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CAPILLARY ZONE ELECTROPHORESIS VERSUS MICELLAR ELECTROKINETIC CHROMATOGRAPHY IN THE SEPARATION OF PHENOLS OF ENVIRONMENTAL INTEREST

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ABSTRACT

The application of capillary electrophoresis techniques to the analysis of phenols is reviewed. Capillary Zone Electrophoresis and Micellar Electrokinetic Chromatography have been primarily employed. The experimental conditions used for determining phenols in environmental samples by these techniques are presented.

INTRODUCTION

Phenolic compounds are important environmental pollutants, due to their high toxicity even at low concentrations ($\mu g \cdot L^{-1}$ range) and common use. Therefore, their concentration in the environment requires constant monitoring. Many important phenolic compounds have nitro groups (NO_2) and halogen atoms (Cl) bonded to the aromatic rings. These substituents may strongly affect chemical and toxicological behavior.[1,2] These compounds originate from such diverse sources as pesticide application, industrial wastes, water supplies, and automobile exhausts. Chlorophenols as pollutants in drinking water, released

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through waste water, have urged the need for methods to monitor these compounds in industrial effluents and natural waters. In addition, the US Environmental Protection Agency (EPA)[3] has listed eleven phenols as organic priority pollutants: phenol; 2-nitrophenol; 4-nitrophenol; 2,4-dinitrophenol; 2-chlorophenol; 2,4-dichlorophenol; 2,4-dimethylphenol; 4-chloro-3-methyl-phenol; 2-methyl-4,6-dinitrophenol; 2,4,6-trichlorophenol and pentachloro-phenol.

The analysis of phenols has been widely studied using Gas Chromatography (GC)[4-6] and High Performance Liquid Chromatography (HPLC).[7-11] The polarity of phenols and their low vapor pressure are factors that complicate GC analysis. In order to enhance the volatility and detectability of phenols, sample derivatization is typically necessary prior to GC analysis. This is why GC methods present some disadvantages, such as long sample preparation time and incomplete recoveries for many phenolic derivatives. On the other hand, the factors that complicate GC analysis do not have adverse effects on HPLC analysis. The mode utilized in HPLC is the reversed-phase mode with isocratic or gradient elution. However, owing to the inherent limited resolving power of conventional HPLC techniques, optimization of phenols separation often involves complex procedures or numerous experiments, especially gradient elution.

Presently, Capillary Electrophoresis (CE) is a major trend in analytical chemistry, and the number of publications has increased exponentially in recent years.[12-17] Initially, CE was primarily applied to the field of biochemical analysis, but it has also proved useful in the separation of pollutants. The need for optimized separations for a wide variety of compounds has promoted several working modes that can be used in CE. Capillary Zone Electrophoresis (CZE) and Micellar Electrokinetic Chromatography (MEKC) have become the most popular modes of CE in environmental applications. These techniques are a good alternative for pollutants unsuitable for GC, and affected by the poor efficiency of HPLC. For this reason, the review of CZE and MEKC capabilities for the analysis of phenolic compounds is the aim of this work. Articles which appeared on the subject from 1984 through February 1996 are included.

ANALYSIS OF PHENOLS BY MICELLAR ELECTROKINETIC CHROMATOGRAPHY

Micellar Electrokinetic Chromatography (MEKC) was developed by Terabe et al.[18-20] In this technique, an ionic surfactant is added to the CZE buffer at concentrations exceeding the critical micelle concentration (cmc) to form micelles, therefore expanding CE's enormous power to the separation of

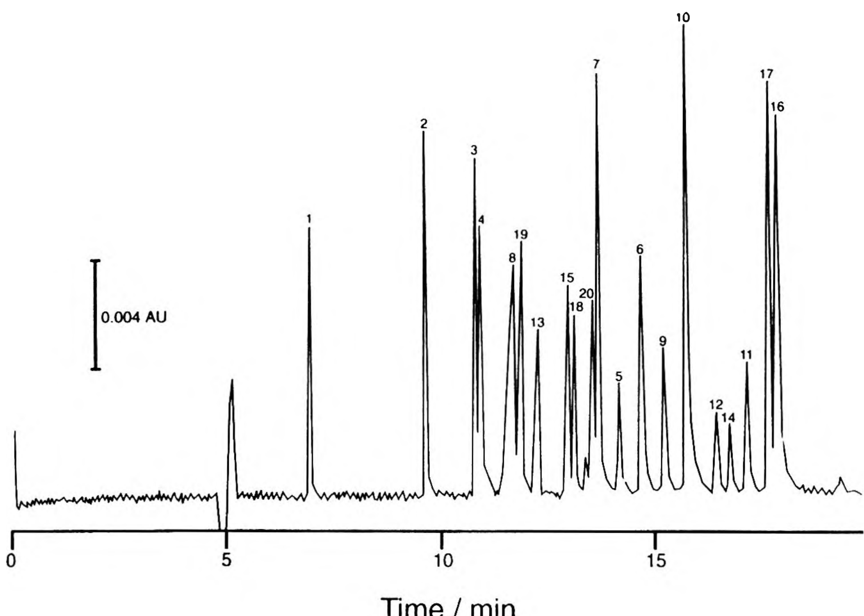

Figure 1. Elcetropherogram of a mixture of all the isomeric chlorinated phenols, including phenol by MEKC. Peaks: (1) phenol; (2) 2-chloro; (3) 3-chloro; (4) 4-chloro; (5) 2,3-dichloro; (6) 2,4-dichloro; (7) 2,5-dichloro; (8) 2,6-dichloro; (9) 3,4-dichloro; (10) 3,5-dichloro; (11) 2,3,4-trichloro; (12) 2,3,5-trichloro; (13) 2,3,6-trichloro; (14) 2,4,5-trichloro; (15) 2,4,6-trichloro; (16) 3,4,5-trichloro; (17) 2,3,4,5-tetrachloro; (18) 2,3,4,6-tetrachloro; (19) 2,3,5,6-tetrachloro; (20) pentachloro. Conditions: micellar solution, 0.07 M SDS, in phosphate-borate buffer, pH 7.0; separation tube, 650 x 0.05 mm i.d.; length of the tube used for separation, 500 mm; total applied voltage, 15 kV; current 28 μA; detection wavelength, 220 nm; temperature, 35°C. Reproduced from (23) with permission of Elsevier Science Publishers.

both charged and uncharged solutes.[21-22] Although anionic surfactants are the most commonly used, especially sodium dodecyl sulphate (SDS), others such as cationic, non-ionic, and zwitterionic have been used too. The micelles are spherical aggregates the hydrophobic groups of which are oriented toward the center of the micelle, and polar or charged groups are along the sphere's surface. Anionic micelles are retarded in the electric field and move at slower velocity than the electroosmotic flow. In this instance, analytes are separated based on their differential partitioning between the buffer phase (which migrates with the velocity of the electroosmotic flow) and the hydrophobic interior of the micelles (micellar phase, which acts as a pseudo-stationary phase). Due to the fact that the micellar phase is moving toward the detector,

an elution window is created and bordered by a column void time (t_o, mobility of the electroosmotic flow) and a micelle migration time (t_{MC}). All analytes must elute between those two limits, t_o and t_{MC}, depending on their partition between the aqueous and micellar phases.

The use of CE for the separation of several *substituted phenols* was first reported by Terabe et al.[18] in 1984. In this initial work on the use of MEKC, up to fourteen phenols were completely resolved within 19 minutes using a borate-phosphate buffer at pH 7.0 (solute molecules were electrically neutral) and with SDS as micellar system.

These authors also studied the separation of all *isomers of chlorophenols* (nineteen) under various conditions of pH and SDS concentration.[23] Complete separation of all isomers was accomplished within 18 min (see Figure 1) under experimental conditions similar to those described previously. In both works, plate numbers ranged from 200,000 to 400,000 and detection limits in the $mg\,L^{-1}$ or nanogram range were obtained with UV detection. The reproducibility and quantitative aspects of the results obtained in the separation of chlorophenols by MEKC were studied.[24] Reproducibility of migration times ($RSD_{n=5}$: 0.3-1.2%) was commensurate with that obtained in HPLC. However, reproducibility of injected amount *(manual gravity flow injection)* was not good ($RSD_{n=5}$: 2-5%, for peak height; and $RSD_{n=5}$: 1-8%, for peak area). Correlation coefficients showed good linear correlations between peak area ($r \geq 0.999$) or peak height ($r \geq 0.99$) and concentrations under two orders of magnitude, when an internal standard calibration method was used.

Good results obtained in the separation of chlorophenols by MEKC were the basis for the first separation of eleven *EPA priority phenols* obtained by Ong et al.[25] in 1990 with MEKC. The authors used the electrophoretic medium described before, phosphate-borate buffer with neutral pH and SDS as surfactant. The separation was obtained within a high analysis time (45 min) with a relatively large inner diameter (180 μm). However, although the resolution was improved when a 50 μm i.d. capillary was used, the analysis time was not shorter.[26] The detection levels were in the nanogram range with UV detection.

Recently, the effects of organic additives (tetrahydrofuran, methanol or acetone) on separations by MEKC have been studied.[27] The results obtained were discussed in terms of MEKC applicability to field screening methods. Methanol and tetrahydrofuran tended to bunch peaks whereas acetone appeared to add selectivity. The best separation of seven priority phenols in less than 20 minutes was under acetone-cholate-borate buffer conditions. The micellar

agent chosen was sodium cholate because bile salts micelles are more stable than conventional SDS micelles in the presence of organic modifiers. Acetone allowed a better resolution by reducing the electroosmotic flow.

Table 1 groups the experimental conditions in which the separation of phenols by MEKC was achieved. It is observed that the electrophoretic medium used is similar in almost all applications, 50-100 mM SDS and borate-phosphate buffer (pH 7). However, sodium cholate with acetone and basic pH can be used for rapid separations. On the other hand, the instrumentation is the same: capillaries of 50 µm i.d. and an effective length of ~ 50 cm, at 10-15 kV, with hydrodynamic injection and on-column UV detector. Finally, it is important to note that all applications include demonstrations of standard separations but not real samples. The reason is the limited sensitivity of UV detectors (> mg L^{-1}).

PHENOLS ANALYSIS BY CZE

Capillary Zone Electrophoresis (CZE) is the most common and simple working mode in CE. The separation by CZE is carried out in a capillary filled with a continuous background electrolyte (buffer).[28-30] The direction and the migration velocity of the analytes are determined by both electrophoresis and electroosmosis phenomena. Analytes are separated based on the difference in their electrophoretic mobilities, which are related to their charge densities, mainly based on differences in solute size and charge at a given pH. Generally, the electroosmotic flow will be higher than the electrophoretic migration velocity of most anionic analyses. Consequently, both cations and anions will migrate in the same direction and can be separated in the same run.

With regard to CZE applicability in the analysis of phenols, it is very interesting to note that, if the suitability of CZE for the separation of the chlorophenols is compared with the results obtained by MEKC,[23] even though the separation by CZE was optimized in terms of pH, buffer concentration, and applied voltage to obtain maximum peak separation, all the isomers of chlorinated phenols could not be resolved by CZE.[31] Therefore, MEKC has greater selectivity than CZE, allowing the analysis of all chlorophenol isomers, as stated previously.

On the other hand, the electrophoretic behavior of the eleven *EPA priority phenols* was studied recently by Li and Locke[32] and a simple analytical method using CZE was established. The effects of pH, buffer concentration, and applied voltage on the separation were investigated, and the main conclusion

Table 1

Experimental Conditions for Separation of Phenols by MEKC

Compound Type*	Buffer**	ΔV	Capillary*** System	Injection Syst/Limits	Detection	Notes	Ref.
Subst'd Phenols							
P; 2-CR; 3-CR; 4-CR 2-CP; 3-CP; 4-CP; 2,3-XY 2,4-XY; 2,5-XY; 2,6-XY; 3,4-XY; 3,5-XY; 4-EP	50 mM SDS 25 mM $Na_2B_4O_7$/ 50 mM NaH_2PO_4 (pH 7.0)	15 kV	50(45) cm x 50 μm	Hydrodynamic (gravity) 4 cm x 5s	UV-270 nm 1-10 mg/L	Sep'ns of std solutes	18
Chlorophenols							
P; 2-CP; 3-CP; 4-CP; 2,3-DCP; 2,4-DCP; 2,5-DCP; 2,6-DCP; 3,4-DCP;; 3,5-DCP; 2,3,4-TCP; 2,3,5-TCP;	70 mM SDS 25 mM $Na_2B_4O_7$/ 50 mM NaH_2PO_4 (pH 7.0)	15 kV	65(50) cm x 50 μm	Hydrodynamic (gravity) 4.5 cm x 5s	UV-220 nm 1-10 mg/L	Optimized sep'n of std solutes	23
2,3,6-TCP; 2,4,5-TCP; 2,4,6-TCP; 3,4,5-TCP; 2,3,4,5-TeCP; 2,3,5,6-TeCP; and PCP	100 mM SDS 25 mM $Na_2B_4O_7$/ 50 mM NaH_2PO_4 (pH 7.0)	15 kV	65(50) cm x 50 μm	Hydrodynamic (gravity) 4.5 cm x 5s	UV-220 nm 1-10 mg/L	Quantitation reproducibility of std solutes	23
Priority Phenols							
P; **2-NP**; **4-NP** **2,4-DNP**; 2-CP; **2,4-DCP**; 2,4-DMP; **4-C-3-MP**; 2-M-4,6-DNP; 2,4,6-TCP and **PCP**	50 mM SDS 100 mM $Na_2B_4O_7$/ 50 mM NaH_2PO_4 (pH 6.6)	10 kV	100(85) cm x 180 μm	Hydrodynamic (gravity) 5 cm x 5s	UV-254 nm 1-10 mg/L	Retention behav. of std solutes	25

(continued)

Table 1 (continued)

Experimental Conditions for Separation of Phenols by MEKC

Compound Type*	Buffer**	ΔV	Capillary*** System	Injection Syst/Limits	Detection	Notes	Ref.
Priority Phenols (cont)							
P; **2-NP**; **4-NP** **2,4-DNP**; 2-CP; **2,4-DCP**; 2,4-DMP; **4-C-3-MP**; 2-M-4,6-DNP; 2,4,6-TCP and **PCP**	50 mM SDS 100 mM $Na_2B_4O_7$/ 50 mM NaH_2PO_4 (pH 6.6)	15 kV	60(50) cm x 50 μm	Hydrodynamic (gravity) 5 cm x 5s	UV-254 nm 1-10 mg/L	Retention behav. of std solutes	26
P; **2-NP**; **4-NP** **2,4-DNP**; 2-CP; **2,4-DCP**; 2,4-DMP; **4-C-3-MP**; 2-M-4,6-DNP; 2,4,6-TCP and **PCP**	100 mM SDS 50 mM boric acid/ (pH 8.35) 10% (v/v) acetone	25 kV	57(50) cm x 50 μm	----	UV-214 nm	Rapid sep'n of std solutes****	27

*P: Phenol; CR: Cresols; XY: Xylenols; EP: Ethylphenol; CP: Chlorophenols; DCP: Dichlorophenols; TCP: Trichlorophenols; TeCP: Tetrachlorophenols; PCP: Pentachlorophenol; CMP: Chloromethylphenol; NP: Nitrophenols; DNP: Dinitrophenols; DMP: Dimethylphenol; C-MP: Chloro-methylphenol; M-DNP: Methyl dinitrophenol.

** Sodium dodecyl sulfate.

***x(y). x: total length, y: effective length

****Application including the separation of solutes in negrite.

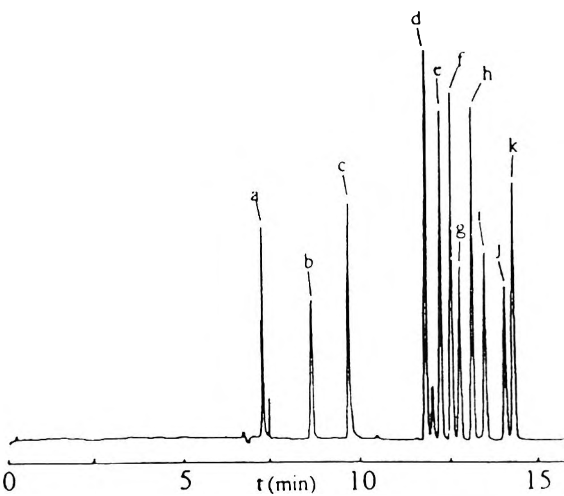

Figure 2. Electropherogram of eleven priority phenols (solute concentration 25 mg L^{-1}) by CZE. Peaks: (a) 2,4-dimethyl-phenol; (b) phenol; (c) 4-chloro-3-methylphenol; (d) pentachlorophenol; (e) 2,4,6-trichloro-phenol; (f) 2,4-dichloro-phenol; (g) 2-methyl-4,6-dinitrophenol; (h) 2-chlorophenol; (i) 2,4-dinitro-phenol; (j) 4-nitrophenol; and (k) 2-nitrophenol. Conditions: phosphate-borate buffer, pH 9.8; separation tube, 100 cm x 75 μm i.d.; length of the tube used for separation, 65 cm; total applied voltage, 22.5 kV; current 53 μA; detection wavelength, 210 nm; vacuum injection time 10 s. Reproduced from (32) with permission of Elsevier Science Publishers.

was that the most critical parameter controlling resolution and separation time was the pH. In this case, CZE provided better results than MEKC, because the eleven phenols can be completely resolved in less than 15 min (Figure 2) analysis time, noticeably shorter compared to the 45 min obtained by MEKC,[26] or the 25 min typically required by HPLC.[33] Optimum conditions included a smaller concentration of the same buffer utilized in MEKC (10 mM phosphate-borate), and basic pH (9.8), for ionization of all phenols except one. Detection was performed with an on-column UV detector and good linearities ($r \geq 0.999$) were obtained for concentrations up to at least 50 mg L^{-1}, with detection limits less than 1 mg L^{-1}.

Comparing the retention behavior between CZE and MEKC, it is interesting to note that the elution order of the eleven phenols found in CZE with a basic buffer (pH ≈10) is opposite to that obtained using MEKC with a neutral buffer (pH ≈7). This is understandable because the separation

mechanisms in CZE and MEKC are basically different. CZE separation is only based on the phenols difference in size and charge at a given pH, whereas in MEKC, it is based on a combination of effects, such as charge/mass ratios, hydrophobicity and charge interactions at the surface of the micelles. In both techniques, the most critical parameter in the separation is the pH, because phenols are weakly basic solutes and the extent of their dissociation, which determines the overall electrical charge of the solute, is governed by the buffer pH.

A new method for the rapid analysis of phenols by CZE was developed in 1995 by Masselter and Zemann.[34,35] In this method, the direction of the electroosmotic flow in a fused silica capillary is reversed by dynamically coating the negatively charged inner surface of the capillary with a layer of either positively charged hemimicelles or polycations, which is formed by adding either a cationic surfactant (cetyltrimethylammonium bromide, CTAB) or a polycation (1,5-dimethyl-1,5-diazaundecamethylene polymethobromide, HDB) to the buffer. A reversal of the electroosmotic flow reduces the analysis time by migration of the anionic analytes in the same direction as the electroosmotic flow *(Coelectroosmotic Capillary Electrophoresis)*. The best separation of several *isomers of alkyl-phenols*, in less than 6 min, is performed using a buffer of low concentration and at high pH value (pH 11, above the pK_A value of the solutes) to achieve the complete dissociation of phenols,[34] with 2-propanol as organic modifier to improve, significantly, peak shape and separation.[35] Other organic solvents (methanol, ethanol, 1-propanol and acetonitrile) have also been studied.[35] The only advantage of this method is the ability to achieve rapid separations of anions at the expense of selectivity and resolution and, although it has been applied only to the separation of several isomers of alkyl-phenols, none of which are priority pollutants, its possibilities could be employed for the rapid analysis of phenols to field-screening methods in simple samples.

The detection system used in all the above-mentioned works has been on-column UV detection, generally employed in CE.[36] This detector is commonly employed in the analysis of phenols because these compounds possess strong absorption in the UV region (210-280 nm). However, despite this detector's acceptable absolute detection limits (in the range of ng solute), the concentration in the peak is relatively high (more than 1 mg L^{-1} for a common solute), because the injection volume in CE is often several nanoliters. This concentration detection ability is not sensitive enough to determine phenols in environmental samples, in which pollutants exist at µg L^{-1} level or lower. Therefore, the use of CE for the analysis of phenols in real samples will not be possible unless enrichment procedures or improved detection systems are employed.

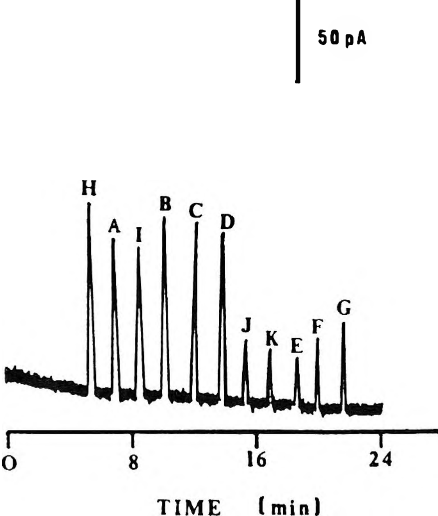

Figure 3. Elcetropherogram of an industrial waste water sample with (A) a 2-chlorophenol concentration of 50 μg L^{-1} by CZE. Peaks: (H) phenol; (B) 2-chlorophenol; (I) 4-chlorophenol; (B) 2,4-dichlorophenol; (C) 2,6-dichlorophenol; (D) o-phenyl phenol; (J) catechol; (K) 2,4,6-trichlorophenol; (E) 2,3,4,6-tetrachlorophenol; (F) 4,5,6-trichloroguiacol; (G) pentachlorophenol. Conditions: 45 mM orthophosphate-15 mM borate buffer, pH 8.0; separation tube, 65 cm x 25 μm i.d.; length of the tube used for separation, 35 cm; total applied voltage, 20 kV; amperotric detection using carbon fibers at +1.4 V versus SCE. Reproduced from (54) with permission of Elsevier Science Publishers.

Several enrichment procedures are being exploited in CE: solid phase extraction with membrane disk[37-39] or in-capillary,[40,41] field amplification injection[42-45] and others based on isotachophoresis.[46-48] Two reviews have been reported recently on referenced procedures.[49,50]

On the other hand, the fact that phenols respond to a sensitive detection method such as electrochemical detection with a microelectrode[51-53] has allowed the separation of *chlorinated phenols in industrial waste* by CZE with on-column electrochemical detection.[54] Seven chlorophenol isomers and three neutral phenols were completely resolved within 24 min (Figure 3) using similar conditions to those described before. Detection was performed in the amperometric mode using a microelectrode (carbon fiber of 10 μm diameter) with an oxidation potential of + 1.4 V vs. SCE. Levels in the μg L^{-1} or picomole range were achieved thermostating the separation capillary. Efficiencies of about 320,000 theoretical plates were obtained, and no

interferences from the impurities present in industrial waste water samples were observed, using only a simple liquid-liquid extraction with chloroform-diethyl ether. Therefore, the use of an on-line electrochemical detector provides excellent sensitivity and selectivity without derivatization.

Chen and Whang[55] also obtained the separation of eleven EPA priority phenols by CZE with on-column amperometric detection. This method has been successfully applied to the analysis of *priority phenols in industrial waste water*. Initially, sodium borate was used as the background buffer (according to previous results). However, large electrophoretic currents (10-100 μA) generated large detector noise, which seriously interfered with amperometric detection (phenomenon reported by other workers[56,57]). In order to minimize this effect, Cyclohexylaminoethanesulfonic acid (CHES) was used as the operating buffer. Due to its zwitterionic nature, electrophoretic currents were only about 1-4 pA. On the other hand, the work electrode potential must be +1.50 V vs. SCE to detect the eleven phenols, although the background stability was poorer than that obtained at +1.10 V, and the carbon fiber electrode durability decreased significantly. But only nine phenols were detected with +1.10 V. The separation of all phenols, obtained within 17 min, presented a number of theoretical plates in the range from 87,000-114,000. Reproducibility results showed satisfactory values in migration times ($RSD_{n=5}$ < 2 %), but not good reproducibilities in the injected amount *(manual gravity flow injection)* with values of $RSD_{n=5}$: 2-9 % for the peak height. However, the results showed good linear correlation ($r \geq 0.99$) between peak height and concentration (over two orders of magnitude), and with concentration detection limits in the $\mu g L^{-1}$ level (10^{-5} - 10^{-7} M). These values were better than those obtained with UV detection but poorer than those of HPLC-amperometric detection.[58]

Finally, laser-induced fluorescence based detection systems have become popular mainly because of their capability to provide extremely high sensitivity (10^{-12} M). However, phenols, as many other compounds, cannot give response because only a few compounds show native fluorescence. In these cases, there are two alternatives: to derivatize non-fluorescent substances[59] or use indirect detection techniques.[60] Briefly, indirect detection consists in the addition of a non-interacting and fluorescing ion to the running buffer to create a constant fluorescence background. When a charged analyte is present, it displaces the fluorescing ion of the same charge due to local charge neutrality, resulting in a decreased background signal even though the analyte does not absorb or fluoresce. This technique was applied by Chao and Whang[61] to the analysis of eleven priority phenols by CZE in NIST standard reference materials and industrial waste waters. In this method, a compromise between optimum peak resolution and satisfactory detection sensitivity must be considered.

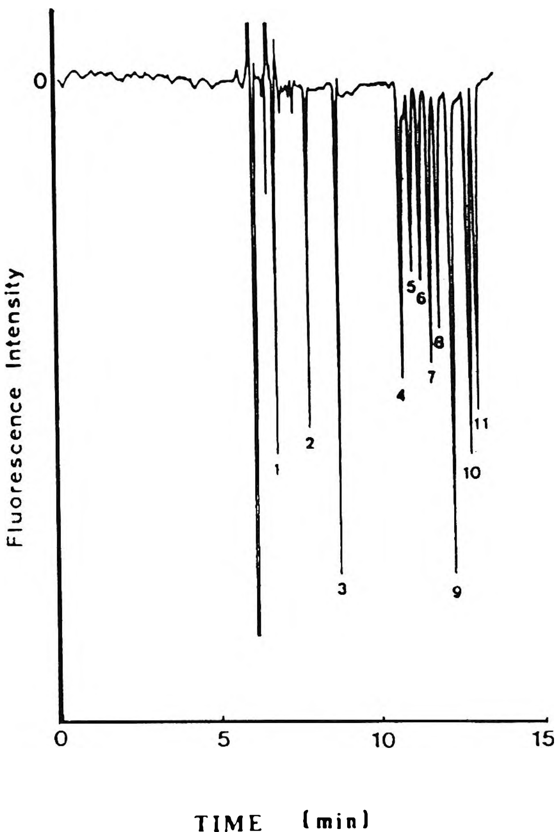

Figure 4. Electropherogram of eleven priority phenols by CZE with indirect fluorescence detection. Peaks: (1) 2,4-dimethylphenol; (2) phenol; (3) 4-chloro-3-methylphenol; (4) pentachlorophenol; (5) 2,4,6-trichlorophenol; (6) 2,4-dichlorophenol; (7) 2-methyl-4,6-dinitrophenol; (8) 2-chlorophenol; (9) 2,4-dinitrophenol; (10) 4-nitrophenol; and (11) 2-nitrophenol. Conditions: buffer, 15 mM borate (pH 9.9) with 1 mM fluorescein; separation tube, 50 cm x 20 µm i.d.; Length of the tube used for separation, 45 cm; total applied voltage, 9 kV; current 2.8µA. Reproduced from (61) with permission of Elsevier Science Publishers.

Firstly, the authors found that a relatively high concentration of electrophoretic buffer (>10 mM) was crucial in the separation of the eleven phenols (the electroosmotic velocity is inversely proportional to ionic concentration[62]), but the increase in the buffer concentration had an adverse effect on the sensitivity of indirect detection. On the other hand, results showed

that the direction of some peaks (positive or negative) was affected by both electric field and background fluorophore concentration. Once the optimal concentrations for the buffer and the fluorophore were chosen (see Table 2), complete separation of the eleven compounds could be achieved in less than 14 min (Figure 4) using a sodium borate buffer at basic pH, as in previous works. The results obtained showed lower analysis time, with better resolution and a higher number of theoretical plates (in the range 99,000-187,000) than those obtained by amperometric detection.[55] The results on reproducibility and quantitative aspects are similar or slightly better, values of $RSD_{n=15} < 1$ % in migration times, $RSD_{n=}$ 2.7-6.3 % for peak height, and linear correlations ($r \geq 0.99$) between peak height and concentration over two orders of magnitude were obtained, with detection limits in the μg L^{-1} range (10^{-6}-10^{-7}M).

Table 2 groups the experimental conditions in which the analysis of phenols by CZE was performed. It is observed that the pH chosen for the electrophoretic medium depends on the type of compounds. The analysis of chlorophenols need a pH between 7 and 8 but, for priority phenols, is more basic (pH ~ 10). Another possibility is a pH 11 when a new method of CZE is used *(Coelectroosmotic Capillary Electrophoresis)*. On the other hand, although in general terms, the buffer used is borate/phosphate, CHES can be utilized. As for the instrumentation, there are several options: capillaries with inner diameter between 20-75 μm, at 9-30 kV, with different injection (hydrodynamic or electrokinetic) and detection systems (UV, amperometric or indirect fluorimetry). It is important to note that detection systems other than UV detectors help to obtain adequate detection limits (in the μg L^{-1} range) to analyze phenols in real samples (industrial waste water), being the most adequate the amperometric to chlorophenols and indirect fluorimetry detection to priority phenols. Finally, the sensitivity obtained by CZE with UV detection is better than that obtained by MEKC (see Table 1), but it is still inadequate for trace analysis of real samples.

CONCLUSION

MEKC techniques were widely used in the analysis of phenols in the past. However, in the last five years, CZE has received more attention. In fact, the theoretical plate number obtained with CZE is higher than with MEKC due to the mass transfer resistance caused by solute partitioning between the bulk buffer and the micelles. Consequently, the sensitivity in MEKC is lower than in CZE.[63] On the other hand, micellar systems are less stable than CZE systems because of the temperature effect on the equilibrium involved. In addition, MEKC optimization is more complicated than in CZE. Two important experimental parameters, pH and micelle concentration, have a great

Table 2

Experimental Conditions for Separation of Phenols by CZE

Compound Type*	Buffer	ΔV	Capillary**** System	Injection Syst/Limits	Detection	Notes	Ref.
Substituted Phenols							
2-MP; 3-MP; 4-MP; 2,3-DMP; 2,6-DMP; 3,4-DMP; 2,3,5-TMP; 2,4,6-TMP	1.25 mM $Na_2B_4O_7$ 15 mM NaH_2PO_4 (pH 11) with 0.001% (v/v) HDB** 40% (v/v) 2-PrOH	30kV	32(24) cm x 50 µm	Hydrodynamic gravity (10 cm x 5s)	UV-254 nm	Sep'ns of std solutes	34 35
Chlorophenols							
2-CP; 2,3-DCP; **2,4-DCP**; 2,5-DCP; **2,6-DCP**; 3,5-DCP; 2,3,4-TCP; 2,3,5-TCP; 2,3,6-TCP;	50 mM Na_2HPO_4/ NaH_2PO_4 (pH 6.9)	18 kV	57(50) cm x 75 µm	Hydrodynamic (Pressure)	UV-214 nm 0.06-0.1 mg/L	Optimized sep'n of std solutes	31
2,4,5-TCP; **2,4,6-TCP**; **2,3,5,6-TeCP**; and **PCP** (Phenol; Catechol; 4-CP; 2-PHP; 4,5,6-TCG)	15 mM Na_3BO_3/ 45 mM NaH_2PO_4 (pH 8.0)	20 kV	65(35) cm x 25µm	Electrokinetic (20kV x 30s)	Amperometric +1.4V *vs.* SCE 1-2 µg/L	Real sample of industr. waste water*****	54
Priority Phenols							
Phenol; 2-NP; 4-NP; 2,4-DNP; 2-CP; 2,4-DCP; 2,4-DMP; 4-C-3-MP; 2-M-4,6-DMP; 2,4,6-TCP; and PCP	10 mM $Na_2B_4O_7$/ Na_3PO_4 (pH 9.8)	22.5 kV	100(65) cm x 75µm	Hydrodynamic (vacuum x 10s)	UV-210 nm 0.3-0.6 mg/L	Optimized sep'n of std solutes	32

(continued)

Table 2 (continued)

Experimental Conditions for Separation of Phenols by CZE

Compound Type*	Buffer	ΔV	Capillary**** System	Injection Syst/Limits	Detection	Notes	Ref.
Priority Phenols (cont)							
Phenol; 2-NP; 4-NP; 2,4-DNP; 2-CP; 2,4-DCP; 2,4-DMP; 4-C-3-MP; 2-M-4,6-DMP; 2,4,6-TCP; and PCP	20 mM CHES*** (pH 10.1)	9 kV	62.5(50) cm x 50μm	Electrokinetic (9kV x 2s)	Amperometric +1.5V *vs.* SCE 0.03-7 mg/L	Real sample of indust waste water	55
	15 mM Na_3BO_3 (pH 9.9) with 1mM fluorescein	9 kV	50(45) cm x 20 μm	Electrokinetic (9kV x 2s)	Indirect Fluorimetry 0.01-0.75 mg/L	Real sample of indust. waste water	61

* MP: Methylphenol; DMP: Dimethylphenol; TMP: Trimethylphenol; PHP: Phenylphenol; TCG: Trichloroguaiacol; CP: Chlorophenols; CMP: Chloromethylphenol; DCP: Dichlorophenols; TCP: Trichlorophenols; TeCP: Tetrachlorphenol; PCP: Pentachlorophenol; NP: Nitrophenols; DNP: Dinitrophenols; MDNP: Methyldinitrophenol.

** HDB: Hexadimethrine bromide.

*** CHES: Cyclohexylaminocthanesulfonic acid.

**** x(y), x: total length, y: effective length.

*****Application including the separation of solutes in negrite and those included in parentheses.

influence on the migration behavior and selectivity in MEKC;[64] but only one important experimental parameter, pH, has a great influence in CZE.[65,66] Despite these drawbacks, MEKC, as opposed to CZE, allows the separation of ions with very similar electrophoretic mobilities, as chlorophenol isomers, because the partition between the aqueous and micellar phases increases the selectivity.

In summary, CZE in conjunction with laser-induced indirect fluorimetry can provide rapid separation and sensitive detection of the eleven priority phenols in real samples. On the other hand, a sensitive detection of chlorophenols can be obtained with amperometric detection, but the separation of all chlorophenols isomers that is possible by MEKC, cannot be achieved by CZE. Finally, it is interesting to note that the separations can be compared to GC separations in terms of resolving power, efficiency, and run time. Moreover, CE techniques do not show peak tailing with the polar nitrophenols and pentachlorophenol, but this appears to be a recurring problem with GC when real sample extracts are injected.

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STUDIES OF THE INFLUENCE OF HYDROGEN CHLORIDE ON THE LIQUID CHROMATOGRAPHIC PROPERTIES OF SILICA-IMMOBILIZED BOVINE SERUM ALBUMIN UNDER NORMAL-PHASE CONDITIONS

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ABSTRACT

The influence of hydrogen chloride on the retention properties of silica-immobilized bovine serum albumin has been studied using a series of simple aromatic phenols and acids. The result of treating the protein with HCl is to mediate hydrogen bonding interactions between the solutes and basic sites in the protein or unreacted amino groups on the surface. The current observations are consistent with previously reported work which examined the influence of HCl on aminopropyl surfaces.

INTRODUCTION

Bovine serum albumin (BSA) is a large flexible "cigar-shaped" protein with a molecular weight of 66,267. The protein has the natural ability to selectively bind a number of ligands including enantiomers such as L-tryptophan and related L-analogs, whereas the corresponding D-isomers do not.[1] A number of investigators have used the silica-immobilized BSA as a

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chiral selective stationary phase in liquid chromatography to resolve racemic compounds.[2-12] The separations are carried out in combination with aqueous buffered eluents to insure that the protein retains much of its native conformation and indigenous binding properties. However, to date, little has been done to investigate silica-immobilized BSA as a normal-phase liquid chromatographic packing.[13,14]

Because proteins are extremely complex biomolecules with tertiary structures stabilized by disulfide bridges. as well as numerous intra- and inter-molecular hydrogen bonding, the number of possible sites where a polar solute molecule may interact, as well as the heterogeneity of these interactions, is great. In the case of BSA, the protein is made up of 582 amino acids with varying α-substituents folded into three domains. To complicate the situation further, the interactions of BSA with numerous organic and inorganic ligands is well known.[15] The protein will bind both anions (chloride, fluoride, bromide and iodide, etc.) and cations (Cu^{++}, Zn^{++}, Ca^{++}, Mg^{++}, etc.).[2]

Since the possibility of hydrogen bonding between ligands and native BSA is known to be an important factor which influence binding, similar strong interactions should potenitally be useful for controling solute retention under normal-phase eleuent conditions. This approach recently has been used to separate phenolic solutes with a ternary mixture of hydrogen chloride-diethyl ether-hexane eluent.

Recently, Ehtesham and Gilpin,[16] have studied the influence of hydrogen chloride on the retention properties of aminopropyl bonded phases under normal-phase eluent conditions by measuring differences in solute retention before and after converting the surface to its corresponding hydrochloride salt. The result of treating the amino groups with HCl was to dramatically decrease the interactions of hydroxylated solutes. In a similar fashion, the influence of hydrogen chloride on the chromatographic properties of silica-immobilized BSA under normal-phase conditions have been examined. The capacity factors for a series of simple hydroxylated and acid containing aromatic compounds have been studied before and after the protein surface was modified by passing a dry HCl-saturated solution of diethyl ether through the column.

The result of this treatment was to mediate the polar interactions which exist between the bonded BSA surface and polar solutes. Thus, solutes which were strongly retained by the unmodified protein could be eluted in a significantly shorter time.

EXPERIMENTAL

Chemicals

All compounds used as solutes as well as the 25% solution of glutaric dialdehyde, the ethanol (anhydrous and denatured with 5% 2-propanol) and the hydrogen chloride (220 g lecture cylinder) were purchased from the Aldrich Chemical Company (Milwaukee, WI, USA). The anhydrous diethyl ether and the HPLC grade 2-propanol, methanol and hexane were from the Fisher Scientific Company (Pittsburgh, PA, USA), the bovine serum albumin (fatty acid free), sodium phosphate (reagent grade) and sodium cyanoborohydride were from Sigma (St. Louis, MO, USA), and the 3-aminopropyltriethoxysilane was from Huls (Bristol, PA, USA). The deionized water was produced in-house using a Millipore (El Paso, TX, USA) model Milli-Q purification system.

Instrumentation

The chromatographic system consisted of a Spectra-Physics (San Jose, CA, USA) model SP8810 precision isocratic pump, model SpectroMonitor III variable wavelength UV detector, and model 4400 Datajet integrator. The samples were introduced using a Rheodyne (Berkeley, CA, USA) model 7125 injection valve with a 20μL loop. In order to insure controlled eluent conditions, the flow rate of the mobile phase was monitored with a Phase Separation LTD (Queensberry, Clwyd, UK) model FLOSOA1 flow meter and the column temperature was controlled in a water bath equipped with a Fisher Scientific model 730 isotemp immersion circulator and a Neslab Instrument (Portsmouth, NH, USA) model EN-350 flow-through cooler.

Column Preparation

The silica-immobilized BSA was prepared as follows. Approximately 2.3 g of Licrospher Si-300 silica (EM Separations, Gibbstown, NJ, USA) were washed with 25 mL of deionized water and the water was decanted off after centrifugation. The silica was dried at 85°C for 4 hours and transferred to a glass reaction vessel. Subsequently, 10 mL of a 10% (v/v) aqueous solution of 3-aminopropyltriethoxysilane were added and the pH of the mixture was adjusted to 3.4 via addition of phosphoric acid. After allowing the reaction to proceed for 3 hours at 75°C, the aminopropyl-silica was washed using six portions of 25 mL deionized water (i.e., the material was centrifuged and the water decanted off between each wash). The product was dried at 100°C

overnight and a small portion of the material was removed for elemental analysis. The remainder (2.4 g) of the modified silica was placed in a flask and 100 mL of a 2.5% aqueous glutaric dialdehyde (10 mL of 25% glutaric dialdehyde and 90 mL 0.05M pH 7 phosphate buffer) and 0.13 g of sodium cyanoborohydride were added. The mixture was allowed to stand for 3 hours after which time the aldehyde activated silica was washed with five 30 mL portions of deionized water and the material freeze dried.

The final protein coupling step was carried out by the drop wise addition of bovine serum albumin (30 mL of 1% BSA diluted with 55 mL of 0.05M pH 7 phosphate buffer) to a flask which contained 2.0 g of the aldehyde-activated silica, 85 mL of 0.05M pH 7 phosphate buffer and 0.1 g sodium cyanoborohydride. The reaction was allowed to proceed 0.5 hour, another 0.1 g of sodium cyanoborohydride was added, and the reaction continued for 12 hours. The resulting silica-immobilized BSA was then washed with 150 mL of deionized water and freeze dried.

A sample of this material was analyzed by Huffman Laboratories (Golden, CO, USA) for carbon, nitrogen and sulfur. The resulting data indicated a coverage of 67 mg of immobilized protein/g of silica.

Approximately 0.5 g of the dried silica-immobilized BSA was added gradually to 30 mL of denatured anhydrous ethanol which was contained in a dynamic packing apparatus. The apparatus was sealed and pressurized to 6000 psi using a Haskel (Burbank, CA, USA) model DST-52 air driven fluid pump with ethanol as the carrier solvent. During this process the 2.1 mm i.d. X 150 mm stainless column were backed in upward fashion.

Chromatographic Studies

The compounds used as test solutes were dissolved in a 17% solution of 2-propanol in hexane at a concentration of ~ 1 mg/mL. Retention data were collected using mobile phases of 100% hexane, 5:95 2-propanol-hexane (v/v) and 10:90 2-propanol-hexane (v/v).

These experiments were carried out on the silica-immobilized BSA before and after it had been treated with a hydrogen chloride-saturated diethyl ether solution. In order to determine the completion of this latter treatment, aliquots were collected at 5 mL intervals, each of these mixed with 25 mL of distilled water and the pH of the water layer measured.

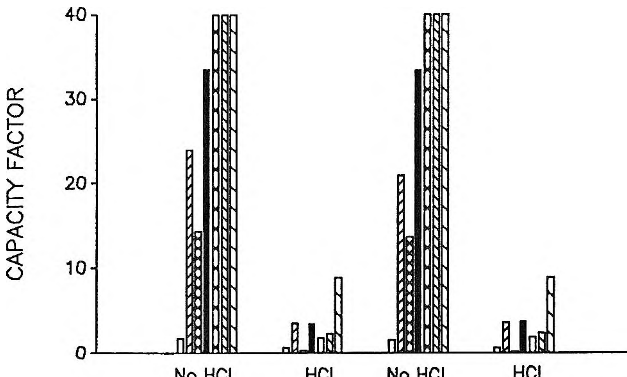

Figure 1. Histogram showing the effect of hydrogen chloride on the retention of the silica-immobilized BSA phase. Mobile Phase: 10% 2-propanol in hexane; Solutes: Graphed in the order presented in Table 2. Note: Bars ending at 40 denote a k' of at least 40 or more. Toluene not shown because k' values are too small to appear on the scale.

RESULTS AND DISCUSSION

Retention measurements were carried out on silica-immobilized BSA before and after treatment with hydrogen chloride. A total of seven sets of retention measurements were made on two different columns cycling between BSA and BSA-HCl. Between each cycle the column was washed with approximately 400 mL of methanol to remove the HCl. As discussed below, this washing was sufficient to remove most of the HCl, but not all, which resulted in incomplete recovery of the columns. In making these measurements, a total of eight organic compounds were selected as test solutes which ranged from a simple aromatic, toluene, to aromatic compounds which contained either one or more polar hydroxyl or carboxyl groups.

An initial set of scouting measurements were made in order to establish eluent conditions of sufficient strength that a majority of the test solutes would elute within one hour. This was done using column 1. A total of three different mobile phases were tried starting with 100% hexane, then hexane with 5% 2-propanol as the polar modifier and finally hexane with 10% 2-propanol. Next, the column was converted to its HCl form under nonaqueous conditions and the measurements repeated for the three mobile phases. The results of this scouting experiment are summarized in Table 1.

Table 1

Retention Data for Silica-Immobilized BSA Before and After Treatment with HCl

	Without HCl	With HCl
Mobile Phase: 10% 2-Propanol in Hexane		
Toluene	0.05	0.03
Phenol	1.57	0.62
Resorcinol	20.9	3.58
Benzoic Acid	13.7	0.20
Methyl 3,5-dihydroxybenzoate	33.4	3.76
3-Hydroxybenozic Acid	Ret.	1.89
4-Hydroxybenzoic Acid	----	2.36
3,5-Dihydroxybenzoic Acid	----	8.86
Mobile Phase: 5% 2-Propanol in Hexane		
Toluene	0.04	0.03
Phenol	3.54	0.70
Resorcinol	99.1	6.96
Benzoic Acid	23.3	0.32
Methyl 3,5-dihydroxybenzoate	Ret.	7.07
3-Hydroxybenzoic Acid	----	3.27
4-Hydroxybenzoic Acid	----	5.10
3,5-Dihydroxybenzoic Acid	----	28.1
Mobile Phase: 100% Hexane		
Toluene	0.08	0.07
Phenol	Ret.	25.4
Resorcinol	----	Ret.
Benzoic Acid	----	Ret

Subsequently, additional experiments were carried out on the same column as well as on a second duplicate column cycling between the BSA and BSA-HCl forms of the surface using hexane with 10% 2-propanol as the eluent. The results from this latter study are given in Table 2.

Table 2

Retention Data Illustrating the Reproducibility of HCl Cycling

		Without HCl	With HCl
Toluene	cycle 1	0.04 ± 0.01	0.03 ± 0.01
	cycle 2	0.05 ± 0.00	0.03 ± 0.00
Phenol	cycle 1	1.57 ± 0.15	0.59 ± 0.04
	cycle 2	1.42 ± 0.18	0.62 ± 0.01
Resorcinol	cycle 1	21.2 ± 3.1	3.49 ± 0.04
	cycle 2	18.8 ± 2.5	3.58 ± 0.06
Benzoic Acid	cycle 1	12.3 ± 3.5	0.27 ± 0.04
	cycle 2	11.1 ± 3.1	0.20 ± 0.07
Methyl 3,5-dihydroxybenzoate	cycle 1	34.8 ± 1.4	3.54 ± 0.00
	cycle 2	28.8 ± 5.3	3.76 ± 0.01
3-Hydroxybenzoic Acid	cycle 1	Ret.*	2.83 ± 0.01
	cycle 2	Ret.	1.89 ± 0.00
4-Hydroxybenzoic Acid	cycle 1	Ret.	2.26 ± 0.03
	cycle 2	Ret.	2.36 ± 0.01
3,5-Dihydroxybenzoic Acid	cycle 1	Ret.	10.7 ± 2.1
	cycle 2	Ret.	8.86 ± 0.06

* Retained with a capacity factor greater than 40.

The variation in capacity factor values obtained between the duplicate columns were within 3-5% for the unmodified BSA and better than 2% for the HCl treated protein. However, comparing the results between the untreated and HCl treated columns, there was a large reduction in retention for the solutes which interact with basic sites. This effect was enhanced as multiple substituents were added to the solute. To further illustrate this dramatic effect the average of the two columns through two complete cycles are presented as a histogram in Figure 1.

The current results are consistent with previously observed reductions in retention for similar hydroxylated solute on aminopropyl bonded phases following conversion of the surface to its hydrochloride salt.[16] The effect of the HCl treatment is to mediate the strong interactions which can occur between the solutes and the basic sites in the protein, as well as possible unreacted basic sites on the surface, since the protein is coupled to the surface via aminopropyl groups. A second feature of the current work, was to illustrate that the protein can be reversibly altered by treatment with HCl as long as eluent conditions are dry. Although there appears to be a small reduction in retention comparing the date from cycle 1 and cycle 2 for both columns (i.e., the retention was always slightly longer in cycle 1 than cycle 2), statistically the results are equivalent within 1 standard deviation unit. The slight reduction, if real, is probably due to trace amounts of HCl retained on the column, even after washing with 400 mL of methanol.

CONCLUSIONS

The strong interactions between silica-immobilized BSA and solutes that contain polar functionality such as hydroxyl and carboxyl groups can be decreased by converting the protein to its HCl form. Such an approach (i.e., the use of protein modifiers) may be useful to create a variety of novel chromatographic supports. It appears that the modification of BSA is reversible assuming that completely anhydrous conditions are maintained. Further experiments are in progress to characterize the normal-phase behavior of silica-immobilized BSA and BSA-HCl, as well as other protein modifiers.

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A FIRST-LEVEL ANALYSIS OF THE ADSORPTION MECHANISM OF ESTERS OF RACEMIC AND MESO-2,3-DIBROMOBUTANE-1,4-DIOIC ACIDS ON SILICA

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ABSTRACT

The thin-layer chromatographic (TLC) retention of eighteen diastereoisomers of type RO_2C-CH(Br)-CH(Br)-CO_2R on silica was studied with 20 computer-selected on the basis of Snyder theory mobile phases having strength, ε, in the range 0.215-0.305 and significantly greater variation in localization, m, and polarity, P'.

A theoretical analysis, using Soczewiński method, the ε values of the mobile phases used and the stereochemical particularities of the compounds, showed that the two ester groups are the main adsorbing groups, shielded by the bromine atoms, which accounts for the relative retention: racemate > meso found in all cases studied. Two mobile phases of intermediate m values lead to best separation of all diastereoisomeric pairs studied.

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H, H, C=C, RO_2C, CO_2R — Z
H, CO_2R, C=C, RO_2C, H — E

type a

CO_2R, Br, H, H, Br, CO_2R — racemate (RR|SS)
CO_2R, H, Br, H, Br, CO_2R — meso (RS)

type b

R = $CH_3, C_2H_5, n-C_3H_7, iso-C_3H_7, n-C_4H_9, sec.-C_4H_9, iso-C_4H_9, n-C_5H_{11}, iso-C_5H_{11}$, cyclohexyl

R = $CH_3, C_2H_5, n-C_3H_7, iso-C_3H_7, n-C_4H_9, sec.-C_4H_9, iso-C_4H_9, n-C_5H_{11}, iso-C_5H_{11}$

Figure 1. Types of the diastereoisomers studied recently[8] and in this paper. One of the enantiomers is represented for the racemate.

INTRODUCTION

In a series of papers,[1] we have reported the TLC adsorptivity of a vast number of conformationally flexible threo-erythro and cis-trans diastereoisomers and rigid Z-E diastereoisomers on, mainly, silica or alumina, with numerous mobile phases. The retention, relative retention and separation of the diastereoisomers have been treated in terms of Snyder theory,[2-4] the Soczewiński method[5] and the Hammett equation.[6,7] Recently, we have studied[8] TLC on silica, of diastereoisomers of type a with an increasing size of group R as a simple case of conformationally rigid diastereoisomers possessing only two equal substituents (see Figure 1).

This paper reports the TLC adsorptivity of the related, but conformationally flexible, diastereoisomers of type **b**, containing two bromine atoms instead of the double bond, enabling us to further compare the role of conformational effects. The mobile phases used were selected on the basis of Snyder theory by a computer program[9] whose general validity in this respect has been recently summarised.[10] The discussion of the data obtained includes the application of the Soczewiński method and a first-level analysis of the adsorption mechanism by a newly proposed procedure.[11]

THEORY

The conversion of the experimental R_F to the TLC retention, R_M, and separation, α, was done by the following equations:

$$R_M = \log k' = \log (1/R_F - 1) \quad (1)$$

$$\log \alpha = R_{M(\text{racemate})} - R_{M(\text{meso})} \quad (2)$$

where k' is the HPLC retention and the subscripts to R_M in the last equation show the configuration of the compound. According to Eq. 2, positive values of $\log \alpha$ correspond to relative retention racemate > meso and vice versa. In addition, a greater absolute $\log \alpha$ value indicates a better separation of the corresponding diastereoisomeric pair.

For the case of retention, R_M, of a sample with mobile phases composed of a non-polar or weakly polar solvent A and a polar solvent B, Soczewiński[5] derived the following equation:

$$R_M = R_{M(B)} - n \log N_B \quad (3)$$

where N_B is the molar fraction of solvent B, $R_{M(B)}$ is the retention when the solvent is pure B and n is the slope of the linear plot. For a given set of mobile phases, n is proportional to the area of solute molecule under adsorption.[12,13]

According to Snyder theory,[2-4] mobile phases are characterised by strength, ε, proportional to the dimensionless Gibbs energy ($\Delta G^o/RT \ln 10$) of adsorption of the mobile phase, localization, m, measuring the specific interactions adsorbent-composing solvents and polarity, P', measuring the interactions sample (solute)-composing solvents (*cf.*, ref. 10). The greater the ε value, the weaker is the sample retention; m and P' tune the selectivity as measured by $\log \alpha$ of solute pairs and strength, respectively.

The dimensionless Gibbs energy of adsorption, Q_i, of a solute group i when $\varepsilon > 0$ is expressed by

$$Q_i = Q_i^o - \varepsilon a_i \quad (4)$$

where Q_i^o refers to the case when pentane is the mobile phase having $\varepsilon = 0$ and a_i is the relative effective area of group i under adsorption. Adsorption of group i is possible if

$$Q_i > 0 \quad (5)$$

Q_i^o and, therefore, Q_i refer to the case where group i is substantially free from interactions with other solute groups. The greater Q_i, the stronger is the adsorption of group i.

Table 1

Data for the Mp's or Bp's and Chemical Shift, δ, of the Proton next to Bromine in ^{1}H-NMR Spectra of the Compounds 1-18 Studied of Type b

R	Configuration	No.	mp °C	bp °C	δ_{CHBr}
CH_3	racemate	**1**	42-43		4.75
	meso	**2**	60-61		4.60
CH_2CH_3	racemate	**3**		137-138/12	4.75
	meso	**4**	56-58		4.60
$CH_2CH_2CH_3$	racemate	**5**		155-160/12	4.78
	meso	**6**		165-166/12	4.70
$CH(CH_3)_2$	racemate	**7**		138-140/12	4.68
	meso	**8**	62-63		4.63
$CH_2CH_2CH_2CH_3$	racemate	**9**		178-182/12	4.73
	meso	**10**		186-188/12	4.65
$CH(CH_3)CH_2CH_3$	racemate	**11**		83-85/0.04	4.70
	meso	**12**		170-172/12	4.60
$CH_2CH(CH_3)_2$	racemate	**13**		90-91/0.03	4.73
	meso	**14**		95-96/0.02	4.68
$CH_2CH_2CH_2CH_2CH_3$	racemate	**15**		117-118/0.10	4.73
	meso	**16**		109-110/0.03	4.65
$CH_2CH_2CH(CH_3)_2$	racemate	**17**		102-103/0.03	4.75
	meso	**18**		113-114/0.03	4.70

The mp's are of samples recrystallized from ethanol.
The bp's were measured under vacuum given in mm Hg.
The δ values are in ppm.
The signal for the CHBr proton is a singlet. Its chemical shift is in a higher field for any meso isomer.

The procedure proposed recently by us[11] for a first-level analysis of the adsorption mechanism is based on calculation of Q_i of all groups i participating in the solute structure under conditions used and discussion of the possible electronic and steric interactions between the adsorbing and non-adsorbing groups i.

EXPERIMENTAL

Compounds **1-18** of type **b** were prepared in good to high yields by the following stereospecific reactions: (a) addition of bromine to maleic anhydride and following esterification gave the racemic compounds and (b) addition of bromine to the corresponding ester of fumaric acid resulted in the meso compounds.

The ^{1}H-NMR spectra of compounds **1-18** were measured on a TESLA 80 MHz spectrometer in acetone solutions with TMS as internal standard. Compounds **1-5, 8, 9** and **18** are known.[14-19]

TLC was performed on silica 60 DG (Readel de Haen, Germany) using the procedure given in ref. 20. The solvents were of analytical-reagent grade. The R_F values were arithmetic means of four to six measurements, showing a reproducibility of ±0.025. The computer program[9] used was LSChrom Ver. 2 for Windows.

RESULTS AND DISCUSSION

The mp's or bp's and data from ^{1}H-NMR spectra of compounds **1-18** are given in Table 1. Table 2 summarizes the mobile phases 1-20 used with their computer - calculated[9] values of ε, m and P', including N_B for the binary mobile phases. The computer choice of mobile phases 1-18 was similar to that described in ref. 10. The mobile phases are composed of two to four solvents comprising eight non-localizing or weakly-localizing solvents and the localizing diethyl ether, ethyl acetate, methyl *tert*-butyl ether, acetonitrile and tetrahydrofurane.[4] Mobile phases 1-10 and mobile phases 11-15 have equal ε of 0.253 and 0.240, respectively. Mobile phases 5 and 16-20 are composed of hexane (solvent A) and ethyl acetate (solvent B) in different ratios, showing molar fraction, N_B, from 0.026 to 0.201 and ε in the range 0.215-0.303. Thus, all mobile phases used have minimum and maximum ε of 0.215 and 0.303, respectively. They show greater variations in m and P', namely $-0.15 \leq m \leq 0.95$ and $0.19 \leq P' \leq 2.75$.

Table 3 includes the data for the experimental R_F values obtained in TLC on silica of compounds **1-18** with mobile phases 1-20. The values of log α derived by Eqns. 1 and 2 and their average values are also included. The tuning effect of P' on ε is seen. For instance, mobile phases 9 and 10 with the greatest P' when $\varepsilon = 0.253$, lead to reduction in retention and higher R_F. The small difference in ε of mobile phases 1-10 and 11-15 (0.013) results in

Table 2

Mobile Phases used in TLC on Silica and the Corresponding Computer-Calculated[9] Values of Strength, ε, Localization, *m* and Polarity, *P′*, including Binary Mobile Phases

No.	Components	Composition (vol %)	N_B	ε	*m*	*P′*
1	Hexane-diethyl ether	90.00 : 10.00	0.122	0.253	0.61	0.37
2	Hexane-1,2-dichloroethane	60.40 : 39.60	0.516	0.253	0.14	1.45
3	Hexane-dichloromethane	44.11 : 55.89	0.721	0.253	0.10	1.78
4	Hexane-Acetone	97.64 : 2.36	0.041	0.253	0.85	0.22
5	Hexane-ethyl acetate	94.65 : 5.35	0.070	0.253	0.57	0.33
6	Hexane-methyl *tert*-butyl ether	95.85 : 4.15	0.045	0.253	0.73	--
7	Hexane-acetinitrile	97.97 : 2.03	0.049	0.253	0.82	0.22
8	Hexane-tetrahydrofuran	92.61 : 7.39	0.113	0.253	0.95	0.39
9	Chlorobenzene-dichloromethane	87.24 :12.76	0.190	0.253	0.01	2.75
10	Toluene-bromoethane	27.99 : 72.01	0.810	0.253	-0.15	2.11
11	Cyclohexane-tetrachloromethane -diethyl ether	83.72 : 10.00 : 6.28	--	0.240	0.54	0.17
12	Cyclohexane-toluene-benzene -diethyl ether	85.75 : 5.00 : 5.00 : 4.25	--	0.240	0.46	0.20
13	Cyclohexane-toluene-benzene -diethyl ether	77.20 : 10.00 : 10.00 : 2.80	--	0.240	0.34	0.43

(continued)

Table 2 (continued)

Mobile Phases used in TLC on Silica and the Corresponding Computer-Calculated[9] Values of Strength, ε, Localization, *m* and Polarity, *P′*, including Binary Mobile Phases

No.	Components	Composition (vol %)	N_B	ε	*m*	*P′*
14	Cyclohexane-toluene-benzene -diethyl ether	68.00 : 15.00 : 15.00 : 2.00	--	0.240	0.17	0.69
15	Cyclohexane-toluene-benzene -diethyl ether	58.57 : 20.00 : 20.00 : 1.43	--	0.240	0.04	0.94
16	Hexane-ethyl acetate	98.00 : 2.00	0.026	0.215	0.54	0.19
17	Hexane-ethyl acetate	96.14 : 3.86	0.050	0.240	0.56	0.27
18	Hexane-ethyl acetate	92.00 : 8.00	0.103	0.270	0.58	0.44
19	Hexane-ethyl acetate	88.00 : 12.00	0.153	0.288	0.58	0.62
20	Hexane-ethyl acetate	84.00 : 16.00	0.201	0.303	0.59	0.79

Table 3

Experimental R_F Values and Derived Values of log α for the Diastereomeric Compounds 1-18 of Type b

R	Config.	Solute No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Avg.
A	Racemate	1	0.27	0.26	0.42	0.18	0.24	0.21	0.08	0.34	0.40	0.56	0.26	0.20	0.28	0.26	0.29	0.11	0.19	0.31	0.46	0.55	0.29
	Meso	2	0.41	0.41	0.51	0.27	0.35	0.29	0.13	0.45	0.51	0.62	0.37	0.31	0.39	0.37	0.39	0.18	0.28	0.42	0.58	0.67	0.40
B	Racemate	3	0.36	0.42	0.50	0.26	0.34	0.26	0.11	0.46	0.47	0.61	0.38	0.26	0.36	0.33	0.36	0.16	0.25	0.40	0.58	0.67	0.38
	Meso	4	0.50	0.48	0.55	0.33	0.42	0.33	0.16	0.53	0.54	0.67	0.48	0.38	0.46	0.44	0.47	0.21	0.33	0.49	0.67	0.76	0.46
C	Racemate	5	0.45	0.50	0.55	0.31	0.40	0.31	0.12	0.54	0.55	0.69	0.48	0.32	0.45	0.43	0.45	0.18	0.29	0.47	0.66	0.77	0.45
	Meso	6	0.56	0.56	0.59	0.38	0.48	0.37	0.18	0.59	0.60	0.72	0.54	0.43	0.51	0.49	0.51	0.24	0.37	0.53	0.73	0.84	0.51
D	Racemate	7	0.47	0.50	0.54	0.31	0.42	0.31	0.13	0.55	0.50	0.66	0.44	0.33	0.42	0.39	0.41	0.20	0.31	0.48	0.68	0.78	0.44
	Meso	8	0.56	0.56	0.59	0.38	0.48	0.37	0.18	0.60	0.56	0.71	0.53	0.42	0.50	0.49	0.50	0.25	0.37	0.55	0.74	0.83	0.51
E	Racemate	9	0.50	0.60	0.62	0.37	0.45	0.35	0.18	0.60	0.64	0.75	0.50	0.38	0.49	0.48	0.51	0.21	0.33	0.52	0.73	0.84	0.50
	Meso	10	0.61	0.61	0.62	0.40	0.51	0.38	0.21	0.63	0.65	0.78	0.58	0.47	0.54	0.53	0.55	0.26	0.40	0.58	0.79	0.88	0.55
F	Racemate	11	0.56	0.63	0.58	0.41	0.48	0.42	0.25	0.58	0.58	0.72	0.53	0.47	0.51	0.47	0.51	0.23	0.35	0.56	0.78	0.87	0.52
	Meso	12	0.61	0.64	0.58	0.44	0.52	0.46	0.28	0.61	0.60	0.74	0.60	0.55	0.57	0.52	0.59	0.28	0.40	0.61	0.82	0.89	0.57
G	Racemate	13	0.53	0.63	0.58	0.38	0.48	0.42	0.23	0.56	0.60	0.75	0.53	0.47	0.54	0.49	0.55	0.20	0.32	0.55	0.79	0.87	0.52
	Meso	14	0.63	0.65	0.60	0.45	0.53	0.47	0.28	0.61	0.65	0.77	0.60	0.54	0.58	0.54	0.59	0.26	0.39	0.60	0.84	0.90	0.57
H	Racemate	15	0.53	0.67	0.59	0.39	0.47	0.45	0.24	0.60	0.68	0.80	0.55	0.48	0.57	0.54	0.60	0.22	0.34	0.55	0.78	0.89	0.55
	Meso	16	0.64	0.70	0.61	0.46	0.55	0.51	0.33	0.65	0.69	0.80	0.63	0.57	0.62	0.57	0.64	0.27	0.40	0.61	0.84	0.91	0.60
I	Racemate	17	0.56	0.69	0.60	0.38	0.47	0.44	0.25	0.62	0.64	0.79	0.58	0.53	0.59	0.55	0.60	0.22	0.35	0.56	0.80	0.89	0.56
	Meso	18	0.66	0.69	0.61	0.46	0.55	0.53	0.34	0.68	0.68	0.81	0.64	0.58	0.63	0.57	0.64	0.28	0.41	0.61	0.87	0.91	0.61

(continued)

Table 3 (continued)

Experimental R_F Values and Derived Values of log α for the Diastereomeric Compounds 1-18 of Type b

R	Config.	Solute No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Avg.
		1-2	0.27	0.30	0.16	0.23	0.23	0.19	0.24	0.20	0.19	0.11	0.22	0.25	0.22	0.22	0.19	0.25	0.22	0.21	0.21	0.22	0.22
		3-4	0.25	0.11	0.09	0.15	0.15	0.15	0.19	0.12	0.12	0.11	0.18	0.24	0.18	0.20	0.20	0.14	0.17	0.16	0.17	0.19	0.16
		5-6	0.19	0.10	0.07	0.13	0.14	0.12	0.21	0.09	0.09	0.06	0.10	0.20	0.10	0.11	0.10	0.16	0.16	0.10	0.14	0.20	0.13
		7-8	0.16	0.10	0.09	0.13	0.11	0.12	0.17	0.09	0.10	0.10	0.16	0.17	0.14	0.18	0.16	0.12	0.12	0.12	0.13	0.14	0.13
		9-10	0.19	0.02	0.00	0.06	0.10	0.06	0.08	0.06	0.02	0.07	0.14	0.16	0.09	0.09	0.07	0.12	0.13	0.11	0.14	0.15	0.09
		11-12	0.09	0.02	0.00	0.05	0.07	0.07	0.07	0.05	0.04	0.04	0.12	0.14	0.11	0.09	0.14	0.11	0.09	0.09	0.11	0.08	0.08
		13-14	0.18	0.04	0.04	0.13	0.09	0.09	0.11	0.09	0.09	0.05	0.12	0.12	0.07	0.09	0.07	0.15	0.13	0.09	0.14	0.03	0.10
		15-16	0.20	0.06	0.04	0.12	0.14	0.10	0.19	0.09	0.02	0.00	0.14	0.16	0.09	0.05	0.07	0.12	0.11	0.11	0.17	0.10	0.10
		17-18	0.18	0.00	0.02	0.14	0.14	0.16	0.19	0.11	0.08	0.05	0.11	0.09	0.07	0.04	0.07	0.14	0.11	0.09	0.22	0.10	0.11
		log α	0.19	0.08	0.05	0.13	0.13	0.12	0.16	0.10	0.08	0.07	0.14	0.17	0.12	0.12	0.12	0.15	0.14	0.12	0.16	0.14	

A: CH_3; B: CH_2CH_3; C: $CH_2CH_2CH_3$; D: $CH(CH_3)_2$; E: $CH_2CH_2CH_2CH_3$; F: $CH(CH_3)CH_2CH_3$; G: $CH_2CH(CH_3)_2$; H: $CH_2CH_2CH_2CH_2CH_3$; I: $CH_2CH_2CH(CH_3)_2$. For composition of mobile phases, see Table 1. The log α values were calculated from R_F by Eq. 1 and 2. The log α values are average values of log α for all diastereomeric pairs obtained with a given mobile phase.

practically equal average R_F of 0.49 and 0.48, respectively. Similarly to a previous study,[10] a linear relationship was found between the average R_F values of the individual compounds of a given configuration with mobile phases 1-10 and 11-15 of equal ε and the corresponding standard deviations, S.D., within R_F.. The plots show S.D. in the range 0.003-0.012. The relative retention racemate > meso was established in all cases studied as seen from the positive log α independently of the size of group R and the variation in ε, m, P' and N_B.

Using linear regression analysis, the retention, R_M, of compounds **1-18** with mobile phases 5 and 16-20 and the corresponding N_B showed a good agreement with Eq. 3. The resulting data for the slope, n, intercept, $R_{M(B)}$, correlation coefficient, r, and S.D. are summarised in Table 4.

Table 5 includes literature data[2] for Q_i°, a_i and calculated by eqn. 4 Q_i values for the groups i participating in the structure of compounds **1-18** under the conditions used specified by the mobile phases 16 and 20 of minimum and maximum ε, respectively.

Application of the Soczewiński Method

The data of Table 4 show that the Soczewiński method is applicable to the cases studied showing average r of 0.957 and $S.D.$ of 0.11. The very close n values for any diastereoisomeric pair support practically equal, within the experimental error, solute areas under adsorption and, therefore, same adsorbing groups for the individual diastereoisomers. For all compounds studied, the absolute n values increase from 1.11 to 1.63 with the increase in the size of group R supporting that the two ester groups are adsorbing in any case. The increase in the area of the ester groups is expected on the basis of their increasing a_i values given in Table 5. The values of the slope, n, and intercept, $R_{M(B)}$, found determine practically parallel plots for any diastereoisomeric pair showing always a stronger retention for the racemic isomer (see Figure 2), which means that the relative retention racemate > meso cannot change with any variation of N_B.

The Relative Retention of Diastereoisomers 1-18 in Terms of a First-level Analysis of the Adsorption Mechanism

As seen from Table 5, the Q_i values of bromine and the ester groups are positive under conditions used. Considerable changes in Q_i are not expected because both bromine and the ester groups have negative inductive effects and

Table 4

Data for the Slope, *n*, Intercept, $R_{M(B)}$, Correlation Coefficient, *r*, and S.D. of the Linear Relationships R_M vs. log N_B * Found for Compounds of Type b with Mobile Phases 5 and 16-20 Composed of Hexane and Ethyl Acetate

R	Configuration	No.	*n*	$R_{M(B)}$	*r*	*S.D.*
CH_3	Racemate	**1**	-1.11	-0.82	0.986	0.05
	Meso	**2**	-1.08	-1.00	0.980	0.06
CH_2CH_3	Racemate	**3**	-1.15	-1.06	0.978	0.06
	Meso	**4**	-1.19	-1.26	0.975	0.07
$CH_2CH_2CH_3$	Racemate	**5**	-1.31	-1.35	0.973	0.08
	Meso	**6**	-1.32	-1.51	0.951	0.11
$CH(CH_3)_2$	Racemate	**7**	-1.28	-1.35	0.966	0.09
	Meso	**8**	-1.29	-1.49	0.962	0.09
$CH_2CH_2CH_2CH_3$	Racemate	**9**	-1.42	-1.59	0.957	0.11
	Meso	**10**	-1.45	-1.74	0.948	0.12
$CH(CH_3)CH_2CH_3$	Racemate	**11**	-1.51	-1.75	0.953	0.12
	Meso	**12**	-1.49	-1.82	0.946	0.13
$CH_2CH(CH_3)_2$	Racemate	**13**	-1.61	-1.84	0.960	0.12
	Meso	**14**	-1.60	-1.95	0.944	0.14
$CH_2CH_2CH_2CH_2CH_3$	Racemate	**15**	-1.60	-1.85	0.939	0.15
	Meso	**16**	-1.61	-1.98	0.941	0.15
$CH_2CH_2CH(CH_3)_2$	Racemate	**17**	-1.62	-1.89	0.945	0.14
	Meso	**18**	-1.63	-2.04	0.930	0.16
Average					0.957	0.11

* N_B is the molar fraction of ethyl acetate in mobile phases 5 and 16-20.

resonance between them cannot occur. In addition, the bromine atom is bulkier than CO_2CH_3 on the basis of their conformational energies of 1.3 kcal/mol and 0.9 kcal/mol,[21] respectively. To evaluate the relative role of the adsorptivity and size of bromine, we calculated the average R_F on same silica of the compounds of type **a** and type **b** established with hexane-ethyl acetate 92:8 (ε = 0.270, mobile phase 19 of ref. 8 and mobile phase 18 of this study). The values found were 0.37 and 0.52, respectively. This shows that compounds **1-18** of type **b** have smaller adsorptivity. The same phenomenon is seen with hexane-ethyl acetate 98:2 with ε = 0.215. Thus, the presence of two bromine atoms

Table 5

Data According to Snyder[2] for the Adsorption Properties on Silica of Groups *i* Participating in the Compounds Studied

Group *i*	Q_i^0	a_i	$Q_i = Q_i^0 - \varepsilon a_i$ ε=0.215	ε=0.303
Br	1.94	1.80	1.55	1.39
CH_3	0.07	1.60	-0.27	-0.41
CH_2	-0.05	0.90	-0.24	-0.32
CO_2CH_3	5.27	10.50	3.01	2.09
$CO_2C_2H_5$	5.22	11.40	2.77	1.77
$CO_2C_3H_7$-n	5.17	12.30	2.53	1.44
$CO_2C_3H_7$-iso	5.29	13.00	2.50	1.35
$CO_2C_4H_9$-n	5.12	13.20	2.28	1.12
$CO_2C_4H_9$-sec	5.24	13.90	2.25	1.03
$CO_2C_4H_9$-iso	5.24	13.90	2.25	1.03
$CO_2C_5H_{11}$-n	5.07	14.10	2.04	0.80
$CO_2C_5H_{11}$-iso	5.19	14.80	2.01	0.71

The data for Q_i^0 and a_i are taken from ref. 2, p. 200 and p. 264 or calculated by summation of the contribution of the composing fragments. The calculated by Eq. 4 Q_i values refer to the mobile phases of minimum and maximum ε value used.

with $Q_i > 0$ does not increase the retention of the compounds of type **b** and, therefore, these atoms are expected to have mainly a shielding role. Consequently, adsorption of any two equal ester groups, hindered by the bromine atoms, is the expected simplified adsorption mechanism. The ester groups and, more precisely, their carbonyls having an increased electron density can form hydrogen bonds with the silanol groups. The two-point adsorption occurs *via* site chelation (see ref. 2, p. 315), *i.e.*, interaction of two adjacent adsorbing groups with a given adsorption site. Conformations suitable for site chelation of the two ester groups are M for the racemates and N for the meso isomers having these two groups in *gauche* position (see Figure 3). For the racemate, another conformation M' with *gauche* esters hindered by each of the bromine atoms is not taken into account because the conformational equilibrium is expected to shift to the conformation which will give best adsorption.

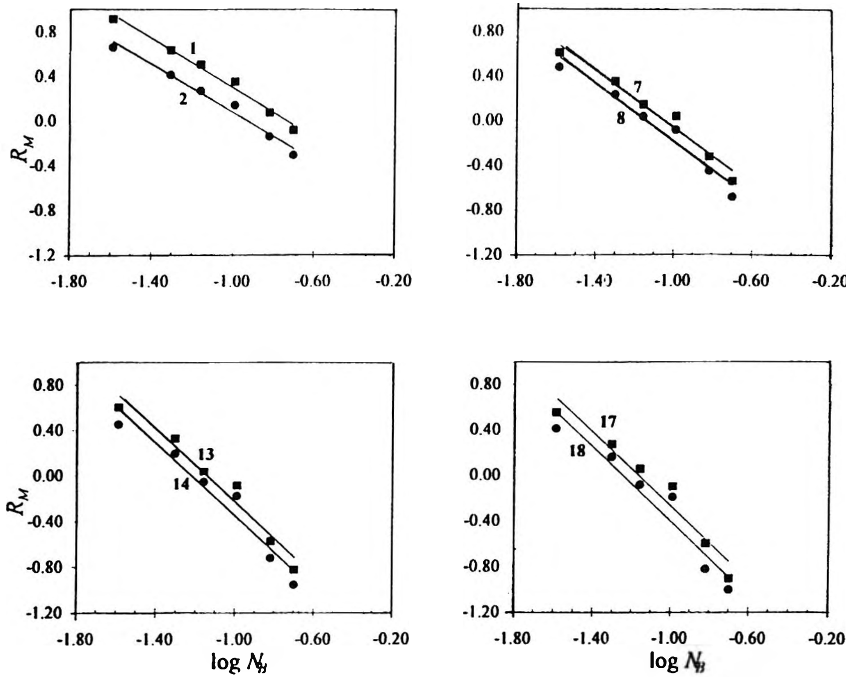

Figure 2. R_M vs. log N_B plots on the basis of the data of Table 2 and Table 3 for diastereoisomers **1, 2, 7, 8, 13, 14, 17** and **18**. N_B is the molar fraction of ethyl acetate in mobile phases 5 and 16-20.

Any racemic compound is expected to show a stronger adsorption because the adsorbing ester groups are not hidered by a bromine atom. On the contrary. such a hindrance is present in the corresponding meso isomer, leading to the relative retention racemate > meso which was found in fact (see Figure 4).

Separation, α, of the Diastereoisomers Studied and Some Comparisons

A fair linear relationship between log α and m was not observed in this study (cf.. refs. 4. 7. 8. 20 and 22). Best separations of all diastereoisomeric pairs studied were obtained with mobile phase 1 and mobile phase 12, showing average log α values. $\overline{\log \alpha}$. of 0.19 and 0.17, respectively (see Table 3). These two mobile phases are of intermediate m values (0.61 and 0.46) and contain the strongly localizing diethyl ether.

Figure 3. Conformations of the diastereoisomers studied.

Figure 4. Schematic illustration of the expected adsorption mechanism on silica for the diastereoisomers studied. A is an adsorption site, probably reactive silanols (see ref. 2, p. 157) suitable for site chelation.

The absence of such a solvent, as in mobile phases 2, 3, 9 and 10, reduces the separation and $\overline{\log \alpha}$ is in the range 0.05-0.08. Mobile phases 12-15 are composed of cyclohexane, toluene, benzene and diethyl ether in different ratios. Best separation was established with mobile phase 12 having the greatest *m* (0.46) because of the greatest content of diethyl ether.

Let us discuss the average separation of any diastereoisomeric pair obtained with all mobile phases used. According to Table 3, the diastereoisomeric pairs **1-2** and **3-4** with group R equal to methyl and ethyl

show best separation and average log α of 0.22 and 0.16, respectively. This probably is due to the stronger adsorptivity of these compounds than that of the remaining compounds with a bulkier group R. The opposite is true for the compounds of type **a.**[1]

Taking into account all log α values found in ref. 8 and this study, we calculated the corresponding average values for the diastereoisomers of type a and type **b** being 0.34 and 0.12, respectively. In our opinion, this reduction in the separation of the diastereoisomers of type **b** is due to changes both in the structure and the conformational stability of the two types of compounds. The presence of the shorter double bond in the compounds of type **a** will lead to an increase in the site chelation of the two ester groups in any Z isomer and a greater difference in the retention of the corresponding E isomer where site chelation is not possible because of the rigid conformation. Such an improvement in the site chelations for the two diastereoisomers of type **b** cannot occur owing to the presence of the longer C(2) - C(3) bond. This assumption is further supported by the fact that the conformationally rigid diastereoisomers of type C_6H_5-CH=C(CO_2R)-C_6H_5, with expected one-point adsorption of the ester group and, therefore, without site chelation, show the same smaller separation[11] as the compounds of type b studied.

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SIMULTANEOUS DETERMINATION OF CAFFEINE AND ERGOTAMINE IN PHARMACEUTICAL DOSAGE FORMULATION BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

A method has been developed for the simultaneous assay of caffeine and ergotamine in the pharmaceutical dosage tablet formulations (Cafergot®) by capillary electrophoresis (CE). The analysis was accomplished by using 25 mM sodium phosphate buffer at pH 6.0 with UV detection at 214 nm. The mean recoveries were 98.9% ± 1.9% and 101% ± 1.03% for the analysis of caffeine and ergotamine respectively. Run time was less than six minutes for the analysis of the two active constituents in the tablets. There were no excipients interferences.

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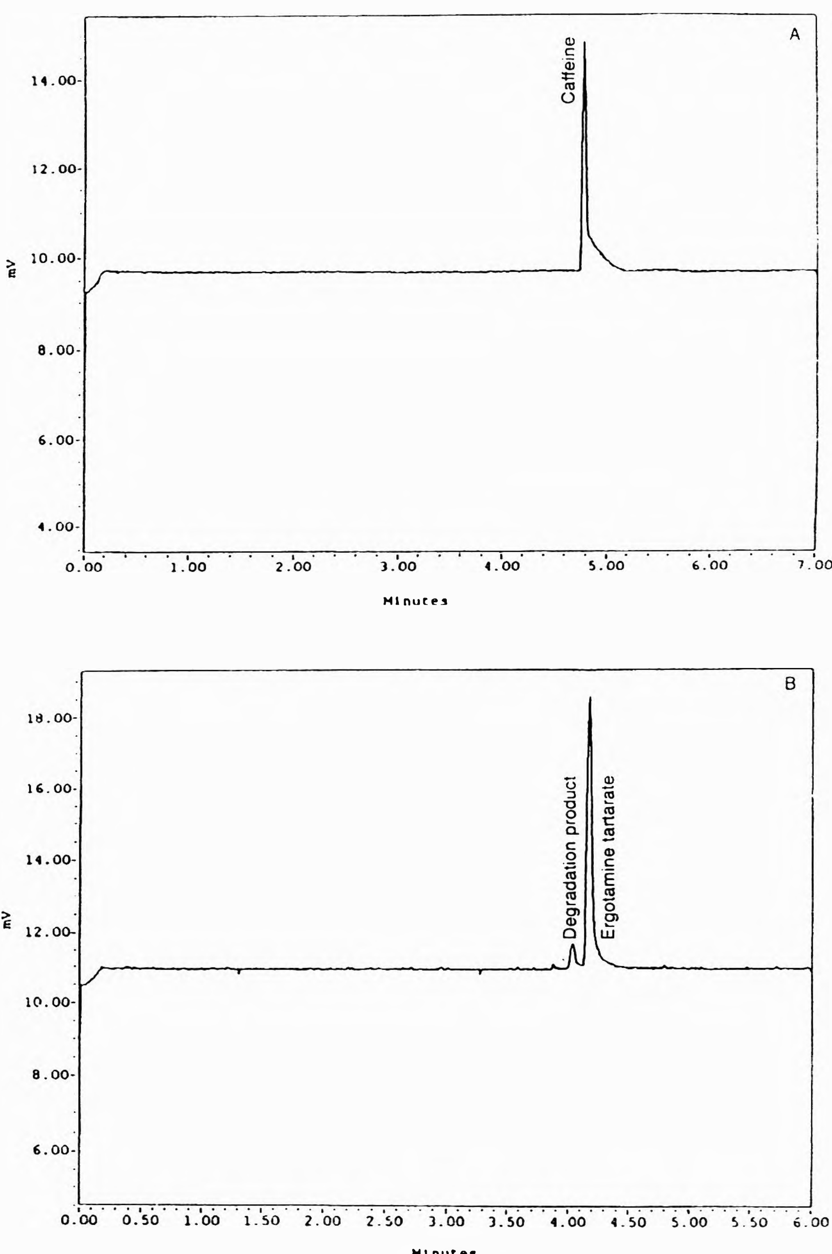
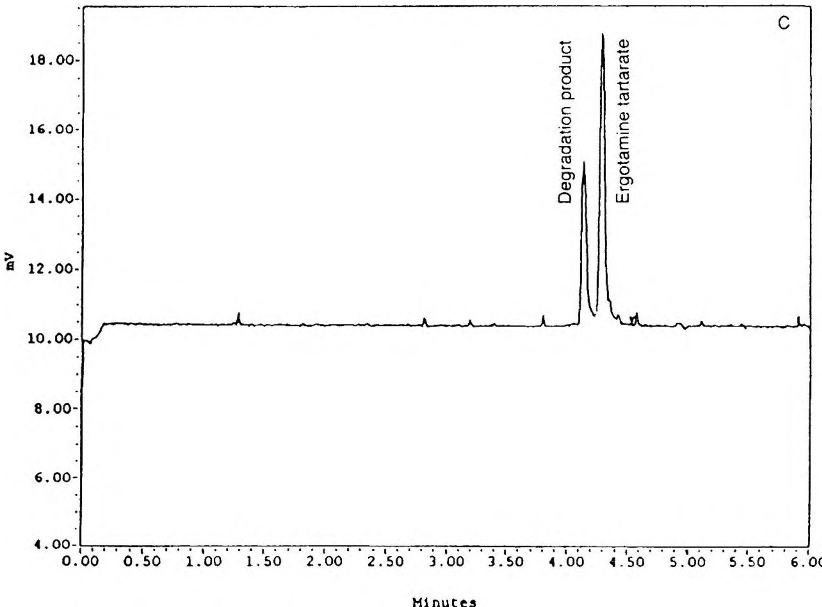


Figure 1. (above & right) Electropherogram of (A) caffeine (B) Fresh solution of ergotamine tartarate (C) Ergotamine tartarate solution after 24 hours.

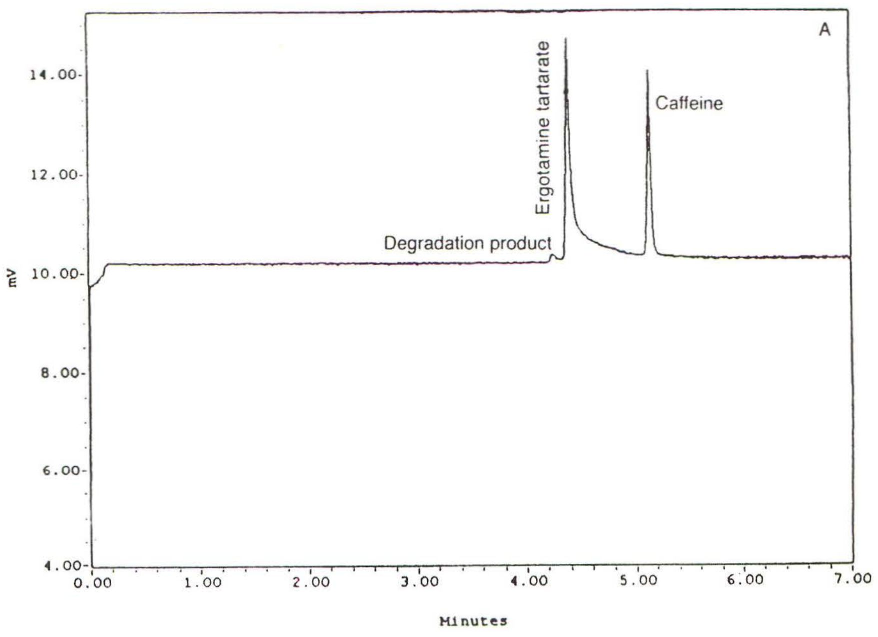


INTRODUCTION

Capillary electrophoresis (CE) has recently emerged as a powerful separation tool for the analysis of a wide variety of complex mixtures.[1] The characteristics of CE modality are reflected in its high separation efficiency, rapid analysis, small sample consumption. Direct detection of analytes and selectivity are remarkable and impressive, which make it very attractive for pharmaceutical analysis.

Ergotamine is considered to be one of the most effective drugs in the treatment of migraine and cluster headaches.[2, 3] A combination of ergotamine and caffeine is clinically used for the treatment of acute attacks of migraine. Saxena and De Deyn reviewed the use of this combination drug in treatment of migraine and its complications.[4]

Determination of ergotamine has been reported using high performance liquid chromatography[5]. Also its analysis in plasma by fluorescence detection[6] and by ion-pair chromatography were published.[7] The analytical profile of


A
Ergotamine tartarate
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Degradation product
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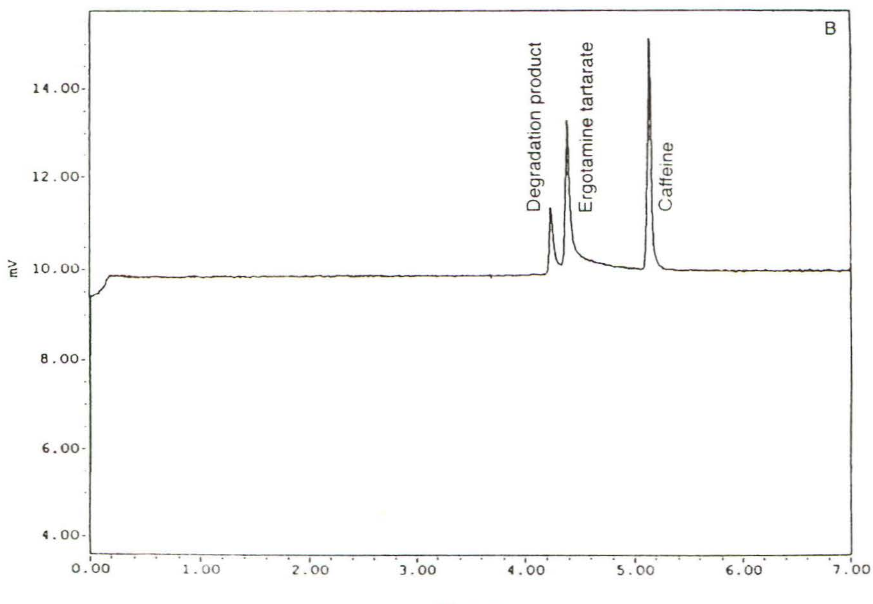
B
Degradation product
Ergotamine tartarate
Caffeine
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Minutes

caffeine was reviewed by Zubair et al[8] which include a compilation of the analytical methods reported in the literature for caffeine analysis. Furthermore, the analysis of caffeine content in various pharmaceutical formulations were also cited[9].

The purpose of this paper is to report, for the first time, a rapid and accurate capillary electrophoresis (CE) method for the simultaneous analysis of ergotamine and caffeine in the pharmaceutical tablet formulation.

EXPERIMENTAL

Chemicals

A standard of ergotamine tartarate was obtained from (GNF, GmbH, Berlin, Germany), caffeine and sodium phosphate were obtained from Sigma (St. Louis, MO. USA). High purity water was obtained from a Milli-Q Water System (Millipore, Bedford, MA). The pharmaceutical dosage forms of Carfergot® tablets (Sandoz Pharmaceuticals, Cambeley Surrey, UK) were purchased from local markets.

Apparatus

A Waters Quanta 4000E capillary electrophoresis system with positive power supply was used (Waters, Milford, MA, USA). The electrophoretic system was controlled by the Millennium 2010 Chromatography Manager (Waters) which was also used for data collection (5 points/sec.) and processing.

All analyses were performed on Accusep polyamide fused-silica capillaries (60 cm x 75 μm ID) obtained from Waters (Milford, MA, USA). The detector time constant was set at 0.3 seconds, all analyses were for 10 second injections, with hydrostatic mode and applied voltage of +20 KV at 30°C with detection at 214 nm.

Figure 2. (left) Electropherogram of caffeine and ergotamine tartarate after extraction from Cafergot® tablets. (A) Fresh solution (B) Solution after 24 hours.

Buffer and Solutions

A standard solution (500 μg/mL) of ergotamine tartarate and (100 μg/mL) of caffeine were prepared by dissolving in high purity water. For establishment of calibration curves of each, five concentrations of ergotamine tartarate in the range of 10-250 μg/mL and of caffeine in the range of 2.5-50 μg/mL were made in running buffer. The buffer used for CE analysis was 25 mM sodium phosphate at pH 6.0. Duplicate runs were made of all concentrations.

Sample Preparations

Two tablets of Cafergot® containing 2 mg of ergotamine tartarate and 200 mg of caffeine were accurately weighed and powdered. An aliquot portion of the powder containing an equivalent of 0.5 mg of ergotamine tartarate, and 50 mg of caffeine was diluted in 10 mL of sodium phosphate buffer and was shaken vigorously for 15 min. followed by centrifugation. An aliquot of the supernatant was then used for the determination of ergotamine tartarate. Further dilutions were made for caffeine determination in order to bring the peak area within scale.

RESULTS AND DISCUSSION

A rapid and simple capillary electrophoresis method has been achieved for the analysis of both ergotamine and caffeine as bulk drug and simultaneously in the pharmaceutical tablet form known as Cafergot®. Detection and separation of these two drugs was accomplished in less than 6 minutes. Retention time for caffeine and ergotamine tartarate as bulk drugs were 4.8 and 4.2 minutes, respectively (Figure 1 A & B).

An unknown peak was observed in the electropheragram of ergotamine tartarate which has a retention time of 4.1 minutes. This peak is believed to be a decomposition or degradation product as its size increases to reach about 50% of the ergotamine peak size within 24 hours (Figure 1 C). This requires the carrying out of the analysis of ergotamine in bulk form or in pharmaceutical tablets, as soon as possible, to avoid the formation of this decomposition product. Shown in Figure 2 A and B are the differences in the electropheragrams after 24 hours.

A regression analysis indicated a linear relationship between the peak area Y and the concentration X, over the range 10-250 μg/mL with correlation coefficient (r) of 0.997 and mean recovery of 101.9% ± 1.03% for ergotamine

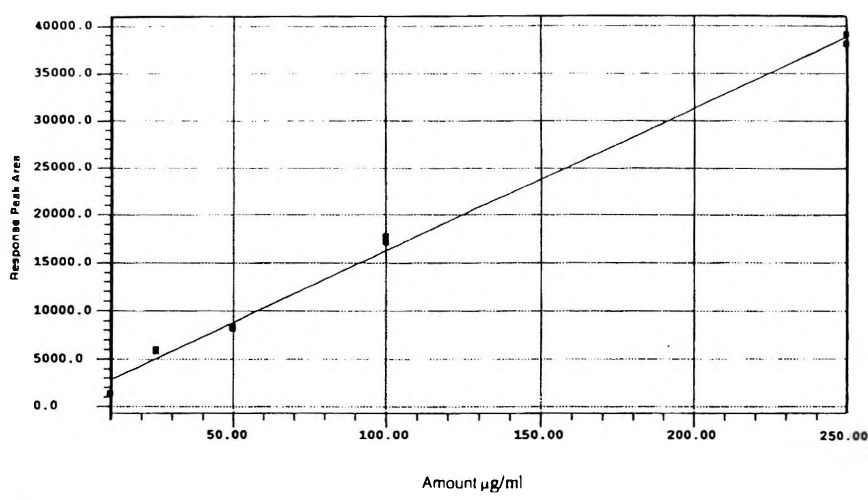

Figure 3. Calibration curve for ergotamine tartarate over the range of 10-250 µg/mL.

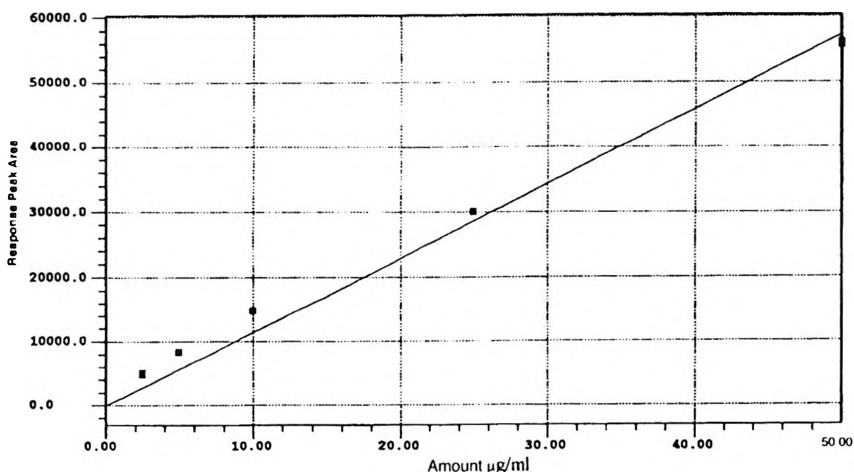

Figure 4. Calibration curve for caffeine over the range of 2.5-50 µg/mL.

tartarate as shown in Figure 3. The calibration curve for caffeine (Figure 4) was linear in the range of 2.5 - 50 μg/mL with correlation coefficient (r) of 0.993 and mean recovery of 98.9% ± 1.9% for caffeine.

The precision and accuracy of the method were verified by analysis of the samples after adding known concentrations of ergotamine tartarate and caffeine to the dosage forms. Tablet excipients did not interfere with the assay of the two drugs.

In summary, the method described permits a simultaneous determination of ergotamine tartarate and caffeine in bulk drug and pharmaceutical tablet forms by CE. This method is simple, rapid, specific and stability-indicating. The proposed method can be used for routine analysis of these drugs and can be used as an alternative tool for the drug quality control laboratories.

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SEPARATION OF TAXOL AND CEPHALOMANNINE BY COUNTERCURRENT CHROMATOGRAPHY

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ABSTRACT

Countercurrent chromatography was used to separate taxol from cephalomannine, on a preparative scale for the final purification of taxol. A solvent system consisting of hexane:ethyl acetate:methanol: ethanol:water in 5:7:5:1:6.5 was found to be suitable in achieving near base-line resolution of the two components. It was found that the amount of methanol in the solvent system was very critical to a successful separation operation.

INTRODUCTION

Taxol[1,2] has been found to be active toward various kinds of cancers and is a major focus of research in chemotherapeutic agents during recent years. Typically, barks, needles or twigs from trees of *Taxus* genus were ground and extracted by a series of polar or non-polar solvents, such as methylene chloride, methanol and, in some cases, supercritical carbon dioxide, to get rid of most

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contaminants and recover taxol.[3,4,5] However, to separate cephalomanine, a contaminant that is most difficult to separate taxol from. which is present in most plant sources in about half as much as taxol, higher resolution methods are required. Column chromatography using silica gel with normal phase elution is widely used.[6]

In preparation of pure taxol in gram quantities for laboratory uses, countercurrent chromatocraphy (CCC) provides a convenient altenative.[7] This technique uses no solid particles and can adjust to the variable conditions of crude extract by adjusting solvent composition to achieve good results. Search for suitable solvent systems is the first step of CCC separation. A successful system was found and reported below.

MATERIALS AND METHODS

Taxol was purchased from suppliers of Yunnan, China in partially purified form. The off-white powder contains about 75 wt % of taxol and 25 wt % cephalomannine. Hexane, ethyl acetate, anhydrous ethanol and methanol were of HPLC grade. The countercurrent chromatography device used was Model CCC-1000 from PharmaTech, Baltimore, USA. Three consecutive coaxial multilayer coil columns were used with a total volume of 320 mL.

An appropriate amount of solvents were mixed and two phases were equilibrated. The top phase was chosen to be the stationary phase and filled the columns first and then the columns were rotated at 1000 rpm. Mobile phase was then pumped in via an HPLC pump at a rate of 1.5 mL/min. After the mobile phase fluid emerged at the exit of the columns, 1.0 mL of sample was injected. The sample contained 6.1 mg of the partially purified taxol, dissolved in 50:50 stationary and mobile phase. Fractions were collected in a size of 6.0 mL, and analyzed by HPLC. A C_{18} reverse phase column (LiChroCART 100, 5μm, Merck) was used, and taxol was detected at 229 mn. The mobile phase used for HPLC analysis was composed of water/methanol (60: 40) at a flow rate of 0.5 mL/min.

Partition coefficients measurement was made for taxol and cephalomannine in liquid-liquid two-phase systems. Partially purified taxol was dissolved in equilibrated two-phase solution. Samples were drawn from two phases and analyzed for taxol and cephalomannine. Samples from the top phase were first vacuumed to dryness and redissolved in the methanol before the HPLC analysis.

Table 1

Partition Coefficients of Taxol and Cephalomannine in the Solvent Systems*

Systems	Hexane	E.A.	MEOH	EtOH	Water	Pt	Pc
1	5	7	4	1	6.5	5.67	4.31
2	5	7	5	1	6.5	1.80	1.42
3	5	7	6	1	6.5	1.09	1.09
4	5	7	7	1	6.5	0.20	0.19
5	5	7	8	1	6.5	0.29	0.27

E.A.: ethyl acetate
Pt: partition coefficient of taxol
Pc: partition coefficient of cephalomannine
* expressed in volume ratio of their constituents

RESULTS

A basic formulation of the solvent system was first chosen, considering past experiences in CCC separation and taxol's general properties, such as its hydrophobicity. The hexane-ethyl acetate-methanol (or ethanol)-water system was commonly used in CCC for the separation of slightly hydrophobic components. The top phase was rich in hexane and the bottom phase was rich in water. Preliminary tests revealed that, in the hexane-ethyl acetate-ethanol-water system (5: 7: 4: 6.5), the elution times for both taxol and cephalomannine were very long compared to the mobile phase elution time. Thus a typical run would require up to 12 h for taxol or cephalomannine to exit the columns. This is caused by the high partition coefficients in this system, i.e. high affinity for the upper phase (stationary phase) by taxol and cephalomannine. Thus, this was not a practical system for the separation. The system using methanol instead of ethanol, e.g. hexane-ethyl acetate-methanol--water (6: 4: 6.5: 3.5) suffered from the opposite problem, i.e., very low affinity for the upper phase by the two components; thus, insufficient resolution. The two components were not separated at the exit, and emerged in one peak.

It seemed reasonable to try using both ethanol and methanol in the solvent system, as their opposite effects on affinity to the upper phase by these two solvents may cancel out. Partition coefficients of taxol and cephalomannine in a series of solvent systems were measured. The results are shown in Table 1.

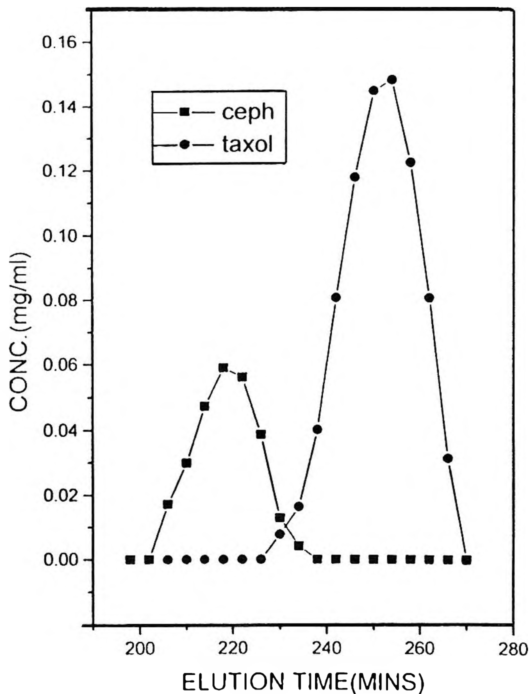

Figure 1. Elution profiles for taxol and cephalomannine. ●: taxol, ■: cephalomannine.

It was somewhat surprising to find that the amount of methanol in this solvent system had a major impact on the partition behavior of taxol and cephalomannine. The reason for this particular phenomenon was not determined.

The solvent system chosen for the CCC separation system was hexane-ethyl acetate-methanol-ethanol-water in 5: 7: 5: 1: 6.5 (system 2 in Table 1), since this was the only one that would result in suitable elution time and separation factor. Rotational speed of the CCC was set at 1000 rpm, the normal operating speed for this model. Mobile phase flow rate was 1.5 mL/min. The stationary phase retention was 264 mL in 320 mL total volume. Thus the mobile phase volume was 56 mL, the elution time for the mobile phase was thus 38 min. As shown in Fig. 1, cephalomannine was ahead of taxol, and near base-line resolution was achieved. The number of theoretical

plates was calculated to be 560 and the resolution was 0.98. The results were consistent with the partition coefficients (taxol: 1.80, cephalomannine: 1.42). Recovery of taxol was 90% with purity above 98%, when the collected fractions were dried under vacuum. These results can be helpful in scaling up the process in larger countercurrent chromatography units, in order to process gram levels of taxol in various kinds of extracts.

ACKNOWLEDGMENT

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DETERMINATION OF THE ALKALOIDS IN COPTIS-EVODIA HERB COUPLE BY CAPILLARY ELECTROPHORESIS

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ABSTRACT

A method combining the techniques of micellar electrokinetic capillary chromatography (MECC) and capillary zone electrophoresis (CZE) has been developed to assay seventeen alkaloids in coptis-evodia herb couple. The MECC method, based on SDS, was applied to analyze three indolequinazoline alkaloids and six quinolone alkaloids in evodia within 30 minutes, and a CZE technique was used to determine eight quaternary alkaloids (dehydroevodiamine in evodia, and seven protoberberine alkaloids in coptis) within 25 minutes. The recovery efficiencies were 96.68-103.19% in MECC and 99.65-103.28% in CZE, with a relative standard deviation of 1.67-4.00% for MECC and 2.33-4.38% for CZE. The calibration curves exhibited good linearity over one order of magnitude of concentration, and their minimum detectable concentrations were approximately 15.78 to 47.33 μg/mL using a 0.75 μm inner diameter column. Contents of the seventeen alkaloids in a methanol-water crude extract of coptis-evodia herb couple could easily be determined by this method.

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INTRODUCTION

Herbs simultaneously used together and in couples are the basic composition units of Chinese herbal formulas and have special clinical significance in Chinese medicine. They are much simpler than complicated formulas in composition, but retain the basic therapeutic features.[1] Coptis (Coptidis Rhizoma) possesses the effects of dispelling heat, drying dampness, purging fire and removing toxin, and evodia (Evodiae Fructus) has the actions of warming middle, dispelling cold, causing vitality to descend and controlling pain.[2] The combined use of these two herbs will enable the healing of hypochondric and costar pain, stomach ache, acid regurgitation, nausea and gastric upset.[1]

The pharmacologically active constituents of coptis are a number of protoberberine alkaloids such as coptisine (**1**), berberine (**3**), berberastine (**4**), epiberberine (**5**), columbamine (**6**), jatrorrhizine (**7**), palmatine (**8**),[3-7] and those of evodia are indolequinazoline alkaloids, dehydroevodiamine (**2**), evodiamine (**9**), rutaecarpine (**10**), carboxyevodiamine (**17**), and quinolone alkaloids, 1-methyl-2-nonyl-quinolone (**11**), 1-methyl-2-[(Z)-6-undecenyl]-4(1H)-quinolone (**12**), 1-methyl-2-undecyl-4(1H)quinolone (**13**), evocarpine (**14**), 1-methyl-2-[(6Z,9Z)-6,9-pentadecadienyl]-4(1H)quinolone (**15**) and dihydroevocarpine (**16**),[8-12] as shown in Figure 1. The former can be analyzed by a standard capillary zone electrophoretic (CZE) method,[13] and the latter have to be separated by both CZE and micellar electrokinetic capillary chromatographic (MECC) methods.[14] The two systems are distinctly different. In order to determine, simultaneously, the chemical constituents of the two herbs, this paper reports a facile and rapid CE analytical method that can be used to help assess not only the herb couple itself but also the quality of Chinese herbal preparations containing the herb couple mentioned herein.

EXPERIMENTAL

Apparatus and Conditions

The analysis was carried out on a Waters (Milford, MA) Quanta 4000 capillary electrophoresis system, equipped with a UV detector set at 254 nm and a 90 cm x 75μm I.D. fused silica capillary tube (Polymicro Technologies, Phoenix, AZ) with the detection window placed 82.5 cm from the injection. The conditions were as follows: sampling time, 2 s, hydrostatic (injection volume, 3.4 nL); applied voltage, 25 kV (constant voltage, positive to negative polarity); and temperature, 24.5-25.0 °C. In CZE, the electrolyte was a buffer

11, R: $(CH_2)_8CH_3$
12, R: $(CH_2)_5CH=CH(CH_2)_3CH_3$
13, R: $(CH_2)_{10}CH_3$
14, R: $(CH_2)_7CH=CH(CH_2)_3CH_3$
15, R: $(CH_2)_5CH=CHCH_2CH=CH(CH_2)_4CH_3$
16, R: $(CH_2)_{12}CH_3$

Figure 1. Structures of the seventeen alkaloids. **1**, coptisine; **2**, dehydroevodiamine; **3**, berberine; **4**, berberastine; **5**, epiberberine; **6**, columbamine; **7**, jatrorrhizine; **8**, palmatine; **9**, evodiamine; **10**, rutaecarpine; **11**, 1-methyl-2-nonyl-quinolone; **12**, 1-methyl-2-[(Z)-6-undecenyl]-4(1 H)-quinolone; **13**, 1-methyl-2-undecyl-4(1 H)-quinolone; **14**, evocarpine; **15**, 1-methyl-2-[(6Z,9Z)-6,9-pentadecadienyl]-4(1H)-quinolone; **16**, dihydroevocarpine; **17**, carboxyevodiamine.

solution of 50 mM maleic acid and 40 mM NaH_2PO_4 and acetonitrile (9:1); run time, 25 min. In MECC, the electrolyte was a buffer solution of 40 mM SDS, 20 mM NaH_2PO_4 and 9 mM $Na_2B_4O_7$ and acetonitrile (3:2); run time, 30 min. Before each run, the capillary was washed with 0.1 N NaOH for 5 min, with water for 5 min and then with buffer for 5 min.

Reagents and Materials

The alkaloids were isolated from Coptidis Rhizoma[3-7] and Evodiae Fructus[8-12] and their structures were elucidated on the basis of spectra such as IR, PMR, CMR and MS. The purities of these compounds were checked by HPLC. Maleic acid was purchased from Wako (Osaka, Japan), sodium dodecyl sulphate (SDS) and 18β-glycyrrhetinic acid from Sigma (St. Louis, MO), benzyltriethylammonium chloride from Merck (Darmstadt, Germany), sodium dihydrogenphosphate from Yoneyama (Osaka, Japan) and sodium borate from Kanto (Kyoto, Japan).

Acetonitrile and methanol were of LC grade (Fisons, Loughborough, England). Deionized water from a Milli-Q system (Millipore, Bedford, MA) was used to prepare all buffer and sample solutions. Coptidis Rhizoma and Evodiae Fructus were purchased from the Chinese herbal market in Taipei (Taiwan).

Preparation of Herb Couple and Chinese Herbal Preparation Extracts

A 1.0 g sample of the pulverized herb couple (containing 0.5 g Coptidis Rhizoma and 0.5 g Evodiae Fructus) was extracted with 70% methanol (6 mL) by stirring at room temperature for 30 min., then centrifuging at 1500 g (Universal, Hettich Zentrifugen) for 5 min. Extraction was repeated three times. The extracts were combined and filtered through a No.1 filter paper. After adding a 5 mL aliquot of internal standard solution, IS_1, (20 mg of benzyltriethylammonium chloride in 1 mL of 70% methanol) for CZE, 2 mL aliquot of internal standard solution, IS_2, (2 mg of 18β-glycyrrhetinic acid in 1 mL of 70% methanol) for MECC, the extract was diluted to 25 mL with 70% methanol. This solution was passed through a 0.45 μm filter and the filtrate was then injected into the capillary electrophoresis system.

2.0 g of the constituent crude drugs of *Pien-tung-wan* (including 1.0 g Coptidis Rhizoma and 1.0 g Evodiae Fructus) was added with a twenty-fold mass of water (40 mL) and boiled mildly for 30 min until the volume becomes halved. After filtration while hot, to the filtrate was added suitable amounts of

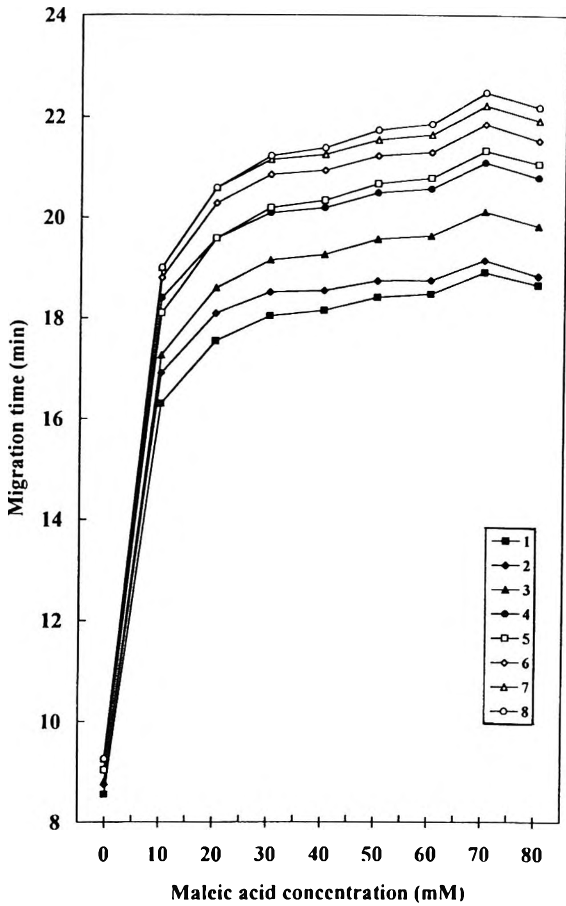

Figure 2. Effect of maleic acid concentration on migration time. All these experiments were conducted at a voltage of 25 kV across the 90 cm x 75 µm I.D. separating tube filled with phosphate buffers of different maleic acid concentrations containing 10% acetonitrile; temperature 24.5-25.0°C; detection wavelength, 254 nm. Symbols are the same as those in Fig. 1.

IS_1 and IS_2, and the resultant solution was diluted with methanol to give a 70% methanol solution. This solution was passed through a 0.45 µm filter and the filtrate was then injected into the capillary electrophoresis system. Similarly, a standard decoction of *Kan-lu-san* (including 2.0 g Coptidis Rhizoma and 1.0 g Evodiae Fructus) was also prepared in the same procedure.

RESULTS AND DISCUSSION

Analytical Conditions

The quaternary alkaloids in Coptidis Rhizoma have been well separated by a CZE technique with the use of acetate as counter ion.[13] However, a similar process was found to fail in the analysis of coptis-evodia herb couple. Under this condition (0.1 M CH_3COONa and methanol in the ratio of 85:15), the peak of dehydroevodiamine not only overlapped with berberine, but also exhibited serious tailing. The alkaloids in Evodiae Fructus were analyzed by two modes, CZE and MECC.[14] Following the evodia CZE condition (40 mM NaH_2PO_4 and acetonitrile in the ratio of 9:1), all evodia alkaloids were eluted along with EOF except for dehydroevodiamine (**2**) which was overlapped with coptis alkaloids; however, using the MECC condition (40 mM SDS, 20 mM NaH_2PO_4, 9 mM $Na_2B_4O_7$ and 40% CH_3CN), the evodia alkaloids (except **2**) in the herb couple were separated as well as those obtained with evodia crude drug itself, without interference. The migration times of those compounds were: **9**, 17.15 min; **10**, 17.47 min; **11**, 18.19 min; **12**, 18.35 min; **13**, 19.10 min; **14**, 19.39 min; **15**, 19.64 min; **16**, 20.30 min; **17**, 20.65 min.

In order to separate the quaternary alkaloids (**1-8**) in coptis-evodia herb couple, a new CZE method was developed with the addition of a dicarboxylic acid to the phosphate buffer in evodia's CZE system. Acetic acid and four dicarboxylic acids, tartaric acid, oxalic acid, succinic acid and maleic acid, had ever been used in this series of trials. From the experiments, maleic acid was found to be the best. Acetonitrile was added to the carrier as organic modifier to achieve a better resolved electropherogram. Other organic solvents such as methanol, ethanol and isopropanol had also been tried as substitutes for acetonitrile, but a much broader peak was obtained.

Effect of Maleic Acid Concentration

Nine electrolyte systems containing 40 mM NaH_2PO_4 and 10% CH_3CN at different maleic acid concentrations, ranging from 0 to 80 mM were used to study the effect of maleic acid concentration on the separability. The results obtained are shown in Figure 2, where the migration times are plotted against maleic acid concentrations. In Figure 2, the migration times of all quaternary alkaloids increased rapidly from 0 to 10 mM maleic acid, showing that there should be a strong interaction between a carboxylic group and a positive nitrogen atom. After that, the migration times were found to increase gradually with the increasing maleic acid concentrations. Usually, the migration times of

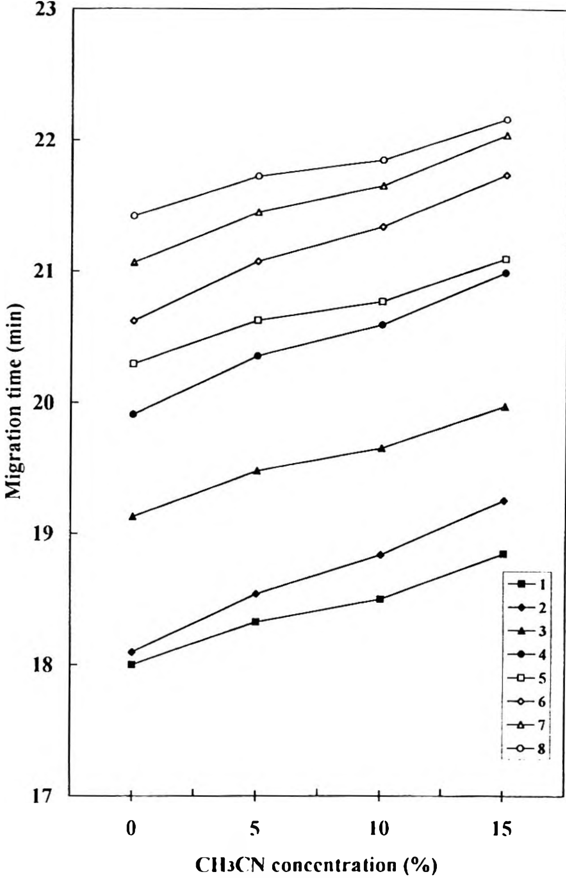

Figure 3. Effect of acetonitrile concentration on migration time. The carriers were 50 mM maleic acid-40 mM NaH_2PO_4 solutions containing 0-15% acetonitrile. Other conditions are the same as those in Fig. 2.

quaternary alkaloids in Coptidis Rhizoma were influenced much more by maleic acid concentration than in Evodiae Fructus. From the results, concentrations at 50 and 60 mM were found to be the best conditions for the separation of these eight alkaloids. However, 60 mM maleic acid gave more baseline noise and, therefore, 50 mM was chosen.

Effect of Acetonitrile Concentration

Four electrolyte systems, containing 50 mM maleic acid and 40 mM NaH_2PO_4 at different acetonitrile concentrations ranging from 0 to 15%, were

used to study the effect of the acetonitrile concentration on the resolution. The results obtained are given in Figure 3. As the acetonitrile concentration increased, the migration times of all quaternary alkaloids became longer, but the resolution of these compounds did vary. When acetonitrile was absent, the resolution value (Rs) between 7 and **8** was 1.19; however, that of **1** and **2** was only 0.50. At 5%, 10% and 15% acetonitrile, the Rs values between **1** and **2** were 1.24, 1.38 and 2.82, respectively, but were 1.35, 0.83 and 0.64 between **7** and **8**. From the calculated results, the best resolution for all the compounds was at 10% acetonitrile. Meanwhile, the addition of acetonitrile to the buffer could also result in a sharper peak and smoother baseline, which came from the reduction of interaction between the silanol group on the capillary wall and the quaternary alkaloids.

Effect of Phosphate Concentration

Six electrolyte systems, at different phosphate concentrations (10-60 mM sodium dihydrogenphosphate), were used to study the effect of phosphate concentration on the resolution. The number of theoretical plates and the resolution values between **1** and **2** and between **7** and **8**, obtained at different phosphate concentrations are listed in Table 1.

Data in Table 1 show that the numbers of theoretical plates for these eight alkaloids, especially those of **1**, **2** and **6**, varied markedly with the change of phosphate concentration of the buffer, and ideal plate numbers could be successfully achieved when the concentrations were higher than 30 mM. However, influence on resolution by changing the phosphate concentration was found to be reverse between **1-2** and **7-8**.

As the phosphate concentration was increased, the Rs values on **1-2** were increased, but that on 7-**8** were decreased. In order to assay all the quaternary alkaloids simultaneously, a concentration of 40 mM was therefore selected.

From the above results, a buffer solution consisting of 50 mM maleic acid and 40 mM NaH_2PO_4 and acetonitrile (9:1) were chosen. Figure 4 is an electropherogram showing the separation of a mixture of the quaternary alkaloids with the following migration times: **1**, 19.39 min; **2**, 19.70 min; **3**, 20.69 min; **4**, 21.55 min; **5**, 21.70 min; **6**, 22.33 min; 7, 22.71 min; **8**, 22.95 min.

Cyclodextrins (CD's) had also been added to the electrolyte in an attempt to get more improvement on resolution, but this approach failed. However, some information about the relationship between the structures of alkaloids and

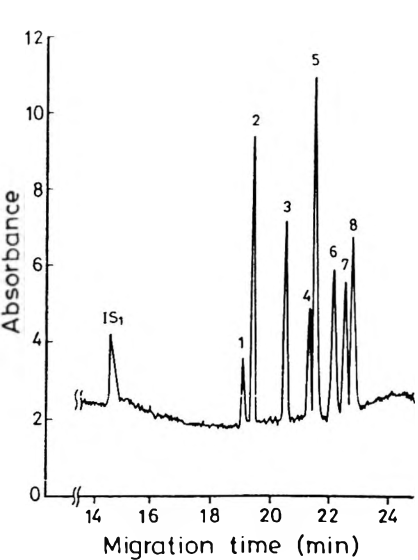

Figure 4. Capillary electropherogram of a mixture of eight quaternary alkaloids in CZE. IS_1 = benzyltriethylammonium chloride; other symbols are the same as those in Fig. 1.

Table 1

Theoretical Plates and Resolutions of the Alkaloids at Different Phosphate Concentrations

	No. of Theoretical Plates ($X10^{-5}$)								Resol'n (Rs)	
Conc'n (mM)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**1-2**	**7-8**
10	1.30	0.83	0.27	0.93	0.75	0.16	0.91	0.63	0.80	1.02
20	1.17	1.20	0.44	1.51	1.12	1.06	1.56	0.70	0.92	1.09
30	0.68	0.75	0.38	1.45	1.08	1.02	1.52	0.91	0.96	0.99
40	1.00	1.39	0.44	1.48	1.14	0.83	1.10	0.82	1.38	0.83
50	1.17	1.25	0.41	1.66	1.12	0.82	1.63	0.95	1.61	0.75
60	1.45	1.39	0.49	1.58	1.41	0.95	1.54	1.00	1.93	0.70

the size of cyclodextrins was obtained. There was almost no influence on all compounds when α-CD was added, but a marked change on the migration times of **1**, **3**, **4**, **6** and **7** was observed when β- and γ-CD were used.

Table 2

Detection Limits and Reproducibility of Migration Time (Mt) and Peak Area Ratio (Ar, Peak Area/IS Area) (n=6) of the Alkaloids

Compound	Detection Limit ng (μg/mL)	Intra-Day RSD, %		Inter-Day RSD, %	
		Mt	Ar	Mt	Ar
1	0.06 (17.43)	0.73	2.33	1.96	3.59
2	0.11 (31.40)	0.77	3.10	1.34	3.17
3	0.06 (16.77)	0.67	3.24	1.68	3.76
4	0.05 (15~97)	0.86	2.89	1.46	4.38
5	0.06 (16.77)	0.87	3.71	1.52	3.71
6	0.06 (16.46)	0.91	2.33	1.53	3.31
7	0.06 (16.46)	0.95	2.77	1.55	3.28
8	0.05 (15.78)	0.95	2.75	1.62	3.07
9	0.13 (39.50)	0.69	2.07	1.52	2.48
10	0.16 (47.33)	0.69	1.67	1.53	2.00
11	0.12 (35.85)	0.75	2.60	1.51	3.46
12	0.12 (35.88)	0.79	2.92	1.51	3.68
13	0.12 (35.65)	0.83	3.33	1.46	4.00
14	0.12 (35.25)	0.89	2.39	1.46	2.68
15	0.12 (35.91)	0.91	3.53	1.47	3.88
16	0.12 (35.85)	0.92	2.69	1.41	2.93
17	0.06 (16.50)	0.66	3.57	1.36	3.78

Compound **1-8** were measured by CZE and **9-17** were determined by MECC.

Obviously, the alkaloids with a highly hydrophilic group on their 2,3-positions, and a dialkoxy group on their 9,10-positions, and the CD with a large-size cavity are the paramount factors for forming a reasonable complex.

METHOD VALIDATION

Precision

The reproducibility (relative standard deviation) of the proposed method, on the basis of peak-area ratios for six replicate injections, was 1.67-4.00% in

Table 3

Data of Linear Ranges and Correlation Coefficients (r) of the Alkaloids

Compound	Linear Range (mg/mL)	Slope	Intercept	r
1	0.016-0.492	14.024	0.434	0.9954
2	0.076-1.216	3.750	0.086	0.9993
3	0.067-2.138	13.495	0.417	0.9950
4	0.015-0.116	13.221	0.147	0.9962
5	0.022-0.350	13.763	0.149	0.9963
6	0.012-0.198	15.790	0.171	0.9962
7	0.012-0.197	13.508	0.147	0.9963
8	0.023-0.720	12.948	0.140	0.9960
9	0.089-0.890	2.611	0.353	0.9999
10	0.085-0.850	2.180	0.102	0.9999
11	0.013-0.130	3.803	0.006	0.9990
12	0.011-0.110	3.485	0.005	0.9985
13	0.017-0.170	3.463	0.005	0.9982
14	0.071-0.710	3.197	0.005	0.9987
15	0.016-0.160	2.969	0.004	0.9991
16	0.021-0.210	3.178	0.005	0.9989
17	0.041-0.410	3.567	0.064	0.9997

Compound 1-8 were measured by CZE and 9-17 were determined by MECC.

MECC and 2.33-4.38% in CZE. and the variation of the migration time of each peak was less than 2% (n=6). The detection limits (S/N=3) for the quaternary alkaloids were 0.05-0.16 ng (15.78-47.33 μg/mL. column inner diameter 75 μm). Detailed data for R. S. D. and detection limits are shown in Table 2.

Linearity

Calibration graphs for the seventeen alkaloids were obtained over a range of one order of magnitude of concentration. Results of the regression analyses and the correlation coefficients (r) are shown in Table 3. The results showed good linear relationships between peak-area ratios (y) and concentration (x, mg/mL).

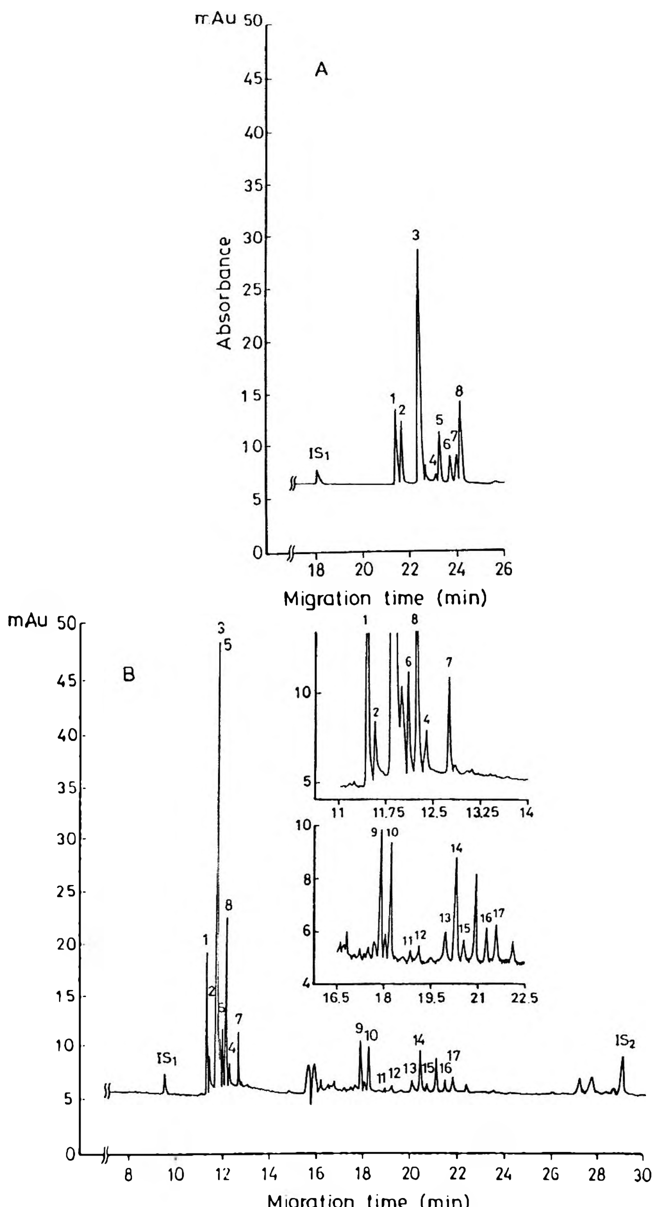

Figure 5. Capillary electropherograms of the coptis-evodia herb couple in (A) CZE and (B) MECC. IS_2 = 18β-glycyrrhetinic acid and other peaks as in Fig. 4.

Accuracy

Suitable amounts (0.01-10.45 mg) of the seventeen alkaloids were added to a sample of coptis-evodia herb couple of known alkaloid content and the mixture was analysed using the proposed procedure.

The recoveries of the alkaloids were 96.68-103.19% in MECC, and 99.65-103.28% in CZE.

Separation of 1, 4, 6, 7 and 8 with MECC; an Alternative Route

Although compounds **1-8** had better to be analyzed by CZE method, Figure 5-B shows clearly that **1**, **4**, **6**, 7 and **8** could also be determined by MECC technique. After comparing the electropherograms in Figure 5-A and 5-B, it was found that MECC provided not only sharper peaks but also shorter migration times than that of CZE. In order to investigate the differences obtained from CZE and MECC systems, the following data were calculated. The Rs values of **1-2, 6-8** and **4-8** were 2.10, 2.09 and 2.00, and the numbers of theoretical plate of **1**, **4**, **6**, 7 and **8** were 541945, 166641, 627312, 317957 and 285733, respectively. The detection limits were 0.10 ng (30.36 μg/mL) for **1**, 0.08 ng (23.96 μg/mL) for **4**, 0.08 ng (23.01 μg/mL) for **6**, 0.08 ng (23.25 μg/mL) for 7, 0.08 ng (24.18 μg/mL) for **8**. Regression equations and correlation coefficients were calculated as follows: **1**, y=15.695x-0.175 (r=0.9995); **4**, y=14.060x-0.035 (r=0.9996); **6**, y=14.773x-0.038 (r=0.9993); 7, y=14.491x-0.035 (r=0.9995); **8**, y=13.894x-0.034 (r=0.9995).

The recoveries were 98.52-103.58%. These data indicate that MECC method could give higher theoretical plate numbers and Rs values, but has lower detection limits than that of CZE. As a result, CZE was able to analyze all the quaternary alkaloids **1-8** simultaneously, and MECC was suitable for the determination of **1**, **4**, **6**, 7, **8** and **9-17** in a single run.

Determination of the Alkaloids in Herb Couple and Chinese Herbal Preparations

When the test solution of the herb couple was analysed by CE under the selected conditions, the electropherograms shown in Figure 5-A and B were obtained. The peaks were identified by comparison of the migration times and by spiking the mixture with a single alkaloid in a subsequent run. By substituting the peak-area ratios of the individual peaks for y in the above

Table 4

Contents (mg/g Crude Drug) of the Alkaloids in the Herb Couple and the Chinese Herbal Preparations (Mean±SD; n=3)

Compound	Herb Couple	*Pien-tung-wan*	*Kan-lu-san*
1	7.43±0.14	4.69±0.27	5.32±0.36
2	17.34±1.00	7.59±0.23	6.35±0.12
3	32.47±0.80	12.17±0.35	17.11±0.39
4	1.24±0.11	1.32±0.02	1.07±0.02
5	4.89±0.27	2.58±0.22	3.11±0.17
6	2.77±0.18	1.86±0.19	1.89±0.22
7	2.76±0.17	2.00±0.22	1.89±0.06
8	10.07±0.52	4.21±0.26	5.93±0.20
9	6.56±0.05	-	-
10	10.15±0.21	-	-
11	0.43 ±0.03	-	-
12	0.77±0.07	-	-
13	1.33 ±0.03	-	-
14	4.96±0.24	-	-
15	1.29±0.14	-	-
16	1.19±0.08	-	-
17	2.38±0.08	-	-

-: The content was below detection limit. Compound **1-8** were measured by CZE and **9-17** were determined by MECC.

equations, the contents of the individual alkaloids in the coptis-evodia herb couple were calculated. Alkaloid contents in the herb couple containing Chinese herbal preparations such as *Pien-tung-wan* and *Kan-lu-san* could also be determined by the same procedure.

The results are given in Table 4. Data in Table 4 show that there are only the water-soluble components, quaternary alkaloids, present in the preparation decoction. Those alkaloids (**9-17**) that were water insoluble or practically insoluble were absent or existed in the amount below detection limits.

This work has successfully demonstrated that, by optimizing parameters such as maleic acid, phosphate and acetonitrile concentration of the electrophoretic media, high resolution separation of a complicated mixture can

easily be achieved. Furthermore, the results obtained indicate that the CZE method proposed for the separation and determination of quaternary alkaloids may be conveniently used for quality assurance of commercially available samples of some Chinese herbal preparations that contain coptis and evodia and also for quality control in pharmaceutical factories.

ACKNOWLEDGEMENT

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PRECONCENTRATION OF ALIPHATIC AMINES FROM WATER DETERMINED BY CAPILLARY ELECTROPHORESIS WITH INDIRECT UV DETECTION

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ABSTRACT

Preconcentration methodology based on adsorption chromatographies for enriching aliphatic amines (C1 to C4 substituted primary, secondary, and tertiary) and alkanolamines in water was studied by free zone capillary electrophoresis (CZE) with indirect UV detection. The solid-phase extraction of amines from water (pH 5) as a preconcentration step was studied for ion exchange solid-phase extraction (SCX) cartridges, cation ion exchange extraction disks, and ion-pairing with C_{18} extraction disks. In the course of the investigations, the electrophoretic properties of the amines was studied in some detail in order to optimize separations and detection limits. The indirect mode is particularly powerful in being able to detect primary through quarternary amines without derivatization. Mobilities of amines were correlated with their Stokes' radii. Increased selectivity for resolving closely related amines under CZE was explored using nonionic surfactants, pH adjustment, and optimized background

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electrolyte. Techniques were developed for obtaining stable baselines in the indirect detection mode. Linearity, sensitivity, and efficiency were explored for this mode of detection for a large set of amines.

INTRODUCTION

Aliphatic amines are toxic substances and irritants to mucous membranes[1] that are among the common chemicals of commerce. They are used as corrosion inhibitors in steam boilers and as starting materials in the manufacture of pharmaceuticals, insecticides, herbicides, fungicides, polymers, surfactants, and rubber accelerators. The related alkanolamines function as solvents and starting materials for surfactants, but they appear to be less toxic than the aliphatic amines.

The many commercial uses and natural occurrences of aliphatic amines (here we refer primarily to C_1 to C_4 alkyl-substituted primary, secondary, and tertiary amines) suggest that ultimately they will appear in the environment as pollutants. Thus, they are target analytes of U.S. EPA Method 8260[2] (also Method 624[3]) where they are classified as volatiles.

The U.S. EPA, EMSL-LV, maintains a continuing interest in analytical methods for amines because of their wide occurrence and toxicity and because there is need for determinative methods for amines as a result of the listing activities for various hazardous wastes under RCRA[4] when amines are suspected to be present.

Aliphatic amines are basic compounds with pK_b values[5] of approximately 5×10^{-4}. The replacement of an aliphatic group, or hydrogen, of an amine with an aromatic ring causes a profound change in the basicity, typically reducing the pK_b by six orders of magnitude. Aliphatic amines and their derivatives are essential to life, functioning as neurotransmitters and hormones[6] and occurring in biological tissues and fluids.[7-10]

Aliphatic amines can present chromatographic problems due to their reactivity and extreme basicity. Derivatization has, therefore, been frequently employed. However, derivatization (e.g., Method 8042)[2] has not achieved as wide adoption in environmental analysis as it has in biological and pharmaceutical analysis. Part of the reason may lie in the large number and complex nature of environmental matrices and the possibility of artifact formation from co-extractives.

Gas chromatography (GC) is frequently used to separate amines using a variety of GC detectors. Amines can be chromatographed underivatized on porous polymers such as HayeSep B, even in the presence of water.[11] Chromosorb 102 treated with KOH[12] and liquid-coated supports using ammonia as carrier gas[13] have been reported. Alternatively, a variety of derivatizing agents have been used for the GC of amines.[14-18]

Liquid chromatography has often been the separation technique of choice for amines due to the variety of derivatizing agents available to introduce chromophores for UV detection or fluorophores for fluorescence detection.[14,19-35] Other techniques used to separate amines include supercritical fluid chromatography,[36] thermospray LC/MS,[37] and ion chromatography.[38]

Applications of capillary electrophoresis to environmental analysis have increased dramatically.[39-46] Usually, these applications have depended on UV detection with separations using free zone electrophoresis or micellar electrokinetic chromatography (MEKC). Applications of CE to inorganic cations and anions have been based on indirect UV detection.[47] Beck and Englehardt developed applications of imidazole and other chromophoric amines as background ions for indirect detection of aliphatic amines and alkali ions in water samples.[48] As pointed out by Kuhr and Yeung,[49] indirect detection methods in CE have their origin in ion chromatography applications.[50] Kuhr and Yeung have discussed the conditions for optimization of indirect UV and fluorescence detection.[51] Kuhr has introduced indirect fluorescence detection under MEKC conditions[52] and further discussed indirect fluorescence detection.[53]

In this work, we evaluate various solid-phase extraction (SPE) adsorbents and systems for the preconcentration of aliphatic amines and some alkanolamines in water. The recoveries from these adsorbent systems are determined using capillary zone electrophoresis (CZE) with indirect UV detection. Three adsorbent systems were examined for the concentration of amines from water: (1) cation exchange[54] solid-phase extraction cartridges, (2) ion-pairing with extraction disks, and (3) cation exchange extraction disks. In the course of the recovery studies, procedures were developed to optimize separations and characterize the electrophoretic properties of amines. Correlations of electrophoretic mobilities with structure were made,[55-56] and practical techniques were developed for minimizing baseline fluctuations. Resolution of amines with similar mobilities was effected by adding nonionic surfactants[57] or through pH selection and optimized matching of mobility of amines with that of the background electrolyte.[47]

EXPERIMENTAL

Chemicals

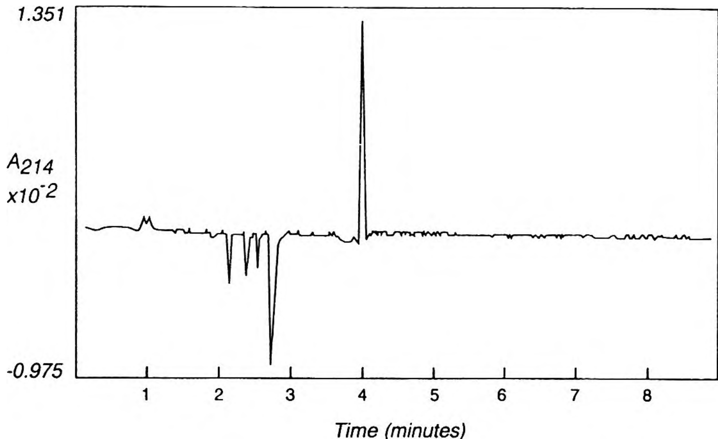
All organic compounds were obtained from Aldrich Chemical Company, Inc. (Milwaukee. WI. USA) unless otherwise specified. Other chemicals were from standard sources of supply. and all were used as received. Deionized water (ASTM Type II) was used for all aqueous solutions. Solutions were freshly prepared for each experiment. Liquid amines were measured gravimetrically for preparation of standard solutions.

Capillary Electrophoresis

A P/ACE Model 2050 Capillary Electrophoresis System (Beckman Instruments. Fullerton. CA, USA) was used for all capillary electrophoretic experiments. The instrument was fitted with a capillary 57 cm X 75-µm I.D.. 50 cm to the detector. with UV detection at 214 nm. The temperature of the capillary was 25°C. and electrophoretic runs were 10 minutes at 30 kV. The capillary was equilibrated with running buffer at the start of each experiment. and washed extensively with acid, alkali, water, and running buffer between runs. Migration times. peak widths, and dynamic reserves were estimated directly from the monitor of the data system (software System Gold. Ver. 6.1). Corrected peak areas. as computed by the data system (peak area multiplied by the velocity of the ion [length to the detector divided by time]). were normalized to the corrected peak area of the internal standard (tetrabutylammonium ion) as a control for the small variations in the nominal volumes of the pressure injections (ca. 5 nL from 1-s injections).

Solid Phase Extraction Studies

Solid phase extraction disks (Bakerbond Octadecyl C18; J.T. Baker, Inc., Phillipsburg. NJ. USA) and SCX cartridges containing 500 mg benzenesulfonic acid and used for cation exchange (Varian Associates Inc.. Sunnyvale. CA. USA) were prepared according to the manufacturers' instructions. An experimental product cation exchange disk was a gift of 3M Industrial and Consumer Sector. New Products Department. St. Paul, MN, USA. Aqueous solutions of analytes were applied at flow rates of about 1 L/h. Increasing flow rates to 1 l/15 min for disk extractions did not adversely affect recovery.



Compound	Migration Time Seconds	Mobility μ_e $10^{-4}Cm^2V^{-1}S^{-1}$	Response Factor Per mM	10^4 Theoretical Plates
propylamine	119	3.76	0.385	5.48
dipropylamine	135	2.83	0.421	3.13
tripropylamine	144	2.39	0.395	5.55
tetrabutylammonium bromide	155	1.81		0.75

Figure 1. Electropherogram demonstrating the separation of propylamine, dipropylamine, and tripropylamine. The background ion was imidazole (5.0 mM, pH 5.0). The internal standard was tetrabutylammonium ion (1.0 mM). Analytes were detected as decrements in absorbance at 214 nm caused by the displacement of the background ion. For other conditions and calculations see text.

RESULTS AND DISCUSSION

Capillary Electrophoresis: Indirect Detection of Organic Amines

Figure 1 shows an electropherogram of propylamine, dipropylamine, and tripropylamine by CZE. A solution containing propylamine (6.0 mM), dipropylamine (3.7 mM), tripropylamine (2.6 mM), tetrabutylammonium bromide (1.0 mM), and imidazole (5.0 mM, pH 5.0) was injected (5.0 nl) into the capillary; thus, the analytes were dissolved in the running buffer. The system peak (displaced imidazole) reached the detector window at 240 seconds. From these times (column 2 of the inset), the apparent mobilities (μ_a) may be

Table 1

Relative Response and Effective Mobility (μ_c) of Selected Organic Compounds

Compound	Slope[1] (response/mM)	Mobility[2] (10^{-4} cm^2 $V^{-1}s^{-1}$)
Morpholine	0.318	3.46
Pyrrolidine	0.305	3.65
Diaminobutane	0.656	4.72
Diamiopropane	0.491	4.81
Diaminopentane	0.555	4.28
Ethanolamine	0.450	3.83
Diethanolamine	1.28	3.10
Triethanolamine	0.991	2.83
Diethylamine	0.306	3.37
Triethylamine	0.342	3.02
Butylamine	0.311	3.33
Dibutylamine	0.363	2.42
Tributylamine	0.385	2.01
Propylamine	0.311	3.64
Dipropylamine	0.380	2.76
Tripropylamine	0.379	2.35
Dimethylamine	0.258	4.33
Triethylamine	0.188	4.17

[1] Slopes (relative response per mM) are based on 5-10 determinations for each compound and were taken from plots like those presented in Figure 2. Linear regression analyses were performed on each data set. Values for R^2 exceeded 0.99 in each case. For other methods, see Figure 1 and text.

[2] Effective mobilities (10^{-4} cm^2 V^{-1} s-1) were calculated from measured migration times by the following equation: μ_a = apparent mobility; μ_c = effective mobility; μ_{EOF} = mobility provided by electroosmotic flow.

$$\mu_a = lL/tV$$

where $\mu_a = \mu_c + \mu_{EOF}$

V = applied voltage

l = effective capillary length (to detector)

t = migration time

L = total capillary length

calculated. The effective mobilities (μ_e; column 3 of inset) are then obtained from familiar equations of CE (see also Table 1).[58] The relationship between the mobility of the amines and the background electrolyte is of interest, and we shall correlate the mobility with an empirical relationship for a large class of amines. The relationship between the response factor (corrected area of analyte ion/corrected area of internal standard ion) of each peak and the concentration of that particular analyte in the test solution injected into the capillary is shown in column 4 (inset). The proportionality between concentration of analyte and the response factor of its peak was constant over a 150-fold concentration range.

Theoretical plate number, or the height equivalent to a theoretical plate (HETP) is a measure of zone broadening and of efficiency of separation and will be discussed in a later section of this paper (cf. Figure 4). The analyte peaks shown in Figure 1 exhibited plate numbers (column 5) from about 10,000 to more than 50,000.

The area of the positive peak which migrates at the EOF, as verified by measuring the migration time of benzyl alcohol, and represents the displaced chromophoric background ion, imidazole, was very nearly equal to the sum of the areas of the negative peaks representing the positively charged cationic analytes and the internal standard. In a series of 15 electropherograms (not presented here), the ratios of the sums of the negative peaks to the sums of the positive peaks were calculated. The average of these ratios was 1.072, and the standard deviation was 0.031.

These results suggest that the analyte ions effect a nearly 1:1 displacement of background electrolyte ions because the analyte ions are dissolved in the running buffer (background electrolyte).[51]

Detector Response is a Linear Function of Analyte Concentration

The usefulness of any determination depends on its ability to measure a target analyte over a useful range of concentrations with a response that is directly proportional to concentration over the entire range. Several experiments demonstrated that the method permitted detection of the analytes of interest over a 150-fold range of concentrations, from 0.02 mM to 3.0 mM, with as little as 5-nL injections (Fig. 2). The mass limit of detection was about 0.1 pmol of analyte injected on-column.

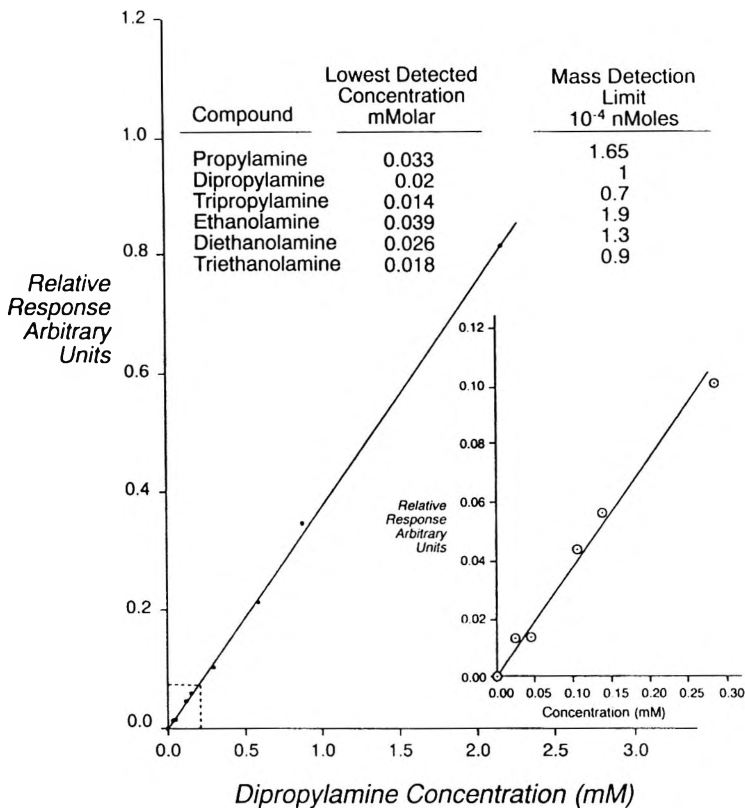


Figure 2. Relative response is a linear function of analyte concentration. Relative responses (corrected area of analyte peak divided by corrected area of internal standard peak) are plotted as a function of the analyte concentrations injected into the capillary. Upper inset gives results for the indicated compounds in similar experiments. Inset at lower right shows enlargement of the plot near the origin surrounded by the dotted line.

The inset in the lower right corner of Figure 2 shows an enlarged plot of that portion of the main figure (dotted outline) representing concentrations of analytes from 0.02 mM to 0.1 mM. Linear regression analyses on data sets like that of Figure 2 gave correlation coefficients (R^2) in excess of 0.99. In the upper inset of the figure are results for six of the organic compounds studied here. Similar analyses were conducted for all of the compounds listed in Table 1, where the slopes of the regressions are given. The detection limit was about 1.5 ppm without preconcentration.

Migration Time and Electrophoretic Mobility Correlations

The electrophoretic mobilities of the organic compounds were calculated from the observed migration times taken directly from data like those presented in Figure 1 and are listed in Table 1. The intrinsic mobility of the internal standard, tetrabutylammonium ion, in these experiments was 1.81×10^{-4} $cm^2V^{-1}s^{-1}$ and varied less than 1% between experiments. In contrast, the value of μ_{eo} in these experiments was quite variable. In a typical experiment, the data permitted 15 determinations of the mobility imparted by the EOF. The average of these was 3.50 with a standard deviation of 0.45.

The mobilities calculated for the organic amines were in accord with expectations, which follow from the basic equation describing electrophoretic mobility:

$$\mu_e = q/(6\pi\eta r)$$

where
μ_e = electrophoretic mobility
q = ion charge
η = solution viscosity
r = ion radius.

The relationship predicts that small, highly charged species would exhibit high mobilities and that large, minimally charged species would exhibit low mobilities.[59] In some cases, the radius must include the solvation sphere of the ion. Figure 3 presents a plot of the reciprocal of the Stokes' radius, based on the empirical formula $1/(m/z)^{0.7}$,[68] of the organic compounds listed in Table 1 as a function of their calculated electrophoretic mobilities. This plot suggests a qualitative inference may be possible in predicting the m/z value of an unknown amine whose mobility falls on the curve between known compounds. The data point representing the ammonium ion (μ_e = 5.8) fell well off the curve, suggesting that the computation used for the organic amines underestimated the effective radius of this ion. These data represent an extension of previous studies to a larger data set of aliphatic amines of environmental interest.

The ionic radii for several inorganic cations (potential interferences to indirect detection of aliphatic amines) can be plotted as a function of their calculated electrophoretic mobilities (not shown). For these ions, the mobility increased with increasing ionic radius.

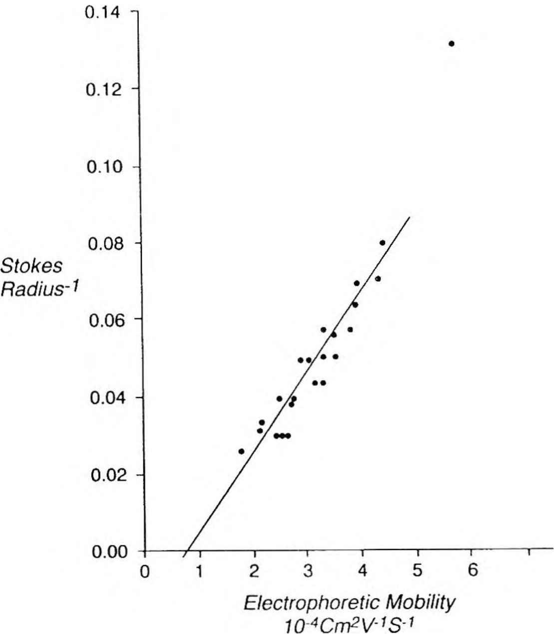


Figure 3. Electrophoretic mobility of several organic amines as a linear function of the reciprocal of their Stokes' radii $[1/(m/z)^{0.7}]$. The mobilities listed in Table 1 are plotted as a function of this parameter for each of the listed compounds. The point lying well off the curve (mobility=5.8) represents the ammonium ion.

One explanation for this, offered by Harned and Owen[60] is that relatively small ions of high charge density would be expected to exhibit relatively large hydration spheres. With increasing radius of given ions, both the hydration number and the radius of the hydration sphere decrease.[61]

Theoretical Plate Number and Efficiency of Separation

The efficiency of separations attainable with electrophoretic methods is determined by a combination of at least two factors. The first is the effective mobility (μ_e), which is determined by charge, size, and resistance to flow of an analyte. The second is dispersion of the analyte ion, which determines the zone length.[59] Dispersion in CE is often dominated by longitudinal diffusion and depends on the molecular diffusion coefficient (D), which can be determined under CZE conditions.[62] Dispersion, or zone length, is also dramatically

influenced by the length of the injection plug and, in the indirect detection method described here, by the difference between the electrophoretic mobility of the analyte and the electrophoretic mobility of the background ion itself, according to the Kohlrausch regulating function.[47,59] Dispersion will be minimal when the mobilities of both the analyte and the background ion are equal or nearly so.[47,63] With indirect detection, peak broadening will increase as the concentration of the analyte approaches that of the background ion.[64] Zone length or broadening of the peak in an electropherogram may be quantified by calculating the plate number according to:

$$N = 5.545\,(t/w_{1/2})^2$$

where t = migration time in seconds

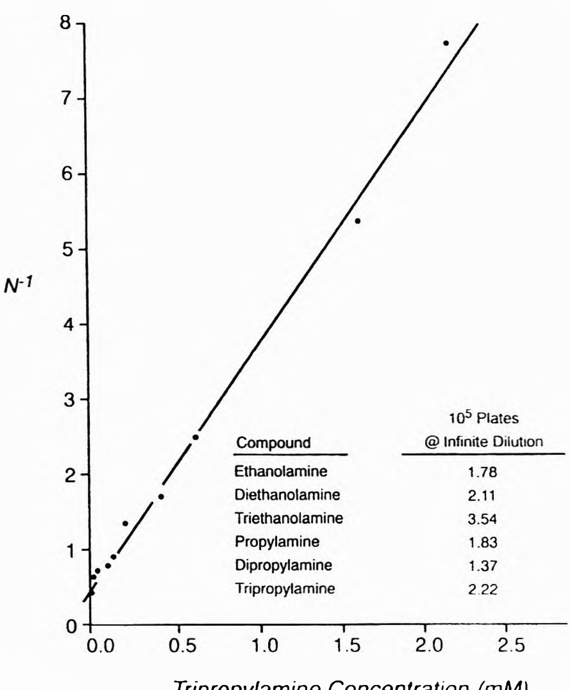
$w_{1/2}$ = peak width, in seconds, at half height

Plate counts of the order of 300,000 are quite satisfactory.[63,65] Zone broadening relative to the concentration of analyte is negligible when the analyte concentration is two orders of magnitude below that of the background ion.

In the work reported here, we have noted the previously observed inverse relationship between theoretical plate number and analyte concentration at constant injection volume. These observations are presented in Figure 4, where the reciprocals of the calculated theoretical plate numbers are plotted as a function of the concentrations of injected tripropylamine solutions. The linear relationship thus obtained permitted extrapolation to infinite dilution of the analyte. From the intercept on the ordinate, we inferred the theoretical plate number at zero analyte concentration. Data for tripropylamine are presented in the figure, and from the ordinal intercept a maximal plate number of 2.22×10^5 was obtained.

Similar plots were prepared for each of the compounds listed in the inset (Fig. 4). As shown, these plate numbers were of the order of 2 to 4 x 10^5, and were comparable to those reported by Gross and Yeung[64] and indicate satisfactory efficiencies.

Maximal theoretical plate numbers were calculated for these compounds based on an assumed value for diffusion coefficients of the order of 1×10^{-5} cm^2s^{-1}. These values were found to vary between 1.8 and 2.9 x 10^6 plates. The plate numbers measured and reported here (1.8 to 3.5 x 10^5) represented 10%



Compound	10^5 Plates @ Infinite Dilution
Ethanolamine	1.78
Diethanolamine	2.11
Triethanolamine	3.54
Propylamine	1.83
Dipropylamine	1.37
Tripropylamine	2.22

Figure 4. Theoretical plate number at infinite dilution. The concentration of analyte injected into the capillary is plotted as a function of the reciprocal of the theoretical plate number observed at that concentration. Extrapolation to zero concentration permits inference of maximal theoretical plate number at infinite dilution. The inset provides results from similar plots obtained with the indicated compounds.

of the maximally attainable efficiency. It is likely that the size of the injection plug employed in these studies (5 nL, about 10 times the size calculated to give 5% broadening) also accounted for a considerable fraction of the reduction from the maximum attainable efficiency.[59]

Figure 5 shows the importance of migration time and illustrates the difficulty in achieving separation of analytes with similar Stokes' radii (cf Fig. 3) and therefore similar electrophoretic mobilities. Butylamine and diethylamine were not separated when run together in buffer with 5.0 mM imidazole as the background ion since their mobilities differ by only 1.2%. Tripropylamine and dibutylamine, whose mobilities differ by about 3%, were just barely separable in this system. This slight difference may be exploited, and better resolution obtained by electrophoresis, if a background ion is used that

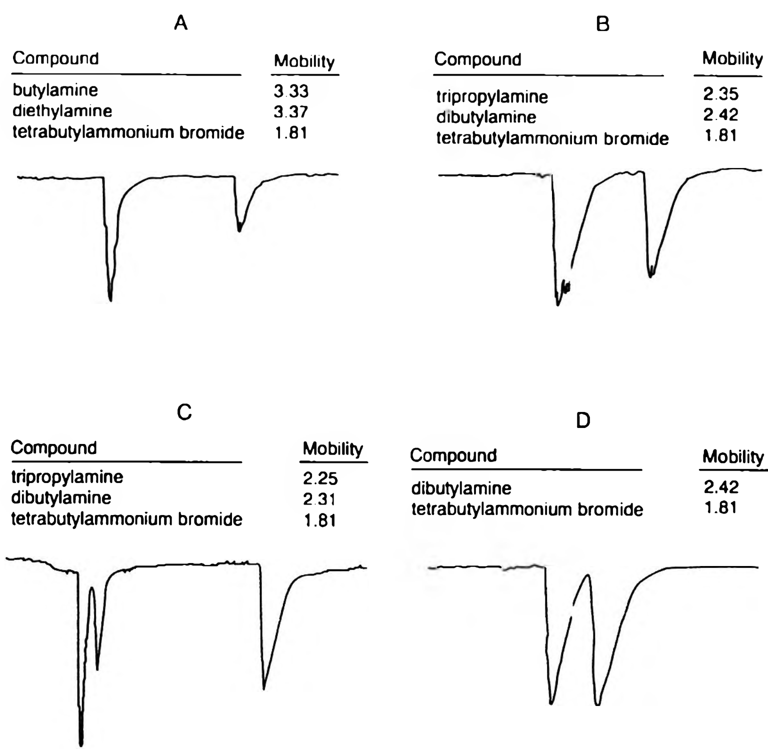


Figure 5. Efficiency of separation depends upon mobility and plate number. Panels A and B show that butylamine and diethylamine are poorly separated and that tripropylamine and dibutylamine are poorly separated in a running buffer consisting of 5.0 mM imidazole at pH 5.0. As shown in panel C, tripropylamine may be separated from dibutylamine by running in buffer consisting of 5.5 mM N-ethylbenzylamine at pH 5.5 and with the detergent POE 20 (2%). Panel D demonstrates that dibutylamine and tetrabutylammonium ion are well separated in either running buffer.

has a mobility more nearly equal to the mobilities of the barely separable pair of analytes. For example, as shown in Figure 5C, when tripropylamine and dibutylamine were run together with N-ethylbenzylamine (pH 5.1, 5.7 mM) as the background ion and in the presence of the nonionic surfactant[57] Tween 20, a considerable improvement in separation was achieved even though no changes in the values of μ_e were observed. The mobilities of dibutylamine and tetrabutylammonium ion differ by 34% and were easily separated in either solvent.

Table 2

Separation of Similarly Migrating Compounds[1]

Compound	Effective Mobility (10^{-4} cm^2 V^{-1}s^{-1}) Imidazole (5.0 mM, pH 5.0)	N-ethylbenzyl amine (5.5mM, pH 5.1)	Benzyltriethyl-ammonium chloride (5.0 mM, pH 10.1)
Tetrabutyl-ammonium ion	1.81	1.81	1.75
Triethanolamine	2.83	N/D	1.75
Dipropylamine	2.76	N/D	2.53
Tipropylamine	2.35	2.35	2.28
Dibutylamine	2.42	2.42	2.29
Butylamine	3.33	N/D	2.23
Diethylamine	3.37	N/D	2.61

[1] See legends of Figure 1 and Table 1.
N/D = not determined.

In Table 2 the results obtained with three buffer systems are compared. Note that triethanolamine and dipropylamine were well separated in benzyltriethylammonium chloride at pH 10.1 but not in imidazole at pH 5.0. Similarly, butylamine and diethylamine were not separated in imidazole but were easily separated in benzyltriethylammonium chloride. These data demonstrate that compounds that are difficult to separate in one electrolyte system may indeed be well separated in another system by taking advantage of slight differences in pK_b values of the target analytes and by optimizing the mobility of the background electrolyte.

This situation is analogous to the recommendation of Terabe et. al. for the separation of weak acids at their respective values of pK_a.[66] To our knowledge, this principle has not been previously applied to the separation of aliphatic amines as shown here.

Addition to the running buffer of nonionic surfactants such as Tween 20 may also enhance resolution of closely migrating analytes. Ionic additives are not recommended in the indirect mode because they may well compete for displacement with the background electrolyte and thereby reduce the response.

Table 3

Recovery of Amines from a More Dilute Aqueous Solution

Disk[1]	------- Percent Recovery[2] -------		
	Propylamine	Dipropylamine	Tripropylamine
1	105	107	108
2	98	112	107
3	96	96	86
4	0	0	0

[1] Empore Bakerbond Octadecyl (C_{18}) extraction disks were prepared following manufacturer's instructions.
[2] The test solution contained per L, 45 μmol of total amines; 150 μmol of dodecylbenzene-sulfonate, sodium salt; and 5.0 mL of methanol. The filtrate from disk 3 was passed through disk 4 to check for amines remaining in solution after the first passage.

Solid Phase Extraction Studies

Experiments were conducted for testing solid phase extraction disks in adsorbing an ion-pairing agent (e.g., decanesulfonate) which would in turn sequester ionized organic amines from aqueous solutions at pH 5.0. These experiments revealed that the recovery of each amine tended to decrease as the volume of applied sample increased. Evidently, the retention of decanesulfonate or the ion pair was insufficient and "wash out" resulted.

Table 3 details an experiment that evaluated sodium dodecylbenzenesulfonate as an ion pairing agent. The average recoveries for propylamine, dipropylamine, and tripropylamine were nearly 100%. The improved recoveries suggest that the adsorbent exhibited a higher affinity for the amine/dodecylbenzenesulfonate than for the amine/decanesulfonate ion pair. The concentration of each amine was 15 μM, just at the limit of detection. The concentration was increased by a factor of 100 by use of the solid phase extraction disk (1 L to 10 mL).

In further efforts to recover propylamines from dilute aqueous solutions, we used experimental cation exchange disks in which the sulfonic acid moieties were chemically bonded to a resin, which in turn was embedded in a Teflon

Table 4

Solid Phase Extraction of Organic Amines from Aqueous Solution: Sulfonic Acid Bonded to Poly(styrenedivinylbenzene) as Cationic Exchange Resin[1]

Compound[2]	Percent Recovery Sample 1	Sample 2	Sample 3	Average
Butylamine	49	67	60	59
Dibutylamine	50	66	76	64
Tributylamine	42	58	46	49
Dimethylamine	12	15	23	17
Diethylamine	10	14	18	14
Triethylamine	12	16	20	16
Propylamine	79	85	83	82
Dipropylamine	72	84	78	78
Tripropylamine	74	75	68	72

[1] Cation exchange disks (47-mm, sulfonic acid bonded to poly(styrene-divnylbenzene) copolymer, hydrogen form) were a gift of 3M, New Products Department, St. Paul, MN 55144. Disks were prepared by first washing with acetone, methanol, water, then very dilute H_2SO_4 (2 drops/ 100 mL), and finally with H_2O until the pH reached5.5. Disks were then eluted with four 5.0-mL protions of 4% NH_4OH in methanol (v/v); and following elution, the disks were regenerated as indicated in the washing procedure.

[2] Samples consisting of 1L, were passed through the disk at a flow rate of approximately 200 mL/min.

matrix. Several analytes were examined (Table 4). The recoveries for propyl-, dipropyl-, and tripropylamine compared well with the best ion-pairing results. Recoveries for some analytes, however, were disappointing. In particular, methylamine and ethylamine were not quantitatively recovered from the disks. Butyl-, dibutyl-, and tributylamine approached quantitative recovery levels, and improvements may result from alternative elution conditions. All analytes were quantitatively sequestered. Recoveries were limited by our inability to elute analytes quantitatively from the disks using the 4% ammonium hydroxide in methanol[54] (i.e., the system best suited for indirect detection). To our knowledge, these data represent the first report for the application of these disks for recovery of aliphatic amines.[67]

CONCLUSIONS

These results have demonstrated the utility of capillary electrophoresis coupled with indirect UV detection to measure the recovery of organic amines in aqueous solutions using various SPE techniques. The limit of detection was in the range of 0.02 mM. The results obtained showed a linear response over a 150-fold range in concentration for the several amines examined, and also that solid phase extraction could routinely achieve concentration factors on the order of 100-fold or more for selected analytes. The indirect detection mode is, however, the least selective and therefore the most subject to interferences. The indirect mode also imposes severe limitations on the choice of electrolytes, their concentrations, additives, and elution systems for solid phase extraction isolation. These restrictions impose practical limitations on the determination of amines in various matrices and to the levels obtainable. The indirect detection mode does offer broad applicability since primary through quarternary amines can be detected under one set of CE conditions. Future work will need to address cleanup techniques for eliminating interferences from inorganic ions such as the alkali metals and alkaline earths using organic solvents and pH. Additional work will examine selective derivatization for laser-induced fluorescence detection.[67]

NOTICE

The U.S. Environmental Protection Agency (EPA), through its Office of Research and Development, funded and performed the research described here. This work has been subjected to the Agency's peer review and has been approved as an EPA publication. The U.S. Government has the right to retain a non-exclusive, royalty-free license in and to any copyright covering this article. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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DETERMINATION OF NATURAL AND SYNTHETIC COLORS IN ALCOHOLIC AND NON-ALCOHOLIC BEVERAGES BY QUANTITATIVE HPTLC

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ABSTRACT

Methods based on high performance thin layer chromatography with scanning densitometry and solid phase extraction were developed for quantification of lycopene and permitted synthetic food dyes in alcoholic and nonalcoholic beverages. Results of analyses of real samples are reported, and the methods were validated by determining the precision of replicate analyses and accuracy (recovery) by analyzing spiked samples.

INTRODUCTION

Thin layer chromatography (TLC) has been used to separate and detect natural colorants added to food products to fraudulently intensify colors, such as lycopene, bixin, canthaxanthin, and beta-apo-8'-carotenal added to products derived from red pepper. The pigments were quantified by scraping the zone

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off the layer and elution from the silica gel with petroleum ether followed by spectrophotometry or by gradient elution high performance liquid chromatography (HPLC) after saponification.[1] TLC combined with solid phase extraction (SPE) has been used for the identification of synthetic color additives (permitted FD&C food dyes) in nonalcoholic[2,3] and alcoholic beverages,[4] but quantification, which is usually performed by column liquid chromatography (HPLC)[5,6] was not carried out.

The purpose of the present study was to develop quantitative high performance TLC (HPTLC) methods for natural and synthetic food colors, to demonstrate the application of the methods to actual samples, and to evaluate their sensitivity, selectivity, accuracy, and precision. A direct-spotting method is described for the determination of lycopene, the natural red pigment in tomatoes, in tomato juice and paste, which would be applicable for quantitative analysis of other sample types[1] to which this pigment had been added.

Methods are also described for the quantification of synthetic food colors in nonalcoholic beverages such as fruit drinks and soda and alcoholic wine coolers by SPE combined with HPTLC. SPE is required for these samples in order to concentrate the colors and remove interferences that cause analyte zones in sample chromatograms that are streaked or do not line up with standards.

EXPERIMENTAL

Standards and Standard Solutions

Lycopene (92.5% purity) and carotene standards were obtained from Sigma Chemical Co. (St. Louis, MO). TLC standard solutions (92.5 ng/µL were prepared in methylene chloride. Standards of the synthetic certified food colors listed in Table 1 were obtained from Warner Jenkinson Co. (St. Louis, MO, USA) with certificates of analyzed purity.

TLC standard solutions were prepared at concentrations of 180 ng/µL in ethanol-water (1:1) for C_{18} solid phase extraction (SPE) of nonalcoholic beverages and for amino-column SPE of alcoholic beverages, and in ethanol-0.2 M sulfuric acid (1:1) for amino-column SPE of nonalcoholic beverages. The Blue No. 1 solutions were diluted 1:10 (18.0 µg/µL) to prepare the TLC standard.

Thin Layer Chromatography

TLC was carried out on 10 x 20 cm Whatman (Clifton, NJ) LHPKDF laned, high performance preadsorbent silica gel plates prewashed by development with methylene chloride-methanol (1:1). Standards and samples were applied to the preadsorbent using a 10 μL Drummond (Broomall, PA) digital microdispenser, and plates were developed in a paper-lined, vapor saturated Camag (Wilmington, NC) HPTLC twin-trough chamber. After drying with warm air from a hair drier or in an oven for ca. 5 min., the areas of the colored zones were scanned with a Shimadzu (Columbia, MD) CS-930 densitometer in the single beam, reflectance mode at the wavelength of maximum absorption for each compound, as determined from the in situ spectra recorded between 370 and 700 nm with the spectral mode of the densitometer. A calibration curve was constructed by linear regression of standard area and weight data, and the weights of analyte in the spotted sample aliquots were interpolated from the curve. Accuracy was determined by adding a known amount of dye to a blank sample not containing that dye or spiking a preanalyzed sample with an additional amount of dye naturally contained. Recovery from these spiked samples was calculated by comparing the experimental concentrations of the dye with the theoretical concentration based on the level of fortification.

Analysis of Samples

Determination of lycopene in tomato juice and paste

Tomato juice was spotted directly with no sample preparation and tomato paste (6.32 g) was dissolved in 30.0 mL of deionized water prior to spotting. The lycopene TLC standard was spotted in 4.00, 6.00, and 8.00 μL aliquots (370, 555, and 770 ng, respectively) to prepare the calibration curve, along with duplicate 5.00 μL aliquots of the tomato juice and paste samples. Development of the plate with petroleum ether-methylene chloride (85:15) for a distance of 7 cm beyond the preadsorbent-silica gel interface required ca. 20 min., and the zones were scanned at 385 nm. The concentration of lycopene in tomato juice (mg/mL) was calculated by dividing the weight interpolated from the calibration curve by the volume of sample spotted.

For tomato paste (mg/g), the interpolated weight was divided by the sample volume spotted and multiplied by the volume of water in which the paste was dissolved; this weight was divided by the weight of paste used.

Determination of Synthetic Food Colors in Non-Alcoholic Beverages by C_{18} SPE

The applicability of the quantitative TLC method was demonstrated by determining Red No. 40 and Blue No. 1 food colors in fruit drinks, and the method was vaildated by analyzing a colorless citrus soda spiked with Red No. 40.

Samples were analyzed by use of a J. T. Baker (Phillipsburg, NJ) glass manifold (no. 7018-00) operated with sufficient vacuum (ca. 15-20 mm of mercury) to produce a flow rate of ca. 3 mL/min. Bakerbond 3 mL light-loaded C_{18} columns (no. 7189-03) were prewashed with 3 mL of isopropanol and 12 mL of deionized water. Columns were inserted into the manifold through stopcock-adaptors, which were adjusted to prevent the column from becoming dry during or after the conditioning stages. Following addition of 5.00 mL of sample with a pipet, the column was washed with 5 mL of 5% aqueous acetic acid to elute polar impurities, dried by drawing vacuum for 10 minutes, and removed from the manifold. Dyes were eluted into a 5 mL graduated vial with a tapered bottom by forcing an appropriate volume of the eluent through the column with gentle pressure from a rubber bulb. The eluent was adjusted to a definite volume prior to spotting for TLC analysis by dilution to a known volume with isopropanol or concentration by evaporation with nitrogen gas flow.

For the determination of Red No. 40 in a fruit punch, the dye was eluted from the SPE column with 2-2.5 mL of isopropanol-water (17:83), and the eluate volume was adjusted to 2.00 mL to prepare the TLC sample. Standard aliquots of 1.00, 2.00, 4.00, and 6.00 µL (18.0-108 ng) and duplicate 2.00 µL aliquots of sample were applied to a silica gel layer, which was developed with butanol-acetic acid-water (4:1:5). Zones were scanned at 500 nm, and the concentration of dye (mg/mL) was calculated by dividing the interpolated weight by the volume of TLC sample spotted, multiplying by the total volume of sample, and dividing again by the volume of beverage placed on the column.

The determination of Blue No. 1 in a raspberry drink was carried out as above except that the dye was eluted from the column with 2.5-3 mL of isopropanol-water (23:77), the eluate was adjusted to 3.00 mL, the 1.00-6.00 µL standard aliquots contained 180 ng-1.08 ug of dye, duplicate 4.00 µL aliquots of sample solution were spotted (delete if same as above), and zones were scanned at 630 nm. For the determination of Red No. 40 and Blue No. 1 in a grape drink, both dyes were eluted from the SPE column with 2.5-3 mL of isopropanol-water (2:8) and the eluate adjusted to 3.00 mL to prepare the TLC standard. After TLC, as described above for the samples containing the

individual dyes, the separated dye zones in the sample chromatograms were scanned at their respective wavelengths of maximum absorption and quantified against calibration curves prepared from standards of each dye chromatographed on the same plate.

To determine the accuracy of the TLC method, a 0.0500 mg/mL spiked sample was prepared by diluting 50.0 µL of a 10.0 µg/µL standard solution of Red No. 40 to 10.0 mL with decarbonated, colorless citrus soda, and a TLC standard was prepared containing 200 ng/µL of the dye in ethanol-water (1:1). Five µL of the spiked sample was analyzed by eluting the SPE column with 5 mL of isopropanol-water (17:83), adjusting the eluate volume to 5.00 mL, and chromatographing duplicate 6.00 µL aliquots of sample together with 1.00, 1.50, and 2.00 µL aliquots of the TLC standard. The theoretical weight in the sample aliquots was 300 ng for 100% recovery, which would match the 1.50 µL standard.

Determination of Synthetic Food Colors in Non-Alcoholic Beverages by Amino Column SPE

The applicability of the method was demonstrated by determining Yellow No. 5 and Blue No. 1 food colors in a lemon-lime drink, and the method was vaildated by analyzing a colorless citrus soda spiked with Blue No. 1.

SPE and TLC were performed in general as described in the last section with the following changes. SPE was performed on Fisher (Pittsburgh, PA) PrepSep NH_2 columns (no. P456), which were prewashed with 5 mL of ethanol-water (1:1) and 10 mL of deionized water. Following addition of 10.0 mL of sample with a pipet, the column was washed with 10 mL of ethanol and 40 mL of water to elute interferences, and the column was not dried by drawing vacuum. The colors were eluted with 3.5-4.0 mL ethanol-0.20 M sulfuric acid (1:1) into a 10 mL vial, and the eluate was blown down just to dryness under a nitrogen stream and reconstituted in 1.00 mL of ethanol-0.2 M sulfuric acid (1:1). The mobile phase was butanol-ethanol-water-ammonia (50:25:25:10), and 1.00, 2.00, 4.00, and 8.00 µL of the TLC standards (18.0-108 ng for Blue No. 1 and 0.180-108 µg for Yellow No. 5) were spotted with duplicate 7.00 µL sample aliquots. Blue and yellow dye zones were scanned at 630 and 420 nm, respectively.

To determine the accuracy of the TLC/amino SPE method, a 0.0500 mg/mL spiked sample of Blue No. 1 in decarbonated colorless citrus soda and corresponding TLC standard were prepared as described above for the Red No.

40 spike. Recovery analyses were performed as for described for the unknown lemon-lime drink except that 5.00 mL of sample was applied to the column; the dye was eluted with 5.0 mL of ethanol-0.2 M sulfuric acid (1:1) and the eluate volume was adjusted to exactly 5.00 mL; and 2.00, 4.00, and 6.00 μL (40.0-120 ng) and duplicate 2.00 μL of sample were applied for TLC.

Determination of Synthetic Food Colors in Alcoholic Beverages by Amino Column SPE

Red No. 40 and Yellow No. 5 were determined in strawberry- and lemon-flavored wine coolers, respectively, using the SPE technique described above for nonalcoholic beverages except that 30.0 mL of sample was applied to the amino column, the column was washed with 10 mL of water instead of 40 mL to elute interferences, the colors were eluted with 4.0 mL of ethanol-0.5 M sulfuric acid (1:1), and the evaporated eluate was reconstituted in 1.00 mL of ethanol-water (1:1). TLC was carried out using 0.50-8.00 μL of standards and duplicate 9.00 μL (yellow) and 2.00 μL (red) aliquots of samples. Mobile phases were butanol-ethanol-water ammonia (50:25:25:10) (yellow) and butanol-acetic acid-water (4:1:5) (red), and zones were scanned at 420 and 500 nm, respectively. Accuracy was determined by analyzing a wine cooler containing Yellow No. 5 and then spiking the sample with a sufficient volume of a 1.83 mg/mL stock solution to double the dye concentration. The analysis was repeated and duplicate 4.5 μL aliquots of the spiked sample were spotted with duplicate 9.0 μL aliquots of the original sample on the same plate. The average scan areas of the two samples would be equal for 100% recovery.

RESULTS AND DISCUSSION

Determination of Lycopene

Lycopene standard formed a red-orange flat, oval-shaped zone with an R_f value of 0.32 when developed with petroleum ether-methylene chloride (85:15) on preadsorbent HPTLC silica gel plates. Development with petroleum ether-acetonitrile-methanol (2:2:4) on a Whatman LKC-18F reversed phase bonded silica gel layer produced a compact lycopene standard zone with an R_f value of 0.24, but directly-spotted samples did not chromatograph as well on the C_{18} plate as on silica gel, which was used in all analyses. No sample preparation was required for analysis of tomato juice or dissolved tomato paste because directly spotted samples produced zones that matched standards in migration distance and shape. The in situ visible spectrum of a standard zone exhibited a

major absorption peak at 385 nm and a smaller peak at 440 nm. This spectrum differed markedly from the spectrum recorded in petroleum ether, which had three roughly equal peaks at 446, 475, and 505 nm.[7] Carotene, which is also present in tomatoes, was separated from lycopene as a yellow v-shaped zone with an R_f value of 0.57. The red zones from the samples and the lycopene standard had identical R_f values and in situ spectra, which confirmed the identity of lycopene in the sample.

For quantification, samples and standards were scanned at 385 nm immediately after TLC because zones faded with increased time of standing due to photochemical air oxidation, resulting in decreased scan areas. The calibration curve prepared from the area of the three standard aliquots had a linear regression correlation coefficient (r value) of 0.99. A sample of tomato juice was analyzed four times and the average concentration of lycopene found was 0.123 mg/mL with a coefficient of variation (CV) of 3.7%, while dissolved tomato paste assayed at 0.483 mg/g with a CV of 0.72%. As another indication of the excellent precision of the quantitative TLC method, the differences between the scan areas of the duplicate 5.00 μL sample aliquots were 1.2% and 1.8%, respectively.

Although the applicability of the new direct TLC method was demonstrated for the analysis of two tomato products, it is equally appropriate for the quantification of lycopene in red pepper products to which it has been added as a coloring agent. The same extraction and solvent partition cleanup methods already described for indirect quantification by spectrometry after scraping and elution of TLC zones and proven to provide quantitative recoveries[1] would be used prior to the much simpler described densitometric TLC method. The method can also be combined with the alumina column chromatographic isolation of lycopene in tomato paste described in university laboratory manuals[7] to form the basis of an excellent organic/analytical student experiment.

Determination of Synthetic Food Colors

The R_f values of 11 synthetic colors encountered in beverages are shown in Table 1 for the two mobile phases used in this study. Red No. 40, Blue No. 1, and Yellow No. 5, which are among the most widely used colors, were chosen to test the quantitative methods for nonalcoholic beverages. With both mobile phases, the dyes formed compact, flat, v-shaped zones that produced narrow, symmetrical scan peaks. Calibration curves typically had r values of 0.96-0.99.

Table 1

R_f Values of Synthetic Colors

Synthethic Color	System 1*	System 2**
Blue No. 1	0.25	0.63
Green No. 3	0.25	0.62
Yellow No. 5	0.06	0.60
Yellow No. 6	0.29	0.66
Amaranth	0.06	0.62
Red No. 3	0.94	0.64
Red No. 4	0.35	0.86
Red No. 40	0.32	0.64

* System 1: butanol-acetic acid water (4 : 1 : 5).
** System 2: butanol-ethanol-water-ammonia (50 : 25 : 25 : 10).
Blue No. 2 fades quickly during TLC development.

Triplicate analysis of fruit punch containing Red No. 40 yielded an average concentration of 0.0620 mg/mL with a CV of 2.0%. Duplicate analysis of blue raspberry drink gave an average Blue No. 1 concentration of 0.00825 mg/mL and a 3.6% difference between trials. Duplicate analysis of grape drink found an average of 0.0923 mg/mL of Red No. 40 with a 1.5% difference between trials and 0.00120 mg/mL of Blue No. 1 with an 8.3% difference. Triplicate analysis of the spiked soda yielded an average concentration of 0.488 mg/mL with a CV of 2.6%. The average recovery was 97.5%. During these analyses, the percent difference in area between scans of duplicate sample aliquots ranged from 0.70-2.8%.

These accuracy and precision results show that the C_{18} SPE/TLC method is truly quantitative when used for the analysis of nonalcoholic beverages. Recovery of Red 40 from the spiked sample was excellent, and visual observations during the analysis of Blue No. 1 indicated that no color was left on the columns. The method will most likely be applicable to beverages containing the other dyes in Table 1. Mobile phases for elution of the dyes from the SPE column are obtained from the identification scheme presented by Young.[3] For analysis of beverages containing a mixture of colors, the compounds must be separated by selective elution from the SPE column, or eluted together and separated on the TLC plate. As an example of separation on a column, 5.00 mL of a colorless soda spiked with Yellow No. 5 (0.0200 mg/mL) and Red No. 3 (0.0700 mg/mL) was analyzed, and the mixture was

completely separated by elution of the yellow dye with 3.0 mL of isopropanol-water (2.5:97.5) and the red dye with the same volume of isopropanol-water (1:1). If a dye mixture is not separated either on the column or the TLC plate, it is still be possible to analyze the compounds individually by scanning at specific wavelengths where one absorbs and the other does not.

For analyses of nonalcoholic beverages using amino-column SPE, standards were prepared in the same ethanol-sulfuric acid solution as used to elute the column and the basic mobile phase (solvent 2, Table 1) so that the sample and standard zones lined up and sample zone streaking was minimized. The dyes are retained on the SPE column because of ionic interactions with the amino groups,[4] rather than hydrophobic interactions as in C_{18} SPE, and the dyes are all eluted together so that selectivity must be achieved by TLC separation or scanning at specific wavelengths.

Duplicate analysis of the lemon-lime drink resulted in identical concentration values of 0.118 mg/mL for Yellow No. 5, and a mean of 0.000747 mg/mL with a difference of 1.2% for Blue No. 1. For these analyses, typical differences between the scan areas of the duplicate sample aliquots was 4.5%. Duplicate analysis of the spiked sample gave an average of 0.0485 mg/mL with a difference of 2.1%, which corresponded to a recovery of 97.0%.

For analysis of wine coolers using amino column SPE, preparation of standards and reconstitution of evaporated SPE column eluates in ethanol-water (1:1) resulted in the best line-up of sample and standard zones and sample least zone streaking. Triplicate analyses of a wine cooler containing Red No. 40 gave an average value of 0.00282 mg/mL with a CV of 8.1%. Analysis of a cooler containing Yellow No. 5 gave a similar dye content value, 0.00192 mg/mL. The spiking experiment on this sample yielded a percent difference of 2.8% between areas of the original and fortified samples, proving the accuracy of the analysis.

The purpose of these studies was to demonstrate the quantitative determination of natural and synthetic food colors using HPTLC and SPE. The optimal approach to analyzing unknown samples involves spotting a series of dye standards providing linear response along with a range of sample volumes, in order to find at least one sample whose scan area is bracketed by standards. Quantification can then be done more reliably by spotting duplicate aliquots of a single sample volume and a smaller range of standard volumes within the linear response region that more closely bracket the sample. Because of this method of in-system calibration, the quantitative results obtained will be at

least as accurate and precise as those achieved by spectrophotometry or HPLC, and the ability to spot multiple samples on the same plate and the small solvent volume used for plate development provide very favorable sample throughput and cost-effectiveness.

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ANALYTICAL AND PREPARATIVE ENANTIOMERIC SEPARATION OF A SERIES OF C5-CYCLOALKYLAMINE-1,4-BENZODIAZEPIN-2-ONE CCK_B RECEPTOR ANTAGONISTS BY CHIRAL HPLC

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ABSTRACT

A series of C5 cycloalkyl benzodiazepin-2-one CCK_B receptor antagonists are reported in which substitution by tolyl-urea at C3 has generated a chiral centre. As only one of these enantiomers is selective for the CCK_B receptor an analytical separation of the enantiomers was developed to monitor the resolution of compounds by chemical means. It was shown that such compounds may be resolved using a Pirkle-type 3,5-(dinitrobenzoyl)-leucine (DNBL) chiral stationary phase (CSP) to give high α and R_S values. Such high enantioselectivities allowed the separation to be scaled-up sufficiently such that gram quantities of each enantiomer could be isolated by preparative chiral HPLC. An investigation is described in which the effect of substituent, mobile phase composition, temperature and the alternate CSP 3,5-(dinitrobenzoyl)-phenylglycine (DNPBG) is discussed for such compounds.

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Figure 1. Structure of L-365,260.

Figure 2. Structure of amidine analogue of L-365,260, where R=cycloalkyl bonded through nitrogen.

INTRODUCTION

Cholecystokinin (CCK) is a 33 amino acid polypeptide hormone which occurs in numerous molecular forms throughout both the central and peripheral nervous systems. CCK exerts a variety of actions on peripheral organs, such as regulating pancreatic secretion and gut motility, and may also function as a neurotransmitter or neuromodulator in the CNS.[1,2] The actions of CCK are mediated by two receptor subtypes designated CCK_A and CCK_B,[3] with the majority of the central receptors being of the CCK_B subtype.

A number of non-peptidic CCK_B receptor antagonists have been reported. Structures based upon the natural product Asperlicin have given rise to a series of 1,4-benzodiazepin-2-ones, notably L-365,260 (Figure 1).[5-7] It was shown that the 3*R*-enantiomer was selective for CCK_B binding over CCK_A.[8] Further modifications of this structure to increase affinity, selectivity and solubility have led to a series of modifications at the C5 position of the benzodiazepine in which the phenyl ring of L-365,260 has been replaced by a cycloalkylamine to form an amidine (Figure 2).[9]

The enantiomeric separation of substituted 1,4-benzodiazepine-2-ones using Pirkle type DNBPG or DNBL chiral stationary phases has already been demonstrated where the C5 substituent was phenyl.[10] However, loss of enantiomeric discrimination was observed in changing to cyclohexyl.

As stereochemistry at C3 is an important factor in determining CCK_B / CCK_A binding selectivity, it is essential to separate the enantiomers of these molecules either to provide enantiomeric purities of resolved material or to preparatively separate racemates. In the present study, the resolution of 1,4-benzodiazepine-2-ones bearing C5-amidine substituents is investigated using both Pirkle DNBPG and DNBL columns. The effect of C5 substituent, temperature and mobile phase composition is considered along with efforts to scale the separation up to provide a preparative method for obtaining individual enantiomers from racemic mixtures.

EXPERIMENTAL

Materials

All compounds described were synthesised in-house with identity and purity confirmed by NMR, MS, HPLC and elemental analysis. HPLC grade methanol, hexane and 1-chlorobutane were obtained from Fisons (Loughborough, UK). Ethanol was obtained from Hayman Limited (Witham, UK).

Instrumentation

An HP1090L series high performance liquid chromatograph was used for the analytical separations (Hewlett Packard, Avondale, USA). The system comprises an autoinjector, consisting of a Rheodyne 7010 injection valve fitted with a 250 µl loop, an autosampler and a binary DR-5 solvent delivery system. Detection was by UV using a built-in filter photometric detector (FPD) and data was processed using an HP DOS ChemStation. Column temperature was regulated using a Violet T-55S column cooler (Flowgen, UK).

Preparative separations were performed using a Shimadzu preparative HPLC supplied by Dyson Instruments (Houghton-le-Spring, UK) consisting of a C-R4A Chromatopac, a SCL-8A system controller, a SIL-8A autoinjector, two LC-8A pumps, a SPD-6A UV detector and a FCV-100B fraction collector.

R - group	L-number	R - group	L-number
N	L-738,416 (**1**)	N	L-744,751 (**6**)
N	L-733,072 (**2**)	N	L-741,597 (**7**)
N, O	L-743,244 (**3**)	N	L-740,104 (**8**)
N	L-741,545 (**4**)	N	L-736,220 (**9**)
N, OMe	L-741,596 (**5**)	N, S	L-740,616 (**10**)

Figure 3. Structure and identification of C5 cycloalkyl substituents.

Chromatographic Conditions

HPLC analysis was performed using columns containing DNBPG or DNBL covalently bonded to silica, supplied by Hichrom (Reading, UK). The dimensions were 250 x 4.6mm i.d. for the analytical columns and 250 x 20mm i.d. for the preparative column with a silica particle size of 5μm. Typical mobile phases were 5% MeOH in 1-chlorobutane or 50% EtOH in hexane. The flow rate analytically was 1.0 mL/min and preparatively 20.0 mL/min. The FPD was set to 230 nm, this being the approximate λ_{max} for these compounds.

Detection for preparative separations was performed at 300nm in an attempt to avoid saturation of the detector. 5μl of a 1mg/mL solution of all compounds were injected for the analytical analyses which were performed at thermostatically regulated temperature.

Table 1

Summary of Capacity Factor, Separation Selectivity and Resolution using a DNBL CSP

Compound L-number	K'_1	K'_2	α	R_S
L-738,416 (1)	1.16	3.95	3.41	16.74
L-733,072 (2)	2.00	3.79	1.90	8.29
L-743,244 (3)	1.13	2.63	2.34	9.57
L-741,545 (4)	1.32	3.03	2.30	10.92
L-741,596 (5)	1.49	3.53	2.37	10.44
L-744,751 (6)	1.68	4.03	2.40	11.42
L-741,597 (7)	1.04	4.32	4.15	17.61
L-740,104 (8)	1.16	2.48	2.14	9.64
L-736,220 (9)	1.31	4.00	3.05	15.22
L-741,616 (10)	1.45	4.00	2.76	13.02

RESULTS AND DISCUSSION

A series of 10 compounds bearing different cycloalkyl C5 substituents was investigated (Figure 3). The effect of varying the C5 substituent was analysed using both the DNBPG and DNBL chiral stationary phases and the α and R_S values for these experiments are presented (Tables 1,2) using a 5% MeOH/ 1-chlorobutane mobile phase. From our previous work with R = cyclohexyl[11] using a DNBL CSP, it was clear that the C5 substituent did not have to be phenyl and hence was not involved in π-π bonding. It was therefore not anticipated that the nature of the C5 substituent would strongly influence the chiral selectivity, as it was felt that this was not significantly contributing towards CSP interactions. Excellent separation selectivities and resolutions were demonstrated for the series with the DNBL CSP proving superior in most cases to the DNBPG, although this was not a general rule. Previous separations of 3-substituted benzodiazepines[10] have shown a similar difference between the phases but not such high stereospecificity. It is suggested that the C3 3-(methylphenyl)-urea substituent contributes significantly to the interactions with the CSP through classical CSP interactions of H-bonding, dipole-dipole and π-π interactions and it is likely that this substituent will dominate the conformation of the molecule to maintain this group psuedo-equatorial.

Table 2

Summary of Capacity Factor, Separation Selectivity, and Resolution using a DNBPG CSP

Compound L-number	K'_1	K'_2	α	R_S
L-738,416 (1)	1.36	1.65	1.21	2.65
L-733,072 (2)	3.31	4.34	1.27	4.54
L-743,244 (3)	1.25	1.40	1.12	1.55
L-741,545 (4)	1.49	1.76	1.18	2.27
L-741,596 (5)	1.65	1.93	1.17	2.88
L-744,751 (6)	2.73	3.80	1.39	5.49
L-741,597 (7)	0.99	1.40	1.42	5.29
L-740,104 (8)	1.32	1.38	1.05	0.66
L-736,220 (9)	1.75	1.86	1.06	0.91
L-741,616 (10)	1.34	1.75	1.31	4.20

In the absence of 'classical' interactions between the CSP and the substituent at C5, one explanation for the differences observed in separation selectivity and resolution upon substituent variation is that the directionality of the classical H-bonds or dipole interactions are altered. If one considers analogue 7, which possesses a cycloalkylamine with 2,6-dimethyl substitution, an α value of 4.15 and an R_S value of 17.61 indicates a significant energy difference between the CSP-interactions of the two enantiomers. Although the amidine group will preferentially try to adopt a planar arrangement, the effect of 2,6-dimethylation will be to partially disrupt the conjugation, hence the substituent will sit slightly out of plane. Assuming that the π-acid/π-base interaction between the benzodiazepine nucleus and the 3,5-(dinitrobenzoyl) group predominates for such CSP's, then this interaction is likely to be restricted by the C5 substituent. Consequently, if one overlays the benzodiazepine portion of each enantiomer, it is found that the directionality of, for example, the C2 carbonyl group of the benzodiazepine is markedly different between these enantiomers, with one projecting above and the other below the plane of the molecule.

This suggests that the carbonyl from only one enantiomer can interact optimally with the CSP, leading to a large difference in interaction energy between enantiomers. This may also be true of other dipoles, H-bond acceptor or H-bond donor group in the molecule. The effect of C5 substitution on

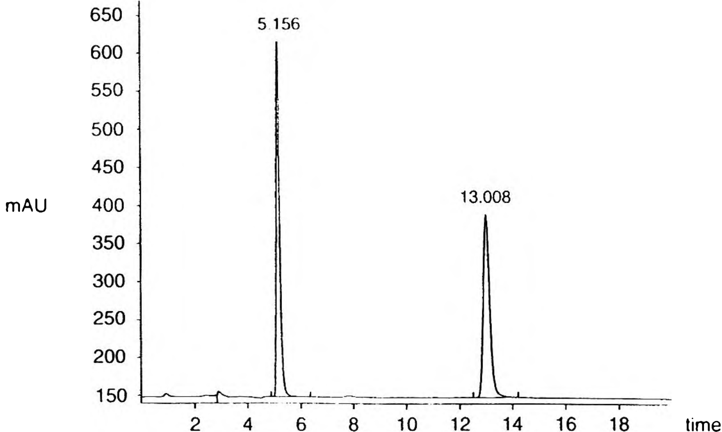

Figure 4. Chromatogram of enantiomeric separation of L-741,597. (Conditions: Hichrom DNBL column (250 x 4.6mm i.d.); mobile phase 5% MeOH in 1-Chlorobutane; Flow 1ml/min; Detect 230nm).

enantiomeric selectivity may therefore be to sterically restrict access to an optimal π-π interaction. The effect of this could therefore be to change the directionality of other substituents on the molecule towards the CSP thereby optimising the energy of interaction for one enantiomer.

It was found that prediction of separation selectivities between the DNBL and DNBPG CSP's was not straightforward. For example, the highest separation selectivity was achieved for 7 on the DNBL phase and this compound also proved to have one of the highest separation selectivities using the DNBPG phase, suggesting that there is some commonality of interactions between the two phases for this compound. However, a major difference between the phases is demonstrated with the C5 pyrrolidine derivative 2. This showed the lowest separation selectivity on the DNBL but one of the highest using DNBPG.

The effect of mobile phase was investigated, as it had been shown that ethanol/hexane mixtures were capable of achieving high separation selectivities, but it was felt that for preparative purposes this would be inadequate due to solubility criteria since these molecules were poorly soluble in such mixtures. Also, since high percentages of ethanol were required for adequate elution times the viscosity of the mobile phase was higher than desired leading to poor mass transport between the phases and consequent

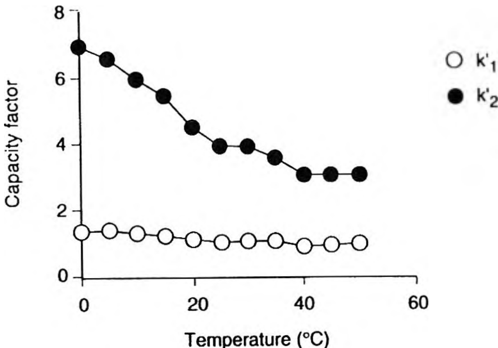

Figure 5. Effect of temperature on capacity factors for L-741,597.

band-broadening particularly of the later eluting enantiomer. Chlorobutane was chosen as the bulk solvent as it displayed increased solubilising properties of the compounds and an increase in polarity over hexane allowing the proportion of organic modifier to be minimised. A typical chromatogram, demonstrating the very high enantioselectivity obtained is shown (Figure 4).

The effect of temperature on capacity factor is demonstrated (Figure 5). The effect of increasing temperature on the capacity factor for the first eluting enantiomer is minimal whereas a steady increase of K'_2 can be demonstrated for decreasing temperature. This is probably due to reducing the degrees of freedom of both the enantiomer and the chiral stationary phase hence the energy of interaction increases leading to an increase in capacity factor. As K'_1 is constant this naturally leads to an increase in α, but the effect of this on R_S is less clear (Figure 6). Resolution appears to reach a maximum at 20-25°C with a rapid decline after this. Resolution would be expected to decrease at higher temperature as K'_2 decreases but not necessarily at lower temperatures. This could be explained by the higher solvent viscocities at lower temperatures, leading again to poor mass-transfer between stationary and mobile phases, resulting in a large increase in the band broadening of both enantiomers even though K'_1 does not change.

Preparative chromatography could be performed for these enantiomers and was indeed the preferred method for resolution of final compounds of this class. This was despite the success of chemical resolution using Edman degradation chemistry of a suitable amino intermediate, as this required further synthetic elaboration to yield the desired compound. Solubility in organic

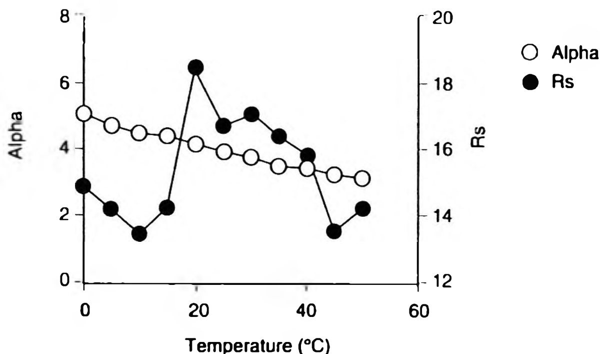

Figure 6. Effect of temperature on separation selectivity and resolution for L-741,597.

solvents was a major issue for these compounds, affecting the choice of mobile phase as described above. It was found that most compounds could be dissolved in a 9/1 chloroform / ethanol mixture as free bases at 10mg/mL. Loadings of 2.5mL, equivalent to 25mg, onto the 20mm i.d. column could then be achieved routinely and the enantiomers separated to baseline. The limiting factor to loading in this case appeared to be volume injected, as larger volumes would cause a large disturbance in peak shape leading to loss of resolution. Where solubility permitted, higher loadings of material in an equivalent volume was achievable with >50mg/run injections being performed. Repeat injections could be made or the process automated if >1g of compound was required. Throughput of this methodology was high with 25mg injections possible every 7 minutes, and peaks were collected in high yield with purities >99% e.e.

As would be expected with the specific stereochemistry of Pirkle phases, the elution order of the enantiomers was entirely predictable with the 3*R* enantiomer always eluting before the 3*S*, making identification of the enantiomer required for CCK$_B$ receptor binding facile.

CONCLUSIONS

In general, the Pirkle DNBL CSP provides the highest selectivity for the 1,4-benzodiazepin-2-ones bearing C5 amidine substituents studied. Use of this CSP allowed facile monitoring of reactions from enantioselective syntheses to determine optical purities and the preparation of material from milligram to gram quantities for biological evaluation.

Although insufficient compounds were available to perform quantitative structural analysis, the compounds that separated well appear to have large bulky groups at C5 that cause this substituent to lie out of the plane of the benzodiazepine portion of the molecule. Additionally it is suggested that the steric effect of the C5 substituents affect the attainment of the dominant π- π interaction and in so doing alter the directionality of interactions of other substituents thereby accentuating the energy differences between the 3*S* and 3*R* enantiomers.

Preparatively, high separation loadings were possible due to the high separation selectivities and good resolutions observed although solubility in suitable organic solvents was a limiting factor. Gram quantities of enantiomers could be prepared using this methodology and this helped to establish chiral HPLC as the method of choice over more classical resolution methods for these molecules.

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IMMOBILIZED IRON(III) METAL AFFINITY CHROMATOGRAPHY FOR THE SEPARATION OF PHOSPHORYLATED MACROMOLECULES: LIGANDS AND APPLICATIONS

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ABSTRACT

This review highlights the advances in Fe(III)-Immobilized Metal Affinity Chromatography [Fe(III)-IMAC] for the separation and characterization of phosphorylated biomolecules. Fe(III)-IMAC has demonstrated a selective interaction with phosphate groups and other hard Lewis bases such as carboxylate and phenol functional groups. These properties make this technique useful for separating and studying macromolecules. Since phosphorylation is a central means of regulation in eukaryotes and prokaryotes, the development of Fe(III)-IMAC is important. Various iron chelating ligands and their properties are discussed. General features of Fe(III)-IMAC are examined and examples of useful separations are considered.

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INTRODUCTION

Immobilized metal affinity chromatography (IMAC) is useful for the separation of biomolecules. The choice of chromatographic support, support activation method, the ligand attached, and immobilized metal ion depends on the properties of the biomolecules to be separated. General IMAC methods and applications have been extensively reviewed.[1-4] Many studies have used divalent metal ions such as Cu(II), Ni(II) and Zn(II) and exploited the affinities for bases such as histidine via metal ion coordination.

Several volumes on general chromatography contain special practical sections describing IMAC protocols.[5-6] Recently, Fe(III)-IMAC has found specific application in the separation of phosphorylated macromolecules and other biological substances.

This review covers the general principles of Fe(III)-IMAC, useful Fe(III) chelating ligands, and the application of Fe(III)-IMAC in adsorption affinity techniques for the separation and study of a variety of phosphorylated biological molecules. The physico-chemical properties of immobilized Fe(III) and its interaction with hard Lewis bases such as phosphate or carboxylate oxygens render this metal ion unique and useful for Immobilized Metal-Affinity Chromatography.

Fe(III)-IMAC: GENERAL PRINCIPLES

First Considerations

Porath and Olin[7] suggested metal-ion "hardness and softness" may be helpful for understanding the mechanisms of Fe(III)-IMAC. At pH's above 7, nickel prefers ligands containing "soft" uncharged sulfur and nitrogen atoms as in cysteine and histidine, while Fe(III) has strong affinity for phosphate, sulfate, carboxylic, and phenolic oxygens. Fe(III) is considered a "hard" Lewis acid, thus it will associate more strongly with "hard" bases such as oxygen.[8] Molecules that have hard bases such as phosphates, carboxylates and phenolic groups have high binding constants. Ligands containing soft bases such as amino and sulfhydryl groups have lower constants. Softer Lewis acids such as divalent metal-ions prefer softer atoms such as nitrogen and sulfur. Although sulfur and nitrogen can be electron donors to Fe(III), these generally form weaker complexes.

O
Support Matrix
O^-
N
3+
OH_2
Fe
O^-
OH^-
OH_2

Figure 1. Octahedral Fe(III)-IDA-water complex.

The strength of the coordinate bond between a Fe(III) ion and an immobilized ligand is controlled mainly by three factors:

1. Pure *electrostatic* interaction controlled by the charge and the ionic radius.
2. The overall affinity of the central metal ion to accept electrons from the l ligand.[9]
3. Metal ion electronic configuration which is capable of stabilization due to distortion of octahedral symmetry (Jahn-Teller effect).[9-10]

Metal Coordination or Ion Exchange?

The flexibility of Fe(III)-IMAC stems from the fact that either ion-exchange or ligand coordination properties can be exploited to "tune" specificity. Factors to consider when designing an Fe(III)-IMAC separation are: the net charge of the metal-ligand complex, the ionic strength of the buffer, the presence of surface accessible protein phosphate groups and the isoelectric point of the protein.

Consider the use of immobilized iminodiacetic acid (IDA) as a chelating ligand. Without coordinated metal ion, negatively charged IDA-bonded stationary phases behave as relatively strong cation exchangers.[11] Sulkowski[12] and others[13] showed that a protein mixture could be separated by chromatography over naked bound IDA and that retention increased in order of increasing isoelectric point. When IDA is chelated to Fe(III), the metal ligand complex has a net positive charge (Fig. 1).

Figure 2 Interaction of Fe(III) and phosphate to form a four-membered ring. (Adapted from reference 19).

This situation is different from that of divalent metal ion-IDA complexes such as Cu(II)-IDA which have no formal charge. For Fe(III) complexes, electrostatic interactions are significant. In this situation, as ionic strength of the buffer is increased, retention of proteins at a pH above their pI (negatively charged) would be expected to decrease due to to shielding of the ligand-metal complex. Proteins of pI 4 to 11 bind to Fe(III)-IDA at pH 6 and low ionic strength.[12]

It has been shown that ion-exchange is the primary binding mechanism for the purification of lactate dehydrogenase by Fe(III)-IMAC.[14,15] It is not displaced from the Fe(III) column by phosphate unless the ionic strength is increased. Ligand-Fe(III) complexes which have no net formal charge bind serum proteins more efficiently than Fe(III)-IDA at low pH, suggesting that nonionic interactions may also play a role in protein-metal complex binding.[7] It may also be useful to consider that steric factors control adsorption or that charge controlled mechanisms occur locally reducing the importance of the net protein charge.

Iron-Phosphate Interactions

The adsorption of phosphate groups to immobilized Fe(III) is not simply the result of electrostatic interactions as negatively charged nucleosides, cyclic mononucleotides and dinucleotides do not bind to IDA-Fe(III).[16] and strength of binding of nucleotides is not affected by the number of phosphates.[17] The high affinity which phosphate has toward Fe(III) is partially explained by the fact that it probably forms two coordinate bonds with the metal ion, whereas, carboxyl groups will form only a single bond (Fig. 2).

The interaction of ferric ion with phosphate has been examined in PEG/dextran two-phase partitioning systems.[18] Affinity for phosphate decreases as alkaline pH is approached. The steep increase in phosphate affinity between pH 3 and 5 is attributed to the ionization of the second acidic oxygen of phosphate. The affinity decreases above pH 5 due to the competitive binding of hydroxyl ions to Fe(III).

Adsorption and Elution

In IMAC, the adsorption of the sample to the resin involves new interactions of the sample with the metal while displacing one or more weakly bound ligands such as water.[3-4,6] When the metal ion binds multiple donor atoms, there is a favorable entropic contribution to the binding energy resulting from displacement of multiple weakly bound ligands (chelate effect).[1,19]

Several methods have been most commonly used for dissociation of phosphorylated molecules from Fe(III)-IMAC columns. They include: pH gradient;[8,20,21] competitive elution with a Fe(III)-binding molecule[22] and elution with Magnesium ion[19] or phosphate ion.[14,23-25]

Mg(II) binds to phosphate groups of the sample, causing desorption. Gradients of NaCl have also been used in Fe(III)-IMAC.[12]

Ovalbumin which contains up to two phosphate groups per molecule, was shown to elute from a Fe(III)-IDA column with a linear gradient of NaCl.[13] This suggested an ionic interaction. However, these experiments were carried out in 25 mM phosphate buffer, explaining the lack of ovalbumin retention. For Fe(III)-IMAC, up to 1 M sodium chloride is often included in the buffer to minimize ion-exchange effects.

NaCl concentrations higher than 1 M do not significantly affect Fe(III)-phosphate interactions.[19] Elution from Fe(III)-IMAC may also be completed by a decreasing salt gradient, because at high salt concentration some proteins may be bound to the stationary phase via hydrophobic interactions.[13]

Samples are easily eluted from iron(III) columns using increasing pH,[13,15,26] or addition of phosphate to the elution buffer to compete off the phosphorylated sample.[27-28] Phosphoserine is also useful as elution agents.[22] Phosphoserine binds to the immobilized Fe(III) displacing the bound sample.

Table 1

Commercially Available Metal-Chelating Supports

Product	Supplier	Support	Ligand	Ref.
Chelating Sepharose Fast Flow	Pharmacia	Cross-linked agarose	IDA	14,19,26
Chelating Sepharose 6B	Sigma	Sepharose 6B	IDA	23
Chelating Amberlite	Sigma	Polystyrene beads	IDA	23
SigmaChrome	Sigma	Poly(hydroxy)-methacrylate	IDA	66
Chelating Superose (a)	Pharmacia	Superose(cross-) linked agarose	IDA	19
Hi-Trap Chelating (a)	Pharmacia	Sepharose, High performance	IDA	19
NTA-Resin	Qiagen	Sepharose-CL-6	NTA	39

(a) Prepacked LC and HPLC columns available.

IRON CHELATING LIGANDS

Almost without exception, commercially available IMAC supports utilize iminodiacetic acid (IDA) as the metal-chelating group. Several available metal chelating supports are listed in Table 1.

Chelating Sepharose 6B® was the first commercially available support to be used for the specific isolation of phosphoproteins by Fe(III)-IMAC.[12,20,29,30] Pre-packed metal affinity columns[31] for fast protein liquid chromatography (FPLC) are also available. These have been used for Fe(III) affinity isolation of phosphopeptides[27] and for model studies of the interactions of Fe(III) ions with phosphorylated amino acids and phosphoproteins.[19]

Table 2

Iron (III) Binding Ligands

Ligand	Coordination	Stability (log K)	Ref.
IDA	3	10.7	7,12,19,37
8-HQ	2	13	37,43,55
Glycylhydroxamate	3	11	37,45,47-48
OPS	3	13	37,50,54
Catechol	2	20	37,67
TED	5	20	7,37
NTA	4	15	37
EDTA	6	25	37
HPO_4^{-2}	2	8.3	37

Numerous volumes are available describing the chemistries and detailed protocols to attach Fe(III)-binding ligands to a natural, synthetic or inorganic chromatographic support.[6,32-34] Fe(III) chelators have been reviewed,[35-36] and only the most common will be discussed. Table 2 shows that Fe(III) prefers binding oxygen and that higher saturation of the six coordination sites forms the most stable complexes. Useful molecules known to chelate iron, the coordination number and their binding constants are tabulated.

Iminodiacetic Acid and its Derivatives

Iminodiacetic acid (IDA) is a "tridentate" iron chelator, with metal coordination through the nitrogen and the two carboxylate oxygen unpaired electrons. Importantly, the metal-ligand complex has 3 coordination sites occupied by weakly bound water which can be displaced by electron rich sites on the molecules to be separated. The iron-IDA complex has a binding constant of log K =10.7 in solution.[37] The iron complex with IDA and water is shown in Fig. 1.

Tris(carboxymethyl)ethylendiamine (TED)

Similar to IDA, TED forms a very stable five-membered ring system with Fe(III) (Fig. 3a). The stoichiometry of the metal-chelate complex is not completely defined.[38]

(a) Tris(carboxymethyl) ethylenediamine

(b) Nitrilotriacetic Acid

(c) 8-Hydroxyquinoline

(d) O -Phosphoserine

Figure 3 (a) Penta-dentate tris(carboxymethyl)ethylenediamine (TED) complex with Fe(III);(b) Fe(III) complexed to nitrilotriacetic acid (NTA)derivatized support; (c) Immobilized 8-hydroxyquinoline complexed with Fe(III); (d) Fe(III) complexed to o-phosphoserine (OPS) derivatized support.

Nitrilotriacetic Acid (NTA)

NTA contains three carboxymethyl groups attached to a tertiary amine. Four coordination sites of the Fe(III) ion are occupied by the carboxylate oxygens and an amino group. The remaining two coordination positions are occupied by water or hydroxyl ion, depending upon pH. The water and hydroxyl sites are labile and can be displaced by ligands and thus are useful for protein interactions (Fig. 3b).

Ni(II)-NTA agarose has become available from QIAGEN Inc. (39). Preliminary experiments have shown that NTA-Fe(III) is useful for separation of phosphorylated ovalbumin (Unpublished data, Fig. 4).

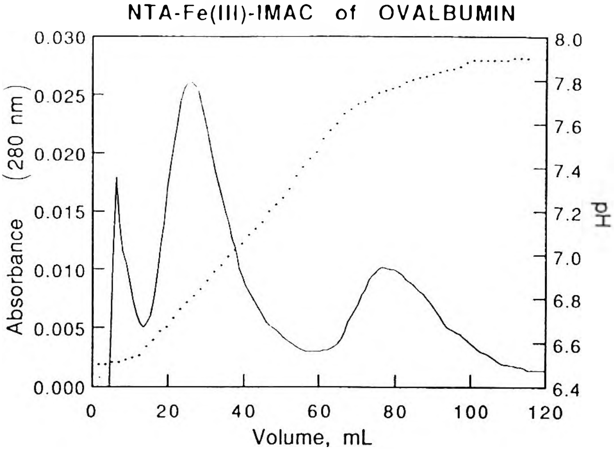

Figure 4. NTA-Fe(III) IMAC of ovalbumin (unpublished result, K.R. Neville and L.D. Holmes).

8-Hydroxyquinoline

8-Hydroxyquinoline (8-HQ) immobilized to silica has long been used for chelation chromatography of transition metal cations.[40-44] The complex of iron and 8-HQ is shown in Fig. 3c.

Hydroxamic Acids

The synthesis and general properties of hydroxamic acids have been extensively reviewed.[36,45-49] They exist as keto-enol tautomeric forms. The keto form is favored at acid pH, and spectroscopic studies indicate that metal complexes are formed with this tautomeric form[48] (Fig. 5). The hydroxamate group is a bidentate chelator that forms a stable 5-membered ring with Fe(III) by loss in free energy during ring formation.

O-Phosphoserine

O-Phosphoserine (OPS) chelation to Fe(III) has been studied by potentiometric titration,[50] and is a useful ligand for Fe(III)-IMAC with an apparent stability constant greater than 10^{13}. The probable structures for the Fe(III)-complex was found to be either ML at low pH and ML_2 at high pH.

R—C(=O)—N(H)—OH ⇌ (Basic pH / Acid pH) R—C(OH)=N(H)—OH

Figure 5. Keto-enol tautomers of a hydroxamic acid.

The chemistries of metal-phosphoserine interactions relating to the role of phosphate esters (e.g. phospholipids) in biochemical processes have been reported.[51-53] The use of OPS-Fe(III) IMAC was recently reported by Zachariou.[54] OPS metal binding is pH-dependent and involves the oxygens in the carboxyl and phosphate groups and the amino nitrogen (Fig. 3d).

EXAMPLES OF Fe(III)-IMAC APPLICATIONS

The usefulness of Fe(III)-IMAC for separation and study of several classes of biomolecules is summarized below.

Amino Acids

Amino acids are zwitter ions and will ionize according to pH. It is interesting to note the contribution nonphosphorylated amino acid residues may make toward the total protein retention in an Fe(III)-IMAC experiment. Cysteine is retained markedly longer on Fe(III)-hydroxymate than any other amino acid.[46] This behavior may be explained by the Lewis base behavior of the sulfhydral group which is chemically similar to hydroxyl and would thus be expected to have appreciable Fe(III) affinity. Cysteine was reported to "destroy" the support in Fe(III)-IDA IMAC due to irreversible binding at low pH.[29] One would anticipate aspartic and glutamic acid residues to show significant Fe(III) interaction through coulombic interactions. Hydrophobic or aromatic amino acids exhibit no special affinity for Fe(III), but phosphoamino acids are strongly retained.[29,46]

Cu(II)-IDA retains amino acids significantly at pH 5 to 6.[13] This is especially interesting for the case of histidine, which strongly interacts with Cu(II).[1,4] Copolymers of glutamate and tyrosine have an affinity for Fe(III)-IDA through carboxylate and phenolic functional groups, although this interaction is weak when compared to phosphate.[16,19] Binding is probably a combination of metal coordination and ion-exchange interactions.

The binding of phosphoamino acids to Fe(III)-IDA Sepharose is very strong compared to that of all amino acids including cysteine, L-aspartic and L-glutamic acids (sulfhydryl and carboxylic group-containing residues). In the absence or presence of high salt, phosphorylated serine, tyrosine and threonine are strongly retained on the IDA-Fe(III) gel at acid pH but not retained at neutral or weakly basic pH.[29] Binding studies using Fe(III)-hydroxamate IMAC demonstrated affinity for surface accessible cysteine, aspartyl and glutamyl residues of proteins.[45-47] Furthermore, binding of proteins to a Fe(III)-8-HQ IMAC resin was demonstrated to be independent of surface accessible histidine, tryptophan or cysteine residues.[55]

Phosphopeptide Studies

The chromatographic behavior of peptides and phosphorylated peptides on Fe(III)-Separose has been analyzed. Basic peptides pass freely through an IMAC column, and acidic peptides are retarded and elute as a broad peak in the pH range 5.5-6.2. Phosphopeptides eluted in the pH range 6.9-7.5.[19]

Certain biological functions of phosphorylation, protein turnover and sequence specificity of protein kinases have been studied by Fe(III)-IMAC of phosphoprotein fragments. Tryptic digests of Photosystem II purified from spinach ^{32}P-labelled chloroplasts were shown to contain phosphothreonine by their retention on Fe(III) chelating Sepharose columns.[20]

Phosphopeptide-mediated intestinal absorption of calcium has been examined Fe(III)-IMAC. Several low abundance caseinophosphopeptides of the pig small intestine have been separated using iron IMAC.[30] Phosphorylated casein proteins are suspected to function as iron transporters and/or storage proteins by forming soluble complexes.[56-59] The physiology of gastric emptying has been studied using Fe(III)-IMAC. Scanff et al.[27] separated low abundance phosphopeptides from a tryptic digest of casein and gastric effluents using an FPLC Fe(III)-Superose HR 10/2 column. Elution was carried out with phosphate buffer.

Secondary, electrostatic interactions were significant, as other non-phosphorylated, strongly basic peptides were also adsorbed to the affinity column. Phosphorylated peptides have been adsorbed with high specificity to Fe(III) immobilized to Whatman paper designed as a counter-part to derivatized Sepharose.[17]

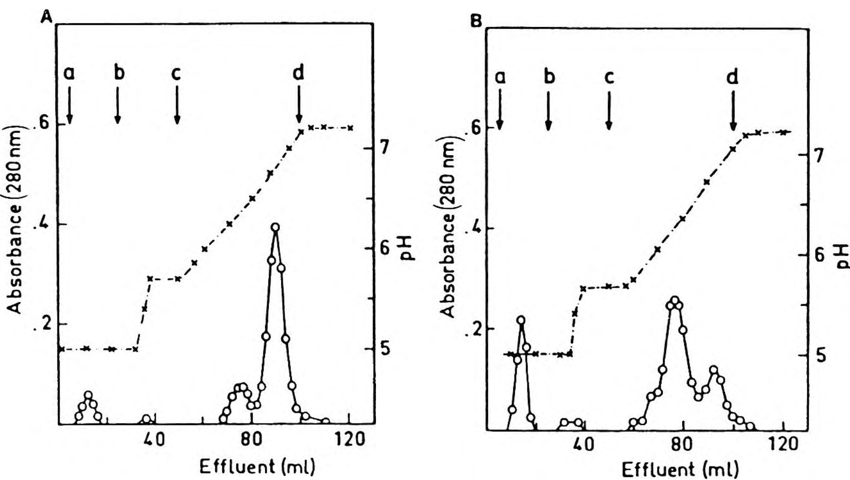

Figure 6. Fe(III)-IMAC of ovalbumin by elution with a pH gradient. (A) Native ovalbumin; (B) partially dephosphorylated ovalbumin. (Used with permission from Ref. 29.)

Phosphoprotein

The first indication that specific phosphate-Fe(III) interactions may be useful for protein separations grew from the work by Porath and Olin comparing the affinities of serum proteins on Fe(III) and Ni(II) columns. The next step was to analyze the Fe(III) binding charateristics of native and dephosphorylated ovalbumin.[29]

At low pH (3.1 and 5.0) the components of native ovalbumin were fractionated according to the number of phosphorylated amino acid residues. Elution of retained proteins was accomplished by pH gradient (Fig. 6).

Similar separation characteristics were found in experiments designed to reduce nonspecific ionic interactions by the use of 1 M NaCl. Enzymatically phosphorylated histone protein showed strong affinity to Fe(III)-IDA.[22] 1 M sodium chloride was used to reduce nonspecific electrostatic interactions. Elution was with a pH gradient. phosphoserine or Mg(II). a phosphate binding ion[59-62] (Fig. 7).

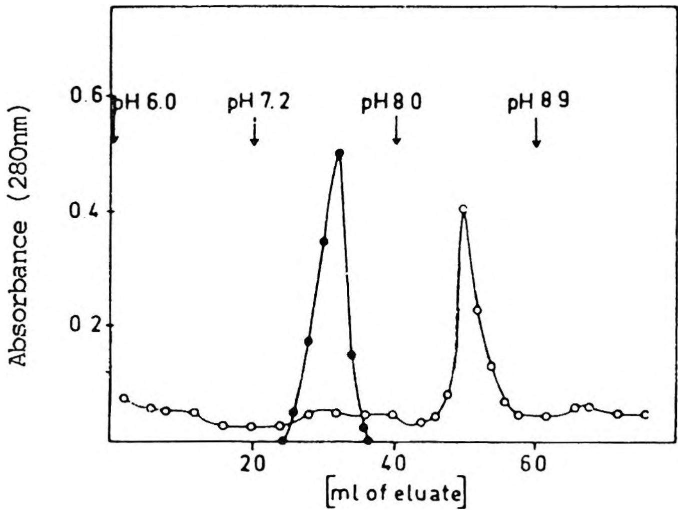

Figure 7. Difference in the pH-dependent elution profiles between phosphorylated (o) and nonphosphorylated histone protein (●) from Fe(III)-IDA gel. (Used with permission from Ref. 22).

Sulkowski[12] studied the affinity of ovalbumin to IDA-Fe(III) and concluded that phosphoproteins display a particularly high affinity for IDA-Fe(III). Secondary interactions involving "nonionic" interactions as well as coulombic interactions were also detected. Ovalbumin has also been purified using metal-affinity partitioning in aqueous two-phase systems.[18] Interestingly, neither TED-Fe(III) or hydroxamate-Fe(III) display any affinity for ovalbumin.[29,46]

Singly phosphorylated pepsin (pI=2.9) was also retained and subsequently eluted with 20 mM phosphate. Furthermore, Kucerova[59] separated human gastric pepsins and pepsinogens by this technique, suggesting that the degree of phosphorylation may be correlated with gastric disease. Michel and Bennett[20] in their studies of the photosynthetic electron transport system, found that phosphoprotein from Photosystem II of spinach was retained on IDA-Fe(III) gel at pH 5.0 and eluted upon increasing the pH to 8.0. Phosphorylation is implicated in photosystem control.[62] Fe(III) immobilized to an agarose-carboxymethyl-picolyamine matrix has been used to isolate insect phospholipoproteins.[28]

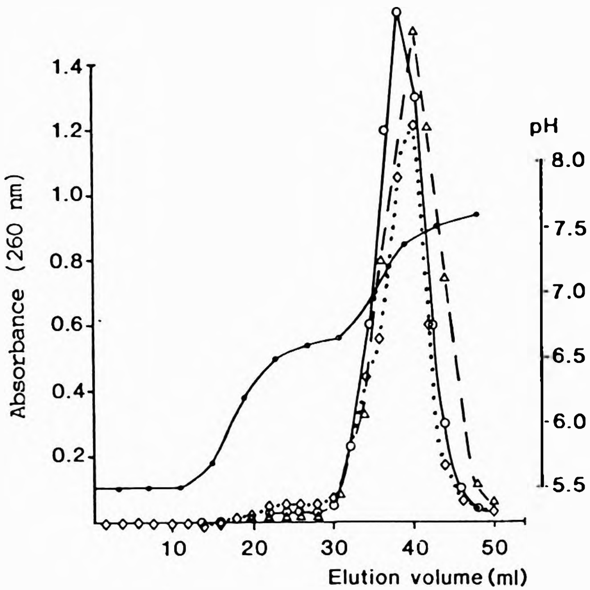

Figure 8. Fe(III)-IMAC elution profiles of adenine nucleotides. AMP (o--o); ADP (Δ--Δ); ATP (◊--◊); pH (●--●). (Used with permission from Ref. 16.)

Specific Fe(III)-phosphate interactions are indicated by protein elution with phosphate buffer but not by high salt. Recently, Fe(III)-IMAC was employed for the separation of microtuble-associated proteins (MAPs).[26]

Nucleotides and Nucleic Acids

Nucleosides, cyclic nucleotides, and dinucleotides which contain only internal or no phosphate groups do not bind to an IDA-Fe(III) column at pH 5.5 in the presence of high sodium chloride.[7,12,16] AMP, ADP and ATP co-elute from an Fe(III)-IMAC gel indicating that adsorption of nucleotides is not based entirely on simple electrostatic interactions (Fig. 8).

Binding of nucleotide was found to be through the terminal phosphate when AMP, ADP and ATP were completely retained by IDA-Fe(III) derivatized chromatography paper.[17] Fe(III)- nucleotide phosphate affinity has been used to probe enzyme active site structural features of several nucleotide binding proteins.[24-25,63] Competition binding studies with photoaffinity-labeled azido analoges of NAD$^+$, ATP, and ADP located peptide sequences at the

adenine binding domains. Also the guanine binding site for glutamate dehydrogenase was identified using this method.[23] Iron(III) IMAC provides a one-step approach for separation of photoaffinity-labeled peptides from non-labeled peptides.

CONCLUSIONS

Fe(III)-IMAC has become a useful technique for the purification and characterization of phosphorylated biomolecules. Phosphorylation modulates enzyme activities in the cell nucleus, mitochondrian, ribosomes and cell membranes. Protein phosphorylation also mediates secretion, molecular transport and membrane permeability.[64-65] The abundance and diversity of phosphorylated molecules and the nature of protein-bound phosphate will drive the development of immobilized iron separation techniques.

Although experiments have reported significant success in achieving phosphate-specific separations, a detailed analysis of the mechanism of adsorption of phosphorylated biomolecules remains to be completed. A wide range of parameters affect chromatographic behavior. Factors such as chelating ligand, support matrix, buffer conditions and temperature require further study in order to optimize analyses. Undoubtedly, a portion of the adsorption behavior is due to general ionic or electrostatic interaction of the metal ion with phosphate or other negatively charged groups. Hydrophobic interactions also must not be ignored. However, Fe(III)-IMAC systems do not interact with phosphoproteins in the same way as ordinary ion exchange resins. Fe(III)-IMAC can be employed to resolve proteins with a wide range of isoelectric points, a task not generally possible in ion exchange chromatography. Fe(III)-IMAC is also effective in probing the accessible surfaces of proteins for the presence of certain amino acids such as aspartate, glutamate and cysteine. This is a useful addition to Cu(II)-IMAC which binds surface histidines.

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NON-IDEAL SIZE EXCLUSION CHROMATOGRAPHY OF EUKARYOTIC PROTEIN SYNTHESIS INITIATION FACTOR 2

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ABSTRACT

Eukaryotic protein synthesis initiation factor 2 (eIF-2), a heterotrimer, was chromatographed on high performance (Superose 6) and standard (Ultrogel AcA 34 and Sephacryl S-300) size exclusion chromatography (SEC) media. The retention volume of eIF-2 on the Superose 6 column was higher than expected from its M_r and elution of a βγ eIF-2 dimer was seen. Conversely, on AcA 34 and Sephacryl S-300 most of the eIF-2 was eluted at its expected position, considering its ellipsoidal structure, as the heterotrimer. Elution of the βγ heterodimeric eIF-2 may therefore be promoted by Superose 6 high performance SEC which therefore provides a means for its rapid preparation for studies in protein synthesis initiation.

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INTRODUCTION

Size-exclusion chromatography (SEC), also known as gel filtration and gel permeation chromatography, is an established technique for the separation and characterization of biopolymers. In general, mild conditions for the mobile liquid phase are employed so as to preserve the structural and functional integrity of the molecules. In ideal, or "pure", SEC, solute molecules are separated solely on the basis of size by equilibrium partitioning via diffusion between the mobile phase and gel pores.[1] The non-ideal behaviour of biopolymers and other substances in size-exclusion chromatography (non-ideal SEC or nSEC) has been characterized for many situations[2-7] and has been effectively utilized to improve the separation of biomolecules.[8] It therefore has considerable potential for exploitation in the chromatographic purification and analysis of biopolymers.

In this study the behaviour of eukaryotic protein synthesis initiation factor eIF-2, a heterotrimer ($\alpha\beta\gamma$) of M_r 122,000, on different size-exclusion chromatographic media was examined. It is shown that the chromatographic behaviour on the high performance medium Superose 6 differs markedly from that on standard media (Ultrogel AcA 34 and Sephacryl S-300), the latter, but not the former behaviour, being in accord with that expected for eIF-2 on SEC. On Superose 6 the interactions between solute and gel matrix responsible for this may have facilitated, together with the high separation efficiencies obtainable with this gel, the elution of a $\beta\gamma$ dimer form of eIF-2. Superose 6 high performance SEC thus affords a means for the rapid and efficient preparation of eIF-2 deficient in the α subunit, i.e. the $\beta\gamma$ dimer.

MATERIALS AND METHODS

The pre-packed Superose 6 column (1.0 x 30 cm) for high performance SEC and Sephacryl S-300 (pre-swollen) were from Pharmacia Fine Chemicals AB, Uppsala, Sweden. The AcA 34 Ultrogel (pre-swollen) was obtained from LKB (Bromma, Sweden). Protein molecular mass markers were from Sigma Chemical Company. Lactate dehydrogenase (pig heart), pyruvate kinase (rabbit muscle), glucose oxidase (*Aspergillus niger*) and hexokinase (yeast) were all acquired from Boehringer Mannheim. The initiation factor, eIF-2, was obtained from rabbit reticulocyte lysate by purification through the phosphocellulose step of a conventional purification procedure,[9] but with the substitution of DEAE-Sephacel for the DEAE-cellulose step since this improved the resolution of eIF-2.

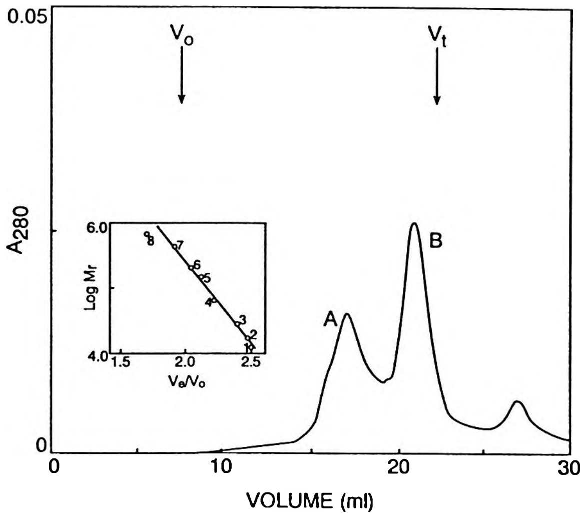

Figure 1. Superose 6 chromatography of eIF-2. The eIF-2 (100 µg in a loaded volume of 0.2 mL), purified through the phosphocellulose step of the conventional purification (9), was applied to the Superose 6 column. The column was equilibrated and developed, at a flow rate of 0.3 mL/min, with the following buffer: 0.1 M KCl; 10 mM potassium phosphate, pH 6.8; 7 mM 2-mercaptoethanol; 10% glycerol. The two major peaks (A and B) eluted from the column were analyzed by SDS-PAGE (Fig. 3). The chromatography was performed another three times and a virtually identical elution pattern was obtained on each occasion. The apparent M_r of peak A was 60,000 and that of peak B 5,000-13,000. These values were obtained by reference to the M_r calibration curve (inset) obtained from chromatography of the following protein M_r markers (M_r values in brackets) on the Superose 6 column: (1) cytochrome c (12,600), 0.3 mg; (2) myoglobin (17,500), 0.3 mg; (3) carbonic anhydrase (29,000), 0.4 mg; (4) bovine serum albumin (68,000), 0.8 mg; (5) alcohol dehydrogenase (150,000), 0.4 mg; (6) β-amylase (200,000), 0.3 mg; (7) apoferritin (443,000), 0.4 mg; (8) thyroglobulin (669,000), 0.6 mg. A very similar plot was obtained when the calibration was repeated.

For standard SEC, AcA 34 (1.6 x 28 cm) and Sephacryl S-300 (1 x 13 cm) columns were used. To maintain a smooth, even and reproducible flow of eluent through the columns the LKB 2150 HPLC pump was employed. The effluent was also monitored continuously with the LKB 2158 Uvicord detector set to measure absorbance at 280 nm.

For all the columns equilibration and development was with the following buffer: 0.1 M KCl, 10 mM potassium phosphate, pH 6.8, 7 mM 2-mercaptoethanol, 10% glycerol. The flow rate was 0.30 mL/min for the

Superose 6 column. For the AcA column the flow rate was 0.17 mL/min and for the Sephacryl S-300 column it was 0.10 mL/min. The chromatography was conducted at 4 °C. The void volumes, V_o, of the columns were determined using blue dextran. The total permeation volumes, V_t, were determined with acetone.

For analysis of column effluent, sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed according to a modified Laemmli method.[10]

RESULTS

High Performance SEC of eIF-2

On the application of eIF-2 to the Superose 6 column, two A_{280} peaks were eluted (Fig. 1). The apparent M_r of each of the peaks was determined from the M_r calibration curve for Superose 6 as indicated. The apparent M_r of 5,000-13,000 (elution close to cytochrome c, but before V_t) for the larger peak and that of 60,000 for the other prominent peak were less than those expected for eIF-2. The recovery in terms of A_{280} units was 60%. Since the actual M_r of eIF-2 is 122,000[11,12] and it elutes from gel filtration columns with a higher apparent M_r than this owing to its ellipsoidal structure,[12-14] the peaks of low apparent M_r obtained for it on Superose 6 chromatography suggest retardation on the column and/or subunit dissociation.

High Performance SEC of other Proteins

Several different oligomeric proteins were chromatographed on the Superose 6 column to determine if any of them underwent clearly detectable subunit dissociation. Individually applied to the column were hexokinase, lactate dehydrogenase, glucose oxidase and pyruvate kinase. On elution a single symmetrical peak was obtained for each protein.

Hexokinase and glucose oxidase eluted in accord with their reported M_r values (Table 1), but the apparent M_r of 85,000 of lactate dehydrogenase was less than its reported M_r of 109,000. For pyruvate kinase the apparent M_r of 114,000 was about half its reported M_r[15] of 237,000. It should be noted that these proteins were loaded at 5 times the concentration of eIF-2.

Table 1

Apparent M_r of Oligomeric Proteins on Superose 6 SEC*

Protein	M_r ($x10^{-3}$)	Apparent M_r on Superose 6 ($x10^{-3}$)
Hexokinase	104	101
Lactate dehydrogenase	109	85
Glucose oxidase	186	166
Pyruvate kinase	237	114

* The proteins (0.5 mg of each in a loaded volume of 0.2 mL) were chromatographed on Superose 6 and their apparent M_r values determined by reference to the M_r calibration curve for Superose 6 (inset to Fig. 1).

Standard SEC of eIF-2

On AcA 34, eIF-2 eluted as two A_{280} peaks. The larger (peak A) was eluted with an apparent M_r of 200,000 and the smaller (peak B) of 68,000 (Fig. 2). The ellipsoidal structure[12-14] of the eIF-2 explains its elution at an apparent M_r value greater than the true M_r of 122,000. SDS-PAGE analysis of the eluted fractions confirmed the elution of the eIF-2 heterotrimer in peak A (not shown). The tailing of the first peak (A) was due, from the SDS-PAGE analysis, to some proteolysis of the β subunit which is a well-documented phenomenon.[16] The occurrence of the second peak (B) was due to a 67 kDa polypeptide present in the eIF-2 preparation (see below). Similar results were obtained on Sephacryl S-300. Care was taken in comparing the behaviour of eIF-2 on the three different SEC media used to ensure that the results on Superose 6 were not due to a lower ratio of amount loaded to column volume compared with the ratios for the AcA 34 and Sephacryl S-300 columns.

SDS-PAGE Analysis of eIF-2 Fractions Eluted on Superose 6 SEC

The two peaks, A and B of the experiment of Fig. 1, were each collected and concentrated by Millipore ultrafiltration. After lyophilization, the peaks were subjected to SDS-PAGE (Fig. 3). Electrophoresis was stopped before the bromophenol blue had run off the gel in order to search for the presence of any low M_r peptides. On staining the gel with Coomassie Blue only very faint bands could be detected for each of the peaks so the portion of the gel on which

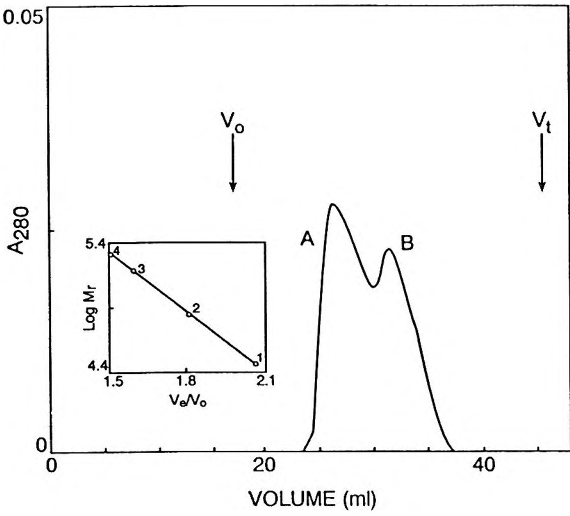

Figure 2. AcA 34 chromatography of eIF-2. The eIF-2 (100 µg in a loaded volume of 0.5 mL), purified through the phosphocellulose step, was chromatographed on AcA 34 (1.6 x 28 cm) at a flow rate of 0.17 mL/min. The column buffer was as for Superose 6 (legend to Fig. 1). The chromatography was repeated twice and similar results were obtained in each case. The apparent M_r of peak A was 200,000 and that of peak B 68,000 by reference to the M_r calibration curve for AcA 34 (inset) constructed using the following protein M_r markers (M_r values in brackets): (1) carbonic anhydrase (29,000), 3 mg; (2) bovine serum albumin (68,000), 6 mg; (3) alcohol dehydrogenase (150,000), 4 mg; (4)β-amylase (200,000), 4 mg. A similar plot was obtained when the calibration was repeated. On chromatography of the eIF-2, 2 mL fractions were collected, concentrated, lyophilized and analyzed by SDS-PAGE (not shown). Peak A consisted of the eIF-2 heterotrimer and peak B a 67 kDa polypeptide. This analysis was also undertaken on the fractions eluted for each of the two repeat chromatographic runs and the results were very similar.

the peaks were run was separated from the portion containing eIF-2 as a marker and the former silver stained. Comparing the bands obtained for each of the peaks with the subunit bands of the marker eIF-2 (Fig. 3), for peak A the β and γ subunits of eIF-2 were clearly evident, but not the α subunit. That loss was of the α subunit was confirmed by measurement of the migration distances of the polypeptide bands relative to that of bromophenol blue, i.e. the R_f values (legend to Fig. 3).

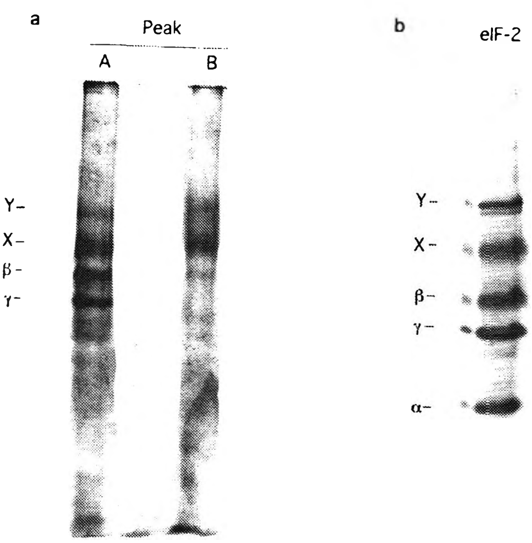

Figure 3. Analysis of peaks eluted on Superose 6 chromatography of eIF-2. The peaks A and B eluted on Superose 6 chromatography of eIF-2 (Fig. 1) were concentrated, lyophilized and then subjected to SDS-PAGE. Thirty μL SDS-PAGE sample buffer was added to each lyophilized peak. The portion of the gel containing the peaks was silver stained (a), and the portion containing marker eIF-2 stained with Coomassie Blue (b). Because of the different staining conditions, the latter was more swollen than the former. R_f values for the bands were as follows: (a) Y=0.30, X=0.37, β=0.44, γ=0.50. (b) Y=0.23, X=0.31, β=0.40, γ=0.45, α=0.58. Similar results were obtained on analysis of the peaks eluted on a repeat Superose 6 SEC of eIF-2.

Two contaminating peptides, X and Y, of apparently higher M_r on the gel than eIF-2β were in the eIF-2 preparation. X could have been the 67 kDa noted previously[17,18] with a presumed function in protein synthesis initiation.[19] Very little could be detected in peak B (Fig. 3) except for the two relatively high M_r contaminating polypeptides, X and Y. Their positions on the gel implied that each had an M_r >60,000, whereas the apparent M_r of peak B from its elution position was 5,000-13,000. Retardation on the column must therefore have occurred. Retardation of eIF-2 was implied also by the apparent M_r of peak A being 60,000 (Fig. 1) whereas if the β and γ subunits eluted in this peak (Fig. 3) were still bonded together a higher apparent M_r (>90,000) would have been obtained. Retardation may have also been implicated in the loss of α subunit from the peak A eIF-2 (Fig. 3).

Peptides, as degradation products, eluted from the Superose 6 column would be expected to be largely lost during concentration by Millipore ultrafiltration since the nominal M_r of the filter unit was 30,000. Free α subunit may have also been lost on concentration because of its M_r, 32,000, being close to the nominal M_r of the filter unit.

DISCUSSION

The deviation of nSEC from "pure" SEC is generally due to one or more of the following effects: (i) electrostatic interactions between the solute molecule and charged groups of the gel matrix; (ii) hydrophobic interactions between solute and stationary phase; (iii) hydrogen bonding between solute and resin; and (iv) steric effects which are dependent on the shape of the solute molecules, i.e. whether spherical, ellipsoidal or rod-shaped, for example.

Assessment of the different chromatographic behaviours of eIF-2 requires consideration of the compositions of the gel matrices. Superose 6 is agarose-based with a 6% agarose composition.[20] AcA 34 is a mixed polyacrylamide and agarose gel. Sephacryl S-300 is a cross-linked copolymer of allyldextran and N, N'-methylenebisacrylamide.

For explanation of the behaviour of eIF-2 on Superose 6, this gel is known to contain small amounts of negatively charged carboxyl and sulphate groups.[20] Superose media in most situations would therefore behave as weak cation-exchange resins.[6] Hydrophobic[5] and aromatic[4] interactions also cannot be ruled out. While the gel hydrophobicity parameter value, which is able to define the relative hydrophobicity of a gel,[21] of 0.084 for Superose 6 may seem relatively low, it is much higher than those of 0.015 and <0.01 for the polyacrylamide-based Biogel A-50M and Sephadex G-100 dextran, repectively.[21] Coulombic and hydrophobic attractions between solute molecules and Superose media are difficult to suppress.[5,22] Retarding aromatic interactions have been claimed to induce the unexpectedly late elution of different hybridoma antibodies when chromatographed on a Superose column.[4] Aromatic amino acids and hydrophobic peptides have been found to undergo retarding interactions with the matrix of a Superose 12 column during SEC of milk protein hydrolysates.[7] The potential for agarose gels to bind biopolymers has been demonstrated by the adsorption of ribosomal RNA,[23] specifically 28 S RNA,[24,25] to Sepharose 4B. Rat liver 60 S ribosomal subunits adsorb to the agarose gels, Sepharose 4B and Biogel A-15M, which could be as a result of interaction between the gel and the 28 S RNA of the subunits.[26] That the binding, in each case, was promoted by conditions of high salt and lower temperatures suggests that the interaction in each instance is hydrophobic.

Notwithstanding the influence of nSEC effects for specific biopolymers, the maximum selectivity and high efficiency of Superose is reported to make it very effective in the resolution of solutes differing in molecular mass by as little as 20%.[20,27] HPLC gel filtration has also been used to study the concentration-dependent association-dissociation of bovine and rat liver glutamate dehydrogenase.[28] It seems likely that in trimeric eIF-2 there is a dynamic equilibrium between the three subunits such that loss of a temporarily dissociated α subunit into a gel pore will lead to further α subunit dissociation to restore equilibrium of the reaction $\alpha\beta\gamma \Leftrightarrow \alpha + \beta\gamma$ in the interstitial volume. Repetition of sequestration of the α subunit by the gel will lead to progressive formation of the βγ dimer. The much lower surface contact between gel and solution in the AcA and Sephacryl gels results in a much less effective sequestration of the α subunit. Superose 6 nSEC interactions may have enhanced sequestration of the α subunit with this matrix.

Elution of the βγ dimer on Superose 6 chromatography of eIF-2 suggests that in addition to enabling rapid isolation of this dimer, the technique may be a very useful tool for study of the association-dissociation equilibrium between α subunit and the βγ dimer in the same way that high performance gel filtration has been used to study the association-dissociation behaviour of bovine and rat liver glutamic dehydrogenase.[28]

ACKNOWLEDGEMENTS

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ERRATUM corrected 23 Jun 97 /AP

H. Guolan, Z. Weihua, Z. Zhiren: "Separation of Positional Isomers of Chlorophenols by Reverse Phase HPLC," J. Liq. Chrom. & Rel. Technol., 19(6), 899-909 (1996).

Figure 3, as originally published, is incorrect. Following is the correct figure.

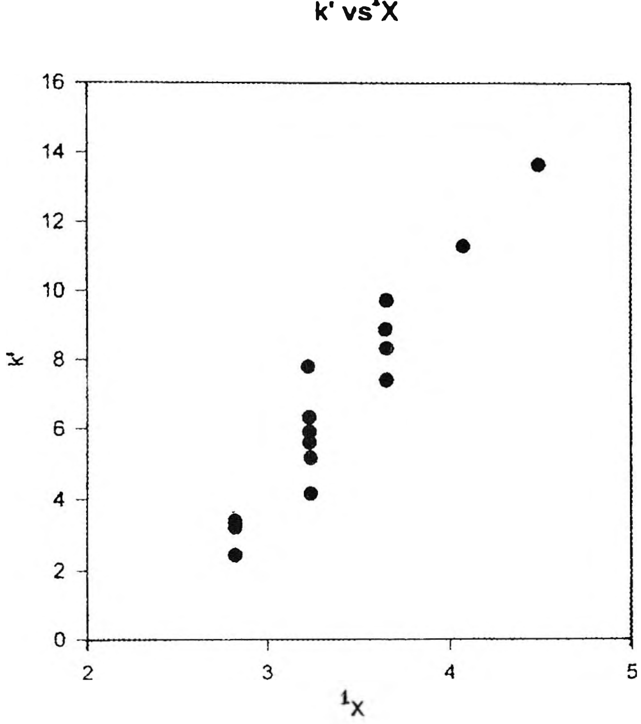

Figure 3. Capacity Factor (k') vs. Molecular Connectivity Index (^{1}X)

J. LIQ. CHROM. & REL. TECHNOL., 20(1), 157–158 (1997)

THE BOOK CORNER

BIOFLUID ASSAY FOR PEPTIDE-RELATED AND OTHER DRUGS, E. Reid, H. M. Hill, I. D. Wilson, eds., Volume 24 in the Methodological Surveys in Bioanalysis of Drugs series, The Royal Society of Chemistry, Cambridge, U.K., 1996, 350 pp., £89.50.

This volume is based on the proceedings of the 11th International Bioanalytical Forum, conducted by the Forum Syndicate of the Chromatographic Society. The symposium was held August 29 to September 1, 1995 in Guildford, U.K.

The apparent purpose of this volume is, simply, to record and disseminate the proceedings. Although some of the contributions are excellently reported, some of the proceedings included in this volume are somewhat abbreviated, sometimes comprising no more than a brief outline of the work that is reported. Expanded, complete papers would, no doubt, have made this a useful reference manual.

An analyte index is included at the end of the book. This would be, perhaps, the most valuable contribution. But, its organization is confusing, requiring more than a quick reference to locate a specific analyte.

The contributions are grouped under the following topics:

Peptide-Type Agents

Agents with Peptide-Type Targets

Various Drug Analytes: Problems and Strategies

Sample Treatment including Protein Removal and SPE

Approaches for Analytical Separation and Detection

Data Acquisition and Validity in Relation to Drug Appraisal

This volume, according to the publisher, is aimed at the staffs of research and professional organizations involved in drug development and bioanalytical research. Although this is the audience to whom the covered subjects would would appeal, one must be cautioned not to expect more than sketchy overviews with minimal references to the published literature. As such, the volume's utility is limited.

Reviewed by
Dr. Jack Cazes
The Cazes Group, Inc.
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Details may be obtained from Dr. Jack Cazes, P. O. Box 970210, Coconut Creek, FL 33097, USA. (954) 973-8516; E-Mail: jcazes@icanect.net.

J. LIQ. CHROM. & REL. TECHNOL., 20(1), 161–166 (1997)

LIQUID CHROMATOGRAPHY CALENDAR

1997

MARCH 16 - 21: PittCon '97, Atlanta, Georgia. Contact: PittCon '97, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

APRIL 13 - 17: 213th ACS National Meeting, San Francisco, California. Contact: ACS Meetings, ACS, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6059; FAX: (202) 872-6128.

APRIL 14 - 19: Genes and Gene Families in Medical, Agricultural and Biological Research: 9th International Congress on Isozymes, sponsored by the Southwest Foundation for Biomedical Research, Hilton Palacio del Rio, San Antonio, Texas. Contact: Mrs. Janet Cunningham, Barr Enterprises, P. O. Box 279, Walkersville, MD 21793, USA.

MAY 5 - 9: 151st ACS Rubber Div Spring Technical Meeting, Anaheim, California. Contact: L. Blazeff, P. O. Box 499, Akron, OH 44309-0499, USA.

MAY 23 - 24: ACS Biological Chemistry Div Meeting, San Francisco, California. Contact: K. S. Johnson, Dept of Biochem & Molec Biol, Penn State Univ, 106 Althouse Lab, University Park, PA 16802, USA.

MAY 27 - 28: IInd Miniaturisation in Liquid Chromatography versus Capillary Electrophoresis Conference, University of Ghent, Ghent, Belgium. Contact: Prof. Dr. W. Baeyens, Faculty of Pharmaceutical Analysis, University of Ghent, Harelbekestraat 72, B-9000 Ghent, Belgium.

MAY 27 - 30: ACS 29th Central Regional Meeting, Midland, Michigan. Contact: S. A. Snow. Dow Corning Corp., CO42A1, Midland, MI 48686-0994.

MAY 28 - 30: 31st ACS Middle Atlantic Regional Meeting, Pace Univ, Pleasantville, NY. Contact: D. Rhani, Chem Dept, Pace University, 861 Bedford Rd, Pleasantville, NY 10570-2799, USA. Tel: (914) 773-3655.

MAY 28 - JUNE 1: 30th Great Lakes Regional ACS Meeting, Loyola University, Chicago Illinois. Contact: M. Kouba, 400G Randolph St, #3025, Chicago, IL 60601, USA. Email: reglmtgs@acs.org.

JUNE 22 - 25: 27th ACS Northeast Regional Meeting, Saratoga Springs, New York. Contact: T. Noce. Rust Envir & Infrastructure. 12 Metro Park Rd. Albany. NY 12205. USA. Tel: (518) 458-1313; FAX: (518) 458-2472.

JUNE 22 - 26: 35th National Organic Chemistry Symposium, Trinity Univ., San Antonio, Texas. Contact: J. H. Rigby, Chem Dept. Wayne State Univ., Detroit. MI 48202-3489. USA. Tel: (313) 577-3472.

SEPTEMBER 7 - 11: 214th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings. 1155 16th Street. NW. Washington. DC 20036-4899.

SEPTEMBER 21 - 26: Federation of Analytical Chemistry & Spectroscopy Societies (FACSS), Cleveland, Ohio. Contact: J. A. Brown. FACSS. 198 Thomas Johnson Dr. Suite S-2. Frederick. MD 21702. USA. Tel: (301) 846-4797; FAX: (301) 694-6860.

OCTOBER 19 - 22: 49th ACS Southeast Regional Meeting, Roanoke, Virginia. Contact: J. Graybeal. Chem Dept. Virginia Tech. Blacksburg, VA 24061. USA. Tel: (703) 231-8222; Email: reglmtgs@acs.org.

OCTOBER 21 - 25: 33rd ACS Western Regional Meeting, Irvine, California. Contact: L. Stemler. 8340 Luxor St, Downey, CA 90241. USA. Tel: (310) 869-9838; Email: reglmtgs@acs.org.

OCTOBER 26 - 29: 8th Symposium on Handling of Environmental & Biological Samples in Chromatography and the 26th Scientific Meeting of the Group of Chromatography and Related Techniques of the Spanish Royal Society of Chemistry, Almeria, Spain. Contact: M. Frei-Hausler. IAEAC Secretariat. Postfach 46. CH-4123 Allschwill 2. Switzerland. FAX: 41-61-4820805.

OCTOBER 29 - NOVEMBER 1: 32nd ACS Midwest Regional Meeting, Lake of the Ozarks, Osage Beach, Missouri. Contact: C. Heitsch, Chem Dept, Univ of Missouri-Rolla, Rolla, MO 65401, USA. Tel: (314) 341-4536; FAX: (314) 341-6033; Email: reglmtgs@acs.org.

NOVEMBER 11 - 15: 5th Chemical Congress of North America, Cancun, Mexico. Contact: ACS Meetings, 1155 16th St, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-6286; FAX: (202) 872-6128.

NOVEMBER 16 - 21: Eastern Analytical Symposium, Garden State Convention Center, Somerset, New Jersey. Contact: S. Good, EAS, P. O. Box 633, Montchanin, DE 19710-0635, USA. Tel: (302) 738-6218.

1998

MARCH 1 - 6: PittCon '98, New Orleans, Louisiana. Contact: PittCon '98, Suite 332, 300 Penn Center Blvd., Pittsburgh, PA 15235-5503, USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 29 - APRIL 2: 215th ACS National Meeting, Dallas, Texas. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

JUNE 10 - 12: 53rd ACS Northwest Regional Meeting, Columbia Basin College, Pasco, Washington. Contact: K. Grant, Math/Science Div, Columbia Basin College, 2600 N 20th Ave, Pasco, WA 99301, USA. Email: reglmtgs@acs.org.

JUNE 13 - 19: 26th ACS National Medical Chemistry Symposium, Virginia Commonwealth Univ/Omni Richmond Hotel, Richmond, Virginia. Contact: D. J. Abraham, Virginia Commonwealth Univ, Dept of Med Chem, P. O. Box 581, Richmond, VA 23298, USA. Tel: (804) 828-8483; FAX: (804) 828-7436.

AUGUST 23 - 28: 216th ACS National Meeting, Boston, Massachusetts. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6218; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 15th International Symposium on Medicinal Chemistry, Edinburgh, Scotland. Contact: M. Campbell, Bath University School of Chemistry, Claverton Down, Bath, BA2 7AY, UK. Tel: (44) 1225 826565; FAX: (44) 1225 826231; Email: chsmmc@bath.ac.uk.

SEPTEMBER 24 - 26: XIVth Conference on Analytical Chemistry, sponsored by the Romanian Society of Analytical Chemistry (S.C.A.R.), Piatra Neamt, Romania. Contact: Dr. G. L. Radu. S.C.A.R., Faculty of Chemistry, University of Bucharest. 13 Blvd. Reepublicii. 70346 Bucharest - III. Romania.

NOVEMBER 4 - 7: 50th ACS Southwest Regional Meeting, Resw Triangle Pk, North Carolina. Contact: B. Switzer. Chem Dept. N Carolina State University. Box 8204. Raleigh, NC 27695-8204. USA. Tel: (919) 775-0800. ext 944; Email: switzer@chemdept.chem.ncsu.edu.

1999

MARCH 7 - 12: PittCon '99, Orlando, Florida. Contact: PittCon '99. Suite 332. 300 Penn Center Blvd., Pittsburgh, PA 15235-5503. USA. Tel: (800) 825-3221; FAX: (412) 825-3224.

MARCH 21 - 25: 217th ACS National Meeting, Anaheim, Calif. Contact: ACS Meetings. 1155 16th Street. NW. Washington. DC 20036-4899. USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 218th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings. 1155 16th Street. NW. Washington, DC 20036-4899. USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

OCTOBER 8 - 13: 51st ACS Southeast Regional Meeting, Knoxville, Tennessee. Contact: C. Feigerle. Chem Dept. University of Tennessee. Knoxville. TN 37996. USA. Tel: (615) 974-2129; Email: reglmtgs@acs.org.

2000

MARCH 5 - 10: PittCon 2000, Chicago, Illinois. Contact: PittCon 2000. Suite 332. 300 Penn Center Blvd., Pittsburgh. PA 15235-5503. USA.

MARCH 26 - 30: 219th ACS National Meeting, Las Vegas, Nevada. Contact: ACS Meetings. 1155 16th Street. NW. Washington. DC 20036-4899. USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 20 - 24: 220th ACS National Meeting, Washington, DC. Contact: ACS Meetings. 1155 16th Street. NW. Washington. DC 20036-4899. USA.

2001

APRIL 1 - 5: 221st ACS National Meeting, San Francisco, Calif. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 19 - 23: 222nd ACS National Meeting, Chicago, Illinois. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2002

APRIL 7 - 11: 223rd ACS National Meeting, Orlando, Florida. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA.

SEPTEMBER 8 - 12: 224th ACS National Meeting, Boston, Mass. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899.

2003

MARCH 23 - 27: 225th ACS National Meeting, New Orleans, Louisiana. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

SEPTEMBER 7 - 11: 226th ACS National Meeting, New York City. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

2004

MARCH 28 - APRIL 1: 227th ACS National Meeting, Anaheim, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 22 - 26: 228th ACS National Meeting, Philadelphia, Pennsylvania. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128.

2005

MARCH 13 - 17: 229th ACS National Meeting, San Diego, California. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

AUGUST 28 - SEPTEMBER 1: 230th ACS National Meeting, Washington, DC. Contact: ACS Meetings, 1155 16th Street, NW, Washington, DC 20036-4899, USA. Tel: (202) 872-4396; FAX: (202) 872-6128; Email: natlmtgs@acs.org.

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Chapter in a Book:

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Contents Continued

Determination of Natural and Synthetic Colors in Alcoholic and Non Alcoholic Beverages by Quantitative HPTLC 101
S. M. Anderton, C. D. Incarvito, and J. Sherma

Analytical and Preparative Enantiomeric Separation of a Series of C5-Cycloalkylamine-1,4-benzodiazepin-2-one CCK_B Receptor Antagonists by Chiral HPLC 111
A. P. Watt, H. M. Verrier, and D. Rathbone

Immobilized Iron(III) Metal Affinity Chromatography for the Separation of Phosphorylated Macromolecules: Ligands and Applications 123
L. D. Holmes and M. R. Schiller

Non Ideal Size Exclusion Chromatography of Eukaryotic Protein Synthesis Initiation Factor 2 143
M. F. Mouat and K. L. Manchester

Erratum 155

The Book Corner 157

Announcement 159

Liquid Chromatography Calendar 161

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES

Volume 20, Number 1, 1997

CONTENTS

Capillary Zone Electrophoresis Versus Micellar Electrokinetic Chromatography in the Separation of Phenols of Environmental Interest 1
A. L. Crego and M. L. Marina

Studies of the Influence of Hydrogen Chloride on the Liquid Chromatographic Properties of Silica-Immobilized Bovine Serum Albumin Under Normal-Phase Conditions 21
H. Gao and R. K. Gilpin

A First-Level Analysis of the Adsorption Mechanism of Esters of Racemic and Meso-2,3-dibromobutane-1,4-dioic Acids on Silica 31
M. Palamareva and I. Kozekov

Simultaneous Determination of Caffeine and Ergotamine in Pharmaceutical Dosage Formulation by Capillary Electrophoresis 47
H. Y. Aboul-Enein and S. A. Bakr

Separation of Taxol and Cephalomannine by Countercurrent Chromatography 57
F.-Y. Chiou, P. Kan, I.-M. Chu, and C.-J. Lee

Determination of the Alkaloids in Coptis-Evodia Herb Couple by Capillary Electrophoresis 63
M.-C. Lee and S.-J. Sheu

Preconcentration of Aliphatic Amines from Water Determined by Capillary Electrophoresis with Indirect UV Detection 79
W. H. Matchett and W. C. Brumley

(continued on inside back cover)

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