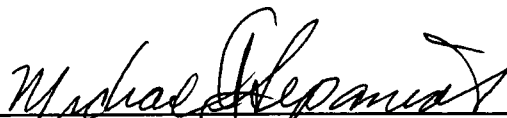


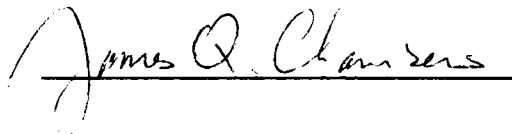
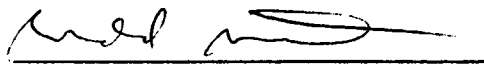
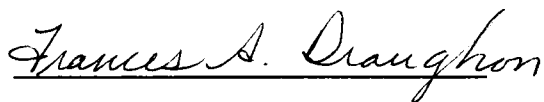
To the Graduate Council:

I am submitting herewith a dissertation written by Roderic O'conor Cole entitled "Applications of Nontraditional Organized Media in Capillary Electrophoresis". I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Chemistry.



Michael J. Sepaniak, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor
and Dean of the Graduate School

**Applications of Nontraditional Organized Media in
Capillary Electrophoresis**

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Roderic O'conor Cole

December 1991

Dedication

To Lori Lea

For her undying love and support

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Abstract

Capillary electrophoresis (CE) is a highly efficient separation technique capable of addressing many types of samples. CE has several advantages over conventional liquid-phase separation techniques: high efficiency, capability for high speed separations, low sample and mobile phase consumption, and selectivity control via solution chemistry. Of these points, two of the less investigated areas are selectivity control and separation speed. Selectivity in CE is largely determined by mobile phase additives. Two forms of CE predominate: capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC). CZE separates charged solutes based on electrophoretic mobility differences and MECC via differential association with electrophoretically-retarded micelles. This work investigates surfactant assemblies and cyclodextrins for suitability as selectivity modifiers in these techniques.

While the potential versatility and efficiency of CE has attracted much attention to these techniques, several problems exist. In MECC, hydrophobic compounds are highly retained and difficult to resolve. Also, selectivity is limited because n-alkyl surfactant-formed micelles are usually employed. This work addresses these issues by investigating bile salt surfactants for use in MECC. Bile salts are biological detergents based on a hydroxy-substituted backbone. These surfactants are shown to produce a general reduction in capacity factor, k' and tolerate the presence of organic solvents to a greater extent than conventional surfactants (thus improving resolution for hydrophobic compounds). In addition, chiral separations are demonstrated for binaphthyl compounds using interactions with bile salt micelles.

High-speed separations (<1 minute) can be achieved in MECC via the use of narrow-bore (25 μ m) capillaries, high applied fields, and the relatively low k' values afforded by the use of bile salts. The high efficiencies attained in MECC are shown to facilitate rapid separations, and important experimental parameters are investigated for aflatoxin analysis.

Cyclodextrins (CDs) can also be employed as mobile phase additives in CE. CDs are cyclic sugars capable of forming inclusion complexes that are influenced by molecular size and isomeric form of the analyte. CDs are demonstrated to be useful for chiral separations of charged compounds in the CZE mode. CD type and concentration are important in determining resolution. CDs are also employed in conjunction with surfactants for the MECC separation of methylanthracene isomers.

Table of Contents

Chapter	Page
1. Introduction.....	1
Historical Perspective.....	2
Introduction to Capillary Electrophoresis.....	6
Capillary Zone Electrophoresis.....	6
Micellar Electrokinetic Capillary Chromatography.....	10
Statement of the Problem.....	14
2. Experimental Considerations.....	18
Apparatus.....	18
Procedures.....	19
Column Construction.....	19
Injection.....	19
Detection.....	20
Absorbance Detection.....	21
Fluorescence Detection.....	25
3. Bile Salt Surfactants in MECC: Hydrophobic Molecule Separations.....	28
Introduction.....	28
Surfactants and Micellization.....	28
Micelle Enhanced Fluorescence.....	31
Surfactant use in Chromatography.....	33
Surfactant use in CE.....	33
Experimental.....	36

	Apparatus.....	36
	Chemicals.....	37
	Procedures.....	37
	Results and Discussion.....	39
4.	Experimental Factors Affecting High Speed MECC Separations.....	52
	Introduction.....	52
	Experimental.....	55
	Apparatus.....	55
	Chemicals.....	56
	Procedures.....	56
	Chromatography.....	56
	Efficiency Calculations.....	57
	Sample Preparation.....	57
	Safety Precautions.....	58
	Results and Discussion.....	58
	Optimization of Analysis Time and Efficiency.....	58
	Detection.....	68
	Analysis of Corn Samples.....	71
5.	Chiral Separations in Capillary Electrophoresis.....	76
	Introduction.....	76
	Experimental.....	78
	Apparatus.....	78
	Reagents.....	79
	Procedures.....	79
	Results and Discussion.....	81

	Bile Salt use in MECC	
	Chiral Separations.....	81
	Use of Cyclodextrins in	
	CZE Chiral Separations.....	89
6.	Concluding Remarks.....	107
	References.....	111
	Vita.....	120

List of Tables

Table	Page
I. n-alkyl amine-NBD Capacity Factor Data for Bile Salts and SDS	41
II. Comparison of MECC and HPLC Operating Costs.....	54
III. Factors Affecting Efficiency in High Speed MECC Separations.....	61
IV. Fluorescence Characteristics of Aflatoxins in Various Solvents.....	70
V. Limits of Detection for Aflatoxins in MECC.....	72
VI. Retention Data for NaC/Methanol Study.....	85
VII. Solubility of Cyclodextrins in Aqueous Solution.....	93

List of Figures

Figure	Page
1. Flow Profiles for Electroosmotic and Hydrostatic Pumping.....	8
2. MECC Column Cross-Section.....	11
3. Representation of the Elution Range in MECC.....	12
4. General Experimental Apparatus for CE.....	18
5. Laser-Etched Absorbance Flowcell Apparatus.....	22
6. Split-Block Flowcell Holder for CE Absorbance Detection.....	24
7. Laser-Based Fluorescence Detection Apparatus for CE.....	26
8. Structures of SDS Monomer and Micelle.....	29
9. Nitropyrene Fluorescence in Various Solvents.....	32
10. Surfactants Studied in MECC.....	35
11. Hydrophobic Compounds Studied.....	38
12. Separation of NBD-amines with NaC (A) and SDS (B).....	40
13. Separation of Aflatoxins with NaDC.....	42
14. Separation of NBD-amines using NaC and SDS with 20% MeOH.....	44
15. Plot of $\lambda_{\max}$ for Rhodamine 6G vs. Surfactant Concentration.....	46
16. CMC Behavior of NaC and SDS vs. MeOH Concentration.....	47
17. Bile Salt Separation of PAH.....	49
18. Separation of B(a)P tetrol and B(a)P	50
19. Void and Analysis Time for MECC vs. Applied Field.....	59
20. Efficiency vs. Applied Field for 25 and	

	50 μm i.d. Capillaries.....	63
21.	Analysis of Corn Meal Extract with MECC.....	65
22.	Efficiency vs. Applied Field for 0 and 10% Acetonitrile.....	67
23.	High-Speed Separation of Aflatoxin Standards.....	69
24.	MECC Separation of an Extract of <i>A. Flavus</i> Cultured Corn.....	74
25.	Bile Salt Separations of Binaphthyl Enantiomers.....	83
26.	Binaphthyl Structures.....	86
27.	Dicarboxylic acid Binaphthyl Separation.....	88
28.	Cyclodextrin Properties.....	91
29.	CZE Separation of Charged Binaphthyls with Native CDs.....	97
30.	Separation of Binaphthyl Enantiomers with G1 α -CD.....	99
31.	Mobility of DNS-Phenylalanine Enantiomers vs. [HP β -CD].....	101
32.	Rapid Separation of DNS-Phenylalanine Enantiomers in Commercial Formulation.....	103
33.	Separation of Methylantracene Isomers.....	105

Chapter 1

Introduction

Separation science plays a critical role in nearly every aspect of chemistry. Applications involve the physical separation of substances, for preparative (isolation) purposes, or analytical scale methods for chemical analysis. Advances in separation science have played a critical role in modern technological development. Moreover, developments in separation science will inevitably play key roles in the solution of future problems involving biotechnology, medicine, and environmental monitoring. Improved methodologies are thus important for technological growth.

In terms of chemical analysis, separations play an important role in the development of new methods. Many substances of interest are often present in complex matrices. The inherent lack of specificity in many well-established quantitative measurement techniques such as ultraviolet absorption spectroscopy make it necessary to isolate the desired analyte prior to measurement. Separation techniques such as chromatography and electrophoresis in their various forms serve to impart a vital element of selectivity to the analytical measurement. Thus, these methods represent a tool of formidable prowess for chemical analysis.

An inspection of the developments in analytical separation science reveals a tendency toward [1] more efficient separations, [2] improved selectivity, [3] increased speed of analysis, and [4] improved detection methodologies. Many of these goals are attained via the use of novel transport phenomena and miniaturization of chromatographic systems. One technique which shows promise in areas 1-3 is capillary electrophoresis. Characteristic of

this technique is high efficiency and novel selectivity. This work describes recent advances in the latter area.

Historical Perspective

Chromatography had its beginnings in 1903 when Tswett used what is now known as column liquid chromatography to separate plant pigments (1). While the first application of separation science is credited to Moses, who used a crude ion exchange resin to purify water, the technique at the time was not considered a field appropriate for active research, and was not actively pursued. Based on Tswett's pioneering concept, paper and thin-layer chromatography were developed in the late 1930s and remained the state of the art for some time. These techniques certainly enjoyed wide application, but were time-consuming and tedious.

The first truly instrumental approach to chromatography manifested itself in the form of gas chromatography (GC) in 1952 (2). Performed primarily on an analytical scale, GC has proven invaluable in the separation of many classes of compounds. This approach was based on columns packed with some solid adsorbent and thermal conductivity detection. Many instrumental developments such as readily available carrier gas supply, syringes, injection systems, and precise temperature control have helped to establish GC as a fundamental analytical tool. While packed-column GC served to forward the concept of instrumental chromatography, this approach suffers problems from low efficiency. Increasing column length to increase plate numbers, and thus improve efficiency is limited by the physical size of the GC oven and, more importantly, the low permeability (high pressure drop) of the packed column.

A major stride forward in GC technology was the development of highly efficient narrow-bore (ca. 200-500 μm) capillary columns which has allowed resolution of substances such as volatile hydrocarbons in mixtures as complex

as petroleum. The open tubular approach allows use of long (30-100) m, narrow bore columns due to low pressure drop and small physical size. In this manner, plate numbers could be greatly increased, and many separations could be achieved without relying heavily on alteration of column selectivity to affect separations. Capillary columns are thus often able to separate solutes based mainly on their extraordinary efficiency. This "get a bigger hammer" approach, while not the most elegant, is certainly effective. In addition, rapid mass transfer characteristics allow operation of these columns at high mobile phase velocities, so analysis times are not excessive. Indeed, columns with lengths in excess of 100 meters have been employed for ultrahigh efficiency in some practical applications (3).

The high resolving power of modern GC has been enhanced by the versatility and sophistication of GC detection methods. Standard to most instruments is the flame ionization detector which offers good sensitivity for a very wide variety of compounds. More specialized detectors with very high sensitivity such as the electron capture detector allow the quantitation of low levels of chlorinated waste products in highly complex matrices. In addition, the mass spectrometer can be coupled with the GC column to allow both sensitive detection and identification of analytes. Recent work describes a non-scanning instrument which is capable of collecting mass spectral data from very efficient chromatographic peaks (4). It is an arguable point that GC technology has reached the highest level of all chromatographic methods. Unfortunately, for all its range of applicability and well-established technology, GC is limited by the fundamental constraint that compounds must be able to exist in the vapor phase at a reasonably low temperature (ca. 300 °C) to be analyzed via this technique.

While many interesting compounds are sufficiently volatile for GC analysis, many compounds of interest are not. Synthetic polymers, proteins,

peptides, amino acids, and other molecules of biological and/or environmental interest exist in complex matrices and require separation prior to analysis. The volatility constraint posed by GC spurred the development of high performance liquid chromatography (HPLC). HPLC, in addition to allowing the chromatographic analysis of nonvolatile compounds, opened myriad avenues of selectivity via utilization of specific solute-mobile phase interactions. While the mobile phase in GC serves only to transport solute bands through the column, its composition has little effect on the separation selectivity. The HPLC mobile phase, on the other hand, can be altered by the addition of various amounts of solvents (both organic and aqueous), cyclodextrins (5), and micelles (6), to name a few. This option allowed the development of techniques such as size exclusion and ion exchange chromatography on an instrumental level.

As in the development of GC, the tendency toward smaller, more efficient columns has also been an area of much interest in LC. Microbore columns have been developed which utilize smaller internal diameters and particles relative to conventional HPLC columns (4.6 mm i. d. X 25 cm long). These columns offer the advantages of higher efficiency and reduced mobile phase consumption, the latter becoming more and more important as high purity solvent costs rise and especially the cost of disposal of solvent-laden waste material. Open-tubular LC (OTLC) columns offer the same advantages as capillary GC columns; low pressure drop and high efficiency via longer columns. While OTLC has this potential, however, greatly reduced solute diffusion in the liquid phase demands the use of extremely narrow bore columns (5-10 μm i. d.) and low mobile phase flow rates to achieve the sought after efficiency. Fabrication, injection, and detection problems, as well as analysis times often on the order of hours, have prevented this technique from

realizing the popularity of capillary GC. Thus, the conventional packed column approach remains the workhorse in modern analytical chemistry.

As in the maturation of GC, the development of sensitive, selective detection schemes was and still is an active area of research. In HPLC, many detection methods emerged based on spectroscopy in the ultraviolet and visible range of the spectrum. In addition to spectrophotometry, highly sensitive techniques such as fluorescence and chemiluminescence have been employed. Electrochemical and mass spectrometric schemes have also proven to be highly selective as HPLC detectors. The development of HPLC thus fostered a new approach to achieving highly selective separations of nonvolatile and ionic substances previously considered intractable. First hand accounts of the history of the development of chromatographic technology and instrumentation have been published (7).

Another family of condensed phase separation techniques separates solutes based on differences in migration rates in an electric field. First practiced in the early 1900s, electrophoresis allows the separation of charged compounds such as peptides and proteins. Solute such as these that possess different mass/charge ratios tend to migrate at different rates in an electric field, often allowing them to be separated. This migration effect, or electrophoretic mobility, is the basis for many forms of electrophoresis. Classically, electrophoresis is performed in some stabilizing media such as paper or gel. The stabilizer serves to minimize band dispersion which arises due to convective and/or thermal forces. Mixtures are introduced onto one end of the stabilizing media, and a fixed voltage (or current) applied for a given duration. Detection is generally accomplished via the development of color or some staining procedure. Electrophoresis has exceptionally high resolving power and spot (solute) capacity in addition to versatility. While highly effective, these

types of techniques are limited by the relatively labor-intensive, insensitive detection methods.

Introduction to Capillary Electrophoresis

Capillary electrophoresis (CE) is a conceptually simple form of electrophoresis whereby a wide variety of compounds can be separated. CE differs from conventional forms of electrophoresis in that no anti-convective stabilizing medium such as paper or gel is used and detection is achieved on-line. Separations are performed in free solution within narrow (ca. 50 μm) diameter capillaries. Capillaries of these dimensions are able to effectively dissipate heat generated by the passage of electrical current thus preventing band dispersion due to thermal gradients and convection (8). Primary advantages of the CE techniques are:

- (1) Simple Apparatus: only column, power supply and detector required
- (2) Small sample amounts required: 5.0 nL injection volume typical
- (3) High efficiency: plate numbers typically in excess of 250,000
- (4) Rapid separations possible: analysis times of < 3 seconds reported
- (5) On-line detection: no lengthy post-separation procedures necessary
- (6) Wide variety of selectivities possible via mobile phase additives

Two primary limitations to CE arise from the capillaries employed: migration and retention times suffer from poor reproducibility and the diminutive size of the capillaries employed make sensitive detection difficult. This work focuses on the solution of problems involving Capillary Zone Electrophoresis (CZE) and Micellar Electrokinetic Capillary Chromatography (MECC), two of the more versatile of the CE techniques.

Capillary Zone Electrophoresis

Capillary Zone Electrophoresis was introduced in 1981 by Jorgenson and co-worker (9). CZE is an extremely efficient separation technique whereby

charged solutes are separated on the basis of differences in their electrophoretic mobility. Samples are introduced as a plug at the inlet end of the separation capillary and high voltage (ca. 10 - 30 kV) is applied. Bulk flow due to electroosmosis (see below) is generated, and solutes migrate according to their electrophoretic mobility. Unprecedented high efficiency (typically >250,000 theoretical plates) allows separations of molecules with very small differences in electrophoretic mobility. At the end of the capillary, solute bands pass by a detector and an electropherogram is recorded. Resolution in CZE is governed by the magnitude of the difference in solute electrophoretic mobility and by the magnitude of electroosmotic flow. Resolution in CZE is given by the expression

$$R_s = \frac{\sqrt{N}}{4} \left[\frac{\mu_1 - \mu_2}{\mu_{ave} - \mu_{osm}} \right] \quad (1)$$

where N is the number of theoretical plates, μ_1 and μ_2 the electrophoretic mobilities of the solutes of interest, μ_{ave} the average of μ_1 and μ_2 , and μ_{osm} the electrophoretic flow mobility. It has been shown that maximum resolution is attained when μ_{osm} is very close to $-\mu_{ave}$, albeit at the expense of analysis time (9). In this situation, electroosmotic flow is very small and the tendency to "swamp out" electrophoretic mobility differences reduced to minimum.

Characteristic of many CE separations is the presence of electroosmotic flow. Electroosmotic flow results from the migration of solvated ions in the applied electric field (10). Fixed sites of negative charge at the capillary walls due to ionized SiOH groups, and counter ions from the solution in the capillary produce an electric double layer potential (zeta potential) within the diffuse region near the capillary wall. Application of high potential causes mobile

solvated ions to migrate in the field, and results in electroosmotic solvent flow. Electroosmotic flow velocity, V_{eo} is given by

$$V_{eo} = -\left(\frac{\epsilon\zeta E}{\eta}\right) \quad (2)$$

where ϵ is the permittivity of the mobile phase, η the viscosity of the mobile phase, E the magnitude of the applied electrical field, and ζ the zeta potential. Equation 2 indicates that V_{eo} , and thus flow rate, is directly proportional to the applied field. Electroosmotic flow is responsible for two primary advantages associated with CZE. First, since the magnitude of the electroosmotic flow is large, neutral, negative, and positively charged solutes all elute at the same end of the capillary. This allows solutes of any charge to be analyzed within a single run, or at least with the same instrumental configuration. Second, the velocity profile of electroosmotic flow is virtually constant across the capillary bore, so resistance to mass transfer in the mobile phase is a negligible source of band broadening under normal operating conditions. Figure 1 shows a simple representation of hydrostatic and electroosmotically-pumped systems.

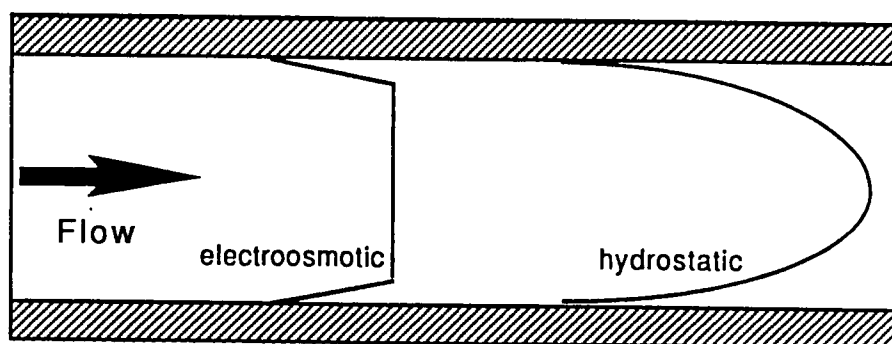


Figure 1: Flow Profiles for Electroosmotic and Hydrostatic Pumping

In the hydrostatic case, solutes must rapidly diffuse across the parabolic flow profile in order to average out velocity differences encountered at different points in the band. Consequently, slow radial diffusion results in band broadening. In gas phase separations, diffusion coefficients are generally high and capillaries of up to 530 μm i. d. may be used without dramatic loss of efficiency. In the liquid phase, however, diffusion coefficients are typically reduced by at least four orders of magnitude and capillaries on the order of 5 - 15 μm i. d. are required for efficient separations employing hydrostatic flow. In addition, liquid phase separations require low flow rates, thus dramatically extending total analysis time. Capillaries of these dimensions pose extremely difficult problems for detection, injection, and fabrication (11). Indeed, it seems that column fabrication in OTLC is often more of an art form than a science. In CZE, rapid diffusion (or short diffusion distance) is not required since the flat flow profile characteristic of electroosmotic flow allows solute molecules within a band to move down the column at a constant velocity. CZE may thus be performed in capillaries of 50-100 μm i. d., significantly relaxing injection and detection constraints. In light of instrumental requirements, CE may well represent a more convenient and practical path to many of the advantages sought after with OTLC.

Efficiency in CZE is given by

$$N = \frac{(\mu_{el} - \mu_{osm})V}{2D} \quad (3)$$

where N is the number of theoretical plates, μ_{el} the solute electrophoretic mobility, μ_{osm} the flow velocity at unit field strength, V the applied voltage, and D the solute diffusion coefficient. Equation 3 assumes the absence on any

contributions to band broadening from injection, detection, thermal-based convection or adsorptive wall interactions (*i. e.* efficiency is limited only by axial molecular diffusion). Under optimum conditions, solutes with low diffusion coefficients such as proteins may potentially exhibit very high efficiency. In practice, however, efficiency for these types of solutes is limited by convection arising from Joule heating (12), and wall adsorption (13, 14). Plate numbers for CZE separations are thus typically on the order of 250,000, although values exceeding this have been reported (9, 15). CZE has successfully been applied to the highly efficient separation of amino acids (9), proteins (14), and peptides (16), to name a few applications. Several review articles have been published (17, 18).

Micellar Electrokinetic Capillary Chromatography

A fundamental limitation of CZE is the requirement that solutes must possess electrophoretic mobility and thus charge to be separated. Neutral molecules have negligible electrophoretic mobility and move with the mobile phase velocity. When mixtures of neutral compounds are introduced into the capillary, all the solutes co-elute at the column dead volume. One approach designed to circumvent this shortcoming is the introduction of tetraalkylammonium salts into the mobile phase for the purpose of forming charged complexes with neutral molecules. The tetraalkylammonium ion/neutral molecule complexes possess different electrophoretic mobilities and can be separated via CZE. This approach was successfully applied to the separation of polyaromatic hydrocarbons (19). While successful, this approach to neutral molecule separations is limited to those solutes which readily form solvophobic complexes with tetraalkylammonium salts.

An elegant solution allowing neutral molecule separations in CE was introduced in 1984 by Terabe and co-workers (20). In this technique, sodium

dodecyl sulfate (SDS) was added to the CZE buffer above its critical micelle concentration. Neutral solutes were separated by differential association with the SDS micelles. Termed micellar electrokinetic capillary chromatography (MECC), this approach has several subtle mechanistic features. Figure 2 shows a cross-sectional view of MECC column dynamics.

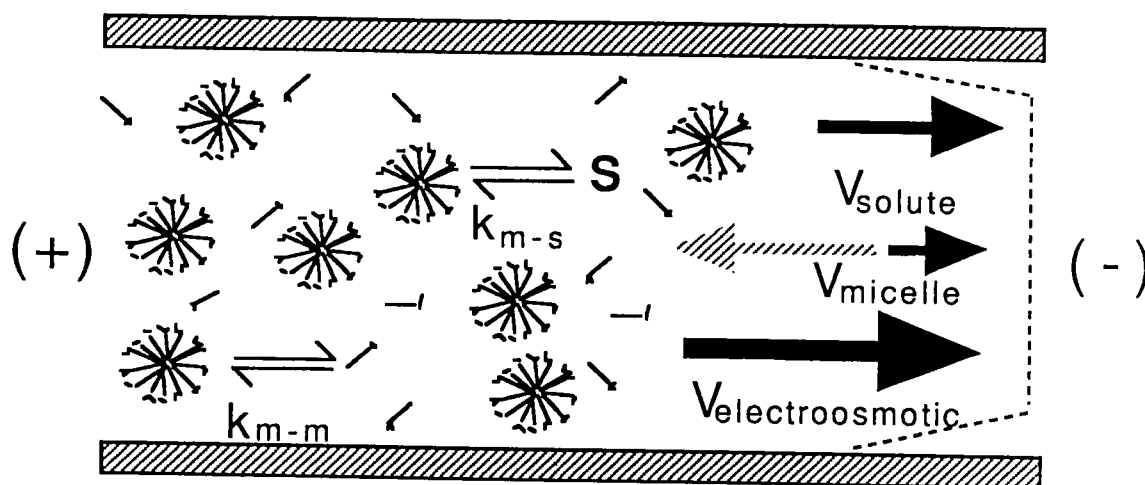


Figure 2: MECC Column Cross-Section

SDS micelles, since they are anionic, possess electrophoretic mobility in an electric field and migrate at a velocity different from the mobile phase. Solutes may differentially interact with the electrophoretically-retarded micellar phase and be separated. In the situation depicted in Figure 2, electroosmotic flow is from the positive electrode to ground. Anionic micelles thus attempt to "swim upstream" against the (stronger) electroosmotic flow, as denoted by the shaded arrow. Since the electroosmotic flow is, in most cases, greater than the electrophoretic mobility of the micelle, micelles introduced with the sample plug are eventually eluted from the capillary, *i.e.* the net micelle velocity is toward

the ground electrode. This creates an elution window in which separation must occur, bounded by the column dead volume and the micelle migration time.

Figure 3 shows a representation the elution window typically experienced in MECC separations.

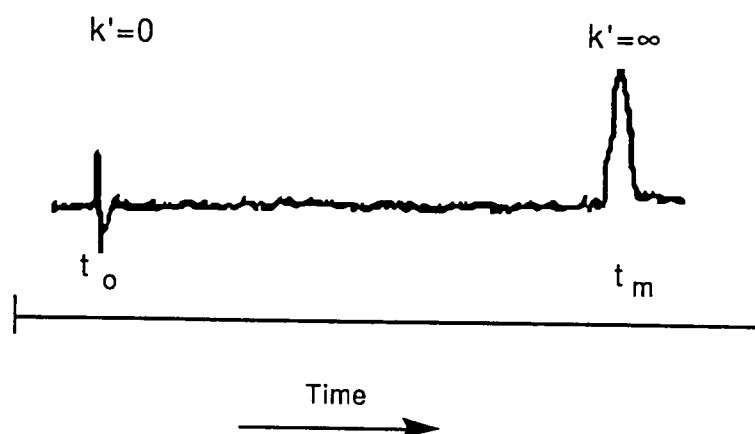


Figure 3: Representation of the Elution Range in MECC.

The column dead time, t_o , is generally marked by a system peak due to solvent disturbance and t_m , the micelle migration time by a compound which associates very strongly with the micelle. Any separation of solutes must occur between these two points; neutral solutes, since they possess no electrophoretic mobility, are not able to oppose electroosmotic flow more effectively than the micelle.

The limited elution range characteristic of MECC has several practical ramifications. First, this constitutes an advantage when dealing with hydrophobic or "dirty" samples, since fresh micellar phase is constantly pumped into the column. Irreversible retention is much less of a problem than in HPLC, and the "stationary" phase is not poisoned by the sample. Additionally, since all neutral solutes eventually pass by the detector, the possibility of "missing" a solute through irreversible retention is eliminated, just as in thin layer

chromatography. Second, the width of the window is dependent on the type of surfactant employed in the mobile phase. If a given surfactant forms micelles with low electrophoretic mobility, the size of the window will be reduced. Conversely, those micelles with stronger mobility have larger windows. The size of the window is measured by the elution range parameter, t_0/t_m . Peak capacity in MECC is given by (21)

$$n = 1 + \frac{\sqrt{N}}{4} \left(\ln \frac{t_0}{t_m} \right) \quad (4)$$

where n is peak capacity, and N the number of theoretical plates. Peak capacity thus increases with increases in either efficiency or elution window size.

Capacity factor (k') values also depend on the size of the elution window.

Solute k' in MECC is given by

$$k' = \frac{t_R - t_0}{t_0 \left(1 - \frac{t_R}{t_m} \right)} \quad (5)$$

where t_R is solute retention time. Note that as t_m approaches infinity, this expression reduces to the conventional expression for k' :

$$k' = \frac{(t_R - t_0)}{t_0} \quad (6)$$

Resolution in MECC is given by (21)

$$R_s = \frac{\sqrt{N}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k'_2}{1 + k'_2} \right) \left(\frac{1 - t_0/t_m}{1 + (t_0/t_m) k'_1} \right) \quad (7)$$

where R_s is resolution between two adjacent peaks and α is selectivity ($\alpha = k'_2/k'_1$). It is important to note the last term in Equation 7 and the resulting dependance of resolution on the size of the elution range. Terabe and co-workers showed that, for two solutes of similar k' , resolution passes through a maximum for k' values of 1 -5 for an elution range parameter value (t_o/t_m) of 0.4 (21). Solute with k' values greater than $k' \sim 5$ begin to suffer losses in resolution from their neighbors. Very hydrophobic compounds, which associate nearly completely with the micellar core over the course of a separation, are generally co-eluted at the micelle migration time. MECC is thus limited to more water-soluble solutes when applied in its purest form. However, several techniques have been developed to partially alleviate this limitation.

Statement of the Problem

Range of application and selectivity in MECC are limited primarily by the n-alkyl surfactants typically employed as pseudostationary phases. Hydrophobic compounds associate strongly with the micellar phase and, as a result, co-elute at the micelle migration time. This problem is partially alleviated by addition of organic modifiers such as methanol and acetonitrile to the mobile phase. Organic modifiers have two important effects on the MECC separation. First, the elution window (and thus peak capacity) is extended. Electroosmotic flow decreases with increasing organic solvent content, presumably due to interactions between solvent and ionized SiOH groups at the capillary walls. This allows the micelles to migrate more effectively in the electric field, leading to a longer t_m , since the micelles are able to better "swim upstream" in this situation. Second, k' values are lowered due to decreased polarity of the mixed aqueous-organic mobile phase. Partition coefficients are altered relative to the

pure water case, and solutes then spend more time in the mobile phase. In some cases, hydrophobic molecules which co-elute at t_m are separated when organic modifier is present in the mobile phase (22, 23). Since resolution maximizes for solutes possessing k 's of 1-5 (21), this approach can be quite effective in achieving separation of more hydrophobic molecules. Organic solvents may also be used in gradient elution MECC (24, 25, 26), in a manner similar to conventional HPLC techniques. While this approach is useful for the optimization of many separations, addition of organic modifier to the MECC mobile phase is limited to approximately 20% (v/v) before efficiency is critically degraded and analysis time increased to prohibitive levels. In order for high efficiency to be maintained in MECC, solutes must be able to interact with micelles of narrow size distribution. Micelle-monomer exchange kinetics must be rapid so that the differences in micelle aggregation number and thus size are "averaged out". The presence of large amounts (>15%) of organic modifier in the mobile phase may alter micellization kinetics, leading to poor efficiency. Thus, the adverse effects of micelle polydispersity set a limit to which organic modifiers may be effectively employed in MECC. In addition to reduced efficiency, electroosmotic flow can be reduced to the point where analysis time becomes excessive (22, 23, 24). Thus, the first aim of this work is to extend the utility of MECC to the separation and analysis of hydrophobic compounds via the use of novel surfactants. Applications involving the use of mixed aqueous-organic mobile phases with novel surfactants and investigations into their effects on the separation will be presented.

In situations where many samples are to be analyzed, an important issue is speed of analysis. High sample throughput translates directly into profit margin in terms of operator salary, mobile phase consumption, and sample turn around time. Conventional HPLC approaches to high-speed separations are

limited by efficiency degradation and high backpressures due to the requirement of high flow rate conditions. OTLC, as discussed above, has the necessary efficiency for the job but requires long columns and low flow rates, a combination not conducive to speed of analysis.

An attractive feature of CE which has received little attention in the literature is that of analysis speed. The combination of strong electroosmotic flow and high efficiency make CE an ideal tool for rapid separations. Separations in both CZE and MECC are typically on the order of 20-30 minutes and, in many cases, less than 10 minutes. Efficiency is a hallmark of CE and is much greater than can be obtained using HPLC, allowing operation under high flow rate conditions. Studies of factors important to rapid separations in MECC are presented for a common class of toxins for which routine analyses are performed. A second focus of this work is thus to explore conditions which optimize speed of analysis for MECC.

A third primary area of study in this work is that of selectivity control and manipulation in CE. Selectivity control in CZE often involves alteration of solute electrophoretic mobility by alteration of the molecules net charge, *i. e* mobile phase pH (27). While successful in some separations, pH alteration changes electroosmotic flow and can lead to extreme efficiency degradation due to wall adsorption, particularly in the case of proteins (14). Organic solvents such as methanol have also been used in improving resolution in CZE (28). As discussed above, addition of surfactants to the CZE mobile phase brings a partitioning mechanism into play. Selectivity in MECC is controlled primarily by the identity of the surfactant employed, but alteration of the mobile phase pH and organic solvent content are also possible routes to alter selectivity. The second primary goal of this work is to enhance the available selectivity in CE via the exploration of various mobile phase additives. By

judicious choice of surfactant, organic solvent, or other reagent, many difficult separations can be achieved in short times. A great strength of CE is that highly selective reagents can be readily added to the mobile phase. The small volumes required allow the use of rare and/or expensive chemicals. Unlike conventional HPLC, complex stationary phase bonding chemistries and great amounts of material are not required. Effects of various additives such as cyclodextrins and their derivatives on both CZE and MECC separations will be presented.

Chapter 2

Experimental Considerations

Apparatus

The general experimental arrangement is depicted in Figure 4. Fused silica capillary columns were purchased from Polymicro Technologies (Pheonix, AZ), inner diameters and lengths are specified where appropriate. Potential to drive the separation process was generated with a Hipotronics (Brewster, N. Y.) model 840A high voltage power supply. Applied voltages ranged from approximately 8 - 40 kV with resulting currents of 10 - 60 μ A. All separations were performed under constant potential conditions.

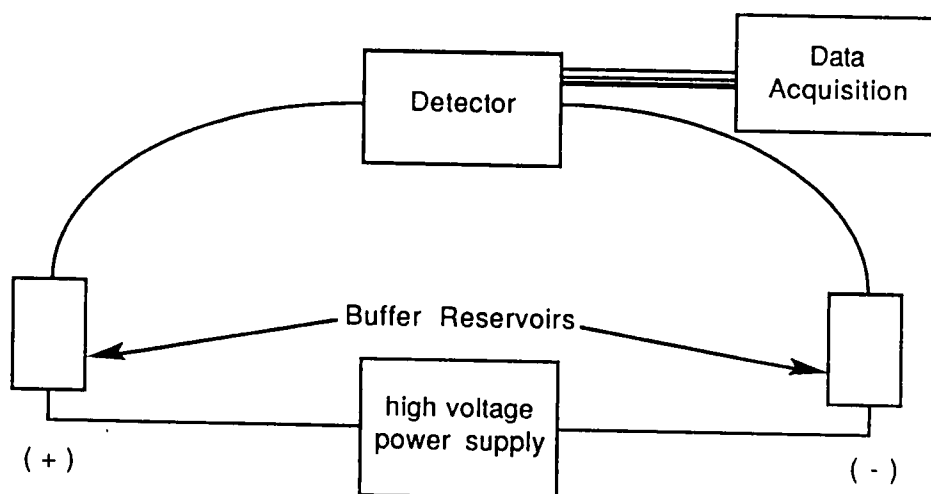


Figure 4: General Experimental Apparatus for CE

Both absorbance and laser-based fluorescence detection schemes were employed in this work and will be described in detail in this chapter. Data was acquired using either strip chart recorders or chromatographic integrators, as specified where appropriate.

Procedures

Column Construction

Capillary columns were constructed by breaking off the desired length and affixing a 16 gauge luer-type needle to the capillary inlet with epoxy. This allowed syringes filled with the appropriate solution to be attached to the needle and hand pressure applied. Prior to filling the capillaries with mobile phase or rinsing solutions, a portion of polyimide coating was removed near the outlet end of the capillary to create a flow cell for optical detection. The appropriate flow cell holder was cemented to the capillary with epoxy or secured via the appropriate fittings.

Injection

Sample plugs were introduced onto the column using both hydrostatic and electroinjection procedures. Hydrostatic injection involves placing the inlet end of the capillary into a vessel containing sample solution and raising the vessel 10-12 cm higher than the outlet end. This proved to be a simple and reliable method and was used for all non-quantitative work. Electroinjection consists of placing the inlet end of the capillary into a sample vessel along with an electrode and applying a low potential (1-5 kV) for a short (10-60 seconds) time. Electroosmotic flow sweeps a plug of sample into the capillary. After injection, the end of the capillary is rinsed with distilled water and replaced in a reservoir containing mobile phase prior to application of running potential. It is important to note that this method is biased toward solutes with high mobility

and steps should be taken to compensate. Electroinjection provides good reproducibility of injection volume and has been well studied (29, 30).

Detection

The high efficiency attained in CE is directly attributable to the narrow bore capillaries employed in the technique. Use of capillaries of these dimensions make commonly employed detection methods problematic and often leads to reduced sensitivity. A variety of detection methods have been employed in CE. The most commonly employed techniques are spectrophotometric absorption and fluorescence. Optical detection methods in CE are performed nearly exclusively on-column; removal of the polyimide coating provides a window transparent to both uv and visible radiation. Other optical detection methods employed to date involve monitoring of the mobile phase refractive index (31), Raman scattering (32), thermal lensing (33), and indirect fluorescence (34).

Non-optical CE detection based on electrochemistry has been developed based on conductivity and amperometry. Conductivity in the non-suppressed mode was achieved for alkali metal ions via use of laser-drilled holes in a capillary for a detection zone (35). Off-column amperometric detection is achieved through the use of a porous glass connection at the outlet end of the capillary to provide electrical connection for the electrophoretic circuit and isolation of the detection electrode (36). Each of these techniques represents negligible extra-column contributions to band broadening.

Mass spectrometric (MS) methods based on electrospray ionization (15, 37) have been developed and provide sensitive detection as well as qualitative information. These interfaces involve coaxial arrangement of separation and reagent delivery capillaries, allowing independent optimization of the separation and ion generation. Ionization occurs immediately after the capillary

terminus, so dead volumes and extracolumn band broadening are negligible. These mechanisms are "soft" ionization techniques which produce abundant molecular ion signals of large thermolabile molecules, thus generating the potential for on-line MS/MS structure elucidation (38). Coupling of the high resolution of CZE with the inherent advantages of MS represents a powerful tool for biochemical analysis.

Absorbance Detection

The most widely used detection modes in CE are based on optical methods. Optical absorbance in the ultraviolet and visible regions of the spectrum has so far proved to be the "workhorse", just as in conventional HPLC. Absorbance is given by

$$A = \epsilon bC \quad (8)$$

where A is absorbance, ϵ the molar absorptivity of the analyte, b the pathlength, and C the molar concentration of the analyte. Absorbance detection in CE suffers from poor sensitivity due to the small optical pathlengths and poor stray light rejection characteristics of the optical arrangements used. However, in many cases, sensitivity is adequate despite this limitation.

In this work, two instrumental configurations were employed for absorbance detection. Early work proceeded using a commercial instrument modified for capillary columns. A Laboratory Data Control (LDC) Model III fixed wavelength HPLC absorbance detector was modified by using fiber optics to transmit uv light emitted from an external Cd lamp (UVP, Palo Alto, CA) through the CE capillary to the signal and reference diodes (Figure 5). The 229 nm wavelength was isolated with an interference filter (Oriel Corp., Stratford, CT) centered at 229 nm, approximately 10 nm FWHM. Key to this arrangement was

the use of laser-etched flowcells developed by a coworker. These flowcells allowed transmission of light through the capillary flow channel and good rejection of stray light (25). These flowcells were fabricated by burning the polyimide coating away from the detection zone, cementing the capillary between two microscope slides, and covering this assembly with a thin coating of flat black paint (Badger Air Brush, Franklin Park, IL). The painted assembly was then affixed to an aluminium holder (constructed in-house) and filled with distilled water. Radiation from an Argon ion laser (Coherent, Palo Alto, CA) operated at 488 nm and 0.8-1.0 watts was focused to a beam waist approximately 50 μm in diameter to burn the paint from the

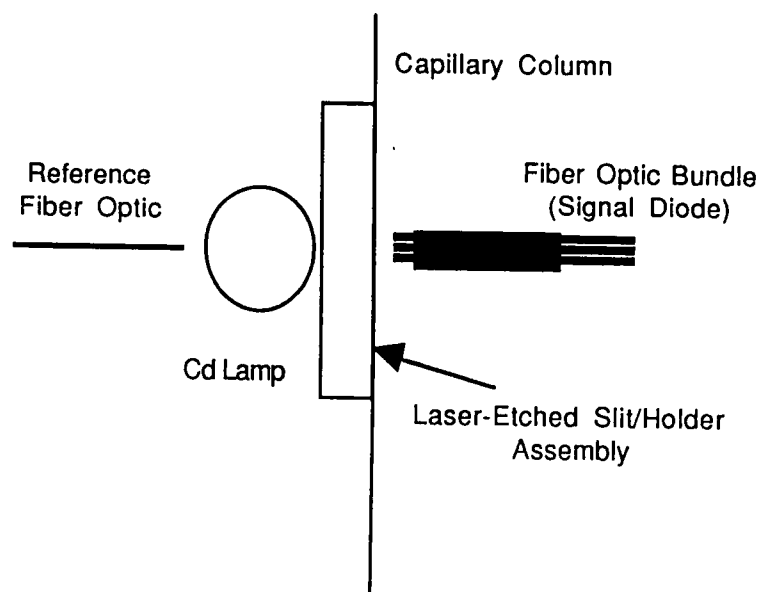


Figure 5: Laser-Etched Absorbance Flowcell Apparatus

flowcell area. The column was translated through the beam via the use of a fiber optic positioner (Newport Corp., Model FP-1). Optical slits were made by

blocking all of the laser-etched slit except for approximately 0.5-1.0 mm with black electrical tape.

Alignment was achieved by placing a bundle of three 400 μm diameter fiber optics in front of the slit and minimizing the absorbance reading (maximizing light transmission) and then balancing the observed absorbance reading to zero with the 200 μm reference fiber (see figure) on the opposite side of the Cd lamp. Normally, this detection apparatus required a warm-up period of 30-45 minutes prior to use to assure a stable baseline.

An alternate method was developed to circumvent the tedious and time consuming laser-etching process. A flow cell holder was constructed from aluminum blocks which allowed quick column replacement and low stray light. This apparatus consisted of two blocks separated by two small pieces of shim stock obtained locally. The shim stock was available in several different thicknesses, allowing a variable sized slit to accommodate different capillary diameters. A groove was cut down the center of the two blocks to allow the capillary to be easily centered on the slit. Capillaries were held in place with a Teflon/sheet metal sandwich affixed to the main assembly with two set screws. A schematic diagram is presented in Figure 6. Stray light levels were evaluated by filling the capillary with a 50/50 solution of isoquinoline/acetonitrile and noting the absorbance reading. The reading was converted to % transmittance ($\text{Transmittance} = -\log(\text{Absorbance})$) and termed the stray light level. Readings obtained with this apparatus were in the range of 1-5%, approximately the same as obtained with the laser-etched slit (25).

More recent work relied on a Linear 204 uv-vis absorbance detector (Linear Instruments, Reno, NV). Capillaries are simply inserted into the optical path and held in place with two ferrule/nut assemblies. Operation and column fabrication is thus considerably less difficult than with the previous system and

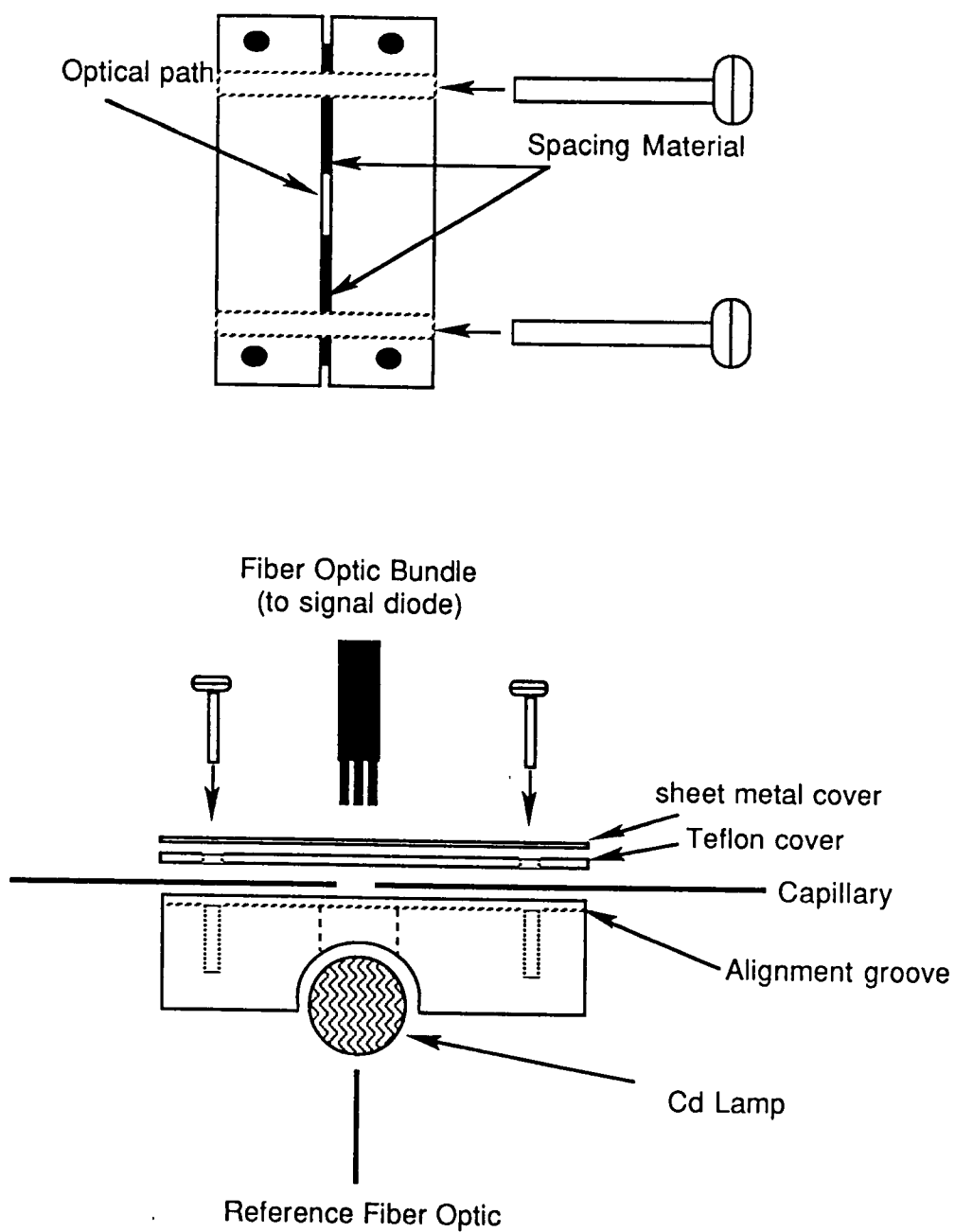


Figure 6: Split-Block Flowcell Holder for CE Absorbance Detection

columns can be rapidly changed. Alignment of the flow cell with the light path was performed by removing the cover from the capillary holder and adjusting the instrument to a visible wavelength. The portion of the column with the polyimide coating removed could then be exactly centered in the light path. In addition to ease of capillary replacement, the Linear 204 instrument has variable wavelength capability, allowing operation at an optimum wavelength for a given application.

Fluorescence Detection

Fluorescence detection is a widely used detection technique in CE due to the relative simplicity and high sensitivity attainable. Fluorescence intensity, I_f , is given by

$$I_f = I_0 \Phi \epsilon b C \quad (9)$$

where I_0 is excitation intensity, Φ quantum yield of the analyte, and $\epsilon b C$ the identical meaning as in Equation 7. Equation 8 is valid for small (0.05) values of absorbance. High sensitivity is attained for fluorescent analytes since emission intensity is linearly related to excitation intensity. Laser-based fluorescence detection is especially useful in CE due to the high excitation powers, collimation, and monochromaticity characteristic of laser emission. Moderate power (5-10mW) laser output beams are easily focused into capillaries as small as 25 μm and represent highly intense excitation powers relative to more conventional sources. The high degree of monochromaticity adds to the available power since wavelength selection devices such as filters and gratings are not often required.

The primary limitation of fluorescence detection is that relatively few molecules exhibit native fluorescence. Derivatization of non fluorescent

analytes is generally carried out prior to injection, since post column reactors often cause band broadening due to excessive dead volume associated with them. In this manner, a wider variety of molecules can be detected via fluorescence. Additionally, laser radiation is limited to discrete wavelengths, further restricting options in terms of native fluorescence and for pre-column derivatization. Laser instrumentation is also significantly more expensive to obtain, maintain, and operate than conventional sources.

Laser-based fluorescence detection was achieved in this work with the experimental arrangement depicted in Figure 7. On-column flow cells were

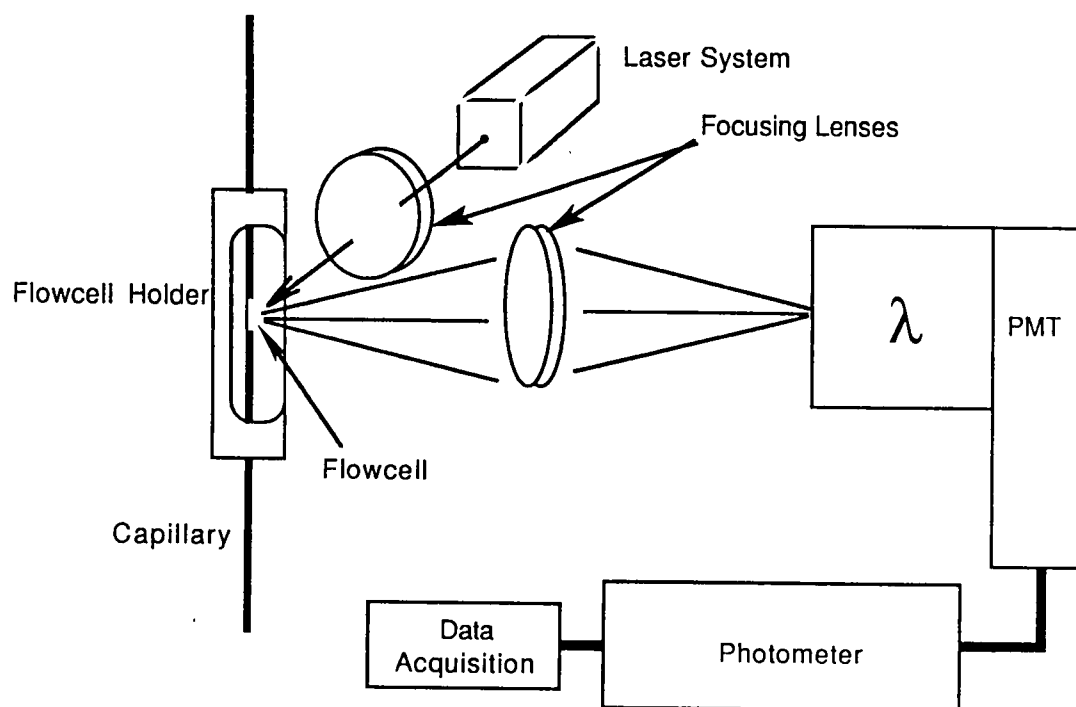


Figure 7: Laser-Based Fluorescence Detection Apparatus for CE

prepared for fluorescence detection by removing a small section of polyimide coating and affixing the resulting flow cell in a cylindrical holder with a u-shaped opening. The holder was then inserted into a fiber optic positioner (Newport Corp., Model FP-1) and aligned with the optical system. Alignment of the capillary is achieved by noting far-field images. Proper focus of the excitation beam is achieved when the transmitted beam is symmetrical (generally oval in shape) when observed in the far field. Positioning of the wavelength selection device (λ in the figure) is achieved by drawing up a concentrated (ca.. 10^{-4} M) solution of fluorophore into the flow cell and focusing the resulting spot of emitted light onto the entrance slit. Specific details concerning laser systems, lenses, and wavelength isolation devices are given where appropriate in later chapters.

Chapter 3

Bile Salt Surfactants in MECC: Hydrophobic Molecule Separations

Introduction

Surfactants and Micellization

Surfactants are molecules which possess distinct hydrophobic and hydrophilic regions, typically an n-alkyl chain connected to a highly polar or ionic group. These molecules may be thought of as possessing a "head" and a "tail" for the polar and hydrophobic regions, respectively. Surfactants may be anionic, cationic, zwitterionic or neutral in nature. They are able to undergo self-association to form dynamic entities known as micelles. In aqueous solution, the surfactant molecules orient their hydrophobic tails in such a way as to minimize contact with water. Termed "normal" micelles, the interior of an n-alkyl type surfactant has a high viscosity and is considered to be similar to a liquid hydrocarbon environment (39). The hydrophilic headgroups are in close contact with the polar solvent. In the case of an ionic surfactant such as SDS, the resulting micelle has a net charge (negative in the case of SDS). A simple representation of the SDS monomer and a cross-sectional view of an SDS micelle are presented in Figure 8.

The inverse situation exists for certain surfactants, when the solvent is apolar and the ionic or polar head groups turn inward away from the bulk solution. Termed "reverse" or inverse micelles, the formation of these aggregates requires the presence of small amounts of water, since the polar head groups congregate around small "pools" of water. An example of a system which forms reverse micelles is Aerosol OT/isooctane system. This work

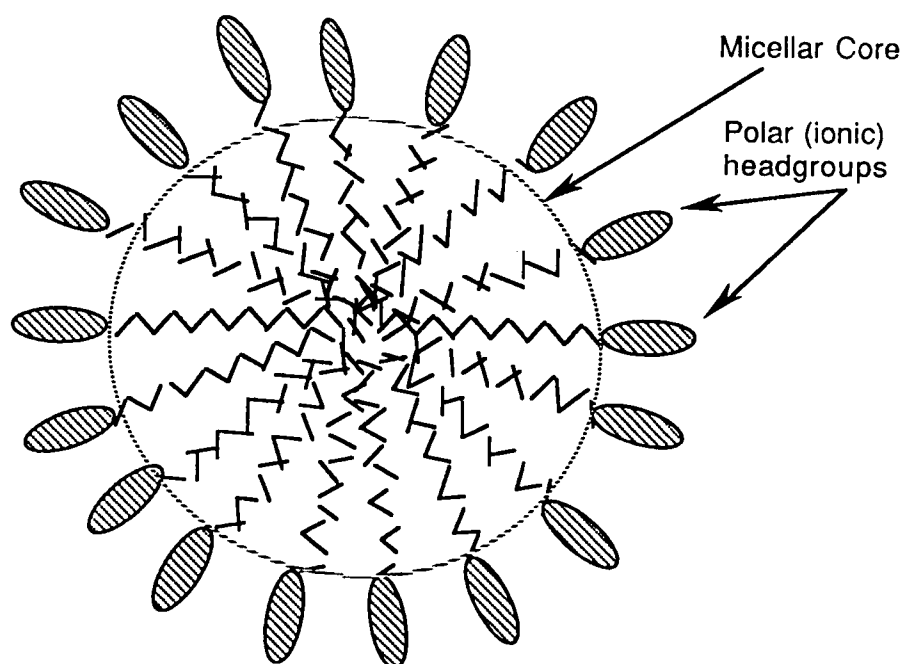
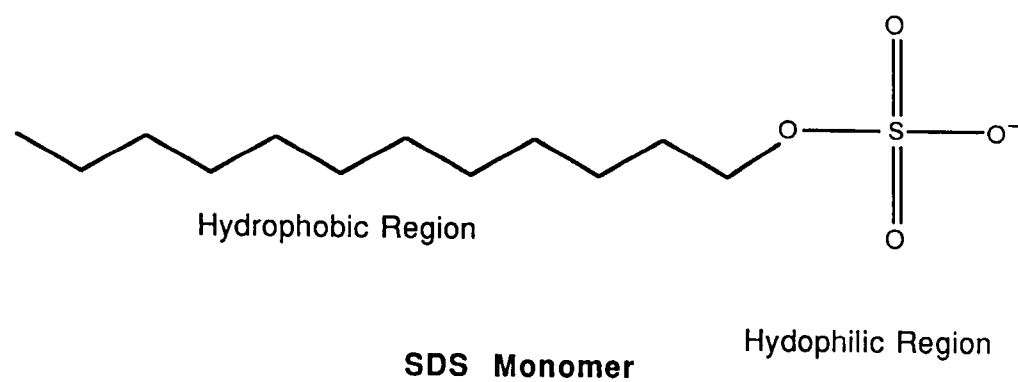


Figure 8: Structures of SDS Monomer and Micelle

deals exclusively with surfactants forming normal micelles in aqueous solution.

Surfactants and the (normal) micellar aggregates associated with them are characterized by three primary parameters, the Krafft temperature, aggregation number and the critical micelle concentration (CMC). The Krafft temperature is that temperature below which micelles cannot form. This temperature is specific for a given surfactant and is a fundamental parameter. All the work reported herein is above the Krafft point for the surfactants studied. Aggregation number (N) is defined as the number of monomers per micelle for a given surfactant. This number is an average value, since the micelle is a dynamic structure. Micelles may thus be polydisperse in nature with a wide range of aggregation numbers (40). Under a specified set of conditions (temperature and pressure), micelle formation is not observed below a certain surfactant concentration. As monomer concentration increases, a point is reached where self-aggregation occurs. Further increases in surfactant analytical concentration result in the formation of more micelles, while the actual free surfactant concentration remains at the CMC. The point at which the CMC is reached is concomitant with a dramatic change in several bulk solution properties such as conductivity, surface tension, viscosity, and light scattering, among others (39). It is important to note that the method of determination of the CMC can influence the value obtained, since different properties are measured. For instance, methods involving monitoring light scattering intensity are sensitive to micelle concentration, whereas methods involving surface tension measurement are sensitive to monomer concentration. In fact, some methods which employ probe molecules may disturb the micellization process to a significant extent (41). Several experimental parameters have an appreciable affect on the CMC and on the size and shape of a micellar aggregate. Solution properties such as ionic strength, pH, and organic solvent content alter the way

strength, pH, and organic solvent content alter the way monomers aggregate. In general, CMC decreases with increasing ionic strength, and identity of the electrolyte (e. g. mono- vs. multivalent cations), and increases with organic solvent concentration. Surfactant concentration is important in determining the type of aggregate behavior. In addition to "regular" micelles, surfactant assemblies such as cylindrical micelles, vesicles, middle and neat phase structures can be formed, depending on the surfactant concentration (39). More detailed discussions on these topics have been published (39, 42).

Micelle Enhanced Fluorescence

Fluorescence intensity of a micelle-solubilized probe is generally enhanced upon micelle formation. Reduction in the local viscosity experienced by the solubilized probe molecule is believed to be primarily responsible for this effect, although other enhancement mechanisms may be responsible (43, 44). Serendipitously, this effect has analytical application in fluorescence detection. In techniques such as MECC, which *requires* interaction of solutes with a micellar phase for separation, micellar enhanced fluorescence can often improve sensitivity. One particularly interesting instance of micellar enhancement involves 1-nitropyrene, a poorly fluorescent molecule due to the presence of an electron-withdrawing nitro group on the pyrene ring. Figure 9 shows a comparison of fluorescence intensity in several solutions. It is interesting to note that the bile salt surfactant (depicted in figure 10), sodium cholate (NaC), shows a significantly greater enhancement than the SDS. Choice of surfactant for use in MECC may thus have ramifications on fluorescence detection sensitivity.

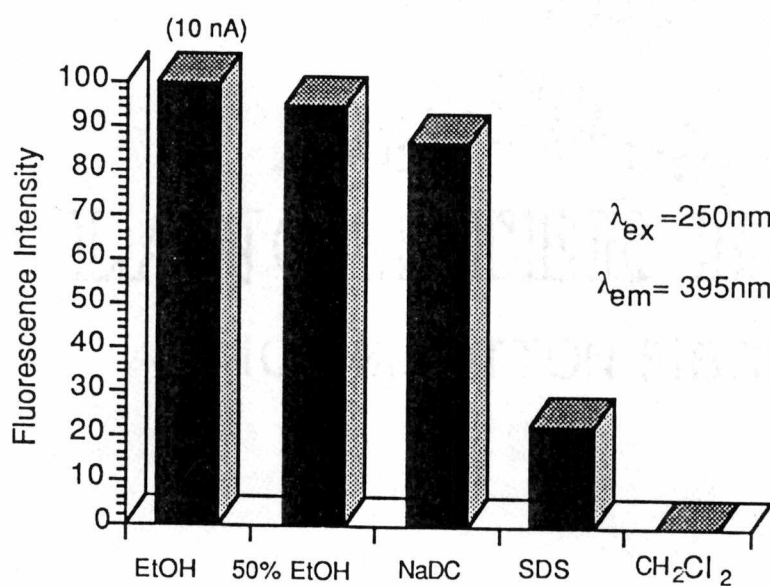


Figure 9: Nitropyrene Fluorescence in Various Solvents

Surfactant use in Chromatography

Micellar solutions have been employed as mobile phase additives in conventional HPLC. In the reversed-phase mode, surfactants such as SDS can be used in gradient elution. Increasing the micelle concentration causes reduction in mobile phase polarity much like increasing organic solvent concentration. Purely aqueous micelle solutions, however, suffer from poor efficiency unless 3-5% 1-pentanol is included in the mixture (42). Two advantages are associated with the use of micellar mobile phases. First, since monomer mobile phase concentration is constant at the CMC value, long equilibration times between gradient runs are not required. Surfactant adsorption to the stationary phase is not altered unless concentrations below the CMC are used. Second, the non-toxic nature of surfactants such as SDS represent a safer situation. Disposal costs are eliminated in most cases, and aqueous surfactant solutions are non-flammable. The diverse types of surfactants available represent alternate chromatographic selectivities.

Surfactant use in CE

Fundamental to chromatographic selectivity in MECC is the structure of the surfactant employed as the pseudostationary phase. The most commonly used surfactant in MECC to date is SDS. SDS forms roughly spherical micelles with an interior similar to a liquid hydrocarbon environment, *i. e.*, very hydrophobic. Chromatographic selectivity roughly parallels that of reversed phase HPLC, in that retention increases for more hydrophobic molecules. Selectivity is often different for molecules possessing ionic or polar functionalities due to the ionic nature of the micelle surface. Ionic interactions with the surface of the micelle can cause coulombic repulsion with like charged solutes, as well as attraction for opposites. In some cases, separation efficiency may be adversely affected as a result of a kinetically slow (relative to simple

solubilization) mechanism involving a polar solute and the micelle exterior (45). Also, solutes possessing polar functionalities can show different selectivities for surfactants with different headgroups. Selectivity differences of cationic (quaternary ammonium) and anionic (sulfonic acid) n-alkyl surfactants have been directly compared (46). As discussed in the introduction, when SDS is used as the pseudostationary phase, separations of extremely hydrophobic molecules such as polyaromatic hydrocarbons (PAH) are often unsuccessful. Hydrophobic species usually co-elute at t_m despite the addition of large ($\geq 20\%$) amounts of organic solvent to the mobile phase. Additives such as cyclodextrins have been used with SDS to separate compounds such as PAH, chlorinated biphenyls and dioxins (47). This work presents an alternate approach involving the use of bile salt surfactants in conjunction with organic solvents.

An alternative surfactant system, the bile salts, have found application in MECC. Bile salts exhibit several useful chromatographic properties such as the ability to recognize specific enantiomeric conformations (48, 49, 50) and increased micelle polarity (48, 51, 52). Bile salts are biological in nature and aid in solubilization of substances such as cholesterol within the body (53). Based on a hydroxyl-substituted steroid backbone, these surfactants possess both hydrophobic and hydrophilic "faces". Figure 10 gives the structures and micellization data for the surfactants compared in this work. While the physical properties of bile salts have been extensively studied (41, 53), there has been some controversy over the structure of their micelles. In 1968, Small introduced a model in which the hydrophobic faces of the monomer units are proximal and the protruding hydroxyl groups face the bulk solution (54). This model, based on logical consideration of space-filling molecular models and interfacial

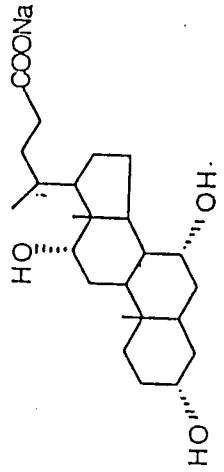
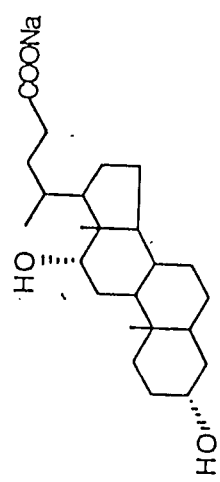
Surfactant	Structure	CMC, mM	N
Sodium Cholate		12.5	3
Sodium Deoxycholate		6.4	4
Sodium Dodecylsulfate	$\text{CH}_3-(\text{CH}_2)_{10}-\text{CH}_2-\text{OSO}_3^- \text{Na}^+$	8.1	62

Figure 10: Surfactants Studied in MECC.

behavior of the monomer units, has recently been expanded in light of more sophisticated experimentation. Recent studies (55, 56, 57) suggest that the aggregates in question form helical micelles with the *hydrophobic* portions of the monomer facing the aqueous solution while the *hydrophilic* portions turn inward. Thus, in some respects, the bile salt micelle can be envisioned as an "inverted" micelle. Studies with fluorescent and spin-label probe compounds lend further support to this argument (57, 58). These studies showed that the interior of the bile salt aggregate is much more polar than that of SDS.

The fact that the bile salt monomer is more polar than SDS leads to a general reduction of k' in MECC (48, 51, 52, 59). This is particularly advantageous in dealing with hydrophobic compounds, as separations which prove difficult with conventional SDS systems are more easily attained with bile salts. In addition, the unique inverted bile salt micelle appears to tolerate high concentrations of solvents such as methanol without drastic loss of efficiency or dramatic increase in analysis time. These desirable characteristics allow separations of hydrophobic compounds never before performed with MECC. This chapter presents several separations of hydrophobic compounds using bile salt mobile phases with high organic modifier content. Investigations of the effects of methanol on the critical micelle concentration for both SDS and sodium cholate, a trihydroxy bile salt, are also reported. Reasons for the tolerance of the bile salt micelles to organic solvent are discussed.

Experimental

Apparatus

Both absorbance and laser-based fluorescence were used in this study. Fluorescence excitation was provided by a Liconics model 423OMB helium-cadmium laser (Sunnyvale, CA) operated at 325 nm. Collection of fluorescence emission was as described in chapter 2. Separation and detection parameters

such as detection wavelengths, column length, mobile phase composition, and applied voltage are given in the text, where appropriate. Absorbance spectra for CMC studies were measured on a Perkin-Elmer model lambda 3B dual-beam spectrometer. The sample cuvette was maintained at 30 °C with a Perkin-Elmer model 0050405 temperature controller bath.

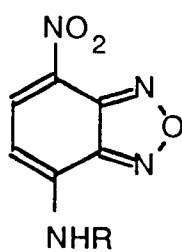
Chemicals

Sodium cholate (NaC), sodium deoxycholate (NaDC) and SDS were obtained from Sigma (St. Louis, MO.). Rhodamine 6G, amines, and 7-chloro-4-nitrobenzo-2,1,3-oxadiazole (NBD-Cl) were purchased from Eastman Kodak (Kingsport, TN.). NBD-Cl derivatization of amines has been described previously (60). All solvents were of HPLC grade and were purchased from Baxter Chemical Co. PAH and aflatoxins were obtained from Aldrich Chemical Company (Milwaukee, WI). Benzo(a)pyrene tetrol was obtained from Midwest Research Institute (Kansas City MO). Structures for compounds studied are depicted in Figure 11.

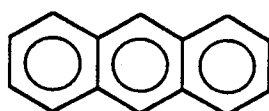
Procedures

Injectons were made by a common syphoning procedure (25). Column pretreatment and equilibration were as described in chapter 2. Due to the very limited water solubility of some of the compounds studied, the samples were dissolved in methylene chloride and extracted into mobile phase or further dissolved in methanol followed by dilution with an approximately 0.1 M surfactant solution. Injectons from pure organic solvent were avoided to prevent problems with electric field distortions and loss of current. Capacity factor values were calculated via Equation 4.

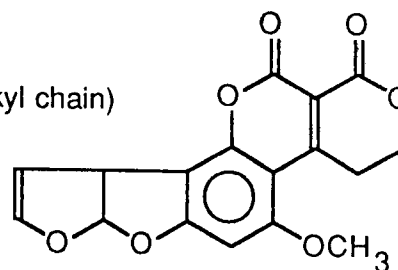
CMCs were estimated via dye solublization as described by Small (41). This procedure consists of measuring the wavelength of maximum absorbance



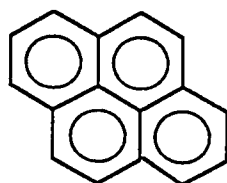
NBD-Amines (R = n-alkyl chain)



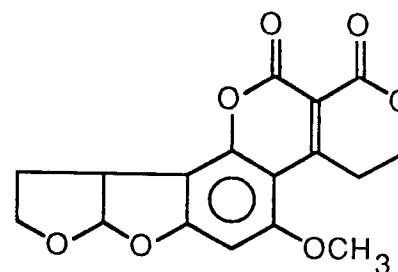
Anthracene



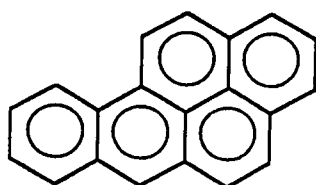
Aflatoxin G1



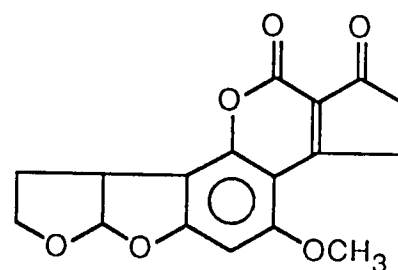
Pyrene



Aflatoxin G2



Benzo[a]Pyrene



Aflatoxin B2

Figure 11: Hydrophobic Compounds Studied

for a dye (Rhodamine 6G) under varying concentrations of bile salt and methanol. The wavelength of maximum absorbance ($\lambda_{\max}$) is measured for each concentration and plotted against the logarithm of the surfactant concentration. All $\lambda_{\max}$ values are the average of three measurements.

Results and Discussion

In first evaluating the bile salts for hydrophobic separations, a well-characterized test mixture (methyl, propyl, pentyl, octyl (C_8), decyl (C_{10}), dodecyl (C_{12}) and tetradecyl (C_{14}) amine) of derivatized n-alkyl amines was employed. The late eluting NBD derivatives (C_8 - C_{14}) serve to gauge hydrophobic compound separation performance as well as to mark the micelle migration time. Figure 12 shows the separation of these compounds with NaC and SDS. Retention time increases with increasing carbon chain length, and elution order is the same for both systems. Note that the late-eluting solutes are partially resolved with the NaC. As mentioned above, the bile salts are relatively polar and, consequently, provide a general reduction in k' values for most solutes. The reduction in k' values is attributed to the presence of hydrophilic hydroxyl groups on the bile salt monomer. Table I shows k' data (obtained via equation 5) for another set of NBD amines for SDS and the bile salts. As the degree of hydroxyl substitution in the bile salt, (hence polarity) increases k' is further lowered. Thus, the trihydroxy sodium cholate (NaC) yields lower values than the dihydroxy sodium deoxycholate (NaDC).

A more significant example of the capabilities of MECC using bile salts is shown in Figure 13; the separation of the aflatoxins G_2 , G_1 , and B_2 with a sodium deoxycholate (NaDC) pseudostationary phase. Aflatoxins are extremely toxic compounds found in spoiled foodstuffs (61). Baseline separation of these compounds has been reported with SDS, but only after

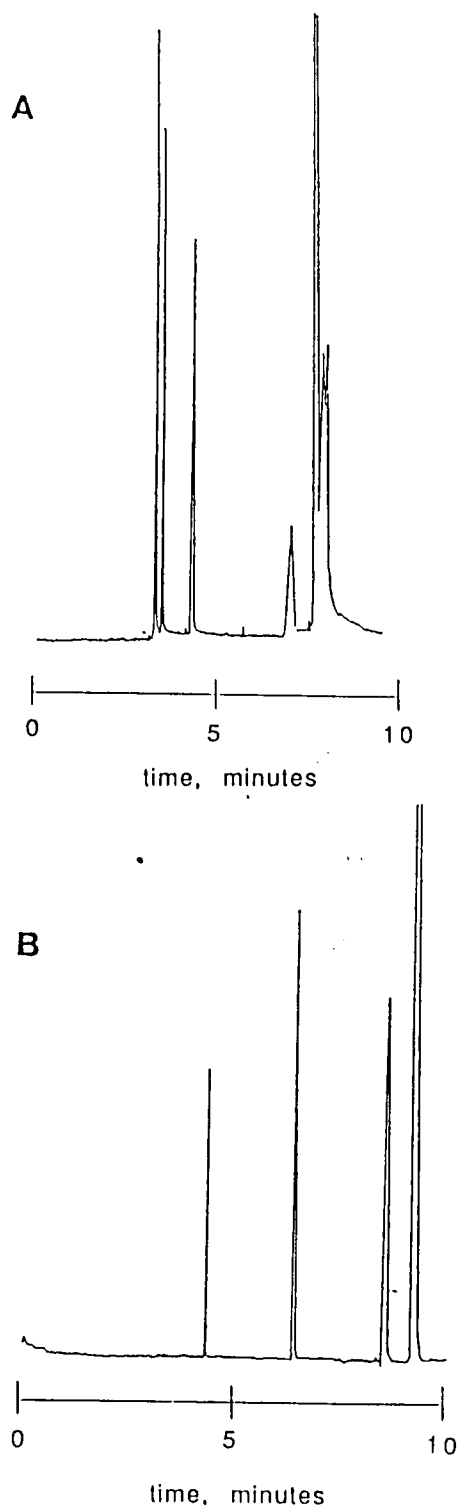


Figure 12: Separation of NBD-amines with NaC (A) and SDS (B).
 Mobile phase: 0.05 M Surfactant, 0.01M Na_2HPO_4 ,
 0.006 M $\text{Na}_2\text{B}_4\text{O}_7$. Applied Voltage: 15kV. Column
 50 cm, 45 cm to detector, 50 μm i. d. Detection: Ar⁺ laser
 Fluorescence, λ_{ex} 488 nm, λ_{em} 540 nm.

Table I: n-alkyl amine-NBD Capacity Factor Data for Bile Salts and SDS

k'	NaCholate	NaDeoxycholate	SDS
Methylamine-NBD	0.56	0.52	0.81
Propylamine-NBD	0.77	0.93	4.32
Pentylamine-NBD	1.72	2.96	32.5
Octylamine-NBD	3.97	5.15	infinite (t_m)

All data aquired at 15 kV, 20 μ A

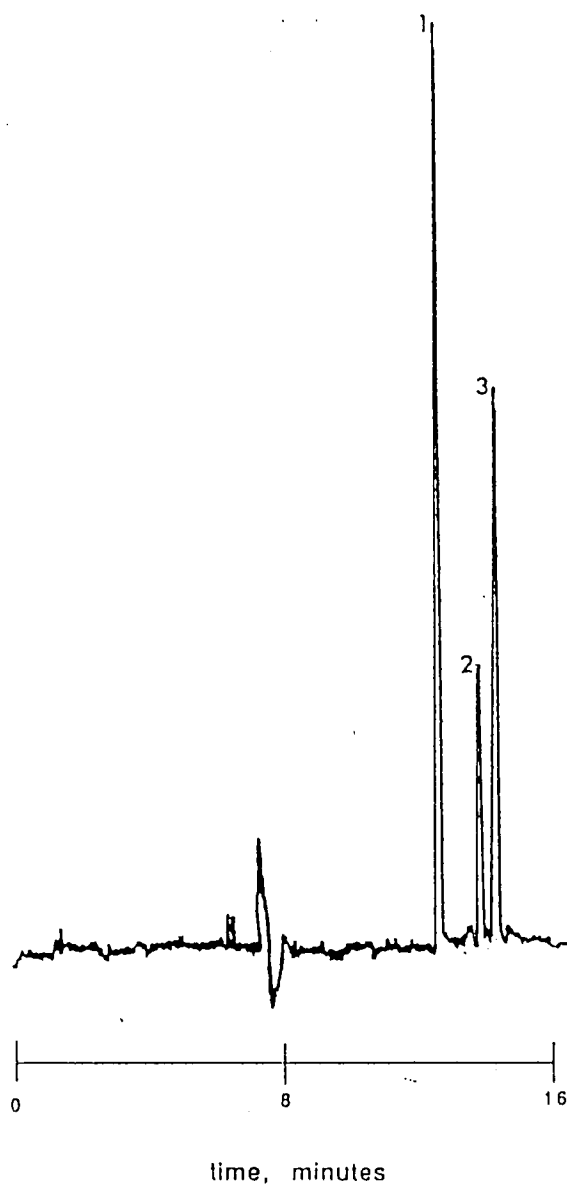


Figure 13: Separation of Aflatoxins with NaDC. Mobile phase: same as Figure 12. Elution Order: G₂, G₁, B₂. Applied voltage 16 kV. Column length 53 cm, 50 cm to detector, 50 μ m i. d. Detection: laser fluorescence λ_{ex} 325 nm, λ_{em} 450 nm.

addition of organic solvent (62). In this case, baseline resolution is obtained for all three compounds without the use of organic modifiers, thus saving analysis time (see introduction). Coupled with the sensitivity of laser-based fluorescence, bile salt MECC offers a rapid assay for these toxins.

Further improvements in resolution are attained when methanol is added to the mobile phase. Figure 14 shows the separation of the aforementioned NBD-amines using a mobile phase containing 20% methanol. With the NaC system (A), all of the more hydrophobic (C_8 - C_{14}) amine derivatives are fully resolved, with reasonable efficiency. In contrast, the SDS system (B) allows resolution of only the C_8 from the C_{10} - C_{14} amine derivatives which co-elute at t_m . In addition, analysis time for the SDS is roughly three times that for the NaC. Efficiency is much poorer with the SDS system, presumably due to the adverse effects of micelle polydispersity. Micelle polydispersity can result in micelles with a range of electrophoretic mobilities. Solutes that associate with these micelles can experience a similar range of velocities and thereby become dispersed. This mechanism is thought to be a significant source of band dispersion in MECC (24, 63). The micelle polydispersity problem is most acute at or near the CMC (24, 48). Addition of organic solvent can lead to the onset of this problem at surfactant concentrations where this is usually not the case. When methanol is added to the mobile phase, the overall solution polarity is decreased. This presumably reduces the affinity of the hydrophobic tails of the SDS monomers for one another, causing an increase in CMC (64). Conversely, the helical structure of the bile salt micelle, since it resembles a reversed or inverted micelle, might be expected to be less vulnerable to disassociation by a reduction in the polarity of the bulk solution. The methanol-modified mobile phase might be favored by the hydrophobic face of the monomer, possibly stabilizing the micelle relative to SDS.

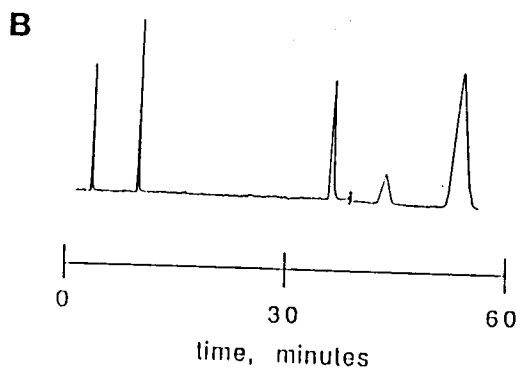
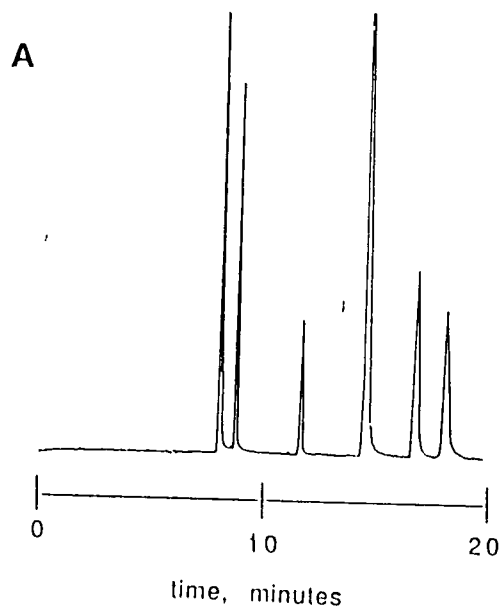


Figure 14: Separation of NBD amines using NaC and SDS with 20% MeOH. Conditions identical with figure 12 except mobile phases are 20% in MeOH. (A) NaC, (B) SDS.

The impact of methanol content on micelle formation was studied by estimating the CMC for both SDS and NaC under MECC mobile phase conditions. Dye solubilization was the method used in this study (41). The wavelength of maximum absorbance was measured between 520 and 580 nm for Rhodamine 6G at several different NaC concentrations. Plots of $\lambda_{\max}$ vs. $\log[\text{NaC}]$ were constructed over a range of methanol concentrations. Figure 15 shows a sample plot. The first shift in $\lambda_{\max}$ is taken as an estimate of the CMC. As the concentration of NaC is increased past the CMC, $\lambda_{\max}$ continues to shift until a plateau is reached. The shape of the plot is assumed to be the result of varying degrees of dye association with the micelle. As more micelles are formed, the dye interacts with increasing strength until a point is reached where the dye is completely micelle solubilized, causing the wavelength shift to reach a maximum. The actual CMC could be taken as this plateau, the first shift, or the midpoint. We have arbitrarily chosen the first break point after the work of Small (41). It should therefore be emphasized that the CMC values presented here are arbitrary estimates of the true CMC. The dependance of the CMC for SDS and NaC on % methanol are shown in Figure 16. The CMC for SDS begins to rise at methanol levels above approximately 10%, while NaC stays nearly constant until 30% methanol. Chromatographic efficiency for SDS reflects these trends (24). As mobile phase methanol content increases, the efficiency with the SDS system shows a steady decrease. Thus, the ability of NaC to tolerate the presence of organic solvent without significant loss of efficiency enhances the capabilities of MECC with regard to the separation of hydrophobic compounds.

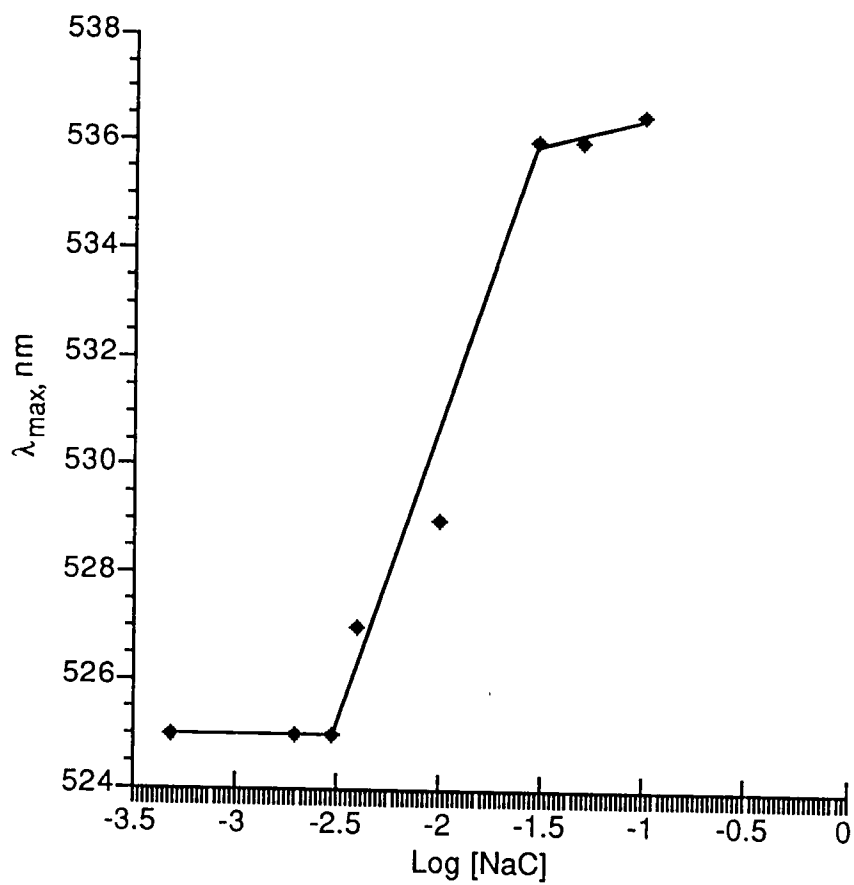


Figure 15: Plot of λ_{max} for Rhodamine 6 G vs. Surfactant Concentration

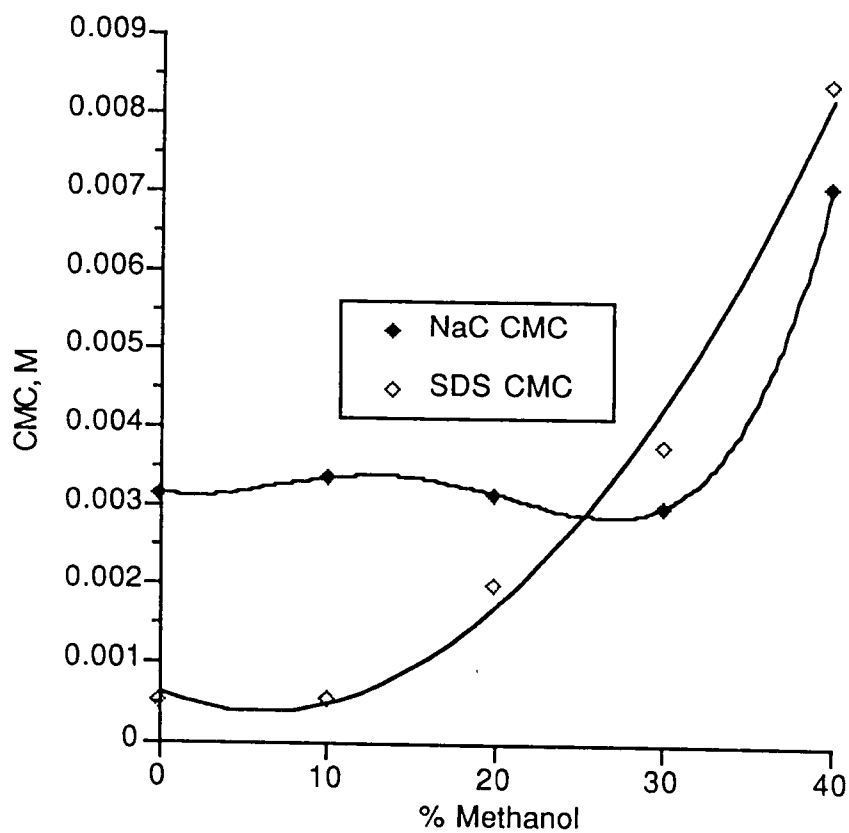


Figure: 16: CMC behavior of NaC and SDS vs. MeOH Concentration

One of the more challenging separation problems for MECC is that of polycyclic aromatic hydrocarbons. To date, there are only two reports of PAH separations with electrophoretic techniques. The earliest approach to hydrophobic neutrals was a CZE separation based on solvophobic interactions leading to charged complexes. Ionic PAH complexes were separated by the usual CZE mechanism (19). A more recent approach involving micellar solutions and cyclodextrins has also been applied to the separation of certain relatively small molecules (47). Bile salt MECC provides relatively efficient separations of PAH as large as pyrene and benzo(a)pyrene (B(a)P), and more general applicability than other electrokinetic-based approaches. Figure 17 is the separation of anthracene, pyrene, and benzo(a)pyrene (B(a)P) with a NaC system. PAH larger than B(a)P can also be addressed by increasing the amount of organic modifier in the mobile phase. Baseline resolution of all three solutes is achieved at only 10% methanol, well in the efficient working range for NaC. At 20% methanol, the resolution is further increased without drastic loss of efficiency, suggesting that PAH larger than B(a)P might be separated.

Substituted PAH such as hydroxy derivatives may also be separated with these systems. While more polar than unsubstituted PAH, these compounds are difficult to address with SDS pseudostationary phases. Hydroxy-substituted PAH play a critical part in determining the carcinogenicity of PAH, and metabolic pathways almost invariably terminate in hydroxy derivatives (65). Figure 18 shows the separation of B(a)P from benzo(a)pyrene tetrol, one of the final forms in the metabolic pathway. This separation suggests that MECC could find application in monitoring ingestion and metabolic kinetics of PAH in biological systems.

Bile salt surfactants clearly exhibit practical advantages over conventional n-alkyl surfactants. Selectivity and applicability of MECC is

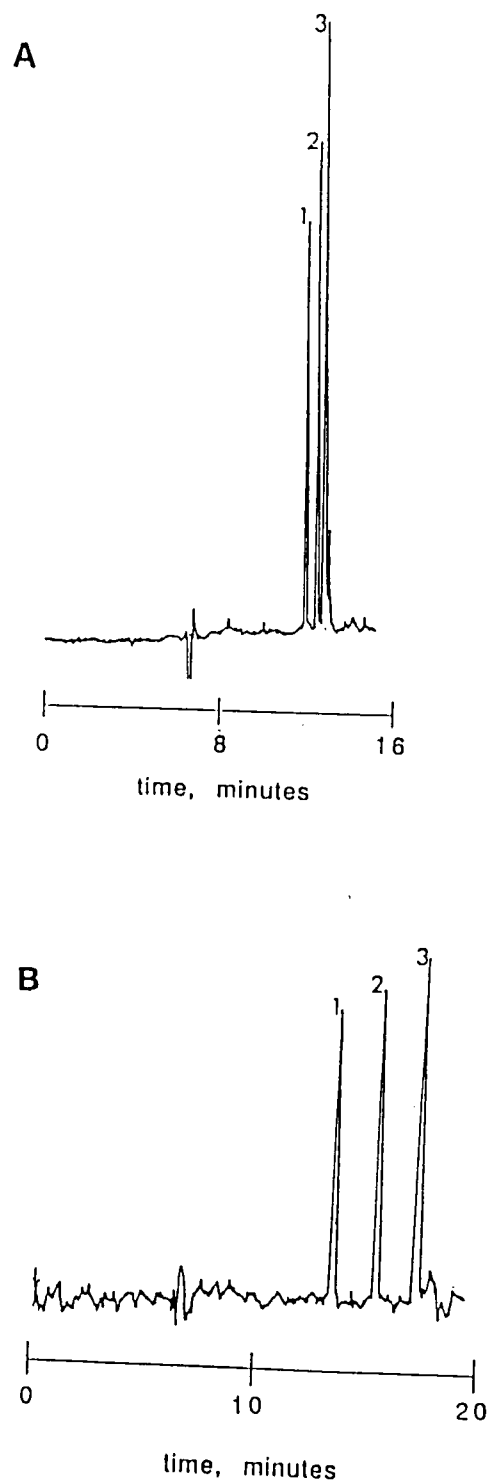


Figure 17: Bile Salt Separation of PAH. Mobile phase 0.05 M NaC, 0.01M Na_2HPO_4 , 0.006 M $\text{Na}_2\text{B}_4\text{O}_7$ with (A) 10% MeOH and (B) 20 % MeOH. Applied Voltage 20kV; Column length 53 cm total, 50 cm to detector, 50 μm i. d. Detection: HeCD laser fluorescence, λ_{ex} 325nm, λ_{em} 450 nm.

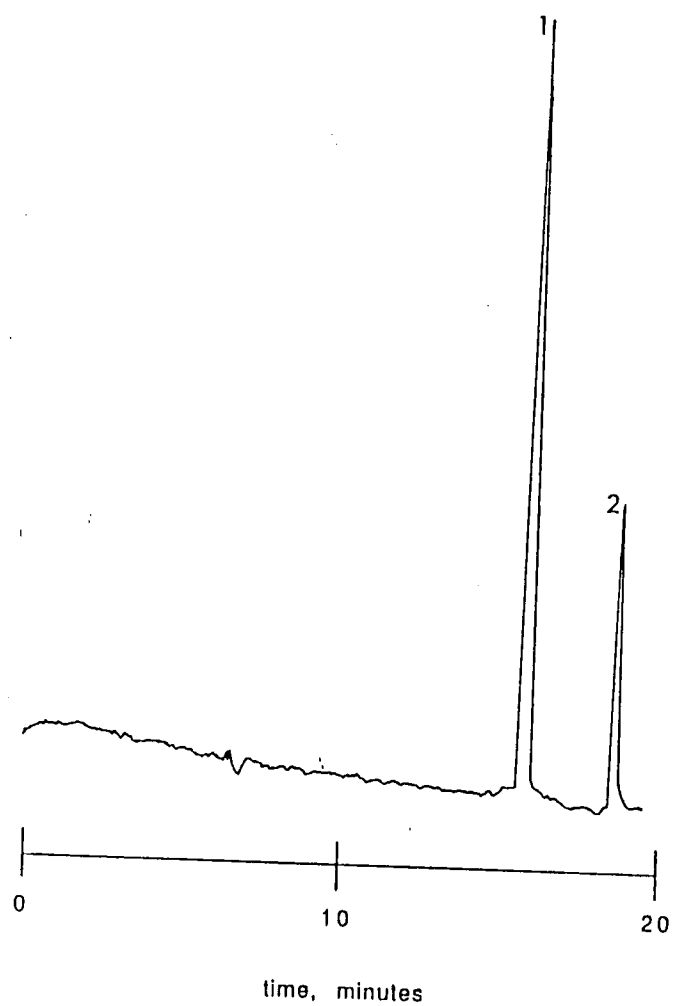


Figure 18: Separation of B(a)P tetrol and B(a)P. Mobile phase 0.05 M NaC, 0.01M Na₂HPO₄, 0.006 M Na₂B₄O₇. Applied Voltage 16kV. Peak Identity: (1) B(a)P tetrol, (2) B(a)P. Other conditions same as in figure 17.

expanded by their use. Unique selectivity is achieved by the highly ordered nature of the micelles; chiral separations are possible (see chapter 5). In addition, the polar interior allows increased resolution for hydrophobic compounds due to general reduction of k' , extending the utility of the technique to previously intractable separation problems. The inverted structure of the aggregates appears to permit relatively undisturbed micelle formation in the presence of organic modifiers, thereby allowing greater peak capacity. In addition, the data presented herein also offers indirect support for the helical structure model of the bile salt micelle.

Chapter 4

Experimental Factors Affecting High Speed MECC Separations

Introduction

It is well established that capillary electrokinetic separation techniques such as capillary zone electrophoresis (CZE) and micellar electrokinetic capillary chromatography (MECC) are capable of highly efficient separations of minute amounts of many classes of compounds (21, 45, 66). The MECC technique addresses the separation of neutral molecules and offers additional selectivity, relative to CZE, for charged analytes via analyte-specific interactions with the micellar phase. In addition to high efficiency, CZE and MECC can be optimized for speed. Recent reports demonstrate the extremely rapid (2 second) separation of fluorescently derivatized amino acids (67, 68). Very small capillary columns (*ca.* 10 μm i. d.) and a unique photoinjection system were employed. This system, while permitting rapid separations, is thus far limited to molecules labeled with fluorescein isothiocyanate (FITC). Rapid (<1 minute) separations are demonstrated herein using capillary electrokinetic methods with conventional injection and detection instrumentation.

Rapid separations are particularly desirable in situations where large numbers of samples are to be analyzed. As applications for MECC and CZE increase, analyses requiring high sample throughput are inevitable. Rapid separations require top performance from the chromatographic system employed. Conventional HPLC approaches generally employ short (~ 3 cm) columns packed with small (~ 3 μm) particles. The combination of high flow rate

and short column provides for short elution times, while the small particle packing diameters provide relatively efficient mass transfer even for moderately high flow rates. Adversely, extremely high flow rates can degrade efficiency resulting in loss of resolution and, due to the use of very small column packing material, produce very high column pressure drops that often result in settling of the packing bed.

In situations where highly efficient liquid phase separations of neutral molecules are required, MECC can be very effective (63, 69). Unlike OTLC, MECC is not limited by mobile phase resistance to mass transfer (10). High efficiency in the former separation technique is only achieved if solutes rapidly diffuse radially across the parabolic flow profile of the mobile phase, thereby averaging the velocity differences encountered. The large flow rates needed for rapid separations exacerbate the mass transfer problem. Electroosmotic pumping produces a relatively flat ("plug-like") flow profile (10), minimizing the need for rapid radial diffusion in CZE and MECC. Moreover, the micelles in MECC are somewhat analogous to conventional packed column particles. The small size of micelles (~ 40 Å in diameter for SDS) and close, uniform (electrostatic) packing produces an ideal packing bed and further contributes to the high separation performance of MECC.

As a result of high efficiency and speed, MECC offers high sample throughput and reduces the amount of solvent used. Table II shows a comparison of HPLC and MECC in terms of analysis time, analyses possible in one day, buffer consumption, and finally, estimated cost in the case of aflatoxin analysis. The speed afforded by electrophoretic methods described here allow a factor of 4 increase in sample throughput while consuming less than 1% of the buffer. In addition to initial purchase price, costs for disposal of solvent waste

Table II: Comparison of MECC and HPLC Operating Costs

	Analysis Time	Analyses/Day ^a	Mobile Phase ^b	Cost/month ^c
HPLC	14-16 minutes	16	550 mL	\$129.00
MECC	1-2 minutes	64	<2 mL	\$ 0.86

^a Assumes a fifteen minute equilibration time after each HPLC run and 5 min. for MECC.

^b Calculated for an eight hour day

^c Calculated for one month, per instrument for HPLC (Acetonitrile/MeOH/H₂O 20/20/60 @ 1.1 ml/min) and MECC (as in fig. 23) mobile phase. Includes only solvent purchase price and approximate disposal costs (\$0.3/lb).

must be taken into account. The CE approach thus represents a mechanism whereby substantial savings and waste reduction could be realized.

One potentially important application of high speed separations is the analysis of mycotoxins, particularly aflatoxins. Aflatoxins are extremely toxic metabolites of fungi such as *Aspergillus Flavus*. These fungi occur in a variety of foodstuffs intended for both livestock and human consumption. Peanuts, corn, grain, rice, soybeans, and oats have all been found to support aflatoxin production (70). As with many other carcinogens, these toxins may be passed up the food chain to humans. For example, toxic aflatoxin metabolites have been found in milk (70). Aflatoxins pose significant health risk even at low concentrations (ppb) and are generally found to target the liver (71). Thus, analyses of aflatoxins is of utmost importance in assuring safe food supplies, but is challenging due to the presence of the toxins at trace levels in complex matrices. Conventional methods for aflatoxin analysis are numerous and include thin layer chromatography, HPLC, and immunochemical methods (72).

The focus of this chapter is the optimization of rapid, sensitive separations in MECC as applied to aflatoxin analysis. Experimental factors which maximize efficiency and minimize analysis time are investigated. Laser-based fluorescence detection is employed and exhibits injected concentration detection limits in the 20-100 ppb range. Considerations for the analysis of aflatoxin in corn meal are discussed.

Experimental

Apparatus

Two laser-based fluorescence detection schemes were employed in the work reported in this chapter. The instrumentation used for studies of analysis speed and efficiency has been recently described in detail (73), and employs a monochromator/ filter combination to isolate the desired emission wavelength

(450 nm, FWHM = 6 nm). An alternative system was used for the determination of detection limits in an attempt to collect a wider portion of the emission spectrum and to increase transmission of signal to the photomultiplier tube. Fluorescence emission was collected at 90° with an f/2 lens and focused onto a 2 mm slit, placed in the center of a light-tight 2 inch cube that contained inlet and outlet apertures. The desired wavelength range was isolated with a bandpass filter (452 nm, FWHM = 20 nm, Oriel Corp, Stratford, CT) that is placed in the inlet aperture of the spatial filter cube. Fluorescence excitation was provided by a helium-cadmium laser (Liconix, Sunnyvale, CA) at 325 nm. Laser power ranged from 7-10 mW. Chromatograms were recorded with both Varian 4270 and 4400 integrators (Palo Alto, CA). Capillaries of 50 and 25 μ m i.d. were purchased from Polymicro Technologies (Phoenix, AZ). Fluorescence spectra were obtained with a Spectrovision DA-10 spectrofluorimeter. Specific spectral acquisition conditions are given where appropriate.

Chemicals

Aflatoxin standards, SDS, and bile salts were purchased from Sigma Chemical Co. (St Louis, MO). All water and solvents used were of HPLC grade and were obtained from Baxter Healthcare Corp. (Stone Mountain, GA). *A. Favus* spores were obtained from Northern Regional Research Laboratories (Peoria, IL.). Corn meal was obtained from a middle Indiana dairy farm.

Procedures

Chromatography

Capillary columns were rinsed with 0.01 M NaOH for approximately 10 minutes before filling with mobile phase. The system was allowed to equilibrate for 10-15 minutes using an applied voltage of 20 kV prior to injection of sample. Applied voltages during chromatographic runs ranged from 8 to 36 kV. Efficiency studies were performed using hydrostatic injection, 7

seconds at a height differential of 10 cm. Chromatograms for calibration curves and corn meal analysis employed electroinjection, 2 kV for 30 seconds.

Capillary columns used in this work were 40 cm in length, 35 cm to detection window.

Efficiency Calculations

Plate numbers were calculated by using the relationship

$$N = 2\pi \left(\frac{t_R h}{A} \right)^2 \quad (10)$$

where N is number of theoretical plates, h peak height counts, A peak area counts and t_R retention time (63). It is essential to note that for some integrators area and height count response factors may not be equivalent and appropriate conversion factors need to be included in the equation. Plate height, H, is calculated as the capillary length to the detection zone divided by N.

Sample Preparation

Standards were prepared by adding one mL HPLC grade acetonitrile to one mg solid aflatoxin and making appropriate dilutions with mobile phase. Stock acetonitrile solutions were stored in the dark at approximately 0° C. Corn inoculated with *Aspergillus Flavus* spores were extracted with two 100 mL portions of chloroform as previously described (74), with the exception that the dry residue was dissolved in HPLC grade acetonitrile. Further cleanup of this solution was provided by the solid phase extraction (SPE) procedure described below.

Corn meal samples were extracted with an 80:20 by volume methanol/water solution. One mL of this solution was diluted to 5.0 mL with 0.5% (v/v) aqueous acetic acid and subjected to the following SPE sample

clean up procedure: 3.0 mL SPE tubes containing 40 μ m LC-CN (Supelco, Bellefonte, PA) bonded phase were preconditioned with 2.0 mL 0.5% (v/v) aqueous acetic acid followed by one minute drying time with air passing through the tube via aspiration. The 5.0 mL diluted extracted sample was placed on the column. The sample was washed by passing 0.5 mL 20% (v/v) tetrahydrofuran in 0.5% (v/v) aqueous acetic acid followed by 2.0 mL of HPLC grade hexane. After a drying period of 2 minutes, the SPE tube was washed with 3.0 mL 25% (v/v) tetrahydrofuran in hexane followed by a 1 minute wash. The aflatoxins were eluted from the SPE tube with two 2.0 mL portions of CH_2Cl_2 /tetrahydrofuran (99:1). The elution solvent was evaporated and the remaining residue dissolved in 200 μ L acetonitrile. A 50 μ L aliquot of this solution was diluted with 200 μ L mobile phase prior to injection. Further details concerning this SPE procedure can be found in product literature available from Supelco.

Safety Precautions

The extremely toxic nature of aflatoxin demands the utmost in attention to safety precautions. Gloves were worn at all times when preparing or handling solutions. Standards were dissolved in an appropriate solvent and transferred via syringe. All working areas and equipment were wiped down with a 5% solution of NaOCl (bleach) to form considerably less toxic derivatives, and all waste solutions were made approximately 5% in NaOCl.

Results and Discussion

Optimization of Analysis Time and Efficiency

Several parameters influence efficiency and analysis time in MECC. The voltage applied across the capillary is the primary factor in determining the velocity of the mobile phase and consequently analysis time. Figure 19 shows

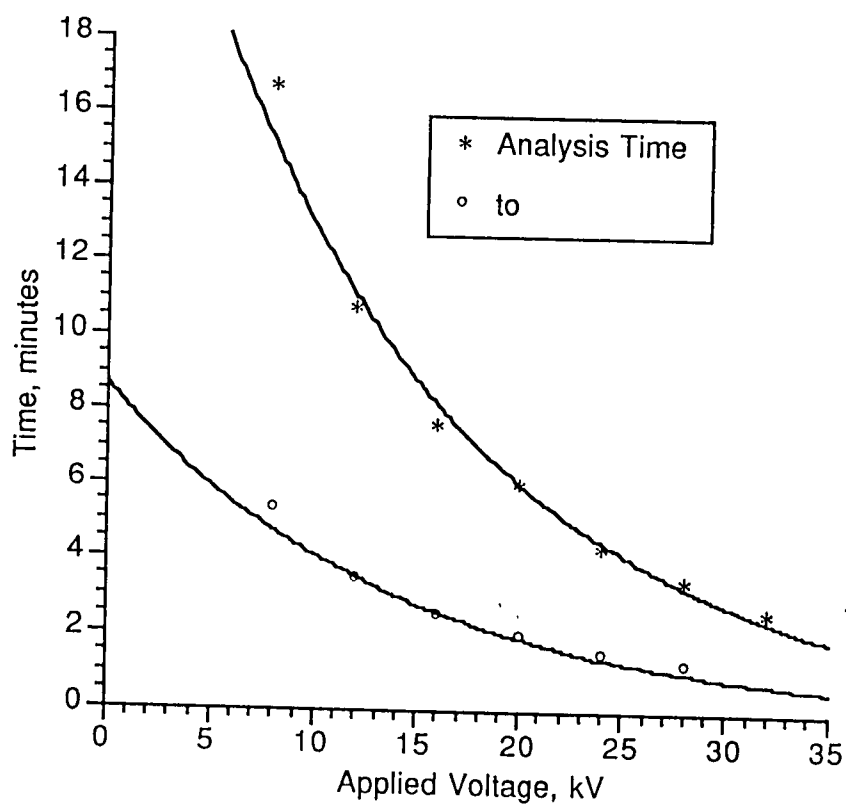


Figure 19: Void and Analysis Time for MECC vs. Applied Field

the dependence of capillary void time, t_0 , (*i. e.*, length of capillary to the detection zone divided by electroosmotic flow velocity) and analysis time for aflatoxin B₁ on applied voltage. As expected, an inverse relationship between applied voltage and flow rate is observed with increasing fields. At higher field strengths, heat dissipation capability of the capillary is exceeded and the temperature of the electrophoretic medium increases. At very high fields the temperature increase is dramatic and in the extreme case, this can cause boiling. Since electrophoretic mobilities and chromatographic partition ratios are temperature dependant, it is not surprising that plots of analysis time vs inverse voltage show deviations from linearity. Graphs of field vs analysis time thus take on the appearance as in Figure 19. Nevertheless, significant reductions in analysis time occur when field strength is increased.

Efficiency in MECC is also dependant on mobile phase velocity, in addition to other parameters. Factors such as buffer and surfactant concentration, column diameter, and detector time constant may all have considerable effects on efficiency. An experimental parameter that is key to separations in MECC is the i. d. of the capillaries employed. The use of small diameter capillaries (i. d. < 50 μm) permits the application of high field strengths while maintaining high efficiency. This is illustrated, along with the effects of other parameters, in Table III. Note that the efficiency data in Table III are reported in number of theoretical plates/minute to give a better idea of the temporal performance of the technique. Retention times for aflatoxin G₁ are less than 5 minutes. The standard conditions used in this work were as follows: buffer composed of 0.01 M Na₂HPO₄ and 0.006 M Na₂B₄O₇, surfactant concentration of 0.05 M SDS or NaDC, capillary 25 μm i. d. by 40 cm long (35 cm to detection zone), and a field strength of 600 V/cm, detector time constant that was limited by the 60 Hz acquisition rate of the integrator employed.

Table III: Factors Affecting Efficiency in High Speed MECC Separations

Experimental Parameter	Sodium Deoxycholate	Sodium Dodecyl Sulfate
Standard Conditions	Plates/minute 128,900	Plates/minute 104,000
Column Diameter ^a 50 μ m	16,000	15,500
Buffer Concentration 0.005 M Na_2HPO_4 0.003 M $\text{Na}_2\text{B}_4\text{O}_7$ 0.02 M Na_2HPO_4 0.012 M $\text{Na}_2\text{B}_4\text{O}_7$	202,300 80,900	154,000 67,000
Surfactant Concentration 0.025M	48,700	82,900
Detector Time Constant 0.1 second	110,300	98,900

All data for Aflatoxin G1. Standard Conditions: Applied Field 600 V/cm, Detector time constant in "out" position, Integrator data acquisition rate 60 Hz, 0.05 M surfactant, 0.01 M Na_2HPO_4 0.006 M $\text{Na}_2\text{B}_4\text{O}_7$ Column: 25 μ m i.d. X 50 cm total length, 40 cm to detector window.
^a Applied Field 300 V/cm

Smaller diameter capillaries effectively dissipate heat, resulting less distortion of the flat flow profile and better efficiency (see below). Applied voltages (or more exactly field strengths) must be high in order to provide large solute migration rates. The use of 50 μm i.d. capillaries limits maximum applied fields to a range roughly 300-400 V/cm due to low electrical resistance and poorer heat dissipation. Application of fields exceeding this value to capillaries with i. d.s $\geq 50 \mu\text{m}$ generally results in degradation of efficiency or boiling of the mobile phase within the capillary.

Figure 20 contains plots of applied field vs. efficiency (plate height) for aflatoxin G₂ using both 50 and 25 μm i. d. capillaries. Van Deemter-like plots such as these can be helpful in studying band broadening mechanisms in MECC (69). At lower fields, efficiency is degraded due to the onset of problems with longitudinal diffusion. Long capillary residence times provide the solute bands with significant opportunity to disperse along the axis of the capillary due to molecular diffusion. As field strength is increased, residence time in the capillary decreases, and an optimum value of field strength is observed (see Figure 20). Efficiency begins to degrade at higher fields due to increased resistance to mass transfer (69) and, more importantly, increased thermal load. This not only produces a general increase in temperature as mentioned above, but also results in radial temperature gradients in the capillary. The temperature gradient can cause band dispersion (12). Radial variances in phase ratio, due to the temperature dependence of critical micelle concentration (CMC), and partition coefficient can also lead to band dispersion. Use of smaller diameter capillaries allows greater potentials to be applied without critical losses in efficiency, facilitating separations under high field conditions. As illustrated in Figure 20, the working range of applied field is much wider for the 25 μm i. d. capillary. Thus, the use of smaller i. d. capillaries is very

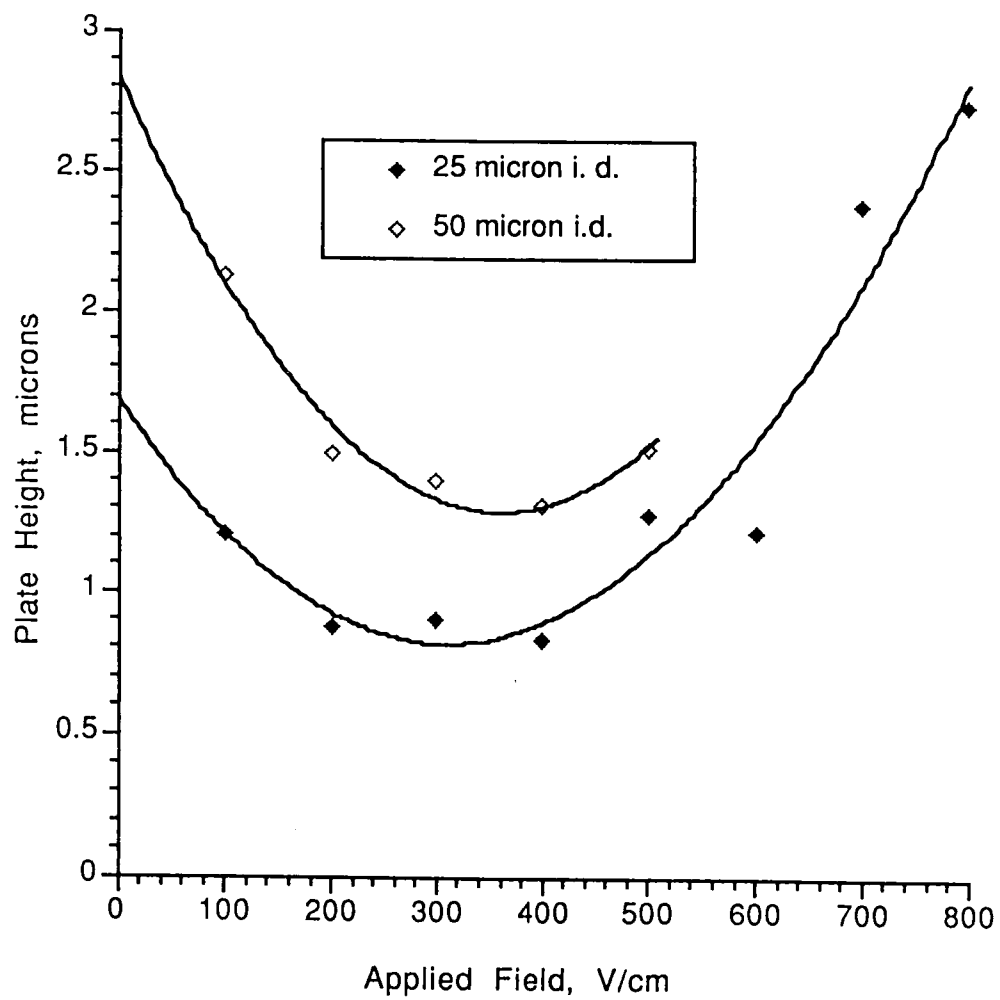


Figure 20: Efficiency vs. Applied Field for 25 and 50 μm i. d. Capillaries

important when performing high-speed MECC separations.

Further evidence of the importance of Joule heating as a source of band dispersion is given by the buffer concentration data in Table III. At higher salt concentrations, current passing through the capillary is greater, and the resulting large thermal load results in band dispersion. Dramatic decreases in efficiency are observed for both surfactant systems when the buffer concentration is increased above the standard conditions case. Working at a relatively low buffer concentration is thus essential for maximizing efficiency.

Surfactant concentration is perhaps the most fundamental adjustable mobile phase parameter in MECC. The working range of surfactant concentration in MECC is bounded at the lower end by the surfactant CMC and at the upper end by the creation of the thermal problems cited above. At the low end of this concentration range micelle-monomer exchange kinetics are such that the adverse effects of micelle polydispersity are generally observed (45, 63, 69). Increasing the surfactant concentration counteracts this effect and efficiency improves as indicated in the table (48, 69). Reducing the standard conditions surfactant concentration by half reduces the efficiency with NaDC by nearly 70%, while SDS shows a 20% reduction. The difference in the magnitude of the reduction of efficiency between the two surfactants may be attributed to the fact that the bile salt has a higher CMC than the SDS. In all of our work, efficiency in MECC degrades dramatically when the surfactant concentration is reduced to near the CMC.

Efficiency may also be affected by injection and detection conditions and instrumentation (29, 63). Efficient, rapid separations necessarily have very small temporal peak variances that require rapid data acquisition and fast detector time constants. Figure 21 A shows the separation of aflatoxins G₂, G₁, B₂, and B₁ from a spiked corn meal sample obtained using an SDS micellar

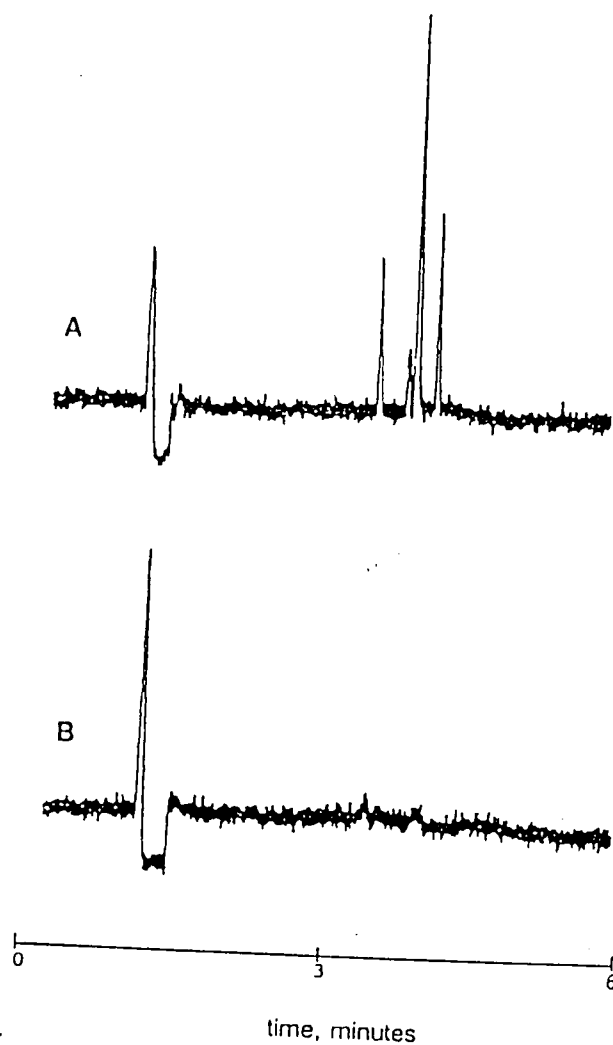


Figure 21: Analysis of Corn Meal Extract with MECC.
A - Corn meal extract spiked with 16 ppm (w/w) aflatoxin standards
B - Unspiked extract
Elution Order: G₂, G₁, B₂, B₁. Mobile phase: 0.05 M SDS,
0.01M Na₂HPO₄, 0.006 M Na₂B₄O₇, 10% Acetonitrile.
Applied Voltage: 20 kV.

phase. Table III shows the effect of time constant on efficiency for aflatoxin G₁. As a consequence of the short time constants that are needed to maintain efficiency in high-speed MECC, baseline noise and limits of detection are increased. Tradeoffs between efficiency and detection must thus be reached.

Retention control also plays an important role in achieving high-speed separations. Optimizing capacity in terms of resolution and analysis time can be critical. Poorly retained solutes elute quickly but may not be well-separated and strongly retained solutes tend to co-elute at the micelle migration time. As discussed earlier, the optimum capacity factor, k' , in MECC falls in the range of 1-5 (21). The results of two approaches to control capacity and optimize separations are reported. First, organic modifiers such as methanol, acetonitrile, and 2-propanol are added to the mobile phase. These solvents have the effects of decreasing mobile phase polarity and extending the elution range (22, 24, 69). In some cases, the solute capacity factor is reduced to more optimal values. In addition to reducing mobile phase polarity, organic solvents generally reduce conductivity, which can improve efficiency at high fields (22, 24). Figure 22 contains plots of applied field vs. plate height for SDS mobile phases containing both 0 and 10% (v/v) acetonitrile. The minimum values of plate height are approximately equal, but plate height does not increase at high fields as sharply for the mixed aqueous-organic mobile phase. Under these conditions, a wider range of (higher) fields can be applied without significant loss of efficiency. This effect is the result of a decrease in dielectric constant of the mobile phase upon addition of the acetonitrile to the mobile phase, which reduces the conductivity of the mobile phase and, hence, the current that is passed at a given voltage. Decreases in mobile phase polarity with the addition of organic solvents generally reduce k' values, causing solutes to spend more time in the faster moving mobile phase. While reduction of electroosmotic flow

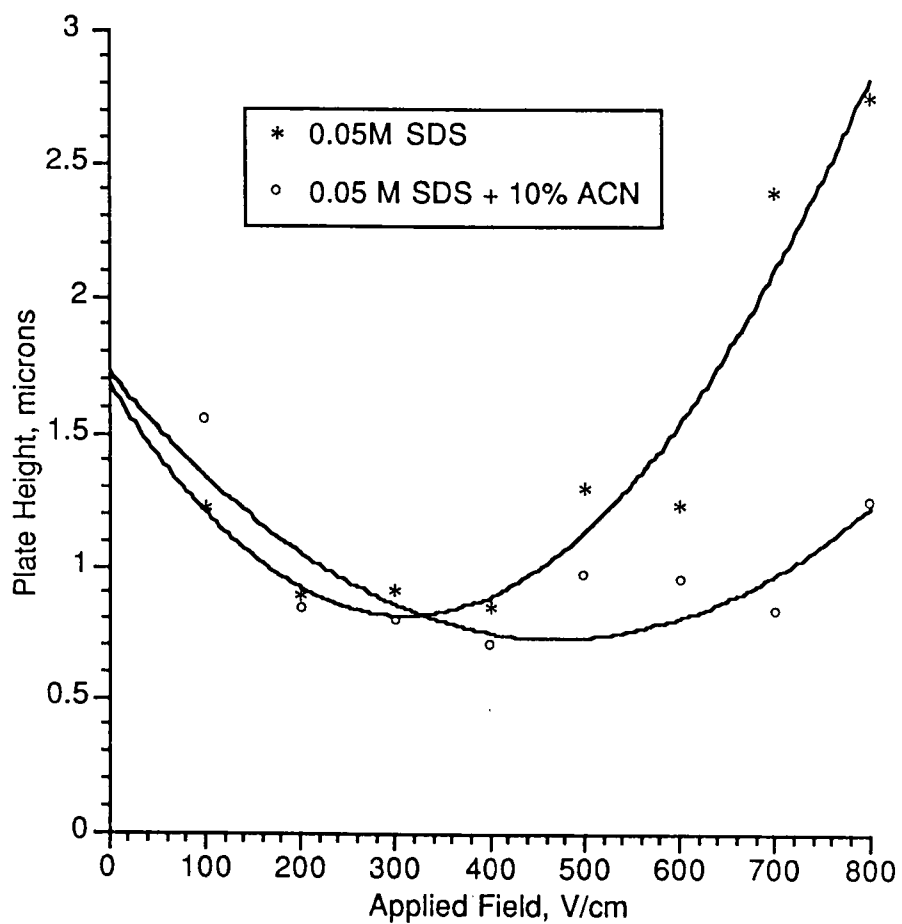


Figure 22: Efficiency vs. Applied Field for 0 and 10% Acetonitrile

and a concomitant extension of elution range is often observed upon addition of organic solvent to the mobile phase, the reduction of solute k' often dominates and they elute more quickly (62). However, it should be noted that depending on the nature and concentration of the organic solvent, electroosmotic flow may be reduced to a point where analysis time is increased, sometimes dramatically (22).

The second approach to control capacity involves the use of bile salt surfactants (48, 59). These naturally-occurring surfactants are based on a hydroxy-substituted steroidal backbone and are more polar in nature than the conventional *n*-alkyl variety, as discussed in detail in chapter 3 (48, 59). Hence, they retain moderately to highly hydrophobic solutes less (k' values are generally closer to the optimum range). Figure 23 shows the separation of the aflatoxins with a bile salt micellar phase optimized in terms of overall resolution and analysis time.

Detection

The very small i. d. capillaries that are essential for rapid MECC separations exacerbate problems with attaining adequate detection sensitivity. In order to preserve the integrity of the narrow solute bands, on-column detection is generally employed. Absorbance detection in small i. d. capillaries is difficult due to very short optical pathlengths, low throughput, and large levels of stray light (25). Laser-based fluorescence detection schemes offer high sensitivity as well as compatibility with capillaries of diminutive dimensions (45, 73, 75, 76). Aflatoxins readily absorb the 325 nm output of helium-cadmium lasers and exhibit native fluorescence, allowing sensitive laser-based detection to be employed (61). Fluorescence detection in MECC is influenced by the type of pseudostationary phase employed, since the emission wavelength and intensity often are affected (62). As discussed in chapter 3, association of solute

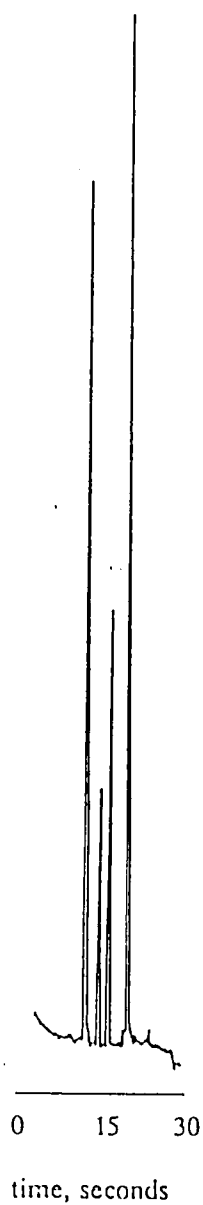


Figure 23: High Speed Separation of Aflatoxin Standards. Mobile phase same as figure 13, with 5% added acetonitrile. Column 40 cm total, 35 cm to detector. Applied Voltage 35 kV.

Table IV: Fluorescence Characteristics of Aflatoxins in Various Solvents

Solvent	Fluorescence Emission Maximum ^a				Relative Intensity at $\lambda_{\max}$ ^b			
	G ₂	G ₁	B ₂	B ₁	G ₂	G ₁	B ₂	B ₁
EtOH	445	448	433	429	68.6	100	6.03	91.4
Water	463	452	440	441	77.4	31.5	100	57.1
SDS	456	454	440	434	100	52.9	69.8	76.2
NaDC	460	452	438	437	89.2	46.9	88.6	100

^a Wavelength data acquired at 10 nm/ min, 1 sec time constant

^b Intensity data acquired at 50 nm/min, 2 sec time constant

with micelles often enhances fluorescence emission (77). The spectroscopic behavior of the aflatoxins in various solvents is shown in Table IV. These data illustrate the importance of considering both the spectroscopic characteristics and the chromatographic properties of the micellar phase in MECC. The varied spectroscopic nature of each individual aflatoxin indicates that multichannel detection might be advantageous, both in terms of identification and increased sensitivity (75).

Calibration plots were generated for all four aflatoxins using an SDS pseudostationary phase. The absence of inner filter related curvature of calibration plots for laser fluorometric detection in narrow bore capillaries is well established (62). Extrapolation of the plots to a S/N of 3 yielded the limits of detection shown in Table V. Injections were made by electroinjection; 2 kV applied for 30 seconds yielding an injection volume in the 25 μm i. d. capillary of approximately 2.5 nL. An internal standard was added to correct for variations in detector response. While these concentration detection limits are not as low as previous reports (61), the weakly fluorescent G_1 and B_1 were quantitated without prior conversion to more fluorescent derivatives. The use of chemical derivitization techniques can result in the formation of highly fluorescent derivatives, leading to improved limits of detection (78). Moreover, the laser used in this work, due to an aging plasma tube, suffered from sporadic changes in both output power and mode structure, which adversely affected detectability and reproducibility.

Analysis of Corn Samples

Aflatoxins are sometimes present in foodstuffs at ppb levels, requiring extraction and concentration. Figure 21 shows the chromatograms resulting from the solid-phase extraction of a corn meal sample and a sample spiked at the 16 ppm (w/w) level. The unspiked sample (B) showed no significant peaks

Table V: Limits of Detection for Aflatoxins in MECC^a

Aflatoxin	Injected Concentration, <u>M</u>	Amount Injected, femtomoles
G ₂	2.85 X10 ⁻⁸	0.070
G ₁	4.36 X10 ⁻⁷	1.1
B ₂	2.64 X10 ⁻⁸	0.065
B ₁	1.26 X10 ⁻⁷	0.031

^a Limits of Detection based on S/N=3;
SDS pseudostationary phase

for any of the four common aflatoxins, a fact which is not surprising due to the cooler climate from which it was obtained. Aflatoxin production is most favored under conditions of high humidity and temperature (70, 71); contamination is thus more of a problem in southern climates. Using the reported workup (the SPE procedure had a reported recovery of roughly 95%), and the LOD for B₁ reported in Table V, the level of the most common aflatoxin, B₁, was less than approximately 50 ppb in this sample.

To assure the presence of aflatoxins and assess the ability of MECC to the toxins in a more complex sample matrix, *Aspergillus Flavus* spores were introduced onto a sample of moist corn kernels and allowed to grow for 2 weeks at 28°C. Figure 24 shows the chromatogram of an extract obtained as described previously. The more complex sample matrix clearly illustrates the need for thorough sample cleanup, as aflatoxin peaks (see arrow for expected retention time of aflatoxin B₁) are not clearly resolved from extraneous fluorescent compounds in the sample. However, if a more selective detection scheme was employed, it might be possible to discern individual aflatoxin peaks in such a mixture. Possible detection schemes include multichannel or time resolved detection (75, 79). Although not pursued in this work, it should be possible to reoptimize the separation to accommodate the more complex sample. The use of longer capillaries to increase plate numbers and/or the incorporation of alcoholic solvents in the mobile phase to significantly increase the elution range could provide the necessary resolution (albeit at the expense of analysis time).

MECC offers a viable method of rapid aflatoxin analysis, and for other compounds requiring routine monitoring. Analysis times on the order of seconds are readily achieved and provide the opportunity for large sample throughput. Significant samples include foodstuff extracts and pharmaceutical

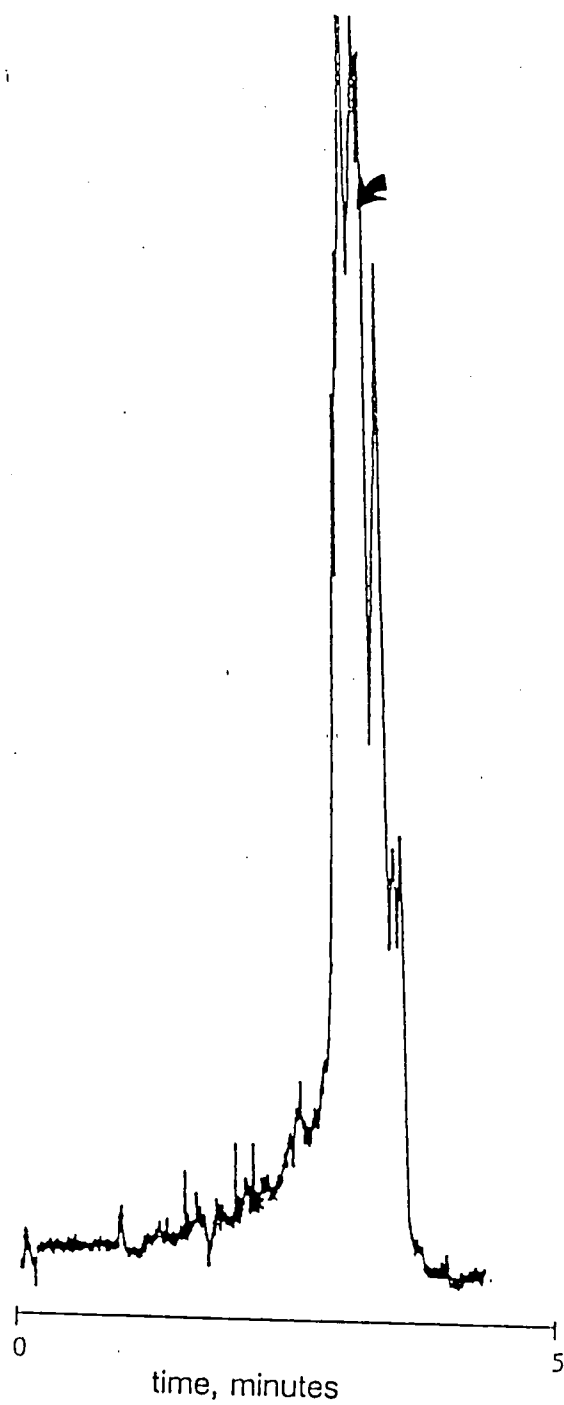


Figure 24: MECC Separation of an Extract of *A. Flavus* Cultured Corn.

formulations. In addition, separations involving enantiomers may well lend themselves to high-speed MECC separation. Detection still remains a limiting factor in applications of rapid MECC and CZE methods, but improvements in laser sources and the development of sensitive alternate schemes will increase the applicability of these techniques.

Chapter 5

Chiral Separations in Capillary Electrophoresis

Introduction

One of the more challenging areas of research activity in separation science is that of chiral separations. Separation of enantiomers is typically a difficult task since these compounds have nearly identical physical properties, the only discernible difference being the direction and amount each species rotates the plane of polarized light. Most enantiomers differ only in their arrangement of functional groups about a carbon atom, one enantiomer being the mirror reflection of the other. This central carbon, or stereogenic center is identified as a carbon atom having four different groups attached to it. Enantiomers may also result from differences in conformation, as with the binaphthyl compounds discussed later in this chapter. Separation schemes must attempt to create interactions specific to one enantiomer, *i. e.* create a dynamic diastereomeric complex. One viable theory of chiral separation mechanisms presents the requirement for a simultaneous three-point interaction between solute and stationary phase for enantiomeric separation (6, 80). In any case, an absolute requirement is that one enantiomer must differentially interact with some phase in the chromatographic system in such a way that the two forms move at different velocity down the column.

Conventional approaches to the separation of enantiomers have involved pre-column derivitization to form diastereomers having different retention characteristics, chiral stationary phases, and in HPLC applications, chiral mobile phase additives. Pre-column diastereomer formation can be a useful approach when only conventional stationary phases are available. Enantiomers are chemically modified to form diastereomers which can be

separated by conventional means. Limitations to this technique include lack of universal applicability, lengthy (and potentially tedious) analysis times, and relatively large sample requirements (6, 80).

The use of chiral stationary phases in some cases allows the direct resolution of enantiomers. This approach involves the chemical coating or bonding of a phase possessing chiral character to the chromatographic support material. Enantiomers moving down the column often differentially associate with the chiral phase and are separated. These phases have been based on molecules such as amino acid derivatives, cyclodextrins, and proteins. Chiral stationary phases have been used with success in GC and HPLC applications (6, 80). HPLC and TLC allow utilization of the mobile phase for selectivity manipulation. As discussed earlier, a wide variety of substances have been added to LC mobile phases for this purpose. Addition of chiral compounds to the mobile phase can result in chiral separation without the use of a chiral stationary phase. Examples include the use of cyclodextrins (5), amino acid-metal complexes (80), and surfactants (81).

CE offers a viable approach to achieving enantiomeric separations. Primary advantages of CE in this area include: (1) low cost, (2) high efficiency and speed, (3) low sample consumption, and (4) wide applicability. First, chiral separations may be performed via conventional HPLC, but often at significant expense. Columns with chiral stationary phases are often priced in excess of \$500, and lifetimes may be relatively short. Additionally, HPLC requires a great deal more solvent than CE, which is important when considering use of a mobile phase additive. Second, efficiency is important for separations in which selectivity is small. The high plate numbers characteristic of CE often allow baseline separation for even the smallest difference in retention. In some cases, the high efficiency available also allows difficult separations to be

performed much more rapidly, as discussed earlier. The small dimensions of the columns used in CE lead to low sample volume requirements, and injection volumes are on the order of nanoliters. In situations involving precious or small samples, CE offers a viable approach. Along these same lines, the total volume of a 50 cm long 50 μm i. d. column is 1.0 μL . This leads to a practical solution volume requirement of approximately 1 mL as compared to an HPLC requirement of roughly 0.5 L for a 20 minute run. These arguments are the same as presented in Table II. Small amounts of material are thus required for many analyses, allowing the use of rare or expensive reagents in achieving unique selectivities. Approaches to chiral separations in CE have been reported in the MECC and CZE modes. This chapter discusses the use of MECC and CZE to enantiomeric separations via the addition of non-traditional organized media to the mobile phase. Applications involving bile salt surfactants and cyclodextrins for chiral separations will be presented.

Experimental

Apparatus

The basic experimental arrangement for CZE/MECC has been described in detail elsewhere (1). Separations in this study were performed either in 50 μm i. d. or 25 μm i. d. fused silica capillaries (Polymicro Corp., Phoenix AZ) with lengths and other physical parameters as noted in the text. High voltage to drive the electrophoresis was provided by a Hipotronics, Inc. (Brewster, NY, USA) model 840A high voltage power supply. Absorbance detection of binaphthyl compounds was accomplished using both laser-etched flow cells and a modified commercial detector, as well as the Linear 204 instrument, as described earlier. Laser-based fluorescence detection of NBD-labeled amines (λ_{ex} 488 nm, λ_{em} 540 nm) was performed using previously described instrumentation with a monochromator/filter combination (69). Detection of

dansylated phenylalanine employed the 325 nm output of a helium-cadmium laser (Liconix, Sunnyvale, CA) operated in conjunction with the filter/slit combination described in the preceding chapter. This arrangement employed a bandpass filter centered on 520 nm, FWHM = 30 nm. Data were recorded with a strip chart recorder (MECC work) or a Varian 4400 integrator (CZE work).

Reagents

The bile salts and native cyclodextrins used in this study were obtained from Sigma Chemical Company. Modified cyclodextrins were obtained from Pharmatec (Alachua, FL) 1,1' bi-2-naphthol (BN(OH)₂) and 1,1' binaphthyl diyl hydrogen phosphate (BNPO₄) were purchased from Aldrich Chemical Company. 1,1' binaphthyl 2,2' dicarboxylic acid (BN(COOH)₂) and 1,1' biphenanthrene 2,2' dihydroxide were supplied by Dr. S. Toda, Ehime University, Japan. Dansyl chloride, 7-chloro-4-nitrobenzo-2,1,3-oxadiazole and polycyclic aromatic hydrocarbons were also purchased from Aldrich. Dansyl-phenylalanine was obtained from Sigma. Dietary supplement capsules (DL-phenylalanine) were obtained locally. Buffer salts and other conventional chemicals were obtained from Baxter Chemical Co. All solutions were made in HPLC grade water, also obtained from Baxter.

Procedures

Prior to use, columns were rinsed with 0.01 M NaOH. The capillaries were then filled with the mobile phase of choice and allowed to equilibrate by applying the running voltage for approximately ten minutes, typically 15-20 kV. Injections were made by a syphoning procedure (25), at an elevation of 10 cm for 10-12 seconds. Exact injection parameters will be specified where needed. All separations were performed using an applied voltage of 15-20 kV, with resulting currents ranging from 20-40 μ A.

Plate numbers (N) were calculated from the peak width at half height via

$$N = 5.5 \left(\frac{t_R}{w_{1/2}} \right)^2 \quad (11)$$

where t_R is the solute retention time and $w_{1/2}$ the peak width at half height. Peak widths were on the order of 0.5-1 mm and were measured with a direct measuring 50X microscope ocular purchased from Edmund Scientific (Barrington, NJ.).

Resolution was calculated via the relation

$$R_s = \frac{2\Delta t_R}{w_1 + w_2} \quad (12)$$

where ΔT is the difference in retention time between the solutes in question and w_1 and w_2 the widths of each peak.

Electrophoretic mobilities were calculated via the relation

$$\mu_{obs} = V \left(\left[\frac{L^2}{t_R} \right] - \left[\frac{L^2}{t_0} \right] \right) \quad (13)$$

where μ_{obs} is observed electrophoretic mobility, L_{col} the column length to the detector, and t_0 the column dead time.

Derivatization of the DL-phenylalanine dose form was performed by first dissolving 1.0 mg of the compound in 5.0 mL 0.01 M disodium phosphate/0.006 M disodium borate buffer, pH 8.5. A 100 μ L aliquot of this solution was added to 100 μ L 1 mg/mL in dansyl chloride in acetone and diluted to 1 mL with acetone. This mixture was allowed to react for 5 hours at room temperature in the dark. The mixture was diluted to 10.0 ml with pH 9 0.01 M disodium phosphate -0.006

M disodium borate buffer and allowed to stand for approximately 1 hour prior to injection.

Results and Discussion

Bile Salt use in MECC Chiral Separations

Surfactants which possess chirality may be employed in the separation of enantiomers. Neutral and charged species are able to interact with the hydrophobic interior of the micellar aggregate, just as described in previous chapters. Chiral surfactants open the possibility of enantiomeric discrimination due to the presence of chiral sites on the aggregate. Synthetic surfactants based on amino acids have been used for chiral separations both by themselves and as mixed SDS micelles (82, 83). Neutral surfactants have also been used in SDS-mixed micelle solutions for the separation of amino acid enantiomers (84). Natural surfactants such as bile salts have also been successfully used to separate enantiomers of amino acids (85), binaphthyl compounds (48, 50), and pharmaceutical compounds (49, 50, 86).

Bile salt micelles, due to their natural optical activity, are able to chromatographically discriminate between enantiomeric forms of certain compounds. The presence of hydroxyl groups on the steroidal bile salt backbone cause the bile salt monomer to possess hydrophilic and hydrophobic faces which induce the formation of aggregates (53). The resulting micelles possess a helical structure, with the hydrophilic region facing the interior of the micelle (56). Approaches to optimize the MECC separation of substituted binaphthyl enantiomers are presented and possible mechanisms for chiral recognition of these solutes will be discussed. Several factors are important in the optimization of binaphthyl enantiomers. First, surfactant concentration is critical in determining retention and efficiency. As an example, consider the chromatograms in Figure 25 which show BN(OH)₂ and BNPO₄ enantiomers

separated under differing mobile phase conditions. In all cases, the neutral BN(OH)_2 eluted prior to the BNPO_4 . At low bile salt concentration (Figure 25A), the enantiomers are not resolved, and efficiency is poor. There is some retention of the solutes, demonstrating that micelles are present, but resolution is limited by poor efficiency. This effect is presumed to be a result of the adverse effects of micelle polydispersity, and has been documented when SDS is used as the surfactant (24). Solute bands are dispersed due to the fact that micelle size, and hence mobility is not uniform. It was anticipated that the small aggregation numbers for the bile salt molecules would exacerbate this problem. The fact that reasonably high efficiency was obtained at higher surfactant concentration (see Figure 25B-D) indicates that, under the conditions employed in this work, either the bile salt micelles are nearly monodisperse or the kinetics of micelle-monomer exchange is rapid on a chromatographic time scale. The magnitude of the elution range parameter, t_0/t_m , dictates the optimal k' for maximum resolution. Under the conditions used to obtain Figure 25A-C, t_0/t_m is 0.4, leading to an optimum k' of 1.5 (9). Increasing surfactant concentration from 0.005 to 0.01 M dramatically improves efficiency and resolution. At the higher bile salt concentration, the efficiency is high (N is approximately 170,000) and the micellar volume is appropriate to yield a k' near optimum. Thus, resolution is enhanced. Increasing the SDC concentration to 0.05 M results in high efficiency but the k' is increased significantly beyond the optimum and resolution is degraded. It should be noted that large increases in surfactant concentration can also produce large electrophoretic currents, thermal gradients within the column, and significant band dispersion (69). In the case of 0.05 M SDC, the addition of methanol alters the electroosmotic flow and extends the elution range which, concomitantly, improves resolution (22). Although it is not easily discerned from Figure 25D, the average k' value is

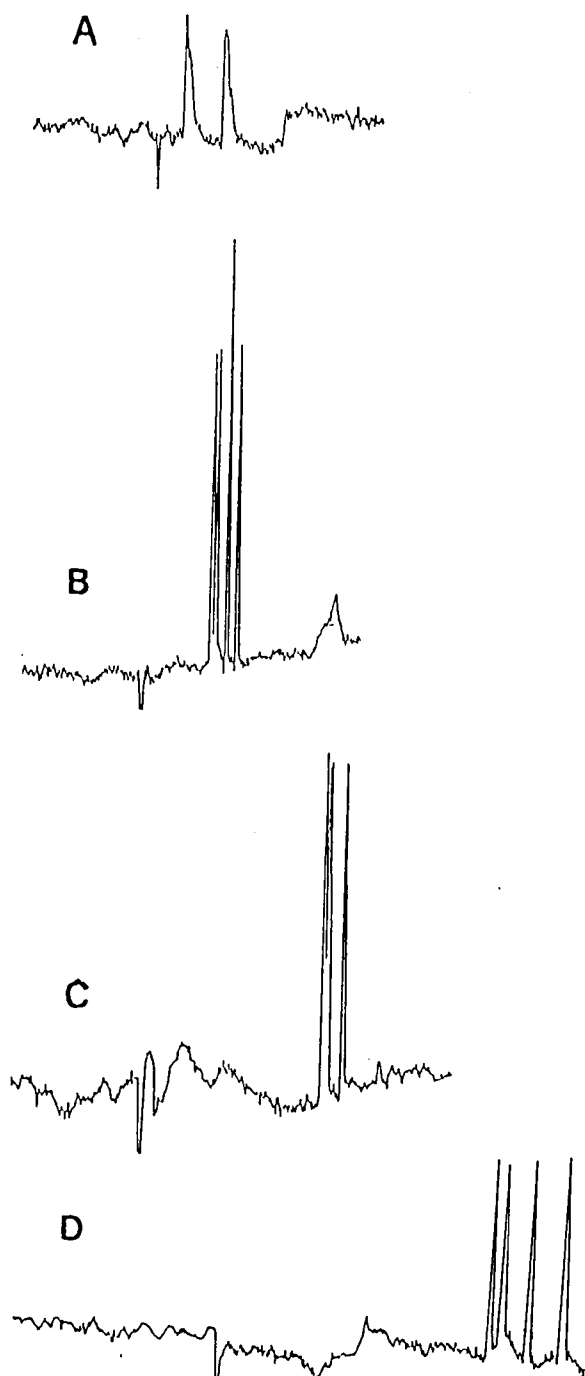


Figure 25: Bile Salt Separations of Binaphthyl Enantiomers. (A) 0.005 M NaDC, (B) 0.01 M NaDC, (C) 0.05 M NaDC, and (D) 0.05 M NaDC with 10% MeOH. Column 60 cm total, 55 cm to detector, 50 μ m i. d. 20 kV applied. Split Block Absorbance detector at 229 nm.

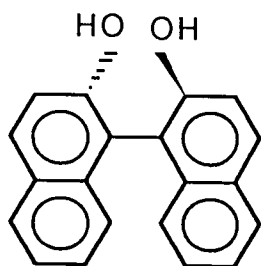
decreased to also provide for better resolution. The effects of adding methanol to a mobile phase containing 0.05 M NaC on the separation of enantiomeric solutes are summarized in Table VI. As methanol content increases, resolution increases and then falls off at 18% methanol. The reduction of resolution, despite apparent improvement in k' can possibly be explained by the fact the compound used to mark t_m (nitropyrene) does not accurately mark the extension in elution range at the higher methanol concentrations. In other words, the k' value at 12% methanol was more near the optimum for resolution of the enantiomers than at 18% methanol. In addition, resolution in MECC depends on N , which in the above data, shows a slight decrease as organic modifier is increased. Thus control of separations with bile salt pseudostationary phases can be effected by surfactant concentration or addition of organic modifiers. The fact that the bile salt systems respond to organic modifier in the same manner as SDS is promising because peak capacity can be increased through elution range extension, and more complex samples can be analyzed using gradient elution techniques (24, 26).

The bile salt micelle allows interactions with solutes which allow at least partial enantiomeric separation of the three binaphthyl compounds depicted in Figure 26. From previous reports, it appears that the bile salts prefer a rigid, planar structure for chiral recognition (49, 50). At this point, it is not possible to describe the exact mode of interaction leading to chiral recognition of binaphthyls, but a likely explanation is that since the micelle itself is present in only one conformation, apparently one binaphthyl enantiomer able to interact more freely with the aggregate than its opposite. In work with the bile salts, the S configuration was retained more than the R.

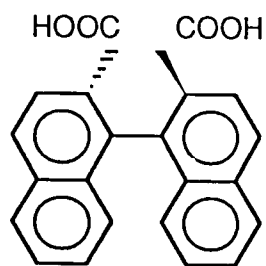
Table VI: Retention Data for NaC/Methanol Study

% Methanol	k' R-BN(OH) ₂	Resolution	N (X1000)	t_o/t_m
0	8.89	0.88	170	0.43
6	6.75	1.03	120	0.41
12	4.69	1.50	130	0.38
18	3.52	1.04	120	0.38

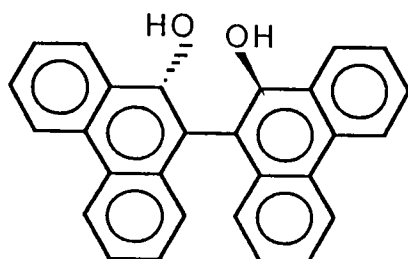
Conditions: 0.05 M, 0.01 M Disodium Phosphate, 0.006 M Disodium Borate, pH 9.0, Applied Voltage 20 kV.



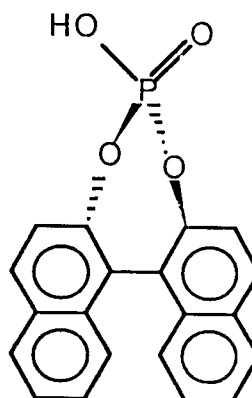
1,1' Bi-2-naphthol



1,1' binaphthyl 2,2' dicarboxylic
Acid



1,1'
biphenanthrene
2,2' dihydroxide



1,1' binaphthyl 2,2' diyl hydrogen
phosphate

Figure 26: Binaphthyl Structures

An important factor in determining the extent of chiral recognition of the binaphthyl compounds by the bile salt micelles is hydrophobicity, due to the dependance of resolution on k' (21). The biphenanthrene compound (see Figure 26) exhibited no enantiomeric separation. This could be attributed to the fact that the compound eluted at or near t_m , well out of the optimal k' window described above. In this case, the k' value would be nearly infinite, and little or no resolution would occur, as the hydrophobic interaction can "wash out" the enantiomeric recognition. Extension of the elution range with organic modifier might partially assuage this problem by reducing k' , but at the cost of analysis time (22, 23, 24), as illustrated earlier.

Another factor of importance in achieving chiral recognition is molecular charge. The BNPO_4 and the $\text{BN}(\text{COOH})_2$ enantiomers were both charged under the alkaline conditions used with NaDC. The singly charged BNPO_4 exhibited the best resolution of all the compounds studied in spite of charge repulsion with the anionic micelle. The doubly charged $\text{BN}(\text{COOH})_2$, however, showed no separation at alkaline pH presumably due to prohibitive charge repulsion between micelle and solute. Mobile phases containing sodium taurodeoxycholate (NaTDC) at pH 4.7 allowed some chiral separation to occur (see Figure 27). The tauro derivatives of the bile salts have very similar characteristics to NaC and NaDC, but the ionized group is an SO_3^- , which remains unprotonated even at low pH. These derivatives show very similar aggregation properties to the unconjugated forms, and chromatographic performance is similar. At the lower pH, the $\text{BN}(\text{COOH})_2$ appears to be at least partially neutralized, thus permitting enhanced interaction with the micelle. Lowering the pH also improved the resolution of the BNPO_4 enantiomers, probably for the same reason. Charge effects may be more complex than the rather simple-minded concept of repulsion between the bulk of the micelle and

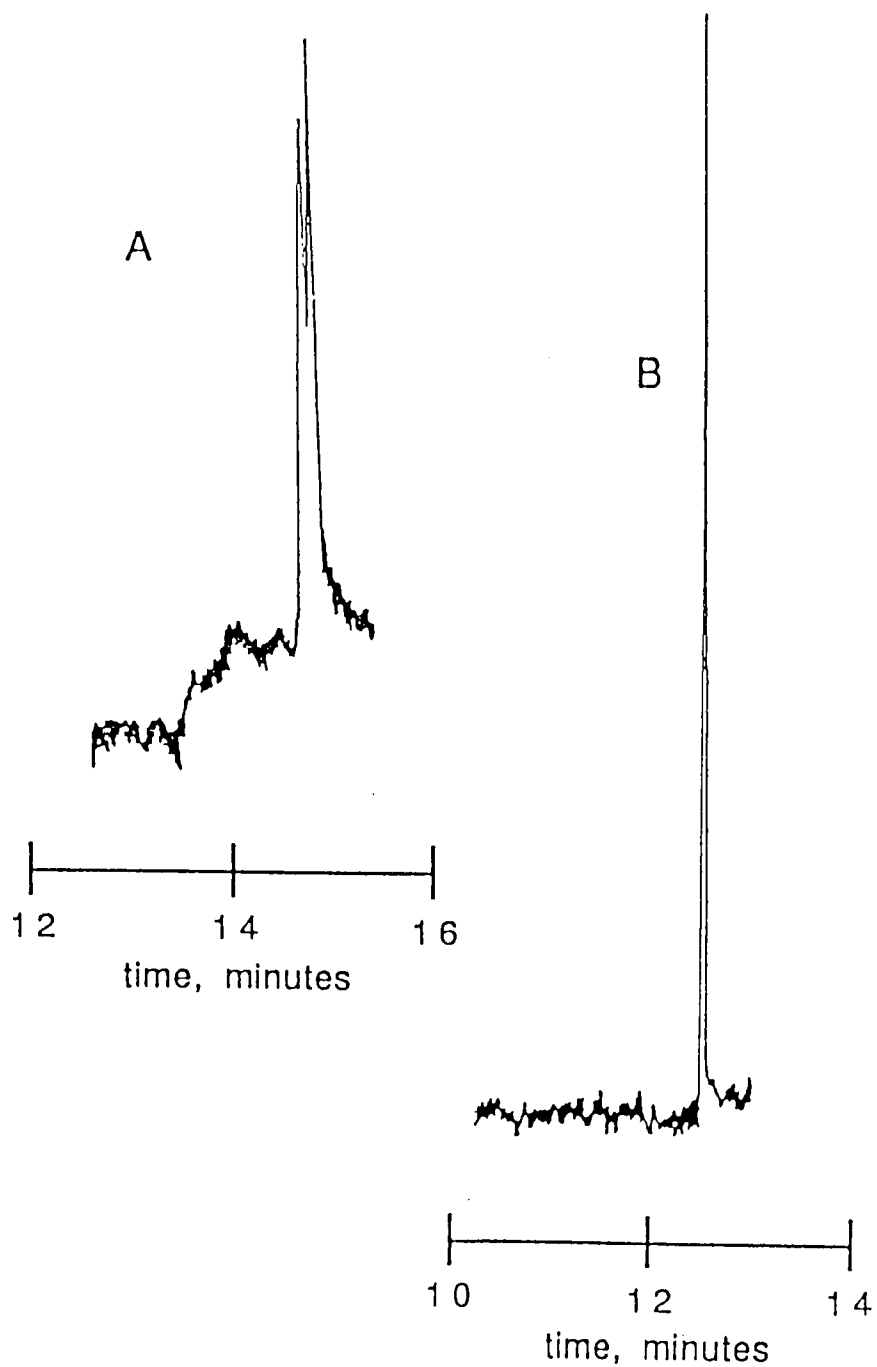


Figure 27: Dicarboxylic Acid Binaphthyl Separation. (A) pH 10 (B) pH 4.7. Mobile phase 0.05 M NaTDC, 0.01 M NaAcetate. Column 60 cm total, 55 cm to detector, 50 μ m i. d. 20 kV applied. Split Block Absorbance detector at 229 nm.

the solute. Since chiral separations require specific interactions with one enantiomer, charge repulsion could perhaps be envisioned as a disturbance of this interaction. For example, the repulsion could cause the solute's approach to the selective site on the micelle to be disrupted, making chiral separation less favorable or impossible.

While the factors discussed above are important, it is likely that other structural features influence binaphthyl enantiomer separation. Factors such as structure rigidity and the angle between the two naphthyl rings may also play a part in the separation process. The fact that the BNPO_4 enantiomers show the highest resolution of all the compounds studied is somewhat surprising considering its negative charge in basic media. One possible explanation of this effect is the rigidity of the molecule due to the phosphate bridge joining the naphthyl rings. Rotation of the naphthyl rings relative to one another is more restricted than with the BN(OH)_2 , and could contribute to the enantiomeric separation of BNPO_4 (87). The magnitude of the angle between the naphthyl rings, as well as rotational dynamics, could also play a significant part in determining chiral recognition of binaphthyl compounds. Studies have shown that the naphthyl ring angle in BN(OH)_2 can vary between 60 and 120 degrees, while locked binaphthyls are separated by approximately 43 degrees (88). In addition, molecular size could be vital in these types of separations due to the helical cavity within the bile salt aggregate (56). Solutes larger than the cavity would be excluded from the micelle. Conversely, smaller molecules are able to more easily access this internal cavity, thus allowing enhanced interaction with the polar portion of the micelle.

Use of Cyclodextrins in CZE Chiral Separations

While chiral surfactants offer a viable approach to chiral separations in CE, an alternate approach to involves the addition of cyclodextrins to the mobile

phase. This is a prime example of the ease with which selectivity can be altered in CE, since a selective reagent is simply added to the mobile phase. This section describes applications of natural and chemically modified cyclodextrins to enantiomeric separations of the binaphthyl compounds and applies the technique to the determination of optical purity for a pharmaceutical preparation of synthetic phenylalanine. Future applications of cyclodextrins in CE mobile phases are presented and discussed.

Cyclodextrins (CDs) are formed by the enzymatic digestion of starch by cyclodextrin transglycosylase. Consisting of cyclic glucopyranose units, CDs are shaped much like a teacup (without handle). The monomeric units are arranged in such a way as to place the hydroxyl groups on the exterior of the structure, thus creating a hydrophobic cavity. The most commonly occurring CDs consist of 6, 7, and 8 glucopyranose units. Designated as α , β , and γ CD, respectively, these substances are able to form inclusion complexes with many molecules. Inclusion complex formation is determined by guest molecule hydrophobicity and, more interestingly, size. Guest molecules of dimensions larger than the CD cavity are excluded from the cavity while molecules of the proper dimensions may enter and form complexes. Inclusion complex formation has proven useful in separations of closely related molecules such as optical and positional ring isomers. Figure 28 shows schematic representations and cavity dimensions for these CDs. In general terms, the CD cavity for the α form will accommodate a single aromatic ring, the β a naphthlene ring, and the γ form can accomodate molecules as large as pyrene. These guidelines, while not exact, provide an estimation of the steric limitations of the various CD cavities.

Two of the more important applications of CDs are in the areas of drug delivery and drug separations. The ability of the cyclodextrin to form inclusion

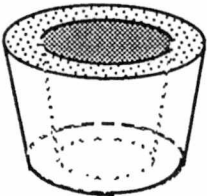
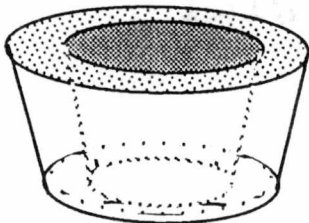
Cyclodextrin	#Glucopyranose units in structure	Cavity Size, nm
 α-CD	6	0.57
 β-CD	7	0.78
 γ-CD	8	0.95

Figure 28: Cyclodextrin Properties

complexes with a variety of host molecules and the water solubility of the CD allows the creation of soluble complexes from insoluble materials. Termed "molecular encapsulation" or "molecular camouflage", this approach to drug formulation represents a viable means of increasing bioavailability. The negligible toxicity of the cyclodextrin allows this approach to be used with a variety of drugs, since, under the appropriate conditions, the drug is displaced from the CD cavity and the CD eliminated from the biological system. One limitation to this approach is the limited water solubility of native CDs. β -CD is generally considered the most useful and is the most widely used form because its cavity dimensions allow complexation of a wide variety of molecules. However, β -CD is nearly an order of magnitude less soluble than the other derivatives. In order to circumvent this limitation, various chemical derivatization schemes were attempted. The most successful modifications involve reactions with the primary hydroxyl groups on the CD mouth. Several of the modifications involving methyl, ethyl, carboxymethyl, and glycosyl groups have resulted in solubilities in excess of 0.2 M. In addition, CD polymers also show enhanced solubility. Use of highly soluble CD derivatives thus allows introduction of a wide range of amounts to be used in CD drug formulation. Table VII compares the solubilities of native and two commercially available derivatives.

The ability of the CD cavity to discriminate between molecules on the basis of size has led to their use as stationary phase materials in chromatography. Conventional approaches involve stationary phases which contain immobilized CDs. These stationary phases have been used in both gas and liquid chromatography (80). Reversed-phase HPLC has employed bonded CD stationary phases for the separation of positional and polycyclic aromatic hydrocarbon ring isomers (80, 89). The natural optical activity of the

Table VII: Solubility of Cyclodextrins in Aqueous Solution

Cyclodextrin	Solubility, <u>M</u>
native α	0.121 ^a
native β	0.0163 ^a
native γ	0.168 ^a
hydroxypropyl- β	>0.2 ^b
1-glucosyl- α	>0.2 ^b

^aFrom Hinze *et al*; *Anal. Chem.* **1989**, *61*, 275.

^bManufacturers' Literature

CD cavity has allowed separation of the enantiomers of amino acids (90), nicotine analogues (91), and pharmaceuticals (92). Mobile phase composition can be used to alter retention on these phases, just as with conventional C-18 phases. Retention decreases with increasing organic solvent, and, as noted by Armstrong, pH, ionic strength, and temperature have a more dramatic impact on retention behavior than with conventional phases (80). Bonded CD stationary phases are commercially available and their use is well-established.

CDs have also found application in separations as mobile phase additives. CDs are ideal for this application since they offer the possibility of unique selectivity and they absorb uv light only slightly. CDs can thus be used at moderate to high concentrations in the HPLC mobile phase without causing problems with high background levels when absorbance detection is employed. Separations of barbiturates (93), and various structural isomers (5) have been reported with CDs present in the mobile phase. As in the case of drug formulation, native CD solubility limits this approach to low concentrations. Various solution additives and derivatives have been investigated for CD solubility enhancement. Hinze has studied the solubility of native CDs in solutions containing urea and sodium hydroxide (94). In solutions containing 8 M urea, β - and γ -CD solubility increased substantially while that of α -CD decreased. Solutions of 1.0 M NaOH also show relatively large increases in the solubility for all of the derivatives. As discussed above, chemical modification of the CD mouth can increase solubility. Two commercially available forms, 1-glucosyl α -CD and hydroxypropyl β -CD are studied for enantioselectivity in this work.

The use of cyclodextrin and cyclodextrin derivatives in CE is a logical extension of existing separation technology. Cyclodextrin use in CE separation schemes offers several important advantages over conventional HPLC

approaches. First, the small volumes of mobile phase required allow even the most expensive derivatives to be routinely used. Most analyses can be run repeatedly with only one mL of mobile phase, provided care is taken not to contaminate the inlet reservoir with sample. Second, problems associated with complex bonding chemistries involved with creating reversed-phase HPLC packings are circumvented. Also, as discussed earlier, the high efficiency afforded by CE allows accomplishment of difficult separations and short analysis times. This section will discuss approaches and applications of CDs as mobile phase additives in both CZE and MECC.

Two approaches may be used in CD-modified CZE to separate enantiomeric isomers. The first involves the separation of charged solutes by CZE. CD inclusion complex formation can alter the velocity of one enantiomer relative to the other. Enantiomeric separations occur with charged compounds in CZE when solutes differentially complex with a mobile phase additive. At low pH (pH 2), electroosmotic flow is small, and charged solutes generally migrate toward the electrode of opposite sign. In this situation, the enantiomer with the highest affinity for the CD migrates more slowly and elutes last (30). At high pH, electroosmotic flow is strong, and the electrophoretic migration of negatively charged solutes is more than compensated for by electroosmotic flow. In this case, the enantiomer forming the strongest CD complex has effectively a lower electrophoretic mobility and elutes from the capillary first.

Second, neutral compounds lack sufficient electrophoretic mobility to effectively migrate in the field, so merely adding a CD to the mobile phase is ineffective, since a neutral molecule/CD complex will migrate through the column at the mobile phase velocity. Neutral molecule separations in CE are effectively addressed with MECC, and chiral separations have been achieved via the use of chiral surfactants of synthetic (82, 83) and natural origin (48, 49,

50, 86). An alternate approach developed by Terabe and co-workers is cyclodextrin-modified MECC (95). In this form of MECC, CDs are added to the micellar mobile phase and selective interactions between CD and enantiomer occur, leading to optical resolution of injected solutes.

Enantiomeric separations with CDs often hinge on favorable interaction between solute and the CD cavity. Strong interaction with the CD does not always result in chiral separation. In some cases, solutes are able to form strong CD complexes without chiral interactions. The most important parameter is the type of CD employed. Figure 29 shows the separation of binaphthyl enantiomers under different conditions. Compounds based on the binaphthyl backbone have been used extensively in chiral synthesis (96). While the basic binaphthyl skeleton is identical between the two derivatives, differences exist in CD complex formation. Interaction with β -CD added to the mobile phase allows separation of the phosphorated form, but not the diacid derivative. Conversely, addition of α -CD to the mobile phase has the opposite effect (Fig. 29B). This is contrary to the observation that β -CD is generally more successful in enantiomeric separations of molecules possessing a naphthyl group (80). In this case, differences can likely be attributed to differences in free rotation about the 1,1' bond joining the naphthyl rings and differences in the angle between them (48). The BNPO_4 enantiomers, due to the bridging phosphate group, are less able to rotate than the diacid forms. As a result, the BNPO_4 enantiomers are not able to access the smaller α -CD cavity. The $\text{BN}(\text{COOH})_2$ enantiomers are able to change conformation more freely in such a way as to interact with the α -CD cavity. Interactions of $\text{BN}(\text{COOH})_2$ with β -CD, however, evidently does not result in a sufficiently snug fit for enantiomeric separation. The results presented in figure 29 A and B indicate the potential utility of using mobile phases containing mixed CDs when separating mixtures of

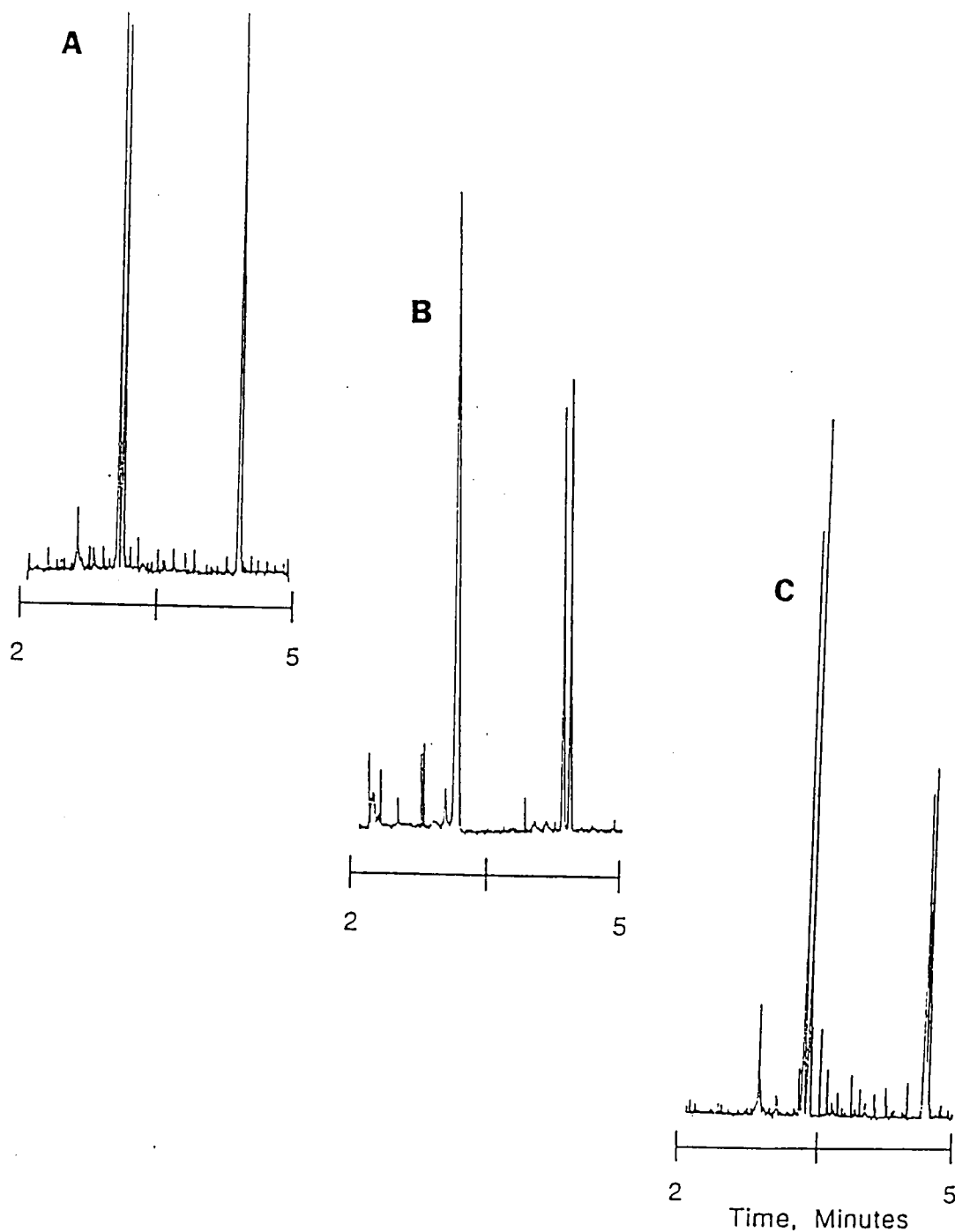


Figure 29: CZE separation of Charged Binaphthyls with Native CDs. Mobile phase 0.01M Na_2HPO_4 , 0.006 M $\text{Na}_2\text{B}_4\text{O}_7$. Column 50 cm total, 40 cm to detector, 50 μm i. d. Applied Voltage 20 kV. Absorbance detection with Linear 204 at 220 nm. Elution order BNPO_4 , $\text{BN}(\text{COOH})_2$.

enantiomeric compounds. Figure 29C shows the partial separation of both sets of enantiomers employing a mobile phase containing equal concentrations of α - and β -CD. Resolution is slightly poorer for each set of enantiomers in this case, most likely due to unfavorable interactions between solute and the improper CD cavity size. This approach shows potential for the use of mixed CD mobile phases. The fact that solutes are excluded from interaction with the CD cavity on the basis of size allows more than one type of selector to be present, since interferences between the two are not detrimental in some cases.

Chemical modification of the CD exterior can affect the enantioselectivity of guest-host complex formation. Figure 30 shows the separation of both charged binaphthyl compound enantiomers using a glycosylated α -CD derivative ($G_1\alpha$ -CD). In this case, interaction between the phosphoralated binaphthyl compound and $G_1\alpha$ -CD allowed enantiomeric recognition to occur. Chemical modification of the CD has been shown to "stretch" the cavity mouth and can change the steric qualities on the host/guest interaction. $BNPO_4$, as discussed earlier, is more rigid than the $BN(COOH)_2$ and is evidently requires a larger cavity for stereospecific inclusion. The cavity expansion afforded by the substitution of the CD primary hydroxyls may explain the separation of the $BNPO_4$ enantiomers demonstrated in figure 30. In the case of the $BNPO_4$ derivative, both enantiomers were available in pure form and the S eluted prior to the R form. These separations serve confirm the importance of the structure of the CD cavity mouth in chiral complexation (80, 97). Other examples of partially successful enantiomeric separations with $G_1\alpha$ -CD include dansyl-phenylalanine and dansyl-tryptophan, but only at high (0.1 M) concentrations. Underivatized α and β -CD were unsuccessful in achieving chiral separation

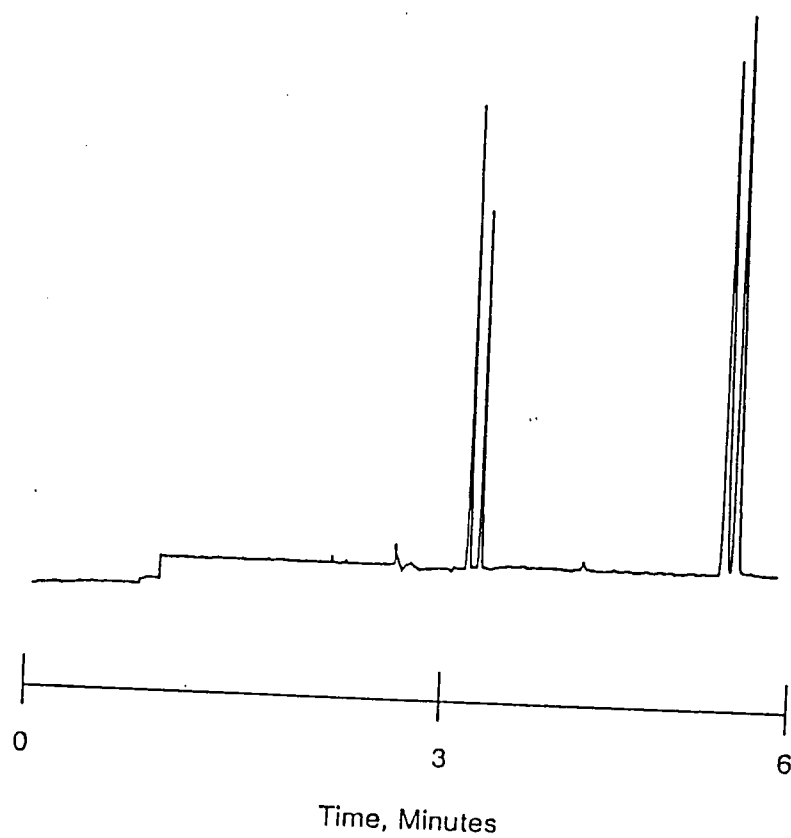


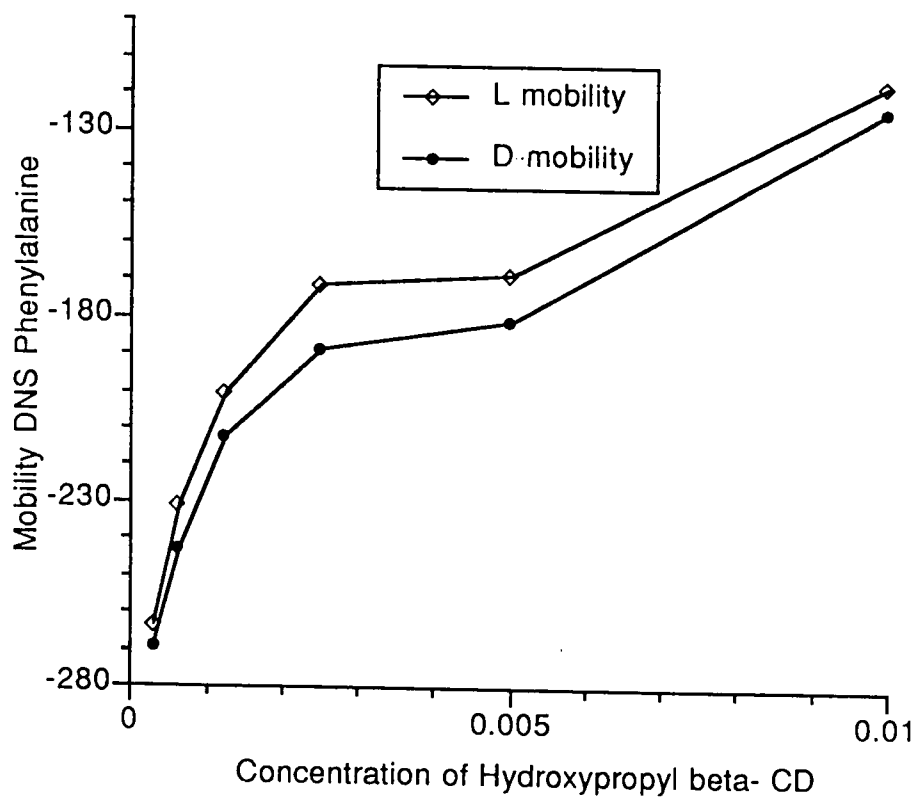
Figure 30: Separation of Binaphthyl Enantiomers with $G_1\alpha$ -CD. Mobile phase 0.01 M $G_1\alpha$ -CD. All other conditions as in Figure 29.

of these compounds.

An alternative form of β -CD, hydroxypropyl β -CD (HP β -CD), was examined for utility in chiral separations of the binaphthyl compounds. Similar results were obtained for this derivative as for G $_1\alpha$ -CD, with the exception that both sets of binaphthyl compounds were separated with HP β -CD in the mobile phase. In addition, the HP β form allowed separation of the enantiomers of DNS-phenylalanine and DNS-tryptophan. While the cavity of the HP β -CD is larger than the G $_1\alpha$ -CD, interactions at the mouth of the CD cavity are apparently critical for the enantiomeric recognition of binaphthyl and dansyl amino acid compounds.

Enantiomeric resolution depends on the concentration of the CD in the mobile phase. Figure 31 shows mobility of DNS-phenylalanine enantiomers as a function of concentration of HP β -CD in the mobile phase. At low (0.0003 M) concentrations, mobilities are similar and enantiomeric resolution is low. As the concentration is increased, the L- form increasingly finds enantiomerically favorable interaction with the rapidly moving CDs. Mobility differences, and thus resolution peaks at 0.0025 M HP β -CD. Further increases in the concentration of HP β -CD decrease resolution and cause a nearly equal reduction in the mobilities of both enantiomers. The guest-host interaction is driven by the tendency of solutes to associate with the hydrophobic interior of the CD cavity. Under some conditions, as in the case with DNS-Phe enantiomers, chiral separation can occur. In other instances (high CD concentration), however, enantioselective interactions are "swamped out" by non-specific hydrophobic association.

A potentially important application of CD-modified CZE is that of pharmaceutical analysis. Optical purity confirmation is important in situations where one enantiomer elicits undesirable physiological response or has toxic



Conditions: 0.01 M Disodium Phosphate/0.006 M
Disodium Borate, pH 9.
25 μ m i. d. column, 50 cm X40 cm to detector.
Applied Voltage 25 kV

Figure 31: Mobility of DNS-Phenylalanine Enantiomers vs. [HP β -CD]

effects. CD-CZE has, in some instances, the ability to provide a rapid means of analyzing routine samples. Figure 32 shows the separation of a nutritional formulation of DL-phenylalanine after derivitization with dansyl chloride. The total analysis time is roughly 90 seconds and the phenylalanine enantiomers show baseline resolution. The use of narrow bore (25 μ m) capillaries and laser-based detection is critical in achieving rapid separation and high sensitivity, as discussed in chapter 4. The application of high-speed CE to optical purity determination shows significant potential in the pharmaceutical area, since many formulations represent relatively simple matrices. In addition, optical purity determination of amino acids are of interest in geologic dating (98) and in and in the synthesis of synthetic antibiotics (99). CE thus offers greatly increased throughput in this area since analysis times can be reduced to 1-5 minutes in most cases. Further applications of this technology to new enantiomeric materials are inevitable.

Cyclodextrins can also be used to alter selectivity in the MECC mode. CD-modified MECC has been reported for the separations of hydrophobic compounds (47, 100). In these applications, native CDs were used in conjunction with large concentrations (4-8 M) urea to effect separations of steroidal compounds and polycyclic aromatic hydrocarbons (PAH). While somewhat successful, the PAH used as test compounds were relatively small (4 ring) and closely related isomers were not included. In addition, it was not made clear exactly what purpose urea served in the separation. Urea addition to the mobile phase has two effects in this situation. First, urea increases the solubility of β and γ CDs. Second, urea adds organic character to the mobile phase, reducing mobile phase polarity. Thus, urea can act much like an organic solvent in reducing k' values to optimum levels in order to improve resolution for hydrophobic compounds. While the chromatographic selectivity

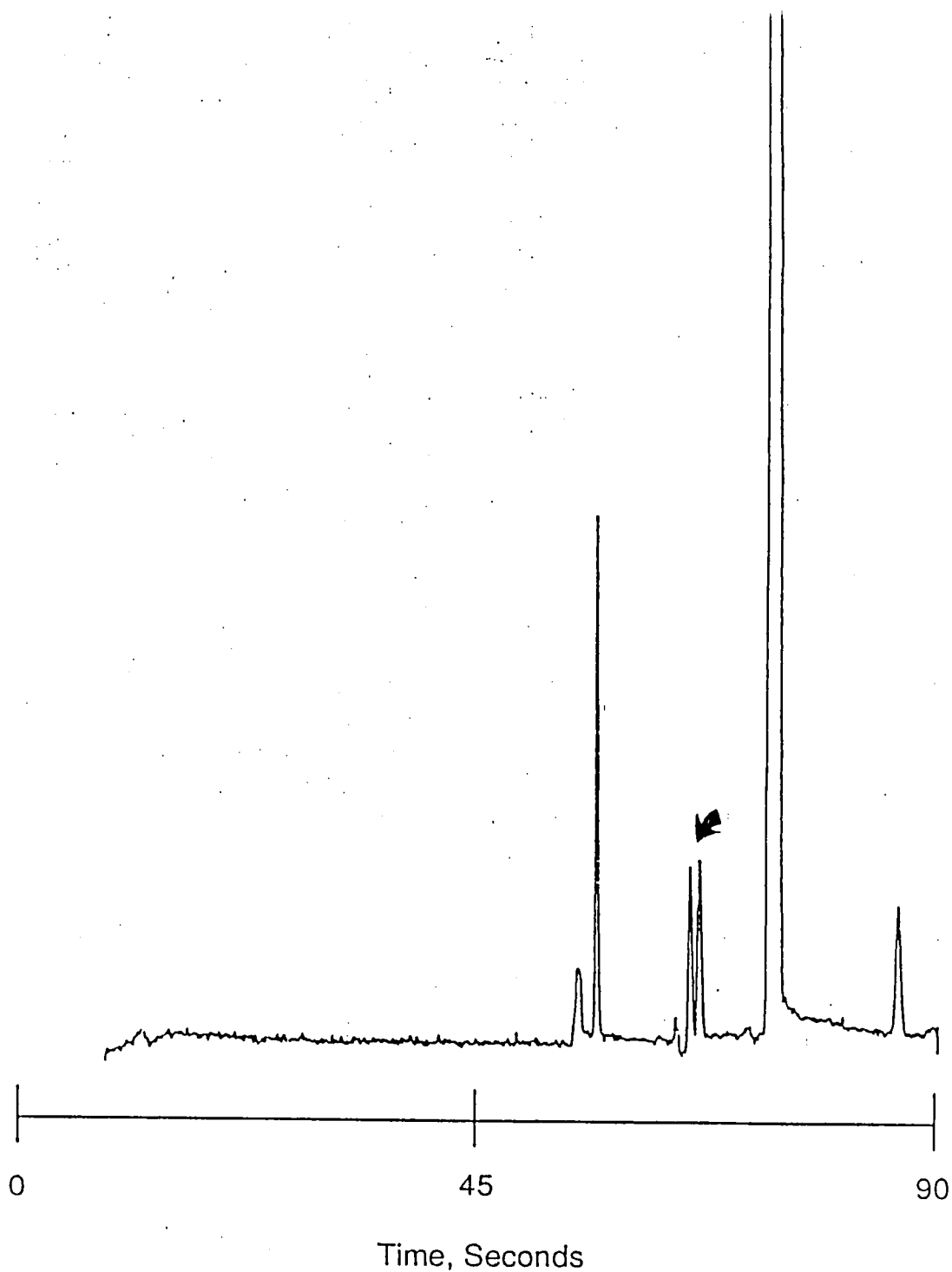


Figure 32: Rapid Separation of DNS-Phenylalanine Enantiomers In a Commercial Formulation. Mobile phase 0.005 M HP β -CD, 0.01M Na₂HPO₄, 0.006 M Na₂B₄O₇. Column 50 cm total, 40 cm to detector, 25 μ m i. d. Detection: Laser fluorescence, λ_{ex} 325 nm, λ_{em} 540 nm. Applied Voltage 35 kV.

shows definite effects due to the presence of CD in the mobile phase, the full extent of the presence (and purpose) of urea is unclear in those particular applications.

Exploratory work with selectivity enhancements via the use of highly soluble CD derivatives has proven to be successful for separations of highly hydrophobic molecules such as methylanthracene derivatives. As a first experiment, a mixture of anthracene, 2-methylanthracene, and 9-methylanthracene was used to gauge separation performance. Under normal conditions (0.05 M SDS, 0% organic solvent), these solutes co-eluted at the micelle migration time. As discussed earlier, a first approach to the optimization of resolution for such compounds is the addition of organic solvents such as acetonitrile to the mobile phase. In this case, a 20% (v/v) acetonitrile mobile phase was required to achieve baseline separation of the three solutes, requiring an analysis time of approximately 45 minutes. Figure 33 shows the partial separation of the same mixture using 0.05 M HP β -CD in the standard SDS system. Resolution occurs as a result of reduction of k' to more optimal values and on differential complexation with the CD cavity. The elution order is thus a function of a solutes' ability to enter the CD cavity. Solutes which are more sterically hindered with protruding functional groups are more excluded from complexation with the CD and spend more time solubilized in the micelle interior. In this case, the anthracene is not sterically hindered to the extent that the 2- and 9- methyl derivatives and elutes prior to them. Use of HP β -CD, in contrast to organic modifier, does not dramatically reduce electroosmotic flow, resulting in the much reduced analysis time in Figure 33. Concentration of the CD is important; below 0.05 M resolution degrades and analysis time increases, while doubling the CD concentration to 0.1 M resulted in reduced analysis time and decreased resolution. It is instructive to note that native β -CD is limited

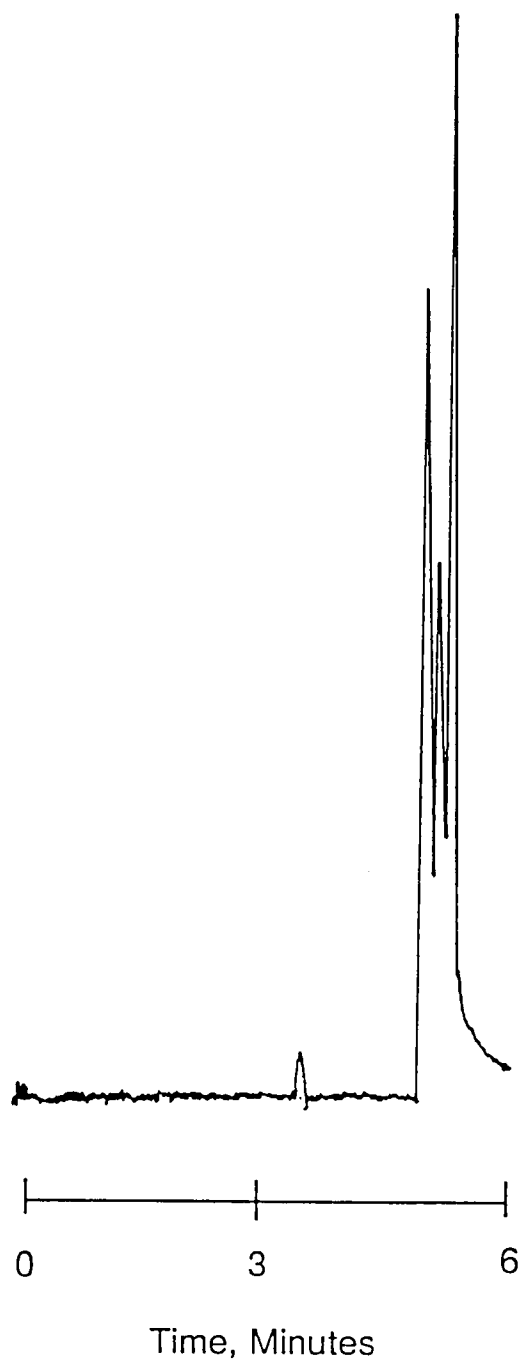


Figure 33: Separation of Methylanthracene Isomers. Mobile phase 0.05 M HPb-CD, 0.05 M SDS, 0.01M Na₂HPO₄, 0.006 M Na₂B₄O₇. Column same as in figure 32. Applied Voltage 25 kV. Detection: HeCD laser fluorescence, λ_{ex} 325nm, λ_{em} 540 nm.

to a much lower concentration (0.01 M) and is not useful in situations where higher concentrations are required. Based on past results, it is expected that improved resolution will result with the intermediate CD concentration coupled with extension of the elution range as a result of organic modifier addition to the mobile phase. Combinations such as this have the potential to be used in separating closely related hydrophobic compounds. Future work involves developing strategies such as these for the separation of 5 and 6-ring PAH, and the investigation of different CD derivatives for both isomeric and enantiomeric compounds.

Chapter 6

Concluding Remarks

Capillary electrokinetic techniques offer a wide variety of modes of chromatographic and electrophoretic selectivity via solute interactions with mobile phase additives. High efficiency, speed of analysis, and instrumental simplicity give the CE approach the potential to become a heavily-used separation technique in a variety of scientific and technical fields. In some ways, CE realizes many of the goals sought after in the development of microcapillary LC. In the terms of selectivity, CE represents an avenue whereby many different compounds may be investigated for use as mobile phase additives due to the fact that selectivity is controlled by changing the composition of the mobile phase. Of the variety of mobile phase additives available, organized media such as surfactants and cyclodextrins have allowed advances in range of applicability, speed of analysis, and selectivity.

Species such as surfactants allow the separation of neutral compounds through micelle formation and constitute the "stationary" phase in MECC. Chromatographic selectivity is strongly influenced by the nature of the surfactant, as illustrated by the bile salts. As an example, consider a comparison of bile salt micelles with SDS micelles. The former are more polar and can tolerate the presence of organic solvents to a greater degree. Chiral separations are also possible, since bile salt aggregates are optically active. While these are certainly advantageous features relative to SDS, selectivity is similar in terms of elution order. Thus, different surfactants may offer unique selectivities in terms of chiral separations. However, general retention trends

are similar in terms of hydrophobicity due to the nature of the surfactant. This is because the surfactant molecule must possess a hydrophobic region for micelles to form. The interior of the normal micelle will thus always be hydrophobic. Polar phases such as used in conventional HPLC will most likely not have their micellar counterparts in MECC.

One potential avenue of research in selectivity is the development of novel chiral surfactants for use in optical purity determinations. MECC is an ideal format for the investigation and utilization of such compounds since even an expensive compound could find practical use due to the small amounts required. New surfactants are needed if growth is to continue in this area.

Another possible application of MECC is the study of micellization processes. A solute's migration behavior and peak shape are directly related to the the composition of the solution, which in turn affects the nature of the micellar aggregate. MECC could potentially be used in the determination of CMC, micellization kinetics, and, through the use of other additives, aggregate structure. Thus, MECC potentially represents a method to study solution chemistry analogous to the inverse GC study of adsorbents.

Cyclodextrins and their derivatives show promise as additives in both CZE and MECC. In CZE, simple addition of CDs to the mobile phase can result in chiral separations of charged compounds. Chiral separations are achieved for compounds which are able to form enantioselective inclusion complexes with the CD cavity. The most notable advantage in this case is that the CD is present in free solution; no bonding chemistry is required to create a stationary phase material as with conventional HPLC bonded phases. Thus, mobile phases containing mixed types of CD can be used to separate mixtures of enantiomeric compounds.

Many potential avenues for novel selectivity lie with CD derivatives. Commercially available derivatives include soluble CD polymers, various alkylated CDs, and ionizable forms of CDs. Each of these derivatives has the potential to add novel selectivities to the CE arsenal. Additionally, chiral co-complexation agents such as camphor derivatives may be explored for use in CD-modified CZE and MECC (101). Neutral compounds can be addressed with CD-type interactions via their addition to the MECC mobile phase. The MECC separation of methylanthracene isomers presented on page 105 shows the utility of CDs as a specific means of reducing k' values for hydrophobic species. As in the CZE case, the various CD derivatives will find uses in MECC.

In addition to organized media, other reagents have potential for use in CE separations. Sugars such as glucose form anionic complexes with borate at alkaline pH and, since they possess electrophoretic mobility, may represent possible pseudostationary phases. Like the bile salts, they are polar and are optically active, hinting at applications involving hydrophobic molecules and chiral separations. Many proteins also show promise in these areas. Proteins such as bovine serum albumin (BSA) have been used as stationary phase materials for chiral separations in HPLC and could be useful in CE. At alkaline pH values, BSA is negatively charged and could be used as a pseudostationary phase material. The diversity of proteins available in pure form (including many affinity proteins) hints at a wealth of possible selectivities.

It is apparent that CE has established itself as a useful analytical technique. Goals to be achieved before the technique enjoys routine use are those of retention time reproducibility, sensitive detection, and instrumental reliability. As in the development of other chromatographic methods, column technology and instrumentation will be critical to the success of CE. Based on the attention CE has thus far received, it is expected that this methodology will

undoubtedly continue to improve in coming years, and progress currently continues in these areas. In terms of selectivity, CE is perhaps one of the more formidable separation techniques, as the possibilities are limited only by solution additives and ability of the researcher to exploit solution chemistries.

References

- (1) Tswett, M. *Travl. Soc. Naturalistes Varisovic* **1903**, 14, 1903.
- (2) James, A. T.; Martin, A. J. P. *Analyst* **1952**, 77, 915.
- (3) Wolley, C. L.; Corman, S. D.; Ervin, N. G.; Long, R. E.; Spock, P. S.; Robillard, M. V.; Shirey, R. E. *Longer Capillary Columns for High Resolution Gas Chromatography*; Sandra, P., Redant, G., Huthig: Monterey, CA, 1990; p 23.
- (4) Sinha, M. P.; Gutnikov, G. *Anal. Chem.* **1991**, 63, 2012.
- (5) Tanaka, M.; Miki, T.; Shono, T. *J. Chromatogr.* **1985**, 330, 253.
- (6) Armstrong, D. W. *Separation and Purification Methods* **1985**, 14, 213.
- (7) Ettre, L. S.; Zlatkis, A. *75 years of chromatography- a dialouge* in Journal of Chromatography Library; Eds.; Elsevier: New York, Vol. 17, p 502.
- (8) Jorgenson, J. W.; Lukacs, K. D. *Clin. Chem.* **1981**, 27, 1551.
- (9) Jorgenson, J. W.; Lukacs, K. D. *Anal. Chem.* **1981**, 53, 1298.
- (10) Knox, J. H.; Grant, I. H. *Chromatographia* **1987**, 24, 135.
- (11) Novotny, M. *Anal. Chem.* **1988**, 60, 500.
- (12) Grushka, E.; McCormick, R. M.; Kirkland, J. J. *Anal. Chem.* **1989**, 61, 241.
- (13) Dougherty, A. M.; Wooley, C. L.; Williams, D. L.; Swaile, D. F.; Cole, R. O.; Sepaniak, M. J. *J. Liq. Chrom.* **1991**, 14, 907.
- (14) Lauer, H. H.; McManigill, D. *Anal Chem.* **1986**, 58, 166.
- (15) Smith, R. D.; Olivares, J. A.; Nguyen, N. T.; Udseth, H. R. *Anal. Chemistry* **1988**, 60, 436.

- (16) Jorgenson, J. W.; Lukacs, K. D. *J. Chromatogr.* **1981**, *248*, 241.
- (17) Schwer, C.; Kenndler, E. *Chromatographia* **1990**, *30*, 546.
- (18) Ewing, A. G.; Wallingford, R. A.; Olefirowicz, T. M. *Anal. Chem.* **1989**, *61*, 292.
- (19) Jorgenson, J.; Walbroehl, Y. *Anal. Chem.* **1986**, *58*, 479.
- (20) Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchiya, A.; Ando, T. *Anal. Chem.* **1984**, *56*, 111.
- (21) Terabe, S.; Otsuka, K.; Ando, T. *Anal. Chem.* **1985**, *57*, 834.
- (22) Balchunas, A. T.; Sepaniak, M. J. *Anal. Chem.* **1987**, *59*, 1466.
- (23) Gorse, J.; Balchunas, A. T.; Swaile, D. F.; Sepaniak, M. J. *J. High Res. Chromatogr. and Chromatogr. Comm.* **1988**, *11*, 554.
- (24) Balchunas, A. T.; Sepaniak, M. J. *Anal. Chem.* **1988**, *60*, 617.
- (25) Sepaniak, M. J.; Swaile, D. F.; Powell, A. C. *J. Chromatogr.* **1989**, *480*, 185.
- (26) Powell, A. C.; Sepaniak, M. J. *J. Microcol. Sep.* **1990**, *2*, 293.
- (27) Fanali, S. *J. Chromatogr.* **1991**, *545*, 437.
- (28) Fujiwara, S.; Honda, S. *Anal. Chem.* **1987**, *59*, 487.
- (29) Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. *Chromatographia* **1986**, *21*, 583.
- (30) Swartz, M. *J. Liq. Chrom.* **1991**, *14*, 923.

- (31) Morris, M. D.; Huang, S.-D.; Demana, T.; Chen, C.-Y. *Anal. Chem.* **1989**, *61*, 1590.
- (32) Chen, C.-Y.; Morris, M. D. *Appl. Spectrosc.* **1988**, *42*, 515.
- (33) Dovichi, N. J.; Yu, M. *Anal. Chem.* **1989**, *61*, 37.
- (34) Yeung, E. S.; Kuhr, W. *Anal. Chem.* **1988**, *60*, 1832.
- (35) Zare, R. N.; Huang, X.; Pang, T. J.; Gordon, M. J. *Anal. Chem.* **1987**, *59*, 2747.
- (36) Ewing, A. G.; Wallingford, R. A. *Anal. Chem.* **1988**, *60*, 258.
- (37) Smith, R. D.; Barinaga, C. J.; Udseth, H. R. *Anal. Chem.* **1988**, *60*, 1948.
- (38) Titus, R.; Gurka, D. F.; D., B. L.; Hinnners, T. A.; Heithmar, E. M.; Henshaw, J. M. *Anal. Chem.* **1988**, *60*, 455.
- (39) Attwood, D.; Florence, A. T. *Surfactant Systems: Their Chemistry, Pharmacy, and Biology*; Chapman and Hall: New York, 1983.
- (40) Zana, R.; Aniansson, E. A. G.; Wall, S. N.; Almgren, M.; Hoffmann, H.; Kielmann, I.; Ulbricht, W.; Lang, J., et al. *J. Phys. Chem.* **1976**, *80*, 905.
- (41) Small, D. M.; Carey, M. G. *J. Colloid Interface Sci.* **1969**, *31*, 382.
- (42) Hinze, W. L. *Organized Surfactant Assemblies in Separation Science in Ordered Media in Chemical Separations*; Hinze, W. L., Armstrong, D. W., Eds.; American Chemical Society: Washington D. C., Vol. 342, p 2.
- (43) Fisher, L.; Oakenfall, D. *Aust. J. Chem.* **1979**, *32*, 31.
- (44) McIntire, G. L. *Critical Reviews in Analytical Chemistry* **1990**, *21*, 257.
- (45) Sepaniak, M. J.; Powell, A. C.; Swaile, D. F.; Cole, R. O. *Fundamentals of Micellar Electrokinetic Capillary Chromatography* in Handbook of

Capillary Electrophoresis; Colburn, J. C. , Grossman, P. D., Eds.; Academic Press: New York, Vol. In Press.

- (46) Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. *J. Chromatogr. Sci.* **1987**, *25*, 514.
- (47) Terabe, S.; Miyashita, Y.; Shibata, O.; Barnhart, E.; Alexander, L. R.; Patterson, D. G.; Karger, B. L.; Hosoya, K., et al. *J. Chromatogr.* **1990**, *516*, 23.
- (48) Cole, R. O.; Sepaniak, M. J.; Hinze, W. L. *J. High Res. Chrom.* **1990**, *13*, 579.
- (49) Nishi, H.; Fukuyama, T.; Matsuo, M.; Terabe, S. *J. Chromatogr.* **1990**, *515*, 233.
- (50) Terabe, S.; Nishi, H.; Fukuyama, T.; Matsuo, M. *J. Microcol. Sep.* **1989**, *1*, 234.
- (51) Nishi, H.; Fukuyama, T.; Matsuo, M.; Terabe, S. *J. Chromatogr.* **1990**, *498*, 313.
- (52) Nishi, H.; Fukuyama, T.; Matsuo, M.; Terabe, S. *J. Chromatogr.* **1990**, *513*, 279.
- (53) Small, D. M. *The Physical Chemistry of Cholanic Acids in The Bile Acids*; Nair, P. P. , Kritchevsky, D., Eds.; Plenum Press: New York, Vol. 1, p 249.
- (54) Small, D. M. *Advan. Chem. Ser.* **1968**, 31.
- (55) Conte, G.; Di Blasi, R.; Giglio, E.; Parretta, A.; Pavel, N. V. *J. Phys. Chem.* **1984**, *88*, 5720.
- (56) Esposito, G.; Giglio, E.; Pavel, N. V.; Zanobi, A. *J. Phys. Chem.* **1987**, *91*, 356.
- (57) Campanelli, A. R.; De Sanctis, C.; Chiessi, E.; D'Alagni, M.; Giglio, E.; Scaramuzza, L. *J. Phys. Chem.* **1989**, *93*, 1536.

- (58) Kawamura, H.; Manabe, M.; Narikiyo, T.; Igimi, H.; Murata, Y.; Sugihara, G.; Tanaka, M. *J. Solution Chem.* **1987**, *16*, 433.
- (59) Cole, R. O.; Sepaniak, M. J.; Hinze, W. L.; Oldiges, K.; Gorse, J. J. *Chromatogr.* **1991**, In Press .
- (60) Murray, G. M.; Sepaniak, M. J. *J. Liq. Chromatogr.* **1983**, *6*, 931.
- (61) Zare, R. D.; Diebold, G. J. *Science* **1977**, *196*, 1439.
- (62) Balchunas, A. T.; Swaile, D. F.; Powell, A. C.; Sepaniak, M. J. *Sep. Sci. Technol.* **1988**, *23*, 1891.
- (63) Terabe, S.; Otsuka, K.; Ando, T. *Anal. Chem.* **1989**, *61*, 251.
- (64) Magid, L. in *Solution Chemistry of Surfactants*; Mittal, K. L., Eds.; Plenum Press: New York, Vol. p 427.
- (65) Becher, G.; Bjørseth, A. *Determination of Occupational Exposure to PAH by Analysis of Body Fluids*. in *Handbook of Polycyclic Aromatic Hydrocarbons*; Bjørseth, A. , Ramdahl, T., Eds.; Marcel Dekker, Inc.: Ney York, Vol. 2, p 237.
- (66) Sepaniak, M. J.; Burton, D. E.; Maskarinec, M. P. *Micellar Electrokinetic Capillary Chromatography in Ordered Media in Chemical Separations*; Hinze, W. L. , Armstrong, D. W., Eds.; American Chemical Society: Washington, D.C., Vol. 342, p 142.
- (67) Jorgenson, J. W.; Bushey, M. M. *Anal. Chem.* **1990**, *62*, 978.
- (68) Jorgenson, J. W.; Monnig, C. A. *Anal. Chem.* **1991**, *63*, 802.
- (69) Sepaniak, M. J.; Cole, R. O. *Anal. Chem.* **1987**, *59*, 472.
- (70) Diener, U. L.; Davis, N. D. *Aflatoxin Formation by Aspergillus flavus in Aflatoxin: Scientific background, Control, and Implications*; Goldblatt, L. A., Eds.; Academic Press: New York, Vol. 7, p 22.

- (71) Smith, D. J., "Analysis of Mold Growth and Aflatoxin Development in Grain Sorghum" Master of Science Thesis, The University of Tennessee, 1989.
- (72) Wilson, D. M. *Arch. Environ. Contam. Toxicol.* **1989**, *18*, 308.
- (73) Swaile, D. F.; Sepaniak, M. J. *Anal. Chem.* **1991**, *63*, 179.
- (74) Draughon, F. A.; Elahi, M. E.; West, D. R. *J. Agric. Food Chem.* **1983**, *31*, 692.
- (75) Sepaniak, M. J.; Swaile, D. F. *J. Microcol. Sep.* **1989**, *1*, 155.
- (76) Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. *J. Chromatogr. Sci.* **1986**, *24*, 347.
- (77) Cline Love, L. J.; Habarta, J. G.; Dorsey, J. G. *Anal. Chem.* **1984**, *56*, 1132.
- (78) Beaver, R. W. *Arch. Environ. Contam. Toxicol.* **1989**, *18*, 315.
- (79) McGown, L. B.; Cobb, W. T. *Anal. Chem.* **1990**, *62*, 186.
- (80) Armstrong, D. W.; Han, S. M. *CRC Critical Reviews in Analytical Chemistry* **1988**, *19*, 175.
- (81) Hinze, W. L.; Williams, R. W.; Fu, Z. *J. Chrom. Sci.* **1990**, *28*, 292.
- (82) Dobashi, A.; Ono, T.; Hara, S.; Yamaguchi, J. *Anal. Chem.* **1989**, *61*, 1984.
- (83) Dobashi, A.; Ono, T.; Hara, S.; Yamaguchi, J. *J. Chromatogr.* **1989**, *480*, 413.
- (84) Otsuka, K.; Terabe, S. *J. Chrom.* **1990**, *515*, 221.

- (85) Terabe, S.; Shibata, M.; Miyashita, Y. *J. Chromatogr.* **1989**, *480*, 403.
- (86) Nishi, H.; Fukuyama, T.; Matso, M.; Terabe, S. *Anal. Chim. Acta* **1990**, *236*, 281.
- (87) Cram, D. J.; Sogah, G. D.; Kyba, E. P.; Gokel, G. W.; de Jong, K.; Koga, K.; Sousa, L. R.; Siegel, L. *J. Org. Chem.* **1977**, *42*, 4173.
- (88) Mislow, K.; Glass, M. A. W.; O'Brien, R. E.; Rutkin, D. H.; Steinberg, D. H.; Weiss, J.; Djerassi, C. *J. Am. Chem. Soc.* **1962**, *84*, 1455.
- (89) Armstrong, D. W.; Ward, T. J. *Cyclodextrin Stationary Phases in Chromatographic Chiral Separations*; Zief, M., Crane, L. J., Eds.; Marcel Dekker: New York, Vol. 40, p 131.
- (90) Armstrong, D. W.; Yang, X.; Han, S. M.; Menges, R. A. *Anal. Chem.* **1987**, *59*, 2594.
- (91) Armstrong, D. W.; Timmons, K. D.; Ward, T. J.; Seeman, J. L.; Secor, H. V. *Anal. Chem.* **1988**, *60*, 2120.
- (92) Gubitz, G. *Chromatographia* **1990**, *30*, 555.
- (93) Sybilska, D.; Zukowski, J.; Bojarski, J.; Szejtli, J. *J. Chromatogr.* **1988**, *436*, 381.
- (94) Hinze, W. L.; Sheng Fu, Z.; Smith, T. K.; Pharr, D. Y. *Anal. Chem.* **1989**, *61*, 275.
- (95) Terabe, S. *Trends Anal. Chem.* **1989**, *8*, 129.
- (96) Noyori, R.; Takaya, H. *Acc. Chem. Res.* **1990**, *23*, 345.
- (97) Armstrong, D. W.; Hilton, M.; Coffin, L. *LC/GC* **1991**, *9*, 646.
- (98) Engel, M. H.; Hagy, B. *Nature* **1982**, *296*, 837.

- (99) Allenmark, S. G. *Chromatographic Enantioseparation Methods and Applications* in Ellis Horwood Series in Analytical Chemistry; Chalmers, R. A. , Masson, M., Eds.; John Wiley and Sons: New York, Vol. p 146.
- (100) Nishi, H.; Matsuo, M. *J. Liq. Chrom.* **1991**, *14*, 973.
- (101) Nishi, H.; Fukuyama, F.; Terabe, S. *J. Chromatogr.* **1991**, *553*, 503.

Vita

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