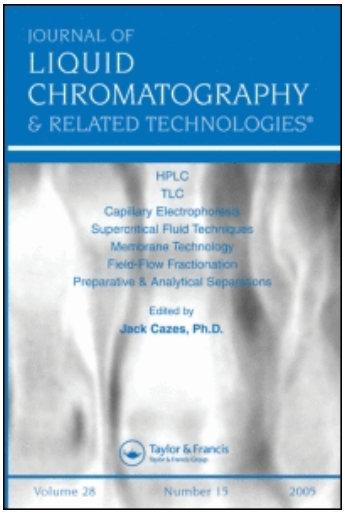

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CAPILLARY ELECTROPHORESIS (CE) METHODS FOR THE SEPARATION OF CARCINOGENIC HETEROCYCLIC AROMATIC AMINES

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ABSTRACT

Recently capillary electrophoresis (CE) has been used for the separation and detection of toxic heterocyclic aromatic amines (HAA) that are present in several environmental samples and food samples. Methods found in the literature were studied and compared in detail, and the conditions for the improved separation of a group of eight HAA are discussed. The effects of buffer pH and the concentration of the organic modifier (methanol and acetonitrile) on the separation and elution order of the HAA were also studied. In addition, for the first time, the running buffer was modified with α-, β-, and γ-cyclodextrin. The overall separation was improved with β-cyclodextrin, and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) gave the strongest interaction.

INTRODUCTION

Heterocyclic aromatic amines (HAA) are an important class of mutagenic/carcinogenic substances mainly found in cooked meat and fish. Sugimura and coworkers,[1] in 1977, found that the smoke and flesh of broiled fish and beefsteak had a high mutagenic activity, which could not be attributed entirely to benzo(a)pyrene. HAA have been found in environmental samples such as airborne particles, rain water, soil, and cigarette-smoke-polluted indoor air.[2] Some of these compounds have also been found in alcoholic beverages such as beer and wine.[3,4] HAA are formed at ng/g levels during the heating process and are the pyrolysis products of amino acids and proteins.[5] The main precursors are believed to be creatine/creatinine, free amino acids, and sugars.[6] All of the known HAA test positive in the Ames/*Salmonella* microsome-mediated mutagenicity test.[7] Many of them are known to induce tumors in the liver, lung, breast, small and large intestines, and other sites.[5] All this suggests that these compounds appear to be ubiquitous environmental pollutants, and their daily exposure to humans is a cause for concern. Thus, very sensitive methods are needed to identify and quantify these compounds down to the ng/g level or even lower.

Several methods have been developed to quantify the HAA. The most common ones use GC-MS,[8,9] HPLC-MS,[10-12] and immunoaffinity chromatography.[13] Recently, solid-matrix luminescence has shown to be useful for the detection of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) at the sub-nanogram level.[14-17] CE has emerged as a convenient method for the separation of HAA.[18-22] In this work, several of the CE and micellar electrokinetic chromatography (MEKC) systems that are found in the literature were compared and attempts were made to improve their efficiency in separating a group of eight HAA. Factors such as pH of the running electrolyte and organic modifier concentration were varied and their effects on the separation of the HAA were studied. Also, cyclodextrin (CD) modified CE was used for the first time to attempt to improve the separation of the eight HAA as well as pairs of the HAA.

EXPERIMENTAL

Chemicals and Reagents

All chemicals and solvents used were of the highest purity available. 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-9H-pyrido[2,3-b]indole (AαC), 3-amino-1-methyl-5H-pyrido[4,3-b]indole acetate (Trp-P-2 Acetate), 2-amino-3-methyl-3H-imidazo[4,5-f]quinoline (IQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-

trimethyl-3H-imidazo[4,5-f]quinoxaline (4,8-DiMeIQx), and 2-amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-f]quinoxaline (4,7,8-TriMeIQx) were purchased from Toronto Research Chemicals Inc., Ontario, Canada. 9-H-Pyrido[4,3-b]indole (Norharman), sodium tetraborate decahydrate, α-cyclodextrin hydrate, β-cyclodextrin hydrate, and potassium chloride (99.999%) were purchased from Aldrich Chemical Company, Milwaukee, WI. Cetyltrimethylammonium bromide (CTAB) was purchased from Sigma Chemical Company, St. Louis, MO. Sodium chloride, hydrochloric acid, and disodium hydrogen phosphate were obtained from Spectrum Chemical Mfg. Corp., Gardena, CA. Citric acid (anhydrous), HPLC grade methanol, HPLC grade acetonitrile, and HPLC grade water were purchased from Fisher Scientific, Fair Lawn, NJ. γ-Cyclodextrin was donated by Cerestar USA, Inc., Hammond, IN.

Instrumentation

All separations were performed on a Beckman P/ACE™ 5000 Series Capillary Electrophoresis System with System Gold® version 8.1 software (Beckman Instruments, Inc., Fullerton, CA). The capillary zone electrophoresis (CZE) separations were all done in the conventional operating mode with the cathode at the detector end of the capillary. For the MEKC separation, the polarities of the electrodes had to be reversed since a cationic surfactant was used. The anode was then at the detector end. An uncoated fused-silica CE column (75 μm I.D., 375 μm O.D., 50 cm length to detector, and total length 57 cm) obtained from Beckman Instruments, Inc., Fullerton, CA was used. The temperature of the capillary was maintained at a fixed level by means of a liquid coolant in the capillary cartridge. All injections were done in the hydrodynamic mode and the solutes were monitored using UV detection at either 214 nm or 254 nm. At the beginning of each day and whenever necessary the capillary was flushed with 0.1M NaOH for 30 min, water for 30 min, and running electrolyte for 30 min. Between runs the capillary was washed with 0.1M NaOH for 2 min and equilibrated by flushing with the running electrolyte for 5 min.

Sample and Buffer Preparation

Stock solutions (20μg/mL) of individual compounds in methanol or of all eight HAA in methanol were prepared and used for further dilutions as necessary. Buffers were prepared fresh each day. Buffer pH was measured using an Orion Research Digital Ionalyzer/501 pH meter (Orion Research Inc., Cambridge, Mass.). The buffers were first filtered through a 0.2 μm filter and sonicated for 15 min to degas the buffers.

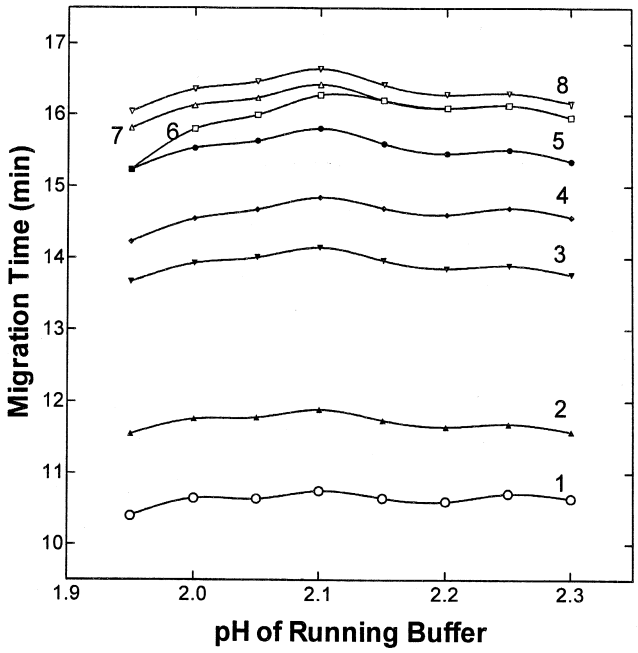

Figure 1. The effect of pH on the migration time. Buffer: Phosphate, Voltage: 20 KV, Temperature:25°C, 1μg/mL of each HAA, 2 s injection and UV detection at 254 nm. Solute identification, (1) IQ, (2) Norharman, (3) Trp-P-2 acetate, (4) AαC, (5) MeIQx, (6) TriMeIQx, (7) DiMeIQx, and (8) PhIP.

RESULTS AND DISCUSSION

Phosphate System

The separation of the eight HAA was studied with a phosphate buffer system developed by Lee and co-workers.[18-20] The buffer consisted of 50 mM Na_2HPO_4, 20 mM citric acid, 30mM NaCl, and 26% methanol. The pH of the buffer was adjusted to the desired level with H_3PO_4.

The applied voltage was 20 KV, the separation temperature was maintained at 25°C, and the absorbance was monitored at 254 nm. Compounds were identified from their migration times obtained by injecting each compound individually.

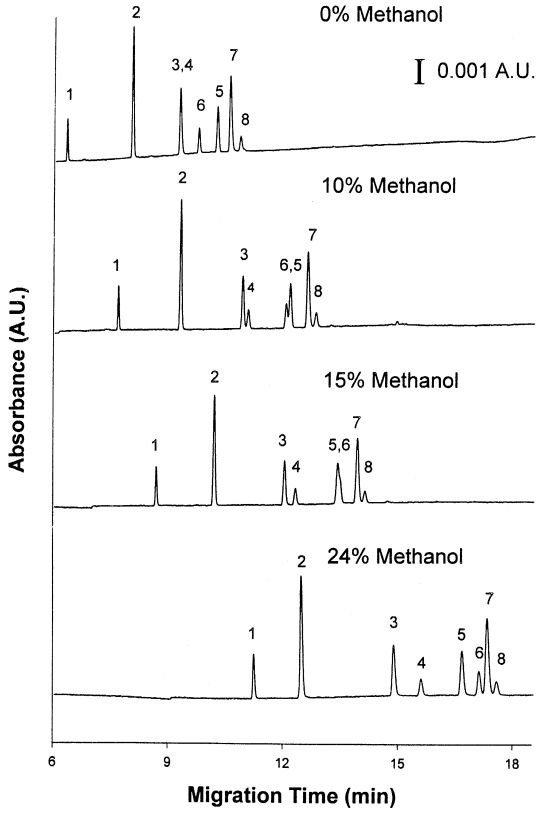

Figure 2. Effect of methanol on the separation. Buffer: Phosphate, pH 2.05, Voltage: 20 KV, Temperature 25°C, 1μg/mL of each HAA, 4 s injection and UV detection at 254 nm. Peak identification same as in Figure 1.

Effect of buffer pH

Since buffer pH has a large influence on electroosmotic flow, it will also have an effect on the separation. The pH of the buffer was varied from 1.90 to 3.00. In general, the migration times changed the greatest in the narrow range of 1.95 to 2.20 (Figure 1). Excellent separations were obtained at pH values of 2.00 and 2.05. In all subsequent work with the phosphate buffer a pH of 2.05 was used. From Figure 1 it can be seen that buffer pH has a large effect on the migration time of TriMeIQx. At a pH of 1.95, it coelutes with DiMeIQx, and at a pH of 2.15, it coelutes with MeIQx. However, the remaining compounds were not affected greatly by pH.

Effect of organic modifier

The effect of methanol and acetonitrile concentration on the separation was studied with the phosphate system. The methanol concentration was varied from 0 to 30%. Figure 2 shows the electropherograms for several different compositions of methanol. For a separation of all eight solutes, 24% methanol in the running buffer gave the best separation. However the last four eluting compounds, MeIQx, TriMeIQx, DiMeIQx, and PhIP, were not separated with high efficiency with 24% methanol. These compounds were better separated with no methanol added to the buffer (Figure 2). In general, with an increase in methanol concentration, the migration times of the compounds increased. This is most likely due to the decreased electroosmotic flow and improved analyte solubility in methanol. The addition of methanol would also be important for the separation of certain pairs of compounds. Trp-P-2 and AαC coelute with no methanol in the running buffer. They are only well separated when the concentration of methanol is 15% or more. With an increase in methanol, there is a reversal in migration order for TriMeIQx and MeIQx. At low concentrations of methanol, TriMeIQx elutes before MeIQx, whereas at a concentration of 15% methanol, they coelute, and at 24% methanol, TriMeIQx and MeIQx are well separated (Figure 2).

Addition of acetonitrile to the phosphate buffer was also studied, and the results were somewhat similar to those of methanol (Figure 3). However, a smaller percentage of acetonitrile (10%) was needed to achieve a complete separation of all eight compounds. Also, adding acetonitrile to the running buffer did not increase the migration times of the compounds. The effect that acetonitrile had on the separation of pairs of compounds like Trp-P-2 and A∝C, IQ and norharman, and TriMeIQx and MeIQx was the same as that of methanol. After comparing the separation results with methanol and acetonitrile, it was concluded that the best separation with the phosphate system was with a pH of 2.05 and 24% methanol (Figure 2).

Lee and co-workers[18-20] used a combined orthogonal array design and overlapping resolution mapping for the optimization of the CZE separation of eleven HAA. First, they used the orthogonal array design to determine the most important factors and interactions. Based on these results, they used the overlapping resolution mapping scheme and obtained global optimum conditions for the separation. They employed these optimum conditions for a CE separation of HAA in rain water samples.[19] In comparing our results with theirs, we have shown that small changes in the buffer pH can have a significant influence on migration times of some of the HAA. In addition, we have shown that methanol and acetonitrile in the running buffer give similar results, but methanol is a better overall organic solvent for the separation of the HAA.

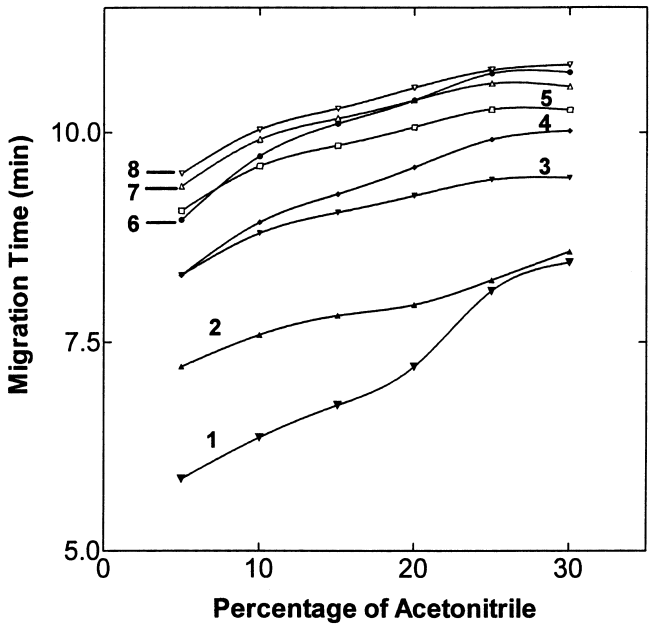

Figure 3. Effect of acetonitrile on the separation. Buffer: Phosphate, pH 2.05, Voltage: 20 KV, Temperature 25°C, 1μg/mL of each HAA, 4 s injection and UV detection at 254 nm. Peak identification same as in Figure 1.

KCl-HCl System

The separation of HAA in a meat extract was studied with a KCl-HCl electrolyte system by Puignou et al.[22] In our work, the running buffer was similar to that of Puignou.[22] It consisted of 10 mM KCl, and the pH was adjusted to the desired level with HCl. The sample solution contained 0.5 μg/mL of each HAA, and it was injected for 2 s. The applied voltage was 20 KV, the separation temperature was maintained at 30°C, and the absorbance was monitored at 214 nm. Compounds were identified from their migration times obtained by injecting the compounds individually.

Effect of pH

Varying the pH for the KCl-HCl system yielded similar results to the phosphate system. The migration time of TriMeIQx increased to a larger extent than that of the other compounds. At a pH of 1.95, it co-eluted with Trp-P-2 and at a pH of 2.25, with MeIQx. Puignou et al.[22] did not investigate TriMeIQx. Our results show importance of pH in separating TriMeIQx from other HAA.

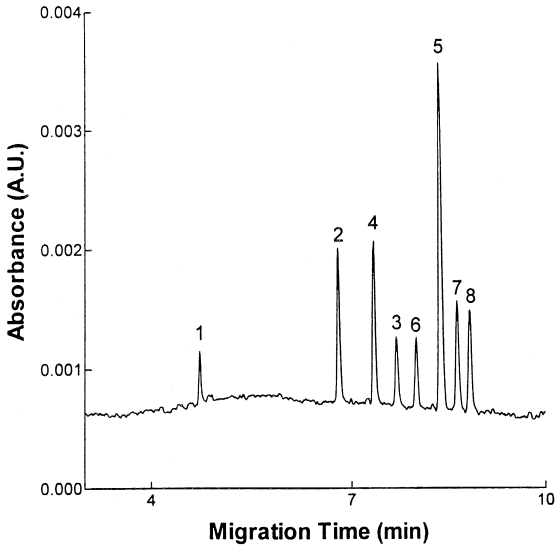

Figure 4. Electropherogram of the separation of all eight compounds. Buffer: KCl-HCl, pH 2.05, Voltage: 20 KV, Temperature 30°C, 0.5μg/mL of each HAA, 2 s injection and UV detection at 254 nm. Peak identification same as in Figure 1.

Effect of organic modifier

The effects of organic solvents with the KCl-HCl system were not studied previously. Addition of methanol and acetonitrile to the running electrolyte using the KCl-HCl system showed no major improvement in the separation. However, the migration behavior of TriMeIQx was the same as with the phosphate system.

At low concentrations of methanol and acetonitrile, TriMeIQx elutes before MeIQx, and at high concentrations it elutes after MeIQx. The best separation with the KCl-HCl system was with a pH of 2.05 and no organic modifier added (Figure 4).

In addition to the KCl-HCl system, Puignou and coworkers[22] used monochloroacetic acid-KOH, formic acid-KOH, glycine-HCl, and citric acid-Na_2HPO_4 as buffer solutions at 0.01 M ionic strength. However, they found the KCl-HCl system to be the best. They investigated the effect of pH, buffer concentration, applied potential, temperature, and injection mode and came up with optimum conditions for the separation of only five HAA. They used these conditions for the determination of HAA in a commercial meat extract.

2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) and 2-amino-3,4-dimethlyimidazo[4,5-f]quinoline (MeIQ) were detected at levels of 10.4 ng/g and 9.3 ng/g, respectively.

Comparison of the Phosphate and KCl-HCl Systems

Both systems were able to separate all eight compounds. However, the KCl-HCl system had several advantages over the phosphate system. The phosphate buffer produced very high currents of the order of 170 μA, which sometimes led to a breakdown of current in the CE instrument. The KCl-HCl electrolyte on the other hand produced much less current (80 μA). Also, the KCl-HCl system had a lower baseline than the phosphate system. This would mean that the KCl-HCl system would give lower limits of detection.

With the KCl-HCl system, a complete separation was achieved in 9 min, whereas 16 min was needed with the phosphate system. The overall separation was better with the KCl-HCl system because with the phosphate system, the last three bands for TriMeIQx, DiMeIQx, and PhIP, were bunched together. Finally, to achieve a complete separation with the phosphate system, 24% methanol had to be added. On the other hand the KCl-HCl system needed no organic solvent.

Micellar Electrokinetic Chromatography (MEKC) System

A micellar electrokinetic chromatography (MEKC) system developed by Olsson et al.[21] was also studied. The running buffer was prepared as follows. A 15 mM sodium tetraborate decahydrate solution was adjusted to a pH of 9.10 with H_3PO_4. Then an amount of CTAB was added to give a 5 mM solution. The final pH was 9.14. Since CTAB is a cationic detergent, the polarities of the electrodes had to be reversed.

Other separation conditions were temperature 25°C, applied voltage 25 KV, and UV detection at 254 nm. The results obtained from using this system were not encouraging. Only IQ, MeIQx, DiMeIQx, and TriMeIQx gave narrow peaks. PhIP and Norharman gave broad peaks with long migration times. Trp-P-2 and AαC did not give any noticeable peaks even when the separation time was 40 min. Olsson et al.[21] used amperometric detection in their work and reported detection limits of 10 μg/L. They also applied their method to a quantitative estimation of HAA in a pan residue extract of fried meat. In their work, they studied quinoline, quinoxaline, and imidazole-type compounds. They did not investigate PhIP, A∝C, and norharman.

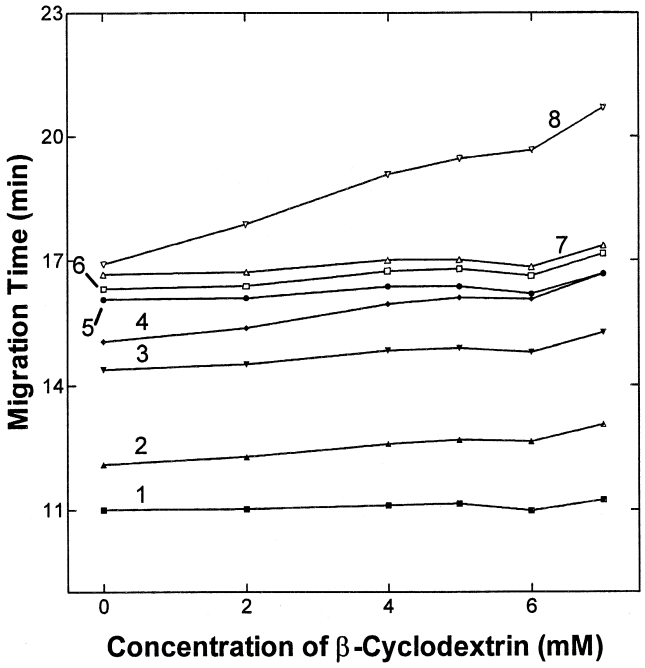

Figure 5. Effect of β-cyclodextrin concentration on migration time. Buffer: Phosphate, pH 2.05, Voltage: 20 KV, Temperature 25°C, 1 μg/mL of each HAA, 4 s injection and UV detection at 254 nm. Peak identification same as in Figure 1.

Cyclodextrin Study

To the best of our knowledge there have been no reports on the use of cyclodextrins in the CE separation of HAA. The addition of α-, β-, and γ-cyclodextrins to the running buffer, using the phosphate and KCl-HCl systems, was investigated with the aim of achieving a better separation of HAA.

Effect of α-cyclodextrin

A concentration range of 0 to 8 mM α-cyclodextrin was employed. With the KCl-HCl system, varying the amount of α-cyclodextrin did not have much of an effect on the separation and on the migration characteristics of most of the compounds. The only compound that interacted with α-cyclodextrin was PhIP. With increasing amounts of α-cyclodextrin the migration time of PhIP increased slightly. The phosphate buffer yielded the same results, but PhIP only interacted to a small extent with α-cyclodextrin.

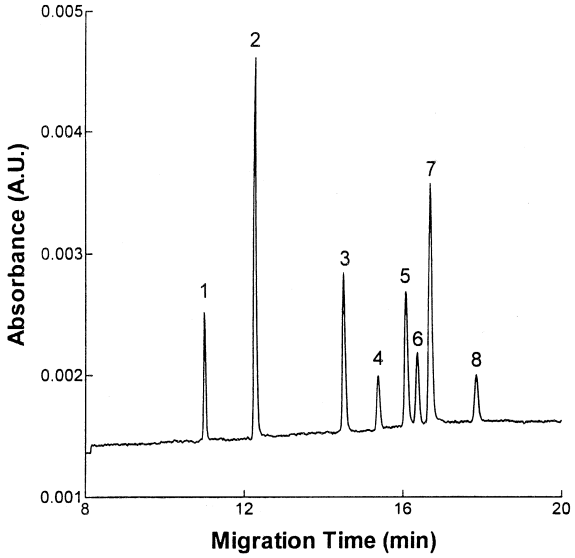

Figure 6. Electropherogram of the separation of all eight compounds with 2 mM β-cyclodextrin. Buffer: Phosphate, pH 2.05, Voltage: 20 KV, Temperature 25°C, 1 μg/mL each HAA, 4 s injection and UV detection at 254 nm. Peak identification same as in Figure 1.

Effect of β-cyclodextrin

The concentration of β-cyclodextrin was varied from 0 to 5 mM for the KCl-HCl system and 0 to 7 mM for the phosphate system. β-cyclodextrin had a small effect on the migration times of AαC and TriMeIQx. However, it had a significant effect on PhIP. Without any β-cyclodextrin PhIP eluted at 8.25 min. With 5 mM β-cyclodextrin PhIP eluted at 10.23 min with the KCl-HCl system. The same results were observed with the phosphate system. As seen in Figure 5 with the phosphate system, β-cyclodextrin had a relatively large effect on the migration times of AαC and PhIP, with PhIP showing the greatest change in migration times. With no β-cyclodextrin, PhIP had a migration time of 16.91 min and with 7 mM β-cyclodextrin, it's migration time was 20.7 min. The best separation was obtained with 2 mM β-cyclodextrin with the phosphate system (Figure 6).

The effect of β-cyclodextrin on PhIP is an important one. To confirm whether a suspected peak is PhIP in an electropherogram of a complex food sample, one could run the separation with the phosphate buffer with and without

β-cyclodextrin added to the buffer, noting the migration times of the suspected peaks for PhIP. If the suspected peak would appear about 4 min later with β-cyclodextrin , then that peak would most likely represent PhIP. The reason why PhIP interacts strongly with β-cyclodextrin is related to the phenyl ring in PhIP which would cause PhIP to fit readily into the β-cyclodextrin cavity. None of the other compounds investigated have this structural feature.

Effect of γ-cyclodextrin

For the experiments with γ-cyclodextrin, the concentration was varied from 0 to 8 mM. Addition of γ-cyclodextrin to both the KCl-HCl and phosphate system had little effect on the migration times of the HAA. Only PhIP showed a slight increase in migration time with increasing amounts of γ-cyclodextrin.

CONCLUSIONS

Three CE methods for the separation of HAA have been studied in some detail. The MEKC system did not give sharp peaks for the compounds, other than the ones with the quinoline and quinoxaline structural features. The other two systems were capable to separating the eight HAA. However, the KCl-HCl system gave a faster, more efficient separation, and needed no organic solvent. For both the KCl-HCl and the phosphate system, the best separation for these eight HAA was obtained at a pH of 2.05. In addition, it was shown that small changes in pH can have a significant effect on the migration time of TriMeIQx.

Organic modifiers were added to the running buffer and with the phosphate system the best separation was with 24% methanol. However, the separation of MeIQx, TriMeIQx, DiMeIQx, and PhIP was better with no methanol present. Acetonitrile had the same general effect on the compounds, but it was not superior to methanol. With the KCl-HCl system, no organic modifier was needed to achieve a separation of the eight compounds.

Cyclodextrins were also studied and an overall improvement in the separation was achieved with β-cyclodextrin. However, more significantly, it was noticed that β-cyclodextrin interacted strongly with PhIP, and this could be explained on the basis of the structure of PhIP.

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