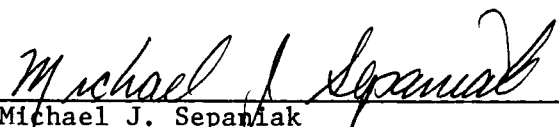
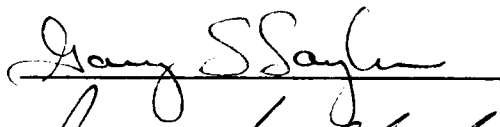
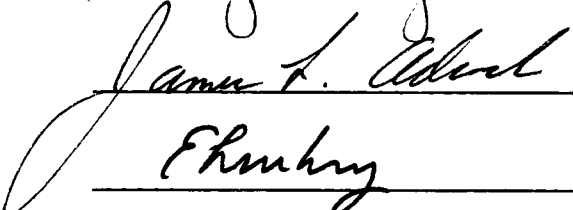


To the Graduate Council:

I am submitting herewith a dissertation written by Dewey Edward Burton entitled "Development and Applications of Micellar Electrokinetic Capillary Chromatography." 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:


Vice Provost
and Dean of The Graduate School

DEVELOPMENT AND APPLICATIONS OF MICELLAR
ELECTROKINETIC CAPILLARY CHROMATOGRAPHY

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Dewey Edward Burton

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Finally, the love and support of dedicated parents are assets in any endeavor.

ABSTRACT

The experimental parameters affecting efficiency and selectivity in micellar electrokinetic capillary chromatography (MECC) were investigated. The use of the technique to separate the metabolites of vitamin B₆ and the quantitation of pyridoxic acid in urine are also presented.

The observed efficiency in a chromatographic technique is dependent upon many experimental factors. The effect of the injection procedures on the observed efficiency was evaluated. The operational parameters of injection voltage and duration of the injection process for electroinjection procedures were examined. Recommendations for injection procedures are discussed for the minimization of injection contributions to band broadening. The effects on the observed efficiency of column parameters were evaluated. Van Deemter type plots were constructed to examine the individual contributions to band broadening in MECC. Column diameter, buffer concentration, applied voltage, and surfactant concentration were studied to elucidate their role in the separation process.

The diversity of available stationary and mobile phases increases the versatility of any chromatographic technique. In MECC the micelles in the mobile phase perform the function of a stationary phase in conventional liquid chromatography. The applicability of four surfactant systems was examined. Test solutes were used to gauge the performance of each micellar system.

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CHAPTER I

INTRODUCTION

Statement of the Problem

Liquid chromatography (LC) has become an indispensable tool for the modern separation scientist. Many nonvolatile or thermally labile analytes can only be satisfactorily analyzed utilizing liquid chromatographic techniques. The advent of high performance liquid chromatography (HPLC) has resulted in the widespread use of liquid chromatography for routine analyses.

A considerable volume of research has been concerned with the improvement of liquid chromatography. Within the last ten years one of the major thrusts of chromatographic research has centered in the area of high resolution liquid chromatography. This research has evolved from the desire to increase the separating power of liquid chromatography. Complex mixtures can be analyzed as more efficient separation techniques are developed. As chromatographic efficiency is increased, more components can be separated in a reasonable amount of time.

Much effort has been expended to increase the efficiency in liquid chromatography. Open-tubular liquid chromatography (OTLC) (1-8) and packed microcolumn liquid chromatography (PMCLC) (9-12) have dramatically increased the attainable efficiency in liquid chromatography. However, the experimental difficulties encountered with these techniques have been formidable.

Column preparation is a very critical aspect of the development of a high resolution chromatographic technique. In OTLC column preparation consists of several tedious steps which include drawing of large diameter stock glass tubing to the appropriate internal diameter (5-50 μm), modification of the inside surface of the capillary in order to increase the available surface area, and finally bonding or coating the inside of the capillary with a suitable stationary phase. Column occlusion during the preparative steps is quite frequent. The probability of a column surviving the numerous preparation and rinsing steps is low. Dynamically modified open-tubular liquid chromatography has alleviated some of the column occlusion problems in OTLC (13). In dynamically modified OTLC the stationary phase is provided through the addition of a surfactant in the mobile phase, thus eliminating the preparation step of bonding the stationary phase to the column wall. Column preparation in PMCLC can also be difficult. It is important with any packed column chromatography to achieve a uniformly packed bed. In general smaller diameter particles ($< 10 \mu\text{m}$) are employed which can increase the frequency of packing irregularities. The reproducibility of packing smaller diameter columns is at present low and the presence of void spaces in the chromatographic bed can severely degrade the efficiency of the column.

The small internal diameters of columns in OTLC place extreme constraints on the detectors. The low volume requirements for detectors have necessitated the development of laser-based detection schemes.

PMCLC utilizes larger diameter columns (CA. 200-400 μm) and the detector requirements are not as stringent.

The injection requirements for OTLC and PMCLC techniques are very demanding. Commercially available "loop" injectors for HPLC have injection volumes which range from 100 nL to 1 mL. For an OTLC column which is 10 m in length and 20 μm i. d., the total column volume is approximately 13 μL . Therefore, most commercial injectors deliver injection volumes which would severely overload these columns. Commercial injectors are available, which are capable of delivering injection volumes less than 100 nL onto the chromatographic column. However, the reproducibility of these injectors in connection with OTLC or PMCLC have not been satisfactory. Attempts have been made to alleviate this problem with moderate success (4, 6, 13).

A final consideration with these high resolution chromatographic techniques is the pumping of the mobile phase. Although the permeability of open capillary columns is high, the pressure drop across columns with very small diameters can be tremendous. Conversely, the permeability of the packed microcapillaries is very low. Thus, the pumping systems have to withstand the extreme back pressures generated with these columns. Syringe pumps are usually employed to meet the operating constraints of the open-tubular and packed microcapillaries and have to be constructed to the specifications for particular experimental designs. Another difficulty encountered with pumping systems for narrow-bore columns is flow control at the low mobile phase flow velocities required for these techniques. Many complex separations in LC

require the use of gradient elution techniques. Problems with mobile phases mixing as well as solvent delivery can be present in these capillary liquid chromatographic techniques.

Micellar electrokinetic capillary chromatography (MECC) is a separation technique which is capable of very efficient separations. Efficiencies over 200,000 theoretical plates/m are routinely attained. The development of MECC has addressed many of the operational problems associated with existing high resolution chromatographic techniques. The column preparation necessary in MECC is minimal compared to other techniques and since the mobile phase is pumped electrokinetically, many of the requirements of the external pumping system are eliminated. The experimental simplicity of MECC coupled with its efficient separations justify the investigation of the operational principles to maximize its utility as a separation technique.

In this dissertation micellar electrokinetic capillary chromatography will be examined as a potential high resolution liquid chromatographic technique. Discussion of the effects of the operational parameters in MECC on efficiency and selectivity will be presented. The analysis of a "real" sample will be evaluated and presented to express the eventual applicability of MECC.

Development of Micellar Separations

The addition of surfactants to chromatographic mobile phases has been used to accomplish two purposes. First, surfactant can be added to the mobile phase in a concentration lower than the critical micelle

concentration (CMC) to obtain surfactant monomers. The ionic surfactant monomers present in the mobile phase can ion-pair with ionic solutes of opposite charge. Reversed-phase ion-pair HPLC has been used to refer to this technique. Second, when the concentration of the surfactant exceeds the CMC, micelles are present in an aqueous phase. Solute may partition with the micelles in addition to interacting with the packed stationary phase. The terms, pseudophase liquid chromatography or micellar liquid chromatography, have been used to describe this technique.

Poor separations of ionic solutes have often occurred in liquid chromatography due to the presence of a variety of ionic species for a particular solute and unfavorable interactions between the ionic analytes and the stationary phase. One ubiquitous characteristic of separations involving ionic species with HPLC is the presence of severe tailing of the eluting peaks. When a surfactant is introduced in mobile phase below the CMC, ion-pairing between the surfactant monomer and the ionic analyte can take place (14-17). Many theories have been proposed as to the mechanism of ion-pair liquid chromatography. One theory holds that discrete ion-pairs are formed between the surfactant monomers and ionic solutes and the ion-pairs then interact with the stationary phase in a manner similar to a nonionic solute. Another theory purports that the hydrophobic "tail" of the surfactant is solubilized by the hydrophobic stationary phase and the ionic "head" group of the surfactant is in contact with the aqueous mobile phase. The theory holds that it is the interaction between the ionic "head" groups

of the surfactant and the ionic solutes that effects the separation. The preponderance of the experimental evidence indicates the mechanism to be a hybrid of these theories (18). Whatever the mechanism, many difficult separations can be accomplished by the use of ion-pairing reagents in HPLC (19, 20).

The use of micellar mobile phases was first reported by Armstrong and Terrill (21). Micellar mobile phases were proposed as an alternative to the use of organic modifiers in thin layer chromatography (TLC). The elimination of potentially toxic organic solvents as well as the minimization of contaminants with purified surfactants were some of the justifications for employing micellar mobile phases. It was observed that the highly selective partitioning obtained with micellar systems could not be duplicated with mixed solvent systems. The major disadvantage was the low sample capacity of the micellar mobile phases relative to the capacity exhibited by mobile phases with organic modifiers (21).

The next logical development for micellar separations was the use of micellar mobile phases in HPLC. Samples containing phenols and polyaromatic hydrocarbons (PAH) were used to demonstrate the utility of aqueous micellar mobile phases in HPLC (22). The same justifications were employed for micellar HPLC as for TLC. Similarly, the sample capacities observed with the micellar mobile phases were less than that for the same separations with organic modifiers. When preparative HPLC was the goal, a means of recovering the solute from the micellar mobile phase had to be implemented (22).

A great deal of research has ensued from the original use of micellar mobile phases in HPLC. Enhancement of fluorescence and phosphorescence signals by micellar mobile phases has generated considerable interest (23, 24). Investigations into the interactions of solutes and micelles have been numerous (25-27) and have resulted in a three phase theory for separations in micellar liquid chromatography (25). The three phase theory considers the equilibria of a solute between the stationary phase, micellar phase, and aqueous phase. The complicated three phase theory was able to explain many of the observed phenomena.

One of the major drawbacks to micellar liquid chromatography has been the poor efficiency of the separations obtained. The work of Dorsey and coworkers (28) addressed the problem of efficiency loss with micellar mobile phases in HPLC. The majority of micellar liquid chromatographic mobile phases has been totally aqueous and it was noted that, "hydrophobic stationary phases exhibit very poor mass transfer characteristics under these conditions" (28). Two steps were taken to lower the resistance to mass transfer in the stationary phase for these micellar mobile phases. First, organic modifiers (alcohols) were added to the chromatographic mobile phase in small quantities to improve the wetting of the stationary phase surface. It was also surmised that the added alcohol might diminish the microviscosity experienced by the solubilized solute, thereby reducing the resistance to mass transfer between the micelle and the aqueous mobile phase (28). A significant increase in efficiency was observed for benzene with the addition of propanol to the mobile phase. Among the common organic modifiers

employed in HPLC, propanol exhibited the largest increase in efficiency for benzene. The optimum percentage of propanol was found to be approximately 10%. The amount of added organic modifier was kept low to avoid disruption of the integrity of the micelles. Second, raising the temperature of the column was investigated. The resistance to mass transfer was expected to decrease as the temperature of the column was raised. However, the observed efficiency remained constant despite the increase in the temperature of the column (28).

Capillary Zone Electrophoresis

Mikkers and coworkers first reported electrophoretic experiments which were performed in narrow-bore teflon tubes (29). The separation of inorganic anions as well as organic anions was performed using the apparatus of Mikkers. The efficiency observed for the separation of selected anions was comparable to that of HPLC. The column dimensions of the narrow-bore teflon tube were 200 μm i. d. and 20 cm in length.

Capillary zone electrophoresis (CZE) was demonstrated by Jorgenson and Lukacs (30) with impressive efficiencies on the order of 400,000 theoretical plates. Borosilicate glass capillaries were utilized with dimensions of 75 μm i. d. and 1 m in length. By the use of open tubular columns band broadening by eddy diffusion or unfavorable interactions with electrophoretic stabilizers was eliminated.

In CZE analytes are separated due to differences in electrophoretic mobilities. Solutes with small differences in their electrophoretic mobilities can be separated by CZE because of the extremely high

efficiencies observed. Equation 1 (30) expresses the relationship between efficiency, N , and the operational parameters of the technique:

$$N = (u * E)/(2 * D) \quad \text{Eq. 1}$$

where u the observed electrophoretic mobility of the analyte, E is the applied voltage, and D is the solute diffusion coefficient of the solute. The parameters u and D are intrinsic functions of the solute molecule. The efficiency of the separation is directly proportional to the applied voltage. When the efficiency, N , is plotted against the applied voltage, E , the predicted linear relationship is observed (30). Electric potentials of tens of kilovolts are used to drive the electrophoretic experiment. At high voltages (> 25 kV) negative deviation from linearity is observed and is attributed to thermal effects generated by the high voltages. The applicability of applied voltages of 100 kV or greater have been predicted (30); however, at present the experimental complications at these extreme voltages have not been addressed. Currently a practical limit on applied voltage is about 40 kV. To take full advantage of the linear relationship between applied voltage and efficiency, steps must be taken to alleviate the thermal problems associated with voltages greater than 30 kV.

The application of high voltages can lead to the generation of a large amount of heat. The excellent heat dissipation characteristics of the capillary columns allow the application of these extreme

voltages. There are, however, limits in which this dissipation is adequate. Heating effects have been observed when power dissipations exceed about 2 watts/meter (33). The heat generated produces a transverse temperature gradient within the column. Since electrophoretic mobility is a function of temperature, solutes in different portions of the column experience differing temperatures and, therefore, have different velocities (30, 33). This velocity distribution can cause band broadening. The voltage required to achieve these powers is dependent upon the length and i. d. of the column and the ionic strength of the mobile phase.

When a high voltage is applied to a capillary containing an electrolyte solution, a flow is induced inside the capillary which is known as electroosmotic flow (31). The specific details of electroosmotic flow will be further discussed in the next section. In the presence of electroosmotic flow the observed mobility, u , of a solute is governed by equation 2 (30):

$$u = u_{el} + u_{osm} \quad \text{Eq. 2}$$

where u_{el} is the electrophoretic mobility of the solute and u_{osm} is the electroosmotic mobility of the solute. Electrophoretic mobility is only observed with ionic solutes; however, all solutes exhibit an electroosmotic mobility. Electroosmotic mobility is similar to electrophoretic mobility and is an intrinsic property of the molecule.

With the high efficiencies generated with CZE neutral solutes can be separated (30, 32). Neutral solutes are transported through the

column via electroosmotic flow and the differences in viscous drag of these neutral solutes, primarily due to size differences, can provide for their separation. However, these differences are usually very small and, consequently, CZE is not very useful for separating structurally similar neutral compounds.

A great deal of research has arisen from the development of capillary zone electrophoresis (30, 32, 34-42). CZE has been applied to the separation of many different classes of compounds. A variety of metal ions (34) and a variety organic ions (30-32, 34-36) have been successfully separated with CZE. Neutral organic molecules pose special problems in CZE and high efficiency is required to separate these analytes (30, 32, 37). Solvophobic interactions of neutral solutes with tetraalkylammonium ions have been explored to aid in the separation of solutes which have no electrophoretic mobility (37). Purely aqueous mobile phases are generally employed in CZE and many compounds of interest display limited or no solubility in these aqueous systems. Nonaqueous or mixed mobile phases have been investigated to aid in the solubilization of organic molecules (37). The physical parameters which control column efficiency and quantitation have also been studied (40). The determination of cinnamic acid in plasma was performed to illustrate the potential usefulness of CZE for real analyses (42).

Theory of Micellar Electrokinetic Capillary Chromatography

Micellar electrokinetic capillary chromatography was first reported by Terabe et al. (43). MECC combines many of the operational

principles and advantages of micellar liquid chromatography (44) and capillary zone electrophoresis (30). MECC (as is CZE) is performed using narrow-bore capillary columns (CA. 25-100 μm i. d. x 50-100 cm) which are filled with an aqueous buffer solution. An electric field is applied longitudinally to the capillary column. As previously mentioned, the application of a high voltage across the capillary column results in electroosmotic flow inside the column (31, 45). Electroosmotic flow provides a means of transporting neutral solutes through the capillary column.

In MECC a surfactant is added to the mobile phase at a concentration above its CMC. The resulting micelles provide an effective mechanism for separating neutral solutes. Neutral solutes are separated based on their differential partitioning between an electroosmotically pumped aqueous mobile phase and the hydrophobic interior of the micelles. The micelles are charged and moving at a velocity which typically is lower than the mobile phase and opposite in direction due to electrophoretic effects.

The mass transport phenomena operative in MECC are depicted in the expanded view of a capillary section shown in Figure 1 (33). The column contains the surfactant/buffer solution. Negatively charged (anionic) surfactant is considered in Figure 1. Primary and secondary sorbed ions generate an electric double layer potential (zeta potential) at the inside surface of the capillary. The larger the zeta potential the greater the disparity in the overall mobilities of positive and negative ions. Because of this disparity a net flow of solvent

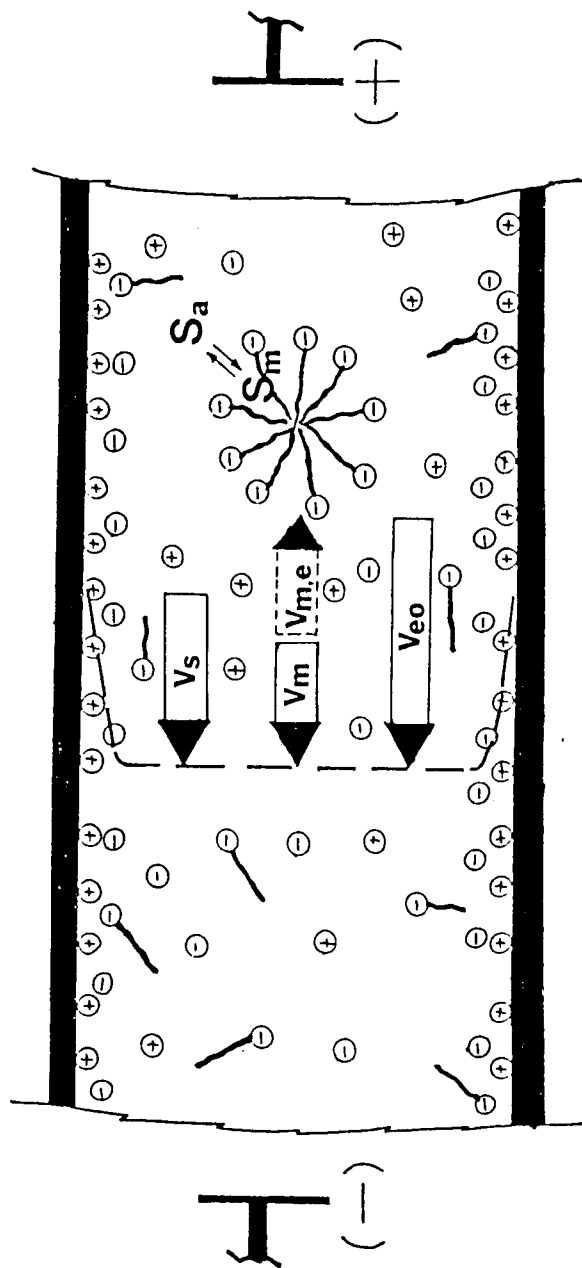


Figure 1. Expanded view of a MECC capillary section showing column dynamics.

Source: Sepaniak, M. J.; Burton, D. E.; Maskarinec, M. P. Proceedings of Symposium on the Use of Ordered Media in Separations, ACS Symposium Series (1986), in press.

(electroosmotic flow) results when an electric field is applied and solvated ions move toward the electrode of opposite sign. In our work with both cationic and anionic surfactants, the electroosmotic flow velocity (V_{eo}) opposes the electrophoretic velocity ($V_{m,e}$) of the micelles and is of a greater magnitude. The net velocity of the micelles (V_m) is the vector sum of V_{eo} and $V_{m,e}$. Consequently, two distinct phases, the aqueous mobile phase and the micellar phase, exist within the column and migrate at different velocities toward the electrode with the same charge as the micelles. V_{eo} is proportional to the magnitude of the zeta potential and the electric field strength, while $V_{m,e}$ is proportional to the micellar electrophoretic mobility and the electric field strength. A solute (S) which partitions between the mobile and micellar phases will have a band velocity (V_s) that is intermediate between V_m and V_{eo} . Solutes which partition differently with the micelles will have different V_s values and are thereby separated.

The dashed line in Figure 1 represents the flow profile present in MECC. The "plug-like" velocity flow profile for electrokinetically pumped capillaries is important in minimizing the resistance to mass transfer within the mobile phase (39). Hydrostatically pumped capillary columns have parabolic flow profiles which tend to severely disperse solute bands unless extremely narrow-bore (i. d.s less than 10 μm) capillaries are employed (3, 31). With a parabolic flow profile, molecules of a solute which are located in different transverse positions in the column experience different flow velocities. The

differing velocities of the solute molecules as they traverse the column lead to band broadening. The "plug-like" flow profiles observed in MECC minimize this phenomenon. Solute molecules located in different parts of the column experience approximately the same flow velocity (except very close to the walls of the capillary where the flow velocity goes to zero).

As previously mentioned, narrow-bore capillaries very efficiently dissipate the heat generated in the electrophoretic processes. Nevertheless, we have observed heating effects when the power dissipation requirements exceed about 2 Watts/meter. Since heat can only be dissipated at the walls of the capillary column, a transverse temperature gradient is produced within the column. Electrophoretic mobility increases with temperature (30), therefore, the "plug-like" flow profile shown in Figure 1 is distorted and the column efficiency is degraded.

Chromatograms in MECC differ from those observed in conventional elution chromatography in that there is generally a limited elution range. Retention times of solutes (t_r) in MECC are given by equation 3 (46):

$$t_r = \frac{t_o}{R + (t_o/t_m)(1 - R)} , \quad \text{Eq. 3}$$

where t_o is the retention time of a solute which is not solubilized by the micelles, t_m is the retention time of a solute which is completely solubilized, and R is the fraction of solute not solubilized by the micelles (i.e., the MECC analog of the retention ratio in conventional

LC). The limited elution range of MECC translates into relatively short separation times, but adversely limits the separation number of the technique. Figure 2 is a representation of a chromatogram in MECC. The two solutes elute from the column between t_o and t_m .

The capacity factor, a measure of the amount of solute in the stationary phase, is a useful parameter in conventional chromatography. The capacity factor, k' , for a neutral solute is given by Equation 4 (43):

$$k' = \frac{t_r - t_o}{t_o (1 - t_r/t_m)}, \quad \text{Eq. 4}$$

Equation 4 is very similar to the equation for the capacity factor in conventional liquid chromatography. The second term in the denominator, $1 - t_r/t_m$, takes into account the limited elution range in MECC. This term does not appear in the equation for conventional liquid chromatography because there is an unlimited elution range with these techniques (i.e., t_r/t_m is effectively zero). As the ratio of t_r to t_m approaches 1, the value of k' approaches infinity. To obtain a k' value of infinity for conventional liquid chromatography, t_r would have to be infinity. The magnitude of k' is related to the amount of time a solute spends inside the micelle. A value of infinity for k' translates into a solute being completely solubilized by the micelle (i.e., the solute velocity is the same as that of the micelles).

One of the ultimate concerns in any chromatographic technique is resolution. Resolution, R_s , in MECC is given by Equation 5 (46):

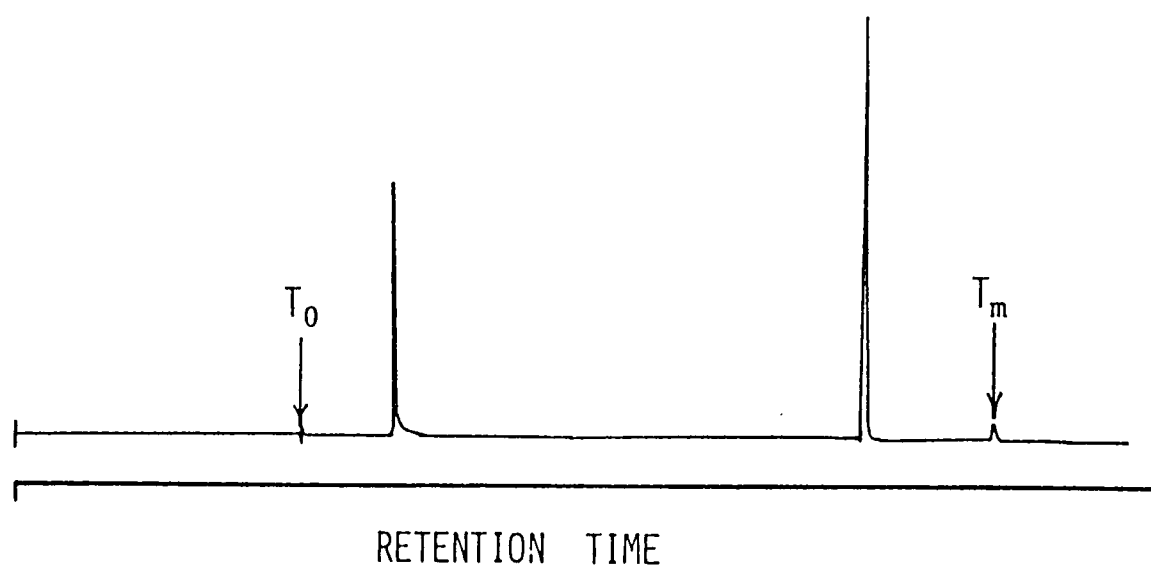


Figure 2. Representation of the elution range in MECC.

$$R_s = \frac{N^{\frac{1}{2}}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k'}{1 + k'} \right) \left(\frac{1 - t_o/t_m}{1 + (t_o/t_m)k'} \right), \quad \text{Eq. 5}$$

where N is the number of theoretical plates for the column, α is the selectivity factor (k'_2/k'_1) for adjacent peaks in the chromatogram. Resolution in MECC depends on four factors, efficiency (N), selectivity (α), capacity (k'), and elution range (as reflected in t_o/t_m). The last factor illustrates an important difference between MECC and conventional chromatography which employs a true stationary phase. Since k' appears in the denominator of the last term of Equation 5, large values of k' tend to degrade resolution. The optimum k' depends on t_o/t_m but is generally in the range of 2-5 (46). As t_o/t_m approaches zero the last term in Equation 5 drops out and the MECC technique resembles conventional elution chromatography. The high efficiencies possible with MECC dramatically increase the resolution capabilities of the technique. Under the proper conditions, column efficiency is outstanding. In one separation of purine compounds we obtained over 600,000 theoretical plates/meter (33). Efficiencies greater than 100,000 theoretical plates/meter are routinely obtained.

In summary micellar electrokinetic capillary chromatography shows promise for the high efficiency separation of a variety of samples which are at least sparingly soluble in water. Fundamental studies of column efficiency and selectivity are presented in this dissertation.

CHAPTER II

METHODOLOGY AND INSTRUMENTATION

Experimental Apparatus

The experimental configuration employed in MECC is shown in Figure 3 (47). The apparatus consists of a length of glass capillary column (25-75 μ m i. d. x 50-100 cm in length) which is filled with a buffer solution containing a surfactant. Each end of the column is immersed in a separate reservoir containing the mobile phase. High voltage is supplied through either a Model R30B (0-30 kV) or a Model R40B (0-40 kV) Hipotronics regulated high voltage DC power supply. Electrical contact with the solutions is provided by either graphite or platinum electrodes. A plexiglass box with an interlock system protects the operator from accidental contact with the high voltage. Detection is performed at the grounded end of the column. The protective box encloses only the high voltage end of the apparatus to facilitate adjustments of detector components. The apparatus also consists of a positioner to align the column, a detector with signal processing instrumentation, and a strip chart recorder. Figure 3 represents a laser-excited fluorescence detection scheme and will be more thoroughly discussed later.

The high voltage power supply provides the electric field which initiates electroosmotic flow inside the capillary column. The applied voltage and resulting current are monitored through meters on the power

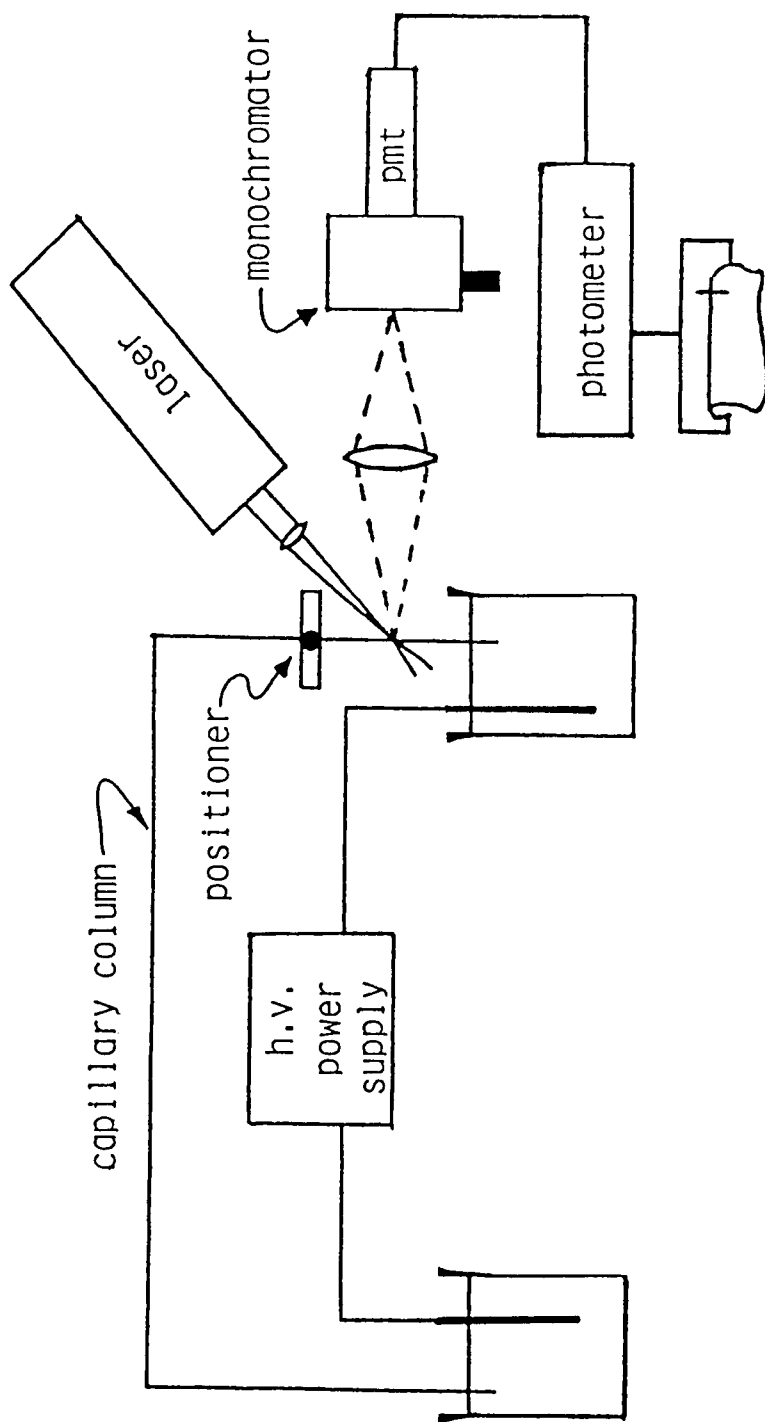


Figure 3. Block diagram of the MECC apparatus with on-column laser-excited fluorescence detection.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. J. Chromatogr. Sci. 1986, 24, 347-351.

supply. The power supply is analogous to the pumping system in conventional liquid chromatography.

Borosilicate glass (30, 36) as well as fused silica (38, 43, 47) capillary columns have been employed for various electrophoretic and micellar separations. In some applications, fused silica columns are necessary if ultraviolet light is used in the detection scheme. Fused silica capillaries transmit uv radiation; however, borosilicate glass absorbs uv radiation and impurities in the glass fluoresce. A background fluorescence is created which is unacceptable for some analyses. Borosilicate glass attenuates the excitation radiation resulting in fewer photons available for absorption. In fluorescence detection the fluorescence signal is directly proportional to the power of the excitation radiation. By attenuating the excitation power, the detection limits for fluorescence detection are adversely affected. Fused silica capillary columns are encased with a polyimide coating which makes them very flexible and easy to manipulate. These capillaries are also commercially available in a variety of sizes and are relatively inexpensive. Borosilicate columns must be drawn to the appropriate size from large i. d. stock tubing (6-7). The fragile nature of the drawn borosilicate capillaries makes them difficult to handle.

Column preparation in MECC is minimal. Borosilicate capillaries are drawn to the appropriate diameter (6-7) with an Alltech capillary glass drawing machine in straight lengths of 75-125 cm. Fused silica columns are simply cut to the appropriate length. The columns are rinsed with 0.1M HCl for one hour to remove any material adsorbed to

the inside surface of the column and to protonate $-\text{SiO}^-$ groups in contact with the solution. No steps are necessary to increase the surface area of the interior of the column as in open tubular liquid chromatography (6-7). Once the rinsing process is completed, the column is filled with mobile phase. The mobile phase is allowed to flow through the column for approximately one hour. After being filled and rinsed with the mobile phase, the column is ready to be used.

Prior to filling, a small section (1-2 mm) of the polyimide coating of the fused silica capillary columns is removed by heat to provide an optical window. Care must be taken that the optical window is free of any debris to reduce the amount of scattered light. Once the polyimide coating is removed from the fused silica, that section of the column is extremely brittle and must be carefully handled. On-column fluorescence detection with borosilicate columns can be performed without any additional preparation.

The columns are filled by gas pressure. A small length of sixteen gauge tubing is fitted with a brass ferrule and appropriate nut. The "fitting" is then epoxied around the detection end of the column. If the epoxy is allowed to completely cure, then the fitting does not have to be removed after the column is filled. A reservoir is connected to a high pressure helium cylinder and the column is connected to the reservoir. The gas pressure can easily fill columns with i. d.s as small as 10 μm .

All surfactants used were of electrophoresis or HPLC grade if available. HPLC grade solvents were used with the exception of water

which was distilled and deionized. All other chemicals were of reagent grade or better and were used without further purification.

Fluorescence Detection

Fluorescence detection has been extensively used with liquid chromatographic techniques (8, 20, 23, 30, 47-56). Fluorimetry is characterized by its high selectivity and sensitivity. The technique can also be easily adapted for LC detection. Many fluorescence detectors are commercially available. Discrimination can be achieved with fluorescence detection by the proper selection of the excitation and emission wavelengths. When a specific class of compounds in a complex mixture is to be separated with a chromatographic technique such as MECC, careful selection of the detection parameters can discriminate against the detection of many undesired components. Detection limits of subpicogram injected quantities of highly fluorescent analytes are easily obtained (47-56).

Figure 3 (47) illustrates the laser-excited fluorescence detection employed in this work. The radiation from either a Cyonics Model 2001 argon ion (Ar^+) laser or an Omnicrome Model 156 helium/cadmium (He/Cd) laser with excitation wavelengths at 488 nm or 325 nm, respectively, is focused into the optical window of a fused silica capillary or directly onto a borosilicate column. A 90 degree geometry is employed for the collection of the resulting fluorescence signal. The fluorescence signals are isolated by an Instruments SA Model H-10 monochromator and

detected with an RCA 1P28 photomultiplier tube. Photocurrents are processed with a picoammeter and recorded on a strip chart recorder.

Lasers have many properties which facilitate on-column fluorescence detection in MECC (47). The excellent beam quality of lasers allows the focusing of radiation into small volumes, which becomes important as the inside diameter of the chromatographic column is decreased. Fluorescence signals are proportional to the intensity of the incident radiation, and lasers can produce the large photon densities necessary for large fluorescence signals. With the use of tunable lasers desired excitation wavelengths can be accessed. Fluorescence detection has also been performed using high intensity arc lamps (30, 48) which has given acceptable results.

The use of very small bore capillaries presents certain experimental difficulties. When laser-based detection schemes are employed, the detector constraints of the small bore columns can be easily accommodated (7). However, the use of commercial absorbance detectors (57) can be difficult due to low photon densities and excessive scattered radiation. The injected quantities of solutes decreases as the column diameter is decreased. For example, the column volume of a 50 μm i. d. and 1 m long capillary is about 8 μL . The injected volume of a solute whose retention time is 10 minutes would be approximately 67 nL (injection: 5 kV for 30 s; separation: 30 kV) assuming a linear relationship between migration velocity and applied voltage which is only approximately valid (39, 47). If the analyte concentration is 1 ppm, then the injected quantity of solute is 67 pg. The small injected

quantities necessitate very sensitive detection schemes. Since fluorescence signals are proportional to the power of the incident radiation, laser-excited fluorescence detection can provide the photon densities necessary to provide adequate detection under these conditions.

Figure 4 shows the separation of four alkyl amines (methylamine, ethylamine, n-propylamine, and cyclohexylamine) which have been derivatized with NBD-Cl (7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole) (49). The 488 nm line of an Ar^+ laser is employed for excitation and the fluorescence is monitored at 535 nm. The use of a 25 μm borosilicate column is acceptable, since the excitation wavelength is in the visible region of the spectrum. The efficiency for the cyclohexylamine is 90,000 theoretical plates/meter. The conditions of the separation are given Figure 4. MECC is able to separate these alkyl amines with similar structures even though each solute is derivatized with a large NBD molecule.

Figure 5 illustrates the fluorescence quenching phenomenon known as the heavy atom effect. In Figure 5A the surfactant is cetyltrimethylammonium bromide and in Figure 5B the surfactant is cetyltrimethylammonium chloride. The other conditions in Figure 5 are identical for A and B. The large peak shown in Figure 5 corresponds to methylamine. The fluorescence signal is quenched by a factor of approximately 70 when the bromide ion is present in the mobile phase. The techniques used to maximize fluorescence signals in MECC are the same as in other liquid chromatographic techniques.

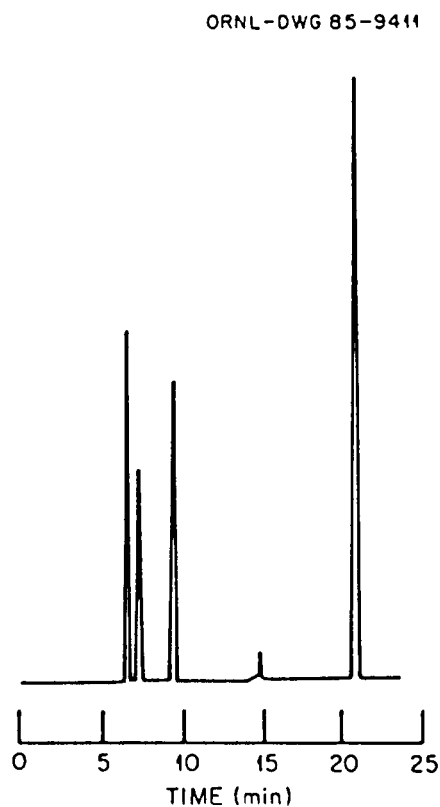


Figure 4. Separation of NBD-Cl derivatized amines. Elution order: methylamine, ethylamine, n-propyl, and cyclohexylamine. Fluorescence detection: excitation, 488 nm; emission, 535 nm. Conditions: 25 μ m x 60 cm borosilicate column; 20 kV, 20 μ A. Mobile phase: 0.01M SDS, 0.05M Na_2HPO_4 in distilled water.

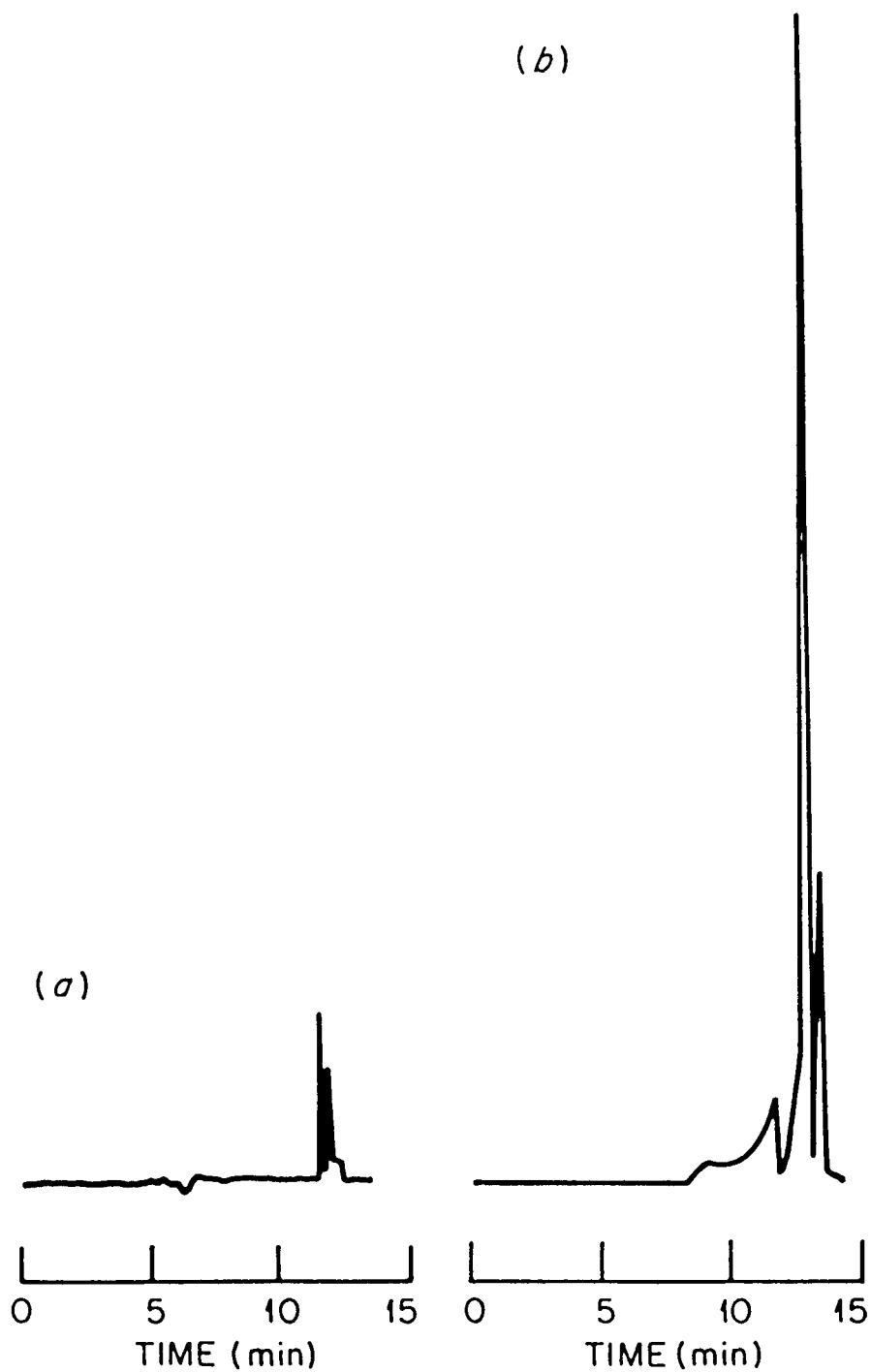


Figure 5. Fluorescence quenching via the heavy atom effect. Mobile phase: (A) 0.01M cetyltrimethylammonium bromide; (B) 0.01M cetyltrimethylammonium chloride. The fluorescence signal for ethylamine in (B) is 70 times greater than in (A). All other conditions are the same as Figure 4.

Absorbance Detection

Absorbance detection has been one of the most widely used liquid chromatographic detection schemes. Since many organic molecules of interest to chromatographers absorb the 254 nm line of a common mercury lamp, the fixed wavelength detector, employing the mercury lamp as a light source, has become a standard. The commercially available uv-visible variable wavelength absorbance detectors has enhanced the versatility of liquid chromatography. When a wide range of solutes is to be analyzed, the flexibility of the variable wavelength detector is invaluable. Although the selectivity and sensitivity of most absorbance techniques is less than fluorescence techniques, absorbance detection is adequate for many analyses.

The flow cell configuration for uv absorbance detection in MECC is shown in Figure 6 (33). A 1 mm section of the polyimide coating is removed from a 1.2 meter fused silica column for on-column uv absorbance detection, such that the separation length of the column is 1 meter. The commercial micro flow cell positioner of a Jasco UVIDEC-100-III is removed from the spectrometer. The micro flow cell (a 2 mm piece of 200 μ m i. d. fused silica tubing) is removed along with the accompanying stainless steel tubing. One sixteen gauge needle is epoxied at each end of the optical window on the capillary column. The column is positioned in each needle such that the column is resting in the beveled portion of the needle. It is important that each needle is epoxied to a small section of the column with its polyimide coating intact, because the bare fused silica portion of the column is extremely brittle. Epoxy should only be placed at the beveled end of the

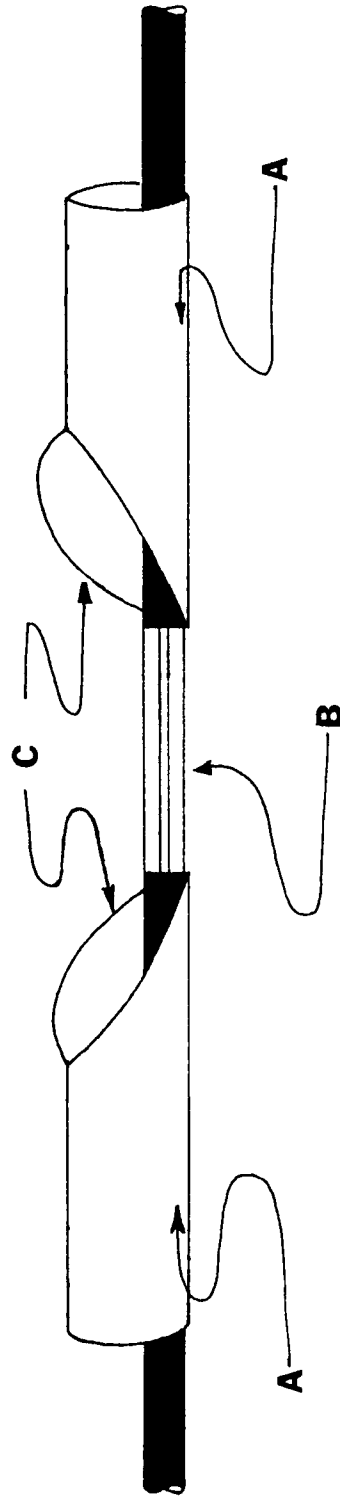


Figure 6. Flow cell configuration for absorbance detection. Flow cell components: (A) 16 gauge hypodermic needle, (B) epoxy, (C) optical window.

column. Epoxying the column in this manner provides enough flexibility to bend the column in the necessary configuration to exit the casing of the spectrometer. Using a microscope the optical window of the column can be aligned in the flow cell positioner with the slit in the positioner. The slit in the Jasco micro flow cell positioner is 200 μm x 2.0 mm. It is only necessary to be certain that the entire flow channel of the column is illuminated. With care the flow cell positioner is reassembled in the spectrometer. No further modifications of the Jasco spectrometer are necessary.

The limits of detection (LOD) for the modified Jasco detector are on the order of 10 ppm for many organic compounds. Although the limits of detection for the modified Jasco uv absorbance detector are above average for conventional absorbance methods, these limits are adequate to detect the test compounds used in this work to study the influences of operational parameters on MECC performance. Modifications would be required if the commercial detectors are to be used for the analyses of "real" samples. The modifications would include better rejection of stray light at the detector and replacement of the photodiode with a more sensitive photomultiplier tube to increase the gain of the detector.

Figure 7 (33) illustrates the separation of a mixture of the five common purines whose structures are given in Figure 8. Detection of the purines is accomplished at 280 nm with the modified uv detector. The concentration of each analyte is approximately 200 ppm. The purines are separated efficiently in less than 20 minutes at an applied voltage of 30 kV. A higher potential could be employed to reduce the

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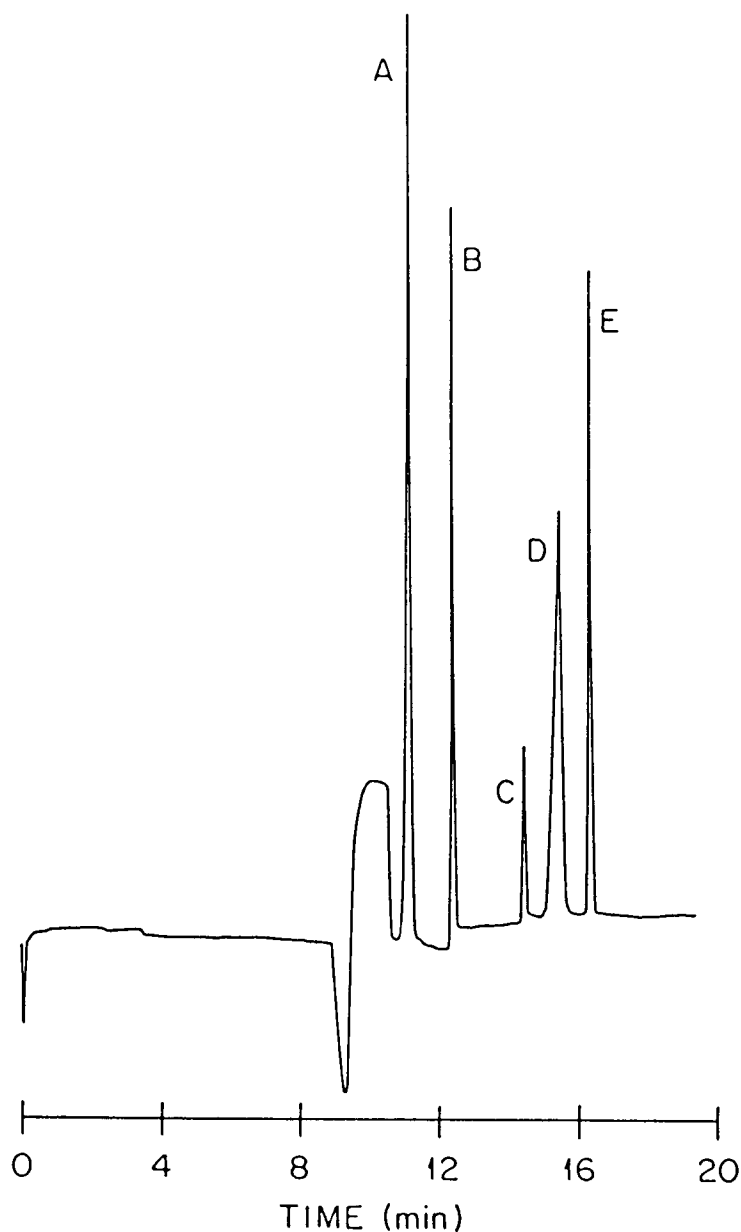
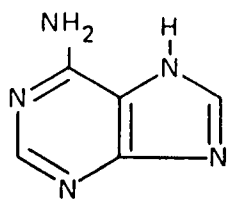
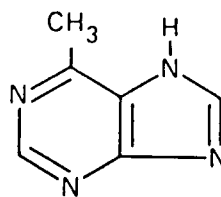


Figure 7. MECC chromatogram of five substituted purines. Elution order: (A) adenine, (B) 6-methylpurine, (C) 2-hydroxypurine, (D) 6,6-dimethylaminopurine, (E) xanthine. Conditions: 75 μm x 1 m fused silica column; 27.5 kV, 70 μA ; detection wavelength, 280 nm. Mobile phase: 0.05M SDS, 0.01M Na_2HPO_4 , 0.006 $\text{Na}_2\text{B}_4\text{O}_7$ in distilled water.

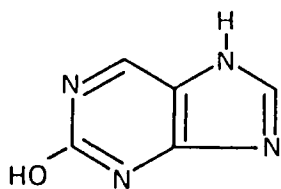
Source: Sepaniak, M. J.; Burton, D. E.; Maskarinec, M. P. Proceedings of Symposium on The Use of Ordered Media in Separations, ACS Symposium Series (1986), in press.



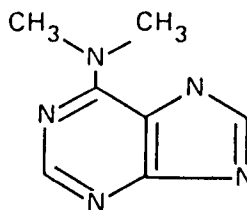
ADENINE



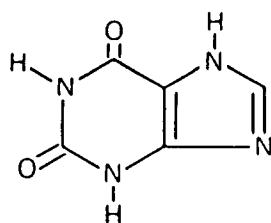
6 - METHYLPURINE



2 - HYDROXYPURINE



6,6 - DIMETHYLAMINOPURINE



XANTHINE

Figure 8. Structures of the substituted purines separated in Figure 7.

separation time, but the resolution between the first two peaks is degraded. The large baseline disturbance at 9.5 minutes is due to methanol in the sample solution.

CHAPTER III

EFFICIENCY STUDIES

Effect of Injection Procedures on Efficiency

Injection is performed in MECC by electrokinetically placing a small volume of sample at the inlet of the column. Since the internal diameter of the column is relatively constant, the injection volume is directly proportional to the length of the column that the sample occupies (L_I). In liquid chromatography the total height equivalent to a theoretical plate (H) is the sum of the individual contributions to band broadening, such as resistance to mass transfer in the mobile phase and axial diffusion. Naturally the width of the solute band at the detector will be greater than or equal to L_I (assuming there is not a concentrating effect at the head of the column). The injection contribution to the total plate height should be controlled by controlling L_I .

For the study of the injection parameters, the mobile phase consists of 0.05M sodium dodecyl sulfate (SDS), 0.01M Na_2PO_4 , and 0.006M $\text{Na}_2\text{B}_4\text{O}_6$. All standards are prepared in the mobile phase. With the addition of the anionic surfactant to the buffer solution to form the mobile phase, the electroosmotic flow is toward the negative electrode. The CMC for SDS is $8.3 \times 10^{-3}\text{M}$ in distilled water.

Sample introduction is accomplished by electroinjection (30). The reservoir at the high voltage (positive) end of the column is removed and replaced with a reservoir containing the sample. Voltage is

applied for a small amount of time, after which the reservoir containing the mobile phase is replaced and the separation is allowed to proceed.

The high efficiencies observed in MECC can be important for many difficult separations. However, the small volumes of the capillary columns (typically a few μL), necessitate careful injection of small volumes of sample. The reproducibility of the injection procedure is quite good. Relative standard deviations of 2.3-6.2% have been previously reported (47).

Figure 9 (57) represents the separation of six substituted purines: theobromine, hypoxanthine, theophylline, caffeine, uric acid, and xanthine. The structures of the purines are shown in Figure 10 (57). The applied voltage is 17.5 kV resulting in a current of 30 μA . Absorbance detection is performed at 280 nm. The concentration of each of the purines is 150 ppm. The retention times of uric acid and xanthine can be decreased by increasing the separation voltage; however, some loss in the resolution of theophylline and caffeine is observed. The separation of the purines could prove difficult by conventional chromatographic methods due to their similar molecular structures.

Two easily controlled parameters in the electroinjection procedure are the applied voltage during the injection process and the amount of time the voltage is applied. For electroinjection the mobile phase reservoir at the high voltage electrode is replaced with a reservoir containing the sample. When the voltage is applied, the sample

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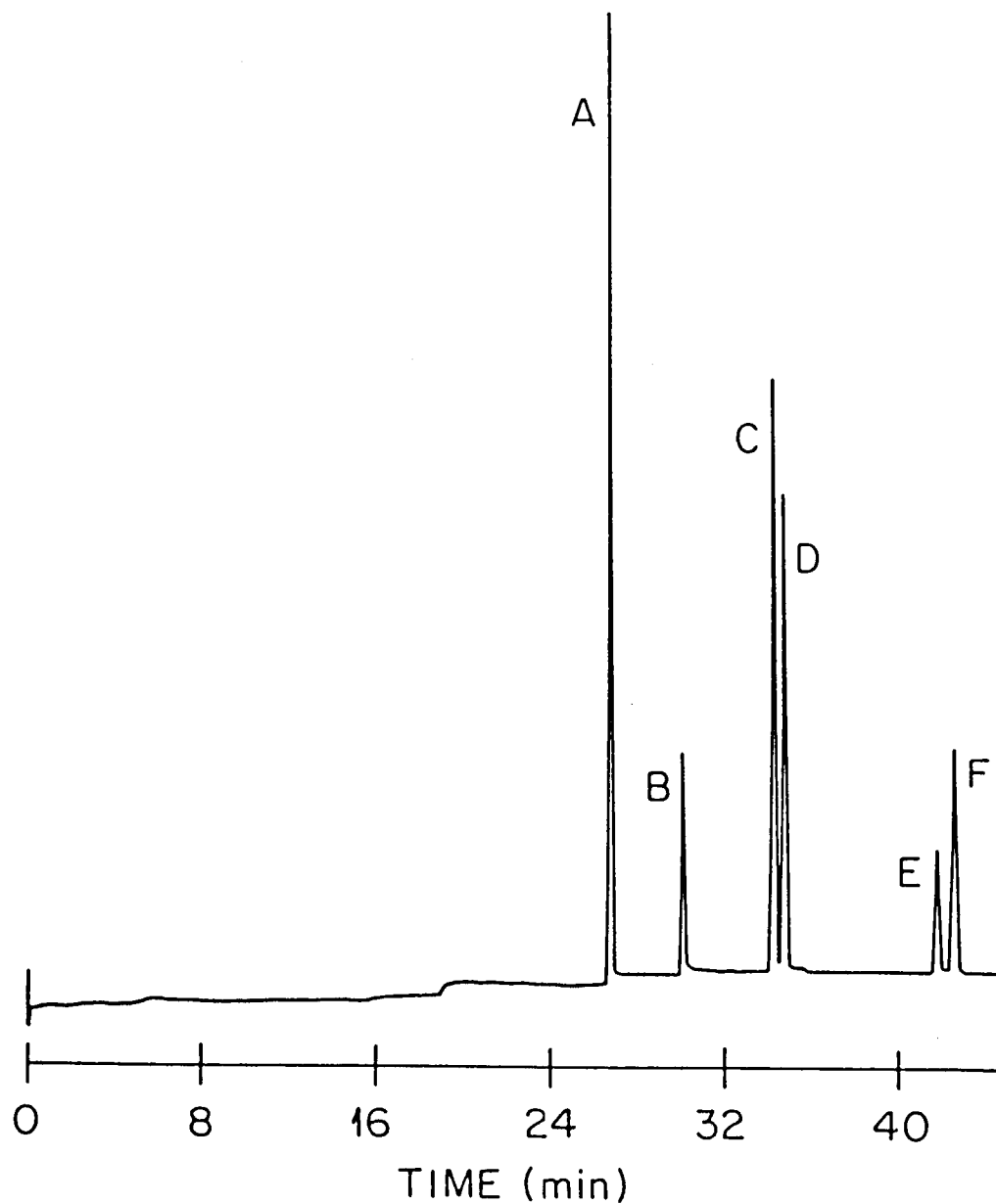
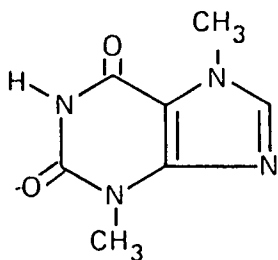


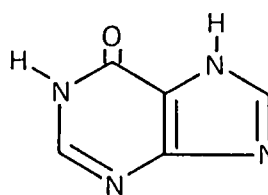
Figure 9. MECC chromatogram of six substituted purines. Elution order: (A) theobromine, (B) hypoxanthine, (C) theophylline, (D) caffeine, (E) uric acid, (F) xanthine. Conditions: 75 μm x 1 m fused silica column; 17.5 kV, 30 μA ; detection wavelength, 280 nm. Mobile phase: 0.05M SDS, 0.01M Na_2HPO_4 , 0.006M $\text{Na}_2\text{B}_4\text{O}_7$ in distilled water.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. Chromatographia in press, October 1986.

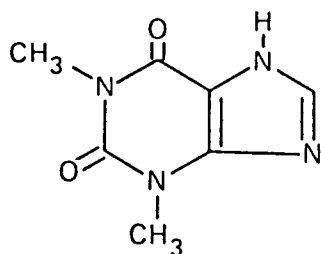
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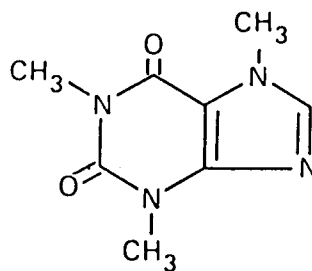
THEOBROMINE



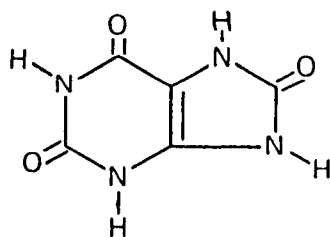
HYPOXANTHINE



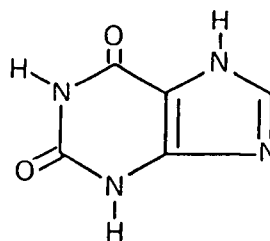
THEOPHYLLINE



CAFFEINE



URIC ACID



XANTHINE

Figure 10. Structures of the substituted purines separated in Figure 9.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. Chromatographia in press, October 1986.

solution electrokinetically migrates into the column. After the replacement of the mobile phase reservoir and the application of the of the separation voltage, the separation process is begun. The injection voltage and duration of the injection control the migration rate of sample into the column. Therefore, the length of the injection plug and consequently the amount of sample injected can be regulated by adjustments in the injection voltage and/or the duration of the injection. Thus, the operator has control of the injection process and its effect on the efficiency of the separation. Injections have been performed by capillary action, where the sample reservoir is elevated above the level of the reservoir at the detection end of the column (30, 43). This injection procedure is not satisfactory in terms of operator control.

The height equivalent to a theoretical plate (H) and the number of theoretical plates (N) were calculated using Equation 6:

$$H = L/N = (L/5.54) * (w_h/t_r)^2, \quad \text{Eq. 6}$$

where L is the length of the column, w_h is the width of the peak at half height, and t_r is the retention time of the solute. Although Equation 6 is generally employed only for gaussian peak shapes, it was used in this work to calculate the plate heights of all peaks in this study irrespective of their shapes.

Table 1 (57) shows the effect of varying the injection voltage, for a constant injection time, on the efficiency (as reflected in the

Table 1

Effect of Injection Voltage on Efficiency

Voltage (kV)	<u>Theobromine</u>		<u>Caffeine</u>	
	N	H (μm)	N	H (μm)
5	76,000	13	95,000	11
10	19,000	53	22,000	45
15	10,000	100	11,000	91
20	5,000	190	6,000	170
25	2,600	380	2,800	360
30	1,700	590	1,800	570

column length: 1 m
column i. d.: 75 μm
injection time: 30 s

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P.
Chromatographia in press, October
1986.

values of H and N) for two test solutes. As the injection voltage was increased from 5 kV to 30 kV, the efficiencies for the test solutes decreased. For caffeine an increase in the injection voltage by a factor of 6 resulted in a decrease in the efficiency by approximately a factor of 50. The conditions of the separations were the same as indicated in Figure 9 with the separation voltage maintained at 25 kV.

Table 2 (57) illustrates the effect on efficiency of varying the injection duration at a constant injection voltage. Analogous to the results in Table 1, the efficiency decreased as the injection time was increased from 10 s to 60 s. The efficiency for caffeine decreased from 240,000 plates/m to 26,000 plates/m as the injection time was increased from 10 s to 60 s. The conditions for the separations represented in Table 2 are identical to those of Table 1. Injections are generally more reproducible if low voltages and long injection times are employed. The relative standard deviations previously reported (47) can be lowered from 2.3-6.2% to less than 1% for the same compounds.

Figure 11 shows the peak shapes for two different injections of caffeine. Peaks A and B represent injections of caffeine at 5 kV for 60 s and 10 s, respectively. Peak A is no longer gaussian in shape and begins to approach a trapezoidal peak shape. The trapezoidal appearance of the peak becomes more prominent as the length of the injection plug increases.

Assuming a linear relationship between applied voltage and migration velocity, which is only approximately valid (58), the value of L_I

Table 2
Effect of Injection Time on Efficiency

time (s)	<u>Theobromine</u>		<u>Caffeine</u>	
	N	H (μm)	N	H (μm)
10	210,000	4.8	240,000	4.2
20	130,000	7.7	150,000	6.7
30	76,000	13	95,000	11
40	38,000	26	59,000	17
50	31,000	32	36,000	28
60	23,000	43	26,000	38

column length: 1 m
column i. d.: 75 μm
injection voltage: 5 kV

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P.
Chromatographia in press, October 1986.

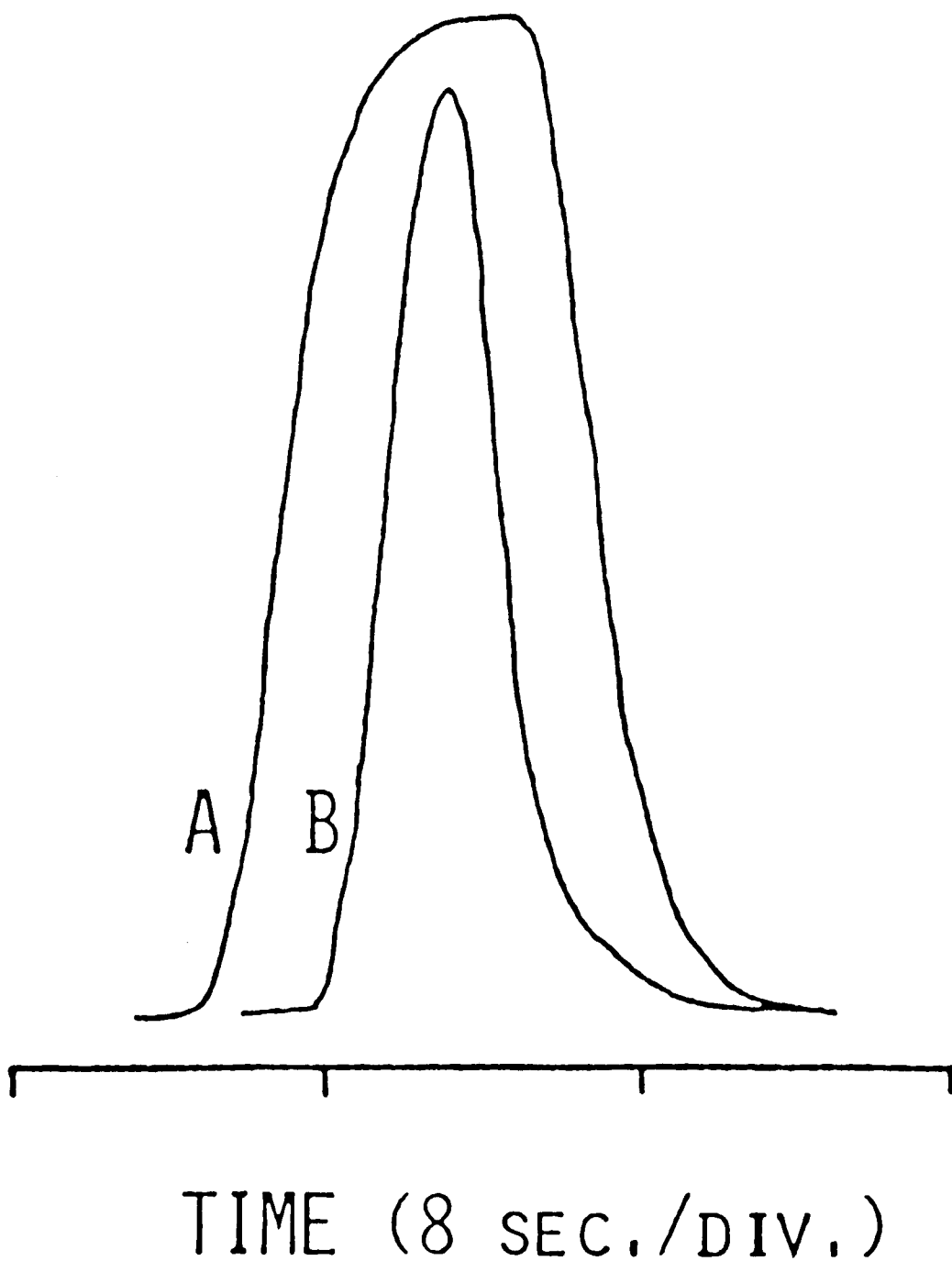


Figure 11. Effect of the injection procedure on the peak shape of caffeine. Injections of caffeine at 5 kV for (A) 60 s and (B) 10 s; separation voltage, 25 kV, 65 μ A; detection wavelength, 280 nm. Mobile phase is the same as for Figure 9.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. Chromatographia in press, October 1986.

for injection A is about 0.8 cm and even if no dispersion occurs would require 13 seconds to completely pass the 1 mm optical window of the column. This is consistent with the peak profile shown in Figure 11. Similarly, a time of approximately 3 seconds would be required for L_I from injection B to pass the optical window. The peak profile for B is considerably greater, indicating significant solute band dispersion within the column. The relatively large optical window also can contribute to the broadening of the peaks in the MECC experiment.

When real samples are analyzed by MECC the composition of the sample solution is not identical to that of the mobile phase. The injection of solutions containing a large amount of organic modifier produced unusual peak shapes. For example, when the concentration of methanol in the sample solution exceeded approximately 20%, shoulders appeared on the peaks. This is illustrated in Figures 12 and 13. In Figure 12 the sample solution is identical to the mobile phase and in Figure 13 the sample solution contains 30% methanol. The conditions for the separations are identical for both figures and are given in Figure 12. All of the peaks in Figure 13 are broader than those in Figure 12. The peaks which elute very close to t_0 are the most distorted because the integrity of the micelles is probably disrupted in the vicinity of the eluting methanol plug. All of the reasons for the misshapen peaks under these conditions are not clearly understood at this time due to the complex processes taking place during the MECC experiment. However, even with these samples, we have observed gaussian peaks shapes when L_I is made very small by adjustments in the

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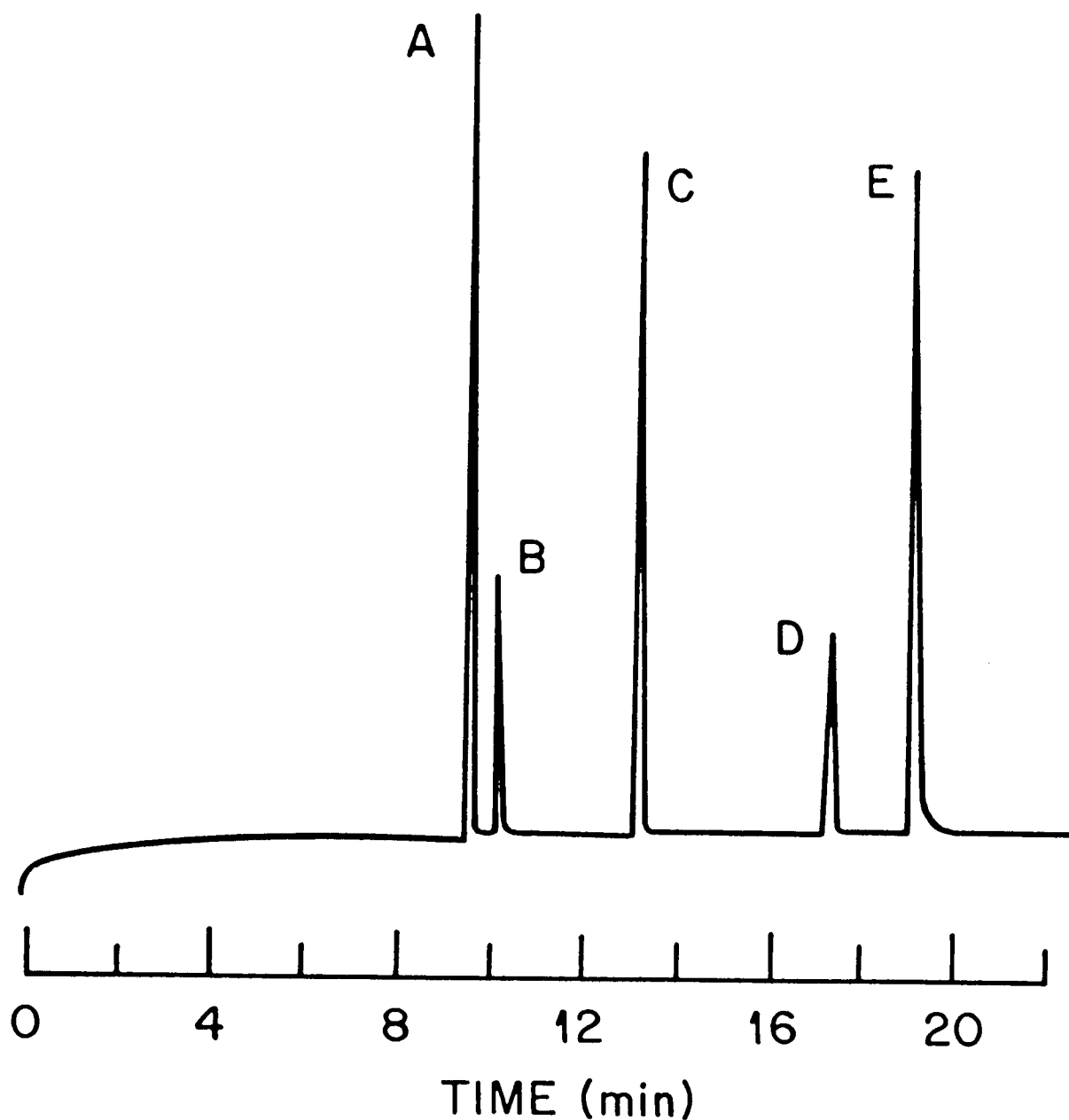


Figure 12. MECC chromatogram with 0% methanol in the sample solution. Elution order: (A) aniline, (B) phenol, (C) nitrobenzene, (D) toluene, (E) 1-aminonaphthalene. Conditions: 75 μ m x 1m fused silica column; 30 kV, 45 μ A; detection wavelength 220 nm. The mobile phase is the same as for Figure 9.

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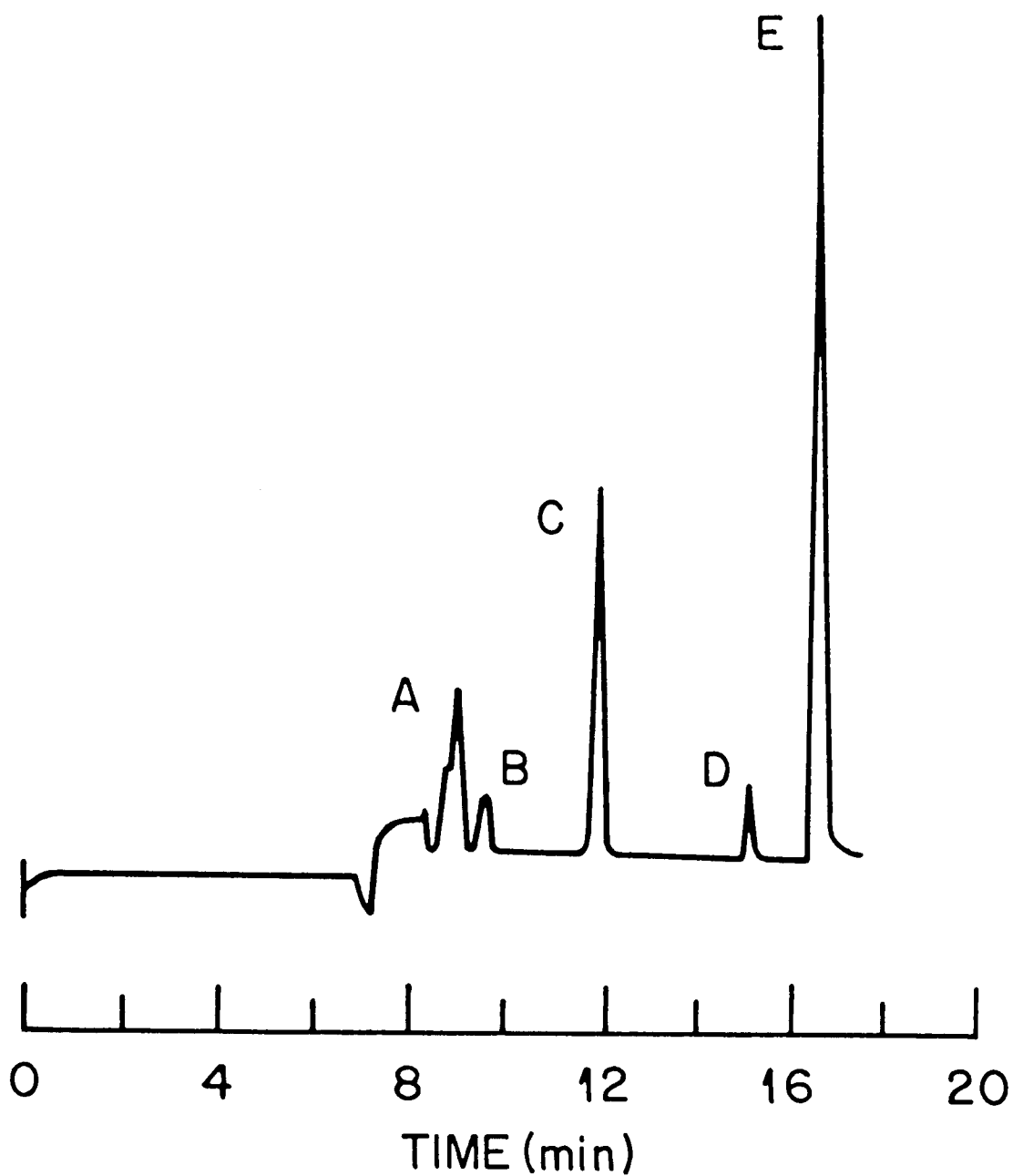


Figure 13. MECC chromatogram with 30% methanol in the sample solution. The conditions are identical to Figure 12.

injection parameters. For purely aqueous samples, such as biological samples, the sample is mixed with an equal portion of the mobile phase and no abnormalities are seen in the peak shapes (47).

The injection procedures can play a definite role in the overall efficiency observed in MECC. The injection parameters are easily controlled by the experimenter and the contribution of the injection process to the total band broadening present in MECC can be easily minimized.

Effect of Column Parameters on Efficiency

The efficiency observed in MECC is in general very high. The experimental parameters in MECC, however, can be adjusted to maximize the efficiency attained by the technique (58). The operational parameters such as applied voltage, concentration of the surfactant, and the concentration and composition of the aqueous buffer can be designated by the experimenter to achieve maximum efficiency. The column diameter can also affect the separation efficiency in MECC. Van Deemter-like plots can be constructed to demonstrate the significance of the contributing factors to band dispersion. The following discussion reflects the work of Sepaniak and Cole (58) and is used by permission.

Ethylamine and cyclohexylamine derivatized with NBD-Cl (49) are used as solutes to illustrate the processes contributing to band dispersion in MECC. Laser-excited on-column fluorescence detection (47) is employed to detect the solutes. The 488 nm line of an Ar^+ laser is used for excitation and detection is performed at 535 nm. Fused silica

columns of 25 μm and 75 μm i. d. and 75 cm in length are employed for the separations.

As in conventional chromatography the total observed height equivalent to a theoretical plate (H) is equal to the summation of the individual band dispersion processes occurring in the MECC experiment. As previously mentioned (57), extra-column processes (principally injection and detection) can also lead to band broadening. With the use of on-column electroinjection (57) and on-column laser-excited fluorescence detection (7), these extra-column contributions to the total plate height can be essentially eliminated. The total plate height (H) for MECC is given in Equation 7 (58):

$$H = H_A + H_S + H_{M,m} + H_{M,c} + H_T, \quad \text{Eq. 7}$$

where H_A is the contribution from axial diffusion, H_S is the resistance to mass transfer in the pseudo stationary phase, $H_{M,m}$ is due to the resistance to mass transfer in the mobile phase (intermicelle), $H_{M,c}$ is the contribution from the resistance to mass transfer in the mobile phase (intracolumn), and H_T is band dispersion related to thermal effects.

As in OTLC, MECC is performed in open capillary columns; therefore, eddy diffusion is not problematic and does not appear in Equation 7. The derivation of exact expressions for each term in Equation 7 is very difficult due to the complication of having two moving phases and the complex nature of the processes present in MECC. In

OTLC the plate height contribution due to axial diffusion is twice the diffusion coefficient (D) of the solute in the mobile phase divided by the linear velocity of the mobile phase (59). Since all solutes regardless of their retention time spend the same amount of time in the mobile phase, the conventional equation of H_A does not depend upon the retention parameter (R). The retention parameter in MECC is the fraction of a solute which is not solubilized by the micelles.

Equation 8 (58) gives the expression for H_A in MECC:

$$H_A = \frac{2 * D * R}{V_{eo} + V_{m,e}(1 - R)} , \quad \text{Eq. 8}$$

The axial diffusion of a solute while solubilized in the micelle is quite small and is neglected in Equation 8. The direct proportionality between the diffusion coefficient as well as the retention ratio and H_A is illustrated by Equation 8. Because of the direct relationship between the applied voltage and the velocity terms in the denominator in Equation 8, an increase in voltage translates into a decrease in H_A . The temperature inside the column also increases with an increase in applied voltage. Since the diffusion coefficient is a function of temperature, excessively high voltages could translate into increased axial diffusion. However, with excessively high voltages the thermal dispersion term, H_T , in Equation 7 will tend to be the dominating factor in the observed plate height.

Although MECC is performed in open capillaries, the columns can behave as packed columns with the micelles functioning as uniformly

sized and evenly distributed particles of packing (58). In packed columns, the resistance to mass transfer in the mobile phase is reduced when smaller diameter particles are employed. With the smaller particles the "diffusion distance" between particles is decreased. Smaller interparticle distances improve the kinetics of mobile phase mass transfer. Above the CMC, when the concentration of the surfactant is increased, the result is a higher concentration of micelles (44). Therefore, the average "intermicellar" distance, the analogous MECC parameter, can be decreased by increasing the concentration of the surfactant. The results of a study of the effect of surfactant concentration on efficiency for a particular MECC system are shown in Table 3. A 100 μm i. d. column was used for this study.

The CMC for SDS is approximately $8 \times 10^{-3}\text{M}$. However, impurities in the SDS reagent and high ionic strength mobile phases can lower the concentration at which micelles are formed. The data in Table 3 (58) indicates some retention occurs at an SDS concentration of $5.0 \times 10^{-3}\text{M}$. Retention time increases as the SDS; hence the micelle concentration is increased. Below $5.0 \times 10^{-3}\text{M}$ SDS the NBD-ethylamine and NBD-cyclohexylamine coelute. The NBD-cyclohexylamine elutes near t_m when the micelle concentration is large. This is reflected in the small changes in t_r when the micelle concentration is changed.

The effect of increasing micelle concentration on efficiency is dramatic, particularly at low surfactant concentrations. One possible explanation for the large improvements in efficiency with increased micelle concentrations is that the intermicelle resistance to mass

Table 3

Effect of Surfactant Concentration on Efficiency

[SDS] (M)	I (μ A)	NBD-Ethylamine		NBD-Cyclohexylamine	
		t_r (min)	N	t_r (min)	N
1.0×10^{-3}	14	4.0	-	4.0	-
5.0×10^{-3}	15	5.0	6,900 ^a	6.6	7,900
1.0×10^{-2}	20	6.3	39,000	9.9	17,000
2.0×10^{-2}	32	7.4	72,000	10.4	30,000
5.0×10^{-2}	59	8.5	65,000	10.7	37,000

a. NBD-ethylamine peak and solvent disturbance only partially resolved.

Source: Sepaniak, M. J.; Cole, R. O. Anal. Chem. in press, February 1987.

transfer, $H_{M,m}$ is a significant factor in the total plate height and it is reduced as the intermicelle distance is reduced. Assuming a CMC of $8 \times 10^{-3} M$ and an aggregation number of 62 for SDS (44), the average intermicellar distance for a $5.0 \times 10^{-2} M$ micelle solution is on the order of tens of nanometers. This distance is so small when compared to the interparticle spaces in a conventional packed column that it would seem that $H_{M,m}$ should be insignificant even at very low micelle concentrations. However, the high efficiency of the MECC technique can add significance to otherwise negligible sources of band dispersion. Furthermore, the magnitude of $H_{M,m}$ will depend on the mobile phase flow profile between the micelles, which to our knowledge has never been studied. The reduction in efficiency for NBD-ethylamine at the highest concentration of SDS in Table 3 is due to thermal related dispersion, H_T , and is discussed later.

At high voltages, the "Van Deemter" plots shown in Figure 14 (58) are similar to conventional chromatography plots in that they show a direct relationship between plate height and applied voltage. This indicates the significance of dispersion due to resistance to mass transfer, which is generally true at high mobile phase velocities in conventional LC. Figure 14 suggests that resistance to mass transfer in the pseudostationary phase H_S is not as important since the high voltage slope of the NBD-ethylamine plot is less than that of the NBD-cyclohexylamine plot. The former solute has a retention ratio (R) which is several times larger and this should result in a larger, rather than smaller, slope for the NBD-ethylamine, providing that H_S is

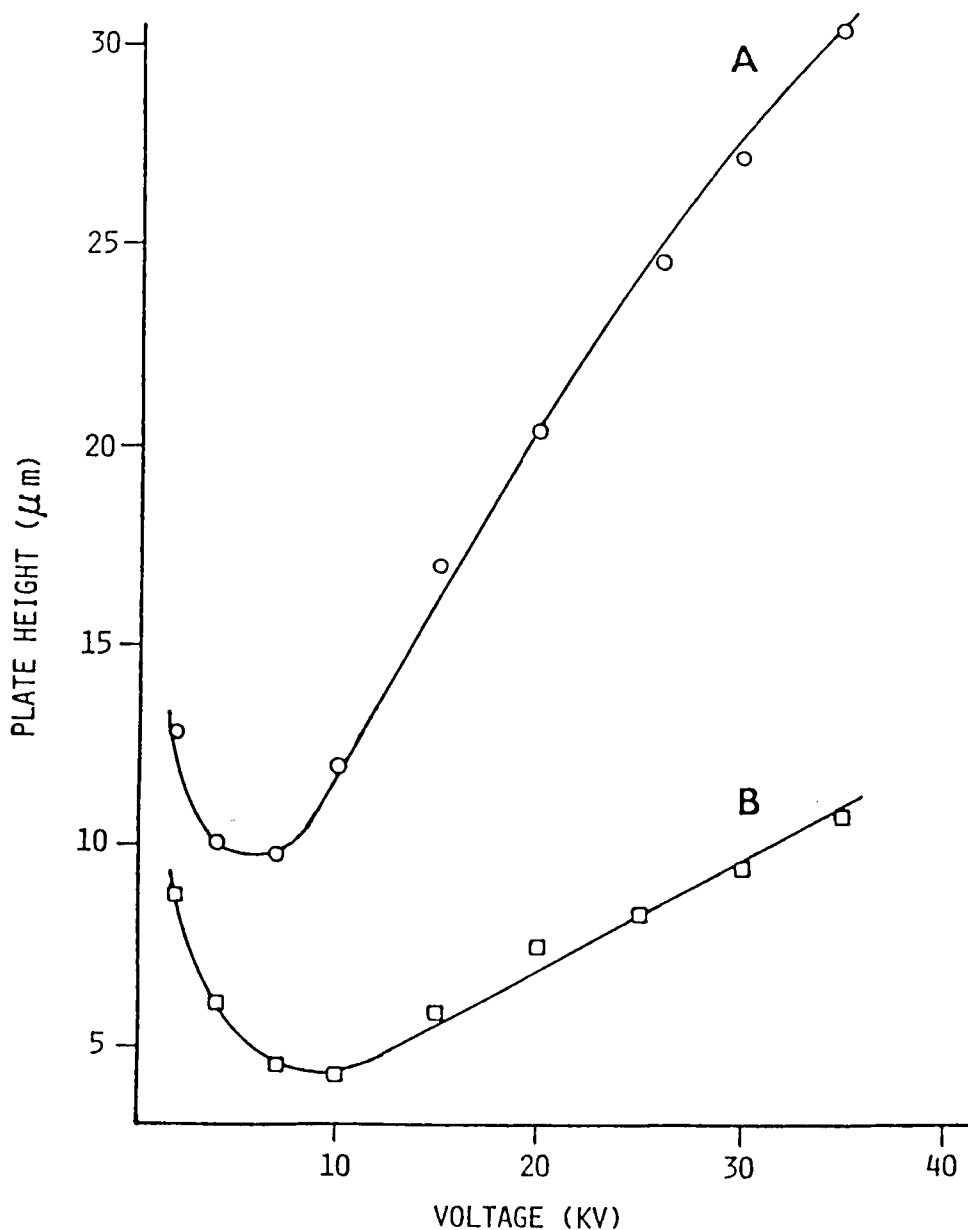


Figure 14. Plate height versus voltage plots for MECC. Test solutes: (A) NBD-cyclohexylamine and (B) NBD-ethylamine. Conditions: 75 μm x 75 cm fused silica column. Fluorescence detection: excitation, 488 nm; emission, 535 nm. Mobile phase: 0.01M SDS, 0.003 Na_2HPO_4 in distilled water.

Source: Sepaniak, M. J.; Cole, R. O. Anal. Chem. in press, February 1987.

significant. The theoretical expression for H_S in conventional chromatography shows only a weak dependence of H_S on R (59). Furthermore, the micelle size should be analogous to stationary phase thickness in conventional partition chromatography and, except in situations when strong solute-micelle electrostatic interactions lead to poor mass transfer kinetics (41), the small dimensions of micelles (e.g., SDS micelles have radii of about 2 nm) should render the H_S term less significant.

When performing separations using true open capillaries, which is not strictly the case in MECC, the major source of band dispersion is generally "intracolumn" resistance to mass transfer ($H_{M,c}$). The transverse velocity profile of the mobile phase is parabolic in hydrostatically pumped capillaries. Dispersion occurs since solutes near the center of the capillary are moving faster than those located near the stationary phase at the walls of the column. The dispersion is less for the plug-like flow profiles of electrokinetically pumped capillaries; however, $H_{M,c}$ can still be significant. If a solute is able to diffuse rapidly across the profile, its dispersion is minimized. Large diffusion coefficients and narrow-bore capillaries are thus desirable.

Figure 15 (58) is a comparison of the NBD-cyclohexylamine peak as a function of applied voltage for (A) 75 μm i. d. and (B) 25 μm i. d. fused silica columns. The current was kept low enough to prevent thermal problems by suitable adjustment of the buffer concentration. At high voltages, the condition for which resistance to mass transfer is

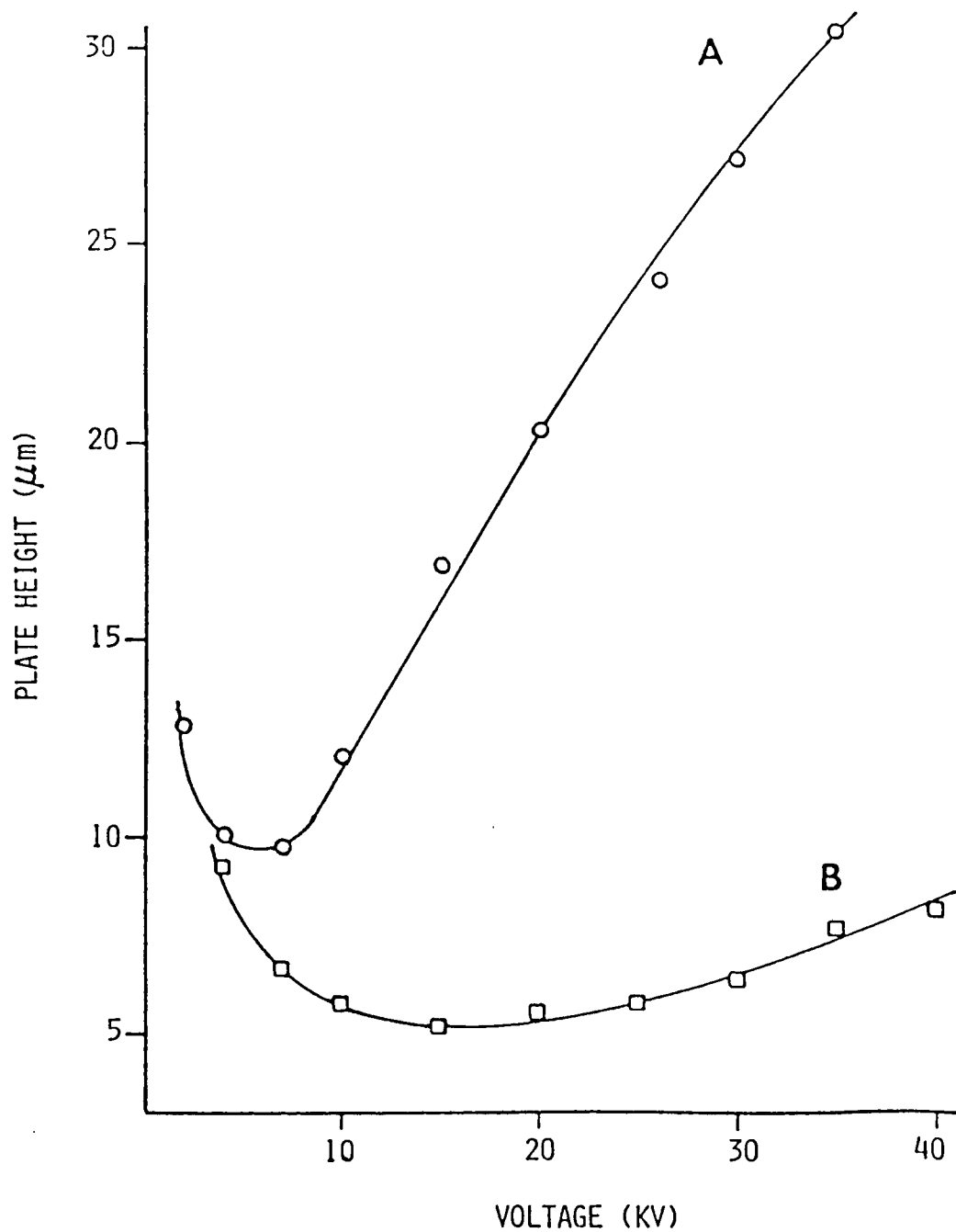


Figure 15. Plate height versus voltage plots (A) 75 μm and (B) 25 μm i.d. columns. Solute: NBD-cyclohexylamine. The conditions are the same as for Figure 14.

Source: Sepaniak, M. J.; Cole, R. O. Anal. Chem. in press, February 1987.

expected to be important, the small i. d. column exhibits much better efficiency, indicating that $H_{M,c}$ is a very significant source of dispersion. The high voltage slope of plot (A) is roughly a factor of 5 greater than for plot (B). The fact that the theoretical quadratic dependence of $H_{M,c}$ on column diameter (59) is not observed could possibly be attributed to the fact that plate height is plotted versus applied voltage rather than mobile phase velocity. Although applied voltage is directly proportional to mobile phase velocity, the relationship is not linear except at low voltages (46).

Figure 14 illustrates the importance of the diffusion processes in determining efficiency in MECC and further indicates the significance of $H_{M,c}$. The solutes might be expected to have similar diffusion coefficients in the aqueous mobile phase. Both $H_{M,m}$ and $H_{M,c}$ should show an inverse dependence on the diffusion coefficient. However, the former should depend on the true solute diffusion coefficient in the mobile phase while the latter should depend on a time weighted, solute/solute-micelle observed diffusion coefficient in the mobile phase. The NBD-cyclohexylamine might then be expected to exhibit much greater intracolumn resistance to mass transfer in the mobile phase $H_{M,c}$, since it spends much more time in the bulky micelle (i.e., has a much smaller observed diffusion coefficient). This is consistent with the high voltage slopes in Figure 14. At low voltages, where H_A should be the most significant source of dispersion, the NBD-ethylamine should have a greater negative slope. Unfortunately, there is not sufficient data at

low voltages to draw any firm conclusions concerning this from Figure 14.

An advantage of CZE over other forms of electrophoresis is the ability of narrow-bore capillaries to limit the electrophoretic thermal load (40) and to efficiently dissipate heat. Nevertheless, when the electric power dissipated per unit length of column is too large (i.e., when voltages and currents are too large) a transverse temperature gradient is created within the capillary. Under these conditions the mobile phase temperature is greater in the center of the capillary than it is near the walls where the heat is conducted to the environment. In general, narrowing the column diameter lessens the temperature gradient. Since electrophoretic mobility increases with temperature, charged solutes which are distributed across the diameter of a capillary assume a dispersive velocity profile, when a temperature gradient exists. It is reasonable to expect the plug-like velocity profile of electroosmotic flow to be distorted similarly, and this should increase the dispersion of neutral solute bands in MECC (i.e., in effect increase $H_{M,c}$).

Another thermal dispersion effect which can occur in MECC relates to the temperature dependence of CMC values. It has been shown that the CMC for SDS increases from approximately $8.4 \times 10^{-3}M$ at $25^{\circ}C$ to $9.8 \times 10^{-3}M$ at $55^{\circ}C$ (60). If the SDS concentration remains constant, increasing the power dissipation requirements would result in a decrease in the concentration of micelles. This would in turn result in an

increase in the $H_{M,m}$ term of Equation 7, thereby decreasing the overall chromatographic efficiency. This effect would be most dramatic when the surfactant concentration is near the CMC.

The limiting power per unit length for the capillaries employed in MECC is roughly 2 Watts/m. Higher values often resulted in a boiling or foaming of the mobile phase, at which point the current ceased and the column had to be refilled with mobile phase. Even at lower powers the thermal dispersion problem discussed above can be significant. This is illustrated by the plot for NBD-cyclohexylamine in Figure 16 (58). Plot B is the same as appears in Figures 14 and 15. However, plot A is obtained using a higher (0.007) buffer concentration. For a given capillary and voltage, a major effect of increasing the ionic strength of the mobile phase is to increase the current and hence the power dissipated. The data point in plot A is at 32.5 kV and 60 μ A while the last data point in B is at 35 kV and 21 μ A. The mobile phase with the higher buffer concentration exhibits better efficiency at intermediate voltages. However, at high voltages the current is excessive as reflected in the very rapid degradation in efficiency as the voltage is increased.

At voltages which yield rapid separations, resistance to mass transfer in the mobile phase, intermicelle ($H_{M,m}$) and intracolumn ($H_{M,c}$), appear to be primary sources of band dispersion. High surfactant concentrations and small capillary i. d.s (but not as small as are necessary for high efficiency in OTLC) are important in

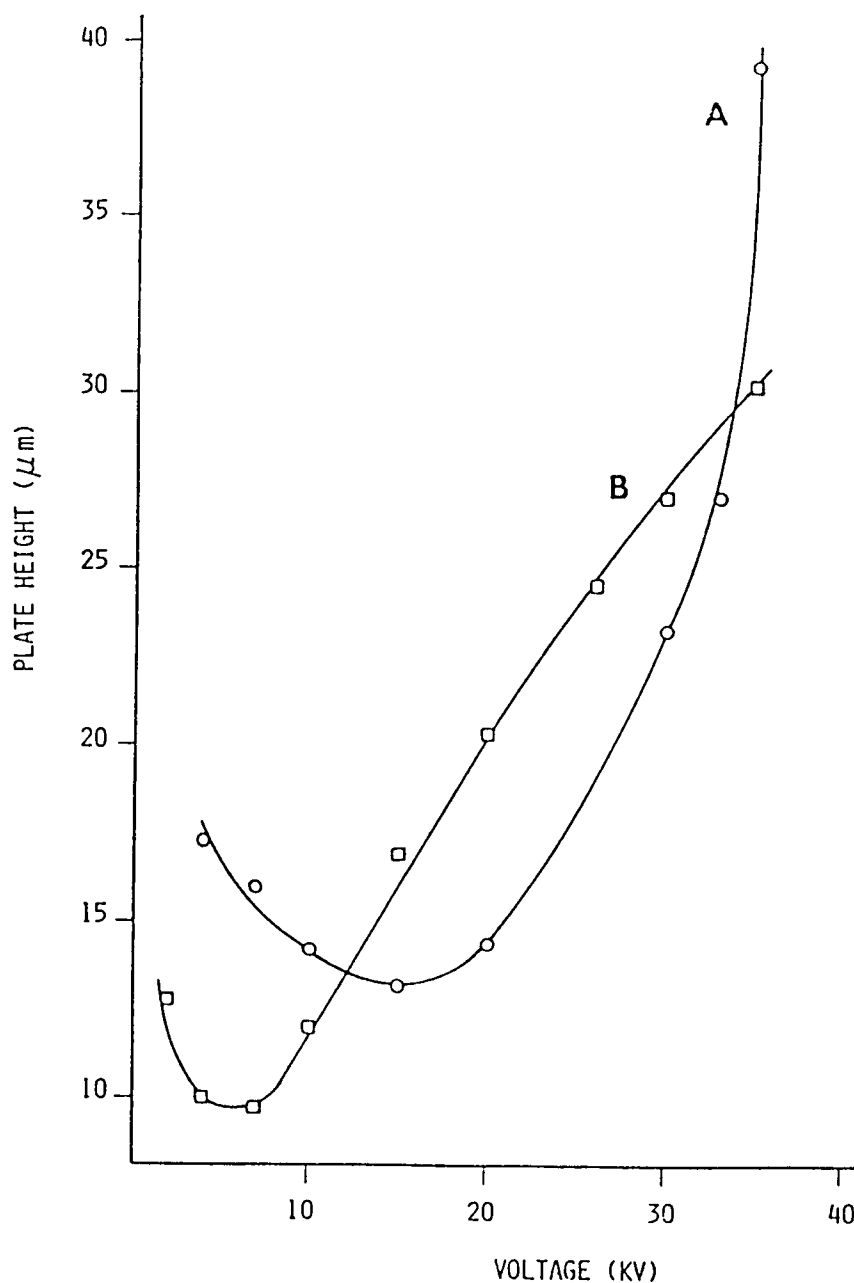


Figure 16. Plate height versus voltage plots with differing buffer concentrations. Solute: NBD-cyclohexylamine. Mobile phase: 0.01M SDS with (A) 0.007 M or (B) 0.003M Na_2HPO_4 . Other conditions same as for Figure 14.

Source: Sepaniak, M. J.; Cole, R. O. Anal. Chem. in press, February 1987.

minimizing $H_{M,m}$ and $H_{M,c}$ respectively. Care must be taken not to exceed the thermal dissipation capabilities of the capillary column by employing high applied voltages and/or high ionic strength mobile phases which results in band dispersion. In general power dissipations of less than 1.0 Watts/m result in efficient separations. The experimental parameters presented can be utilized for efficiency optimization for any MECC system; however, the extent to which these experimental parameters effect efficiency may vary for any particular MECC system.

Summary

The observed efficiency in MECC is dependent on a variety of operational parameters. The maximization of column efficiency requires the optimization of the injection process, the separation conditions, and the detection scheme. Many of these parameters are easily controlled by the operator and simply require attention to the specific aspects of any particular MECC system. Due to the complexity of the processes occurring during the MECC experiment, recommendations for improved efficiency can only take the form of guidelines. Each MECC system should always be considered individually. However, the potential for very efficient separations exists with this technique and the realization of those efficiencies is dependent upon the implementation of these guidelines.

CHAPTER IV

SELECTIVITY STUDY

MECC separations have primarily employed sodium dodecyl sulfate (43, 47, 57, 58, 61) and to a lesser degree cetyltrimethylammonium chloride (33) as the "pseudostationary" phase. A cyclodextrin derivative (62) and dodecyltrimethylammonium bromide (63) have also been used to obtain separations; however, the properties of alternate micellar systems have only been partially explored (46). In conventional HPLC the choice of stationary phases is somewhat limited. For the majority of analyses, the "C-18" stationary phase has been adequate. There is a wide range of "pseudostationary" phases (surfactants) available in MECC. The range of appropriate surfactants is, however, limited by solubility. Moreover, thermal problems can result if large concentrations of a soluble surfactant are required for aggregation (33). Nevertheless, many surfactants are commercially available and appropriate surfactants can be tailor-made for particular separation problems (62).

The conversion from a reversed-phase to a normal-phase system in HPLC requires a new column as well as a conditioning of the chromatographic system. The conversion to a new "pseudostationary" phase in MECC is accomplished by merely rinsing and refilling the existing fused silica capillary column with the appropriate micellar solution. The effort and expense in changing the "pseudostationary" phase in MECC is minimal.

Surfactants are generally categorized with respect to the charge on the surfactant molecule (anionic, cationic, zwitterionic, or nonionic). Two common anionic surfactants (sodium decyl sulfate, STS, and sodium dodecyl sulfate, SDS) and two common cationic surfactants (dodecyltrimethylammonium chloride, DTAC, and cetyltrimethylammonium chloride, CTAC) were selected as "pseudostationary" phases. A group of twelve test solutes was used to explore the properties of each micellar system. The separation characteristics of these four surfactants were studied at various voltages. The utility of each surfactant for MECC is discussed based on the retention characteristics observed in this study.

Experimental Procedures

Changing of the mobile phase was accomplished by rinsing the column with distilled water, then with 0.1M HCl, and finally with the new mobile phase. The rinsing procedures were particularly critical when cationic surfactants were replaced with anionic surfactants or vice versa because precipitation of the surfactants could cause column occlusion. The polarity of the high voltage power supply had to be adjusted with respect to the surfactant employed. With anionic surfactants the high voltage electrode was positive (detection at the negative electrode) and with cationic surfactants the high voltage electrode was negative (detection at the positive electrode). The aqueous buffer for all mobile phases was 0.01M Na_2HPO_4 and 0.006M $\text{Na}_2\text{B}_4\text{O}_7$ with the surfactant concentration at 0.05M. All standards were prepared (at a concentration of approximately 200 ppm) in the appropriate mobile phase with the addition of 10% methanol.

The uv absorbance of all test solutes was monitored at 220 nm. Methanol was used to mark the retention time of a neutral unretained solute, t_o , and sudan III, a water-insoluble dye, was used to determine the retention time of the micelles, t_m (43). Due to its small absorptivity at 220 nm, sudan III was monitored at 345 nm, which also provided an unambiguous baseline deflection for methanol.

Experimental Results

The diversity of available stationary and mobile phases enhances any separation technique. To date MECC has been performed with purely aqueous mobile phases; therefore, the choice of the proper surfactant can be critical to attaining the desired separation. The understanding of micelle characteristics, such as micellar size and solubilizing properties, can prove important in surfactant selection.

Surfactants with alkyl tail lengths from ten carbons, C-10, to sixteen carbons, C-16, were chosen for this study. Surfactants with alkyl tails of less than eight carbons do not generally form micelles (64). The CMC for STS is 0.013M in distilled water; however, it is well known that the CMC for a surfactant is lowered by raising the ionic strength of the solution (64). Impurities in the surfactants themselves can also affect the observed CMC. CMC values increase dramatically as the surfactant's tail length decreases. As the concentration of surfactant is increased to exceed the CMC for the smaller surfactant molecules, the current in the MECC column becomes prohibitively high. The large power dissipation requirements (< 2

Watts/meter) observed with high concentrations of free surfactants can tax the heat dissipation capabilities of the capillary columns and degrade efficiency (58). Conversely, anionic surfactants with alkyl tails longer than C-14 display limited solubility in water. The cationic surfactants with anions show increased solubility (larger CMC) with respect to the same surfactants with bromide counter ions.

Capacity factors were determined using Equation 4 (p. 15). It should be noted that as t_r approaches t_m then k' approaches infinity, consequently k' is not a good measure of analysis time. For example, with a DTAC mobile phase a k' of 29 is obtained for 1-naphthol in 17 minutes at 30 kV. It has been previously determined that an optimum k' value is approximately 2 (46).

The test solutes used in this study (see Table 4) contained acidic, basic, or electron-withdrawing functionalities. Size differences and polarity were also considered in the selection of test solutes. Electrical neutrality for the solutes was desirable at the pH of the mobile phase to eliminate complications due to electrophoretic effects. The capacity factors shown in Table 4 for the test solutes with three micellar "pseudostationary" phases were obtained at a separation voltage of 20 kV.

As a broad generalization, retention order in MECC is consistent with the solubility of the analytes in water. This can be seen for the individual groups of analytes in Table 4. For example, toluene is the most soluble among the neutral solutes considered in Table 4, anthracene is the least soluble, and naphthalene is of intermediate

Table 4

Test Solute Capacity Factors with Three Surfactants¹

Compound	SDS	DTAC	CTAC
ACIDIC			
Phenol	0.60	1.4	2.4
1-Naphthol	8.0	36.	7.2
BASIC			
Aniline	0.46	0.48	0.58
Benzidine	3.9	5.0	3.6
1-Amino-naphthalene	5.9	8.3	4.7
ELECTRON-WITHDRAWING			
Nitrobenzene	1.6	1.2	1.4
2,4-DNT ²	3.4	2.7	2.9
1-Nitro-naphthalene	70.	17.	5.8
NEUTRAL			
Toluene	4.4	2.4	3.2
Naphthalene	70.	14.	7.3
Anthracene	N/D ³	17.	8.7
t_o	12.0	11.8	17.2
t_m	36.2	28.2	44.4

1. voltage: 20 kV

2. 2,4-DNT: 2,4-dinitrotoluene

3. N/D: not detected

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. J. Chromatogr. Sci. submitted for publication.

solubility. Their elution order is consistent with this order of solubility. Furthermore, benzene would be expected to elute prior to toluene based on solubility considerations and as observed in our previous experiments benzene does have a smaller k' than toluene in all of the micellar systems that we have investigated. However, specific micelle-solute interactions can be present which could result in deviations from a predicted order of elution based solely on solubility in the mobile phase.

Proposing explanations for phenomena observed in the MECC experiment is difficult due to the complex nature of the processes present. The micellar literature is not in complete agreement on the mechanisms for solubilization of solute into these aggregates. The observed behavior of aggregate solutions can differ depending upon the method of investigation. For example, CMC data for a given micellar system can vary with its method of determination. Thus exact explanations for the solubilization characteristics of the micellar systems used herein are not presented. However, attempts were made to identify trends which should be of practical value in the utilization of MECC.

The capacity factors for the test solutes with three of the micellar systems at 20 kV are given in Table 4. Several trends are obvious from the data presented in Table 4. CTAC (C-16) yields the smallest k' values for the multiring solutes. 1-Nitronaphthalene shows the largest decrease in k' of the test solutes for CTAC with respect to SDS. For higher molecular weight test solutes the CTAC mobile phase under these conditions is more desirable in terms of the observed

capacity factors. The solubility of large molecular weight solutes in the aqueous phase can be problematic. The free cationic surfactants tend to better solubilize these larger molecules in solution. For example, anthracene is shown in Table 4 as not detected for the anionic surfactant systems, presumably due to limited solubility. It is curious that the k' values for the relatively large solute naphthalene are smaller for the cationic surfactant systems. This may indicate that while the free anionic surfactant does not sufficiently improve solubility in the aqueous phase, the large solutes are solubilized to some degree by the anionic micellar phase. A micelle system other than CTAC or limited amounts of an organic modifier (e.g., propanol) in the mobile phase may be required for the separation of larger molecules with similar structures. However, from the standpoint of maximizing resolution with respect to k' and solubilization capabilities, CTAC appears to be the mobile phase of choice.

Phenol, an acidic compound, shows increased retention with the cationic mobile phases. Phenol is quite soluble in the aqueous mobile phase and has a k' value of 0.64 with the SDS system, but is retarded on the column with the CTAC and DTAC mobile phases possibly due to ionic type interactions with the cationic surfactants. Conversely, the basic compounds experience similar k' s with the anionic and cationic micellar systems. For nitrobenzene and 2,4-dinitrotoluene (electron-withdrawing substituents) very little difference is noted between the anionic and cationic mobile phases. For solutes with functionalities

which would not be expected to interact in an ionic fashion with the micelles the choice of surfactants may not be as critical.

Benzoic acid was included in this study but was omitted from Table 4 for clarity. At the pH of the micellar solutions benzoic acid exists in an ionized form. Neutral species are separated based upon their differential partitioning between the aqueous mobile phase and the hydrophobic interior of the micelles, however, the separation of ionic species involves a combination of electrokinetic and electrophoretic effects (61). The test solutes were separated with an STS micellar system, but because of experimental difficulties, which will be discussed later, the STS data was omitted from Table 4.

Figures 17-19 show the separation of eight of the test solutes using SDS, DTAC, and STS as the micellar phases, respectively. The conditions of the separation in Figures 17-19 are identical and are given in Figure 17.

The elution orders in Figures 17 and 19 (the anionic surfactant systems) are the same with the exception of the benzoic acid which is more retained in the case of the STS micellar system. The behavior of benzoic acid could be due in part to the electrophoretic mobility of the benzoate anion (its predominant form at this pH). Electroosmotic flow velocities for the anionic surfactant systems are approximately equal and in opposition to the electrophoretic flow of the micelles and the benzoate anion. The smaller STS micelles appear to have a greater electrophoretic velocity than the SDS micelles as evidenced by a larger t_m value with the STS system. The electrophoretic velocity of the

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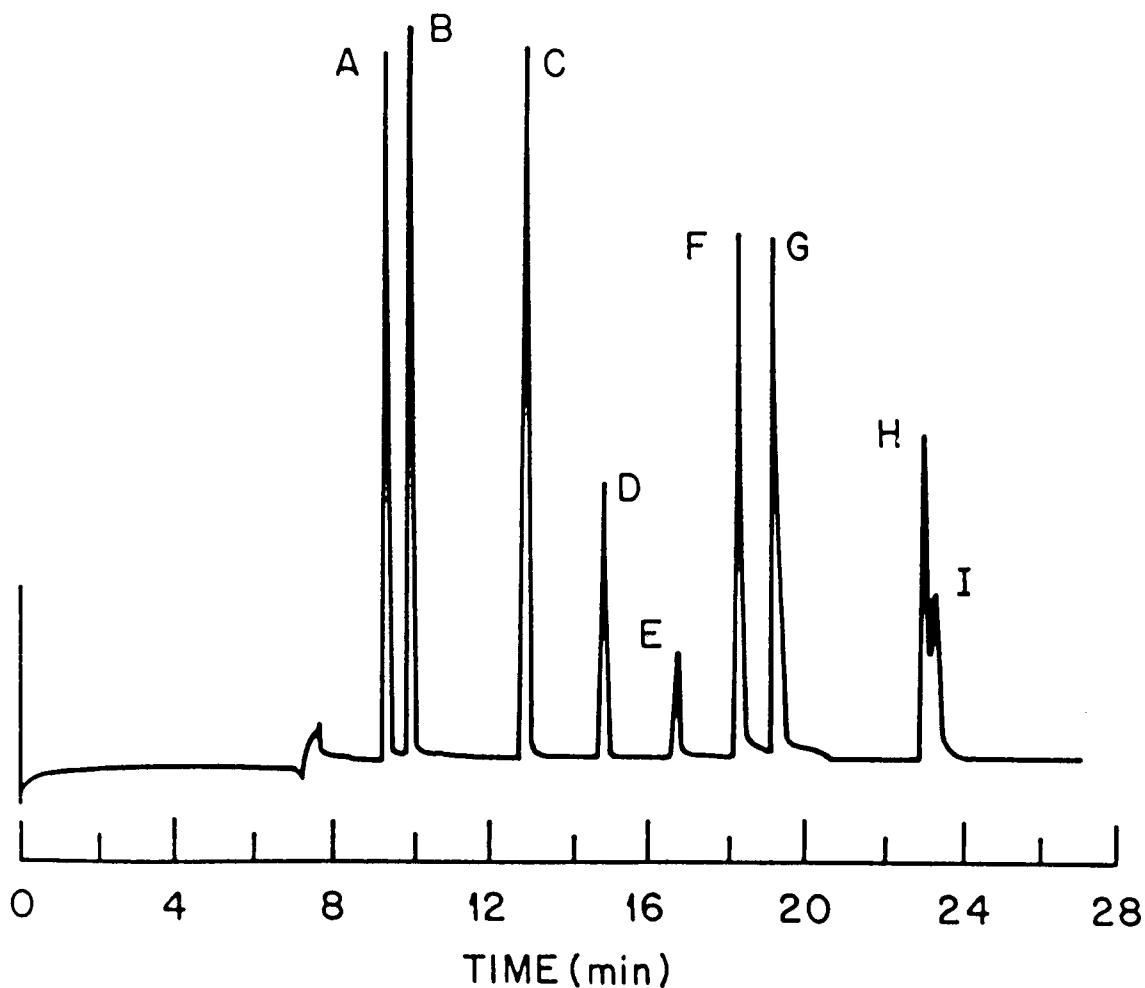


Figure 17. Separation of test solutes with sodium dodecyl sulfate mobile phase. Solutes: (A) aniline, (B) phenol, (C) nitrobenzene, (D) benzoic acid, (E) toluene, (F) 1-aminonaphthalene, (G) 1-naphthol, (H) 1-nitronaphthalene, (I) 2-nitronaphthalene. Conditions: 1m x 60 μ m i. d. fused silica column; 30 kV, 45 μ A; detection wavelength, 220 nm. Mobile phase: 0.05M SDS, 0.01M Na_2HPO_4 , 0.006M $\text{Na}_2\text{B}_4\text{O}_7$ in distilled water.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. J. Chromatogr. Sci. submitted for publication.

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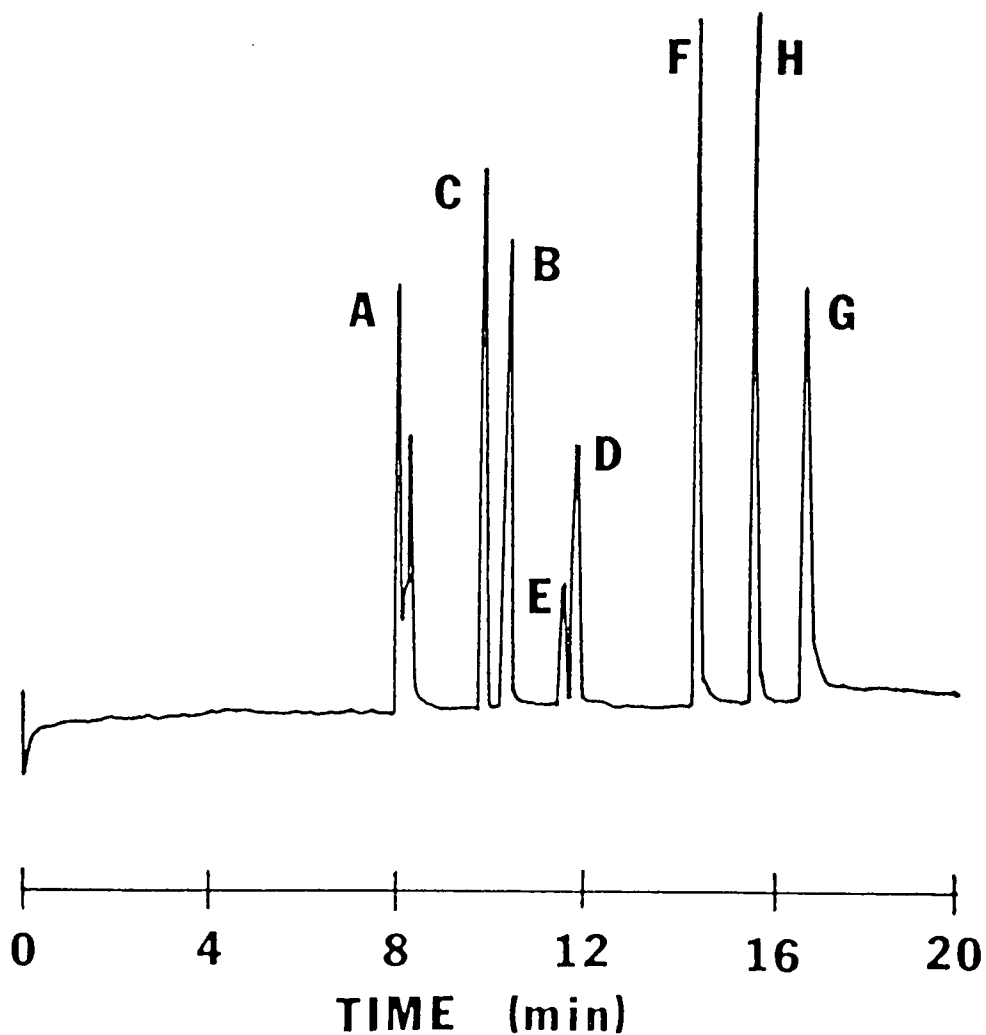


Figure 18. Separation of test solutes with dodecyltrimethylammonium chloride mobile phase. All solutes and conditions are the same as for Figure 17. Mobile phase: 0.05M DTAC, 0.01M Na_2HPO_4 , 0.006M $\text{Na}_2\text{B}_4\text{O}_7$ in distilled water.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. J. Chromatogr. Sci. submitted for publication.

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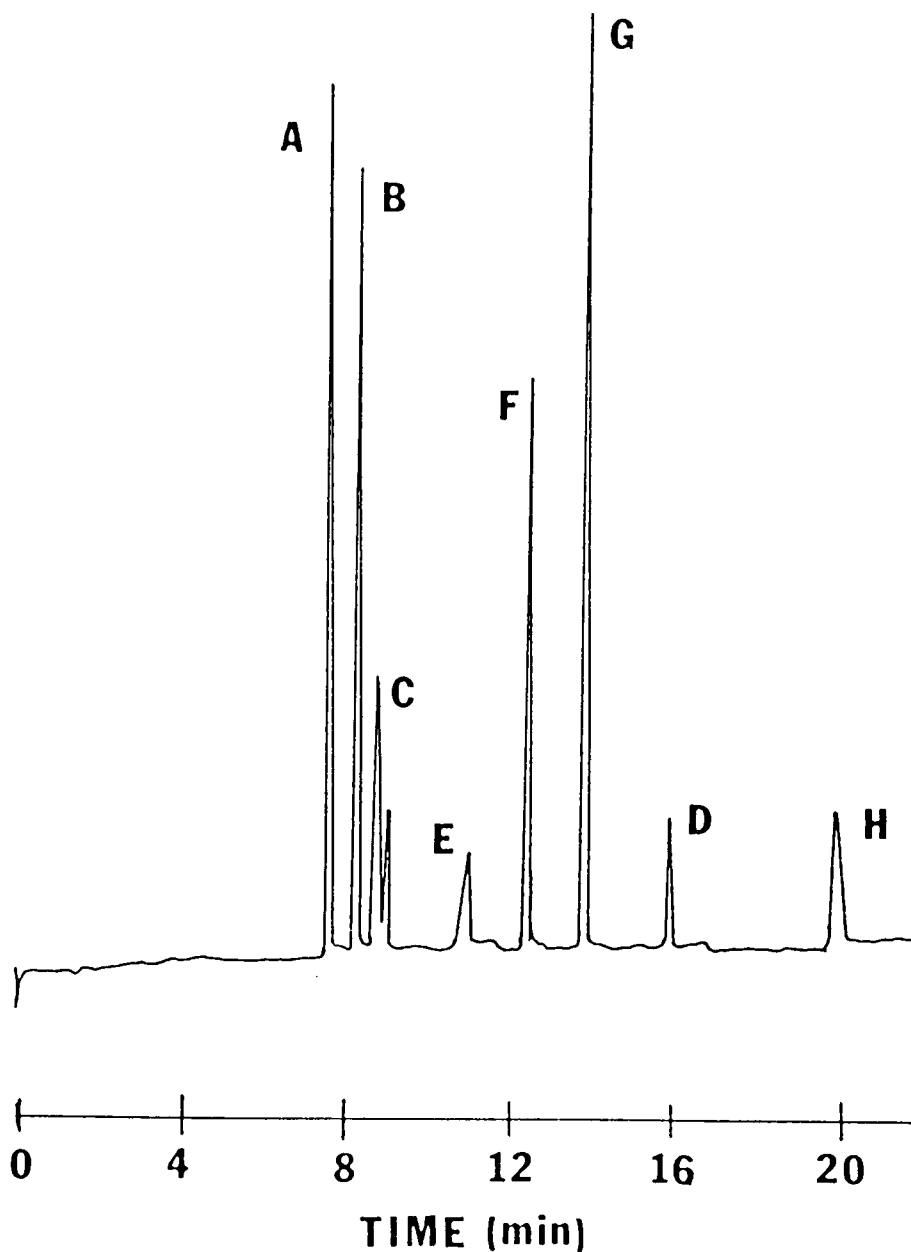


Figure 19. Separation of test solutes with sodium decyl sulfate mobile phase. All solutes and condition are the same as for Figure 17. Mobile phase: 0.05M STS; 0.01M Na_2HPO_4 , $\text{Na}_2\text{B}_4\text{O}_7$ in distilled water.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. J. Chromatogr. Sci. submitted for publication.

benzoate anion is expected to be equal in the SDS and STS experiments. The greater benzoate retention on the STS system indicates that, despite expected repulsions between like charges, there is some micellar solubilization of the anion. Thus, charged and neutral solutes can be eluted in a single analysis within the range defined by t_o and t_m .

For the separation in Figure 19 the capacity factors for each of the solutes are less than those in Figure 17. This is possibly due to poor partitioning into the smaller STS micelles. In Figure 17 there is partial separation of the 1-nitronaphthalene and 2-nitronaphthalene isomers. The SDS is the only system which resulted in the separation of these two isomers.

The selectivity demonstrated in Figure 18 for the cationic DTAC system differs from that of the anionic systems. Nitrobenzene and phenol as well as 1-nitronaphthalene and 1-naphthol have switched their order of elution. The same elution order is seen for the CTAC as well as DTAC micellar systems. The acidic components are more retained with the cationic micellar phases. The interactions between the acidic solutes and the cationic micelles are stronger and are possibly of an ionic type as shown by the capacity factors in Table 4. This is consistent with results from micellar thin layer chromatography, where it was shown that 1-naphthol eluted close to the solvent front with CTAC mobile phases (i.e., naphthol was solubilized by the micelles and not the stationary support) and eluted near the origin with an SDS micellar

system (i.e., naphthol interacted with the stationary phase and was not solubilized by the micelles) (44).

Table 5 shows the effect of varying the separation voltage on the capacity factors for the SDS, DTAC, and CTAC micellar systems. The solutes listed in Table 5 correspond to the solutes separated in Figures 17-19. For STS, SDS, and DTAC the capacity factors decrease as the applied voltage is increased (46) due to joule heating of the mobile phase. Temperature increases on the order of tens of degrees celsius have been reported inside the MECC column (46). However, in this work with the CTAC system capacity factors decrease as the applied voltage is increased. The joule heating of the mobile phase can affect the CMC of a surfactant. For SDS as the temperature is increased from 35° C to 55° C, the CMC increases from approximately $8.4 \times 10^{-3} \text{ M}$ to $9.7 \times 10^{-3} \text{ M}$ (60). This effect would be most dramatic in MECC when the concentration of the surfactant is close to its CMC. The high temperatures of the mobile phase can also influence the thermodynamic partitioning of the solutes between the mobile phase and the micelles.

The elution range in MECC is an important concern. Since t_m represents the retention time of a nonionic solute (sudan III) which is totally solubilized by the micelles and t_o represents the retention time of a nonionic solute (methanol) which is not solubilized by the micelles, then all nonionic solutes will be expected to elute between t_o and t_m . Therefore, in MECC there is a limited elution range for nonionic solutes. The potential problem of a limited elution range can be addressed from two different perspectives. The separation number,

Table 5
Test Solute Capacity Factors at Different Voltages

Compound	<u>SDS</u>		<u>DTAC</u>		<u>CTAC</u>	
	15 kV	30 kV	15 kV	30 kV	15 kV	30 kV
Aniline	0.47	0.29	0.60	0.40	0.55	0.62
Phenol	0.59	0.53	1.8	1.3	2.1	2.9
Nitrobenzene	1.7	1.4	1.7	0.96	1.4	2.1
Benzoic acid	2.6	2.7	3.0	2.3	3.2	5.5
Toluene	4.4	3.7	3.0	2.1	2.9	5.0
Aminonaphth.	7.0	5.1	12.	6.3	3.9	8.0
Napthol	8.1	6.4	36.	29.	5.0	18.
Nitronaphth.	56.	48.	25.	12	4.7	17.
t_o	16.8	7.0	16.8	6.2	23.0	10.2
t_m	51.6	19.7	39.6	14.4	64.0	24.2

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. J. Chromatogr. Sci.
submitted for publication.

n , is the maximum number of peaks which can be separated within a range of retention times and is expressed by Equation 9 (46):

$$n = 1 + \frac{\sqrt{N}}{4} \ln \frac{t_m}{t_o} , \quad \text{Eq. 9}$$

where N is the average chromatographic efficiency. Equation 9 shows that n is dependant on both efficiency and the ratio t_m/t_o . Since efficiencies in excess of 200,000 theoretical plates/meter are routinely attained, extending the elution range (increasing t_m/t_o) can be utilized to increase the separation number. However, for particular separations the selectivity of the micellar phase could supersede the desire for an extended elution range.

Sodium decyl sulfate (STS) was investigated in an effort to increase the elution range of MECC. STS exhibited the largest elution range of the surfactants examined. STS has the largest electrophoretic mobility and since electrophoretic velocity opposes electroosmotic velocity, the net velocity of the STS micelles toward the detection electrode is the slowest. For all of the voltages studied, STS had the largest elution range. Unfortunately, under these mobile phase conditions the STS micellar solutions did not yield sufficient reproducibility to be considered useful. With the STS micellar system the retention time for a given test solute was not reproducible and would increase as replicate injections were performed. Furthermore, the peak shapes of the late eluting solutes began to become increasingly asymmetric as a function of time. Reproducibility was improved for the STS

system by rinsing the column with the mobile phase every two hours. The SDS, DTAC, and CTAC mobile phases did not exhibit this type of irreproducibility.

CHAPTER V

VITAMIN B₆ ANALYSISHistorical Review

The development of an analytical technique is not complete without the demonstration of its utility in handling "real" samples. The large numbers of theoretical plates available with MECC would be somewhat less impressive if the technique could not be applied to a genuine analysis. The usefulness of an analytical technique can be questioned if reasonable applications for the technique are not present. The separation and quantitation of pyridoxic acid in urine was chosen to demonstrate the ability of MECC to perform real analyses.

MECC is well suited for the analysis of biological samples, since they are often in an aqueous matrix. In many biological samples the analytes of interest may be both neutral and ionic. MECC can be used to separate the charged and neutral analytes. The large number of theoretical plates and the good sample capacities afforded by MECC should allow for its bioanalytical use.

Metabolites of pyridoxine (PN, vitamin B₆) exist in the body in several forms. Pyridoxal (PL) and pyridoxamine (PM) are precursors to important coenzymes. Pyridoxamine-5-phosphate (PMP) and pyridoxal-5-phosphate (PLP) are important coenzymes, PLP being essential in the metabolism of tryptophan (66). Pyridoxic acid (PI) is the major metabolite excreted in the urine. The structures of vitamin B₆

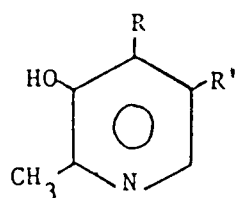
and its metabolites (referred to as B₆ vitamers) are given in Figure 20.

Many methods have been employed in an attempt to separate and quantitate B₆ vitamers in biological fluids. Liquid chromatographic procedures have been outlined which involve ion-exchange chromatography (67, 68), HPLC methods using multiple columns (69), ion-pair reversed-phase HPLC (19-20, 70-71) and flow injection analysis (72). B₆ vitamers have also been separated electrophoretically (73). Fluorescence detection has been used most often (20, 67-74); however, absorbance (19) and phosphorescence (75) detection schemes have also been employed.

Biological matrices which have been analyzed for the B₆ vitamers include serum (20, 67-68), milk (71, 76), various tissues (70), and urine (68). Other samples of interest have been food (74, 77) and commercial vitamin tablets (78).

Separation of B₆ Vitamers

Figure 21 shows the separation of the six B₆ vitamers. These standards are dissolved in the mobile phase. The efficiencies for PL, PI, PMP, and PLP are 60,000 theoretical plates/m and 30,000 theoretical plates/m for PM and PN. The applied voltage is 30 kV, which resulted in a current of 70 A. The MECC technique exhibits the selectivity and efficiency needed to separate the B₆ vitamers. The retention time of PLP could be decreased, to shorten analysis time, by applying higher voltages.



VITAMER	R	R'
PN	CH ₂ OH	CH ₂ OH
PM	CH ₂ NH ₂	CH ₂ OH
PL	CHO	CH ₂ OH
PI	CO ₂ H	CH ₂ OH
PMP	CH ₂ NH ₂	CH ₂ OPO ₃ H ₂
PLP	CHO	CH ₂ OPO ₃ H ₂

Figure 20. Structures of the B₆ vitamers.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. J. Chromatogr. Sci. 1986, 24, 347-351.

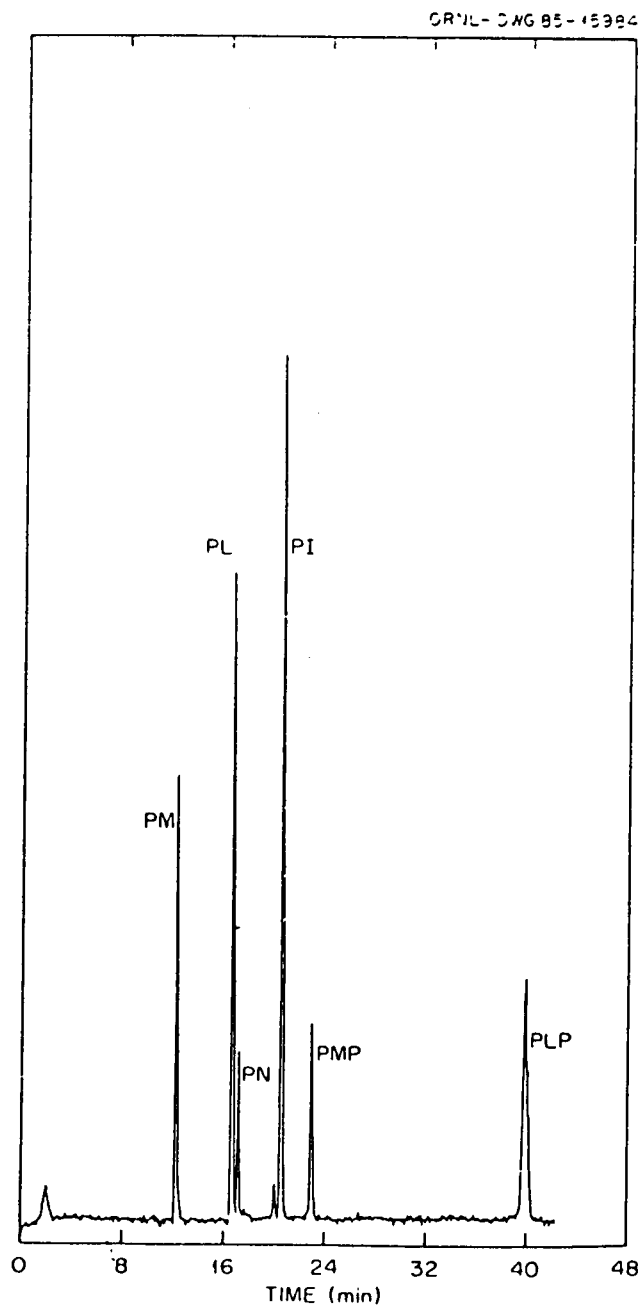


Figure 21. MECC chromatogram of pyridoxine and its metabolites. Fluorescence detection: excitation, 325 nm; emission, 430 nm. Conditions: 75 μm x 1 m fused silica column; 30 kV, 70 μA . Mobile phase: 0.05M SDS, 0.01M Na_2HPO_4 , 0.006M $\text{Na}_2\text{B}_4\text{O}_7$ in distilled water.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. J. Chromatogr. Sci. 1986, 24, 347-351.

Laser-excited on-column fluorescence detection is employed for the B₆ vitamers (see Chapter II). Excitation is performed with the 325 nm line of a He/Cd laser and the fluorescence is monitored at 430 nm.

The limits of detection obtained in this work are shown in Table 6 (47). The quantities listed are injected quantities as determined by the procedure given below. They were determined by injecting serial dilutions of a concentrated standard mixture, then preparing calibration plots (peak height versus amount injected) which were extrapolated to a signal to noise ratio (S/N) of 3 to assign limits of detection. The calibration plots range from the lowest detectable injected quantity to injected quantities approximately three orders of magnitude higher. The correlation coefficients for the calibration plots are also shown in Table 6.

The injected quantities are determined using Equation 10 (47):

$$\text{AMT INJ} = V_c * C_{\text{std}} * t_i / t_r , \quad \text{Eq. 10}$$

where V_c is the column volume, C_{std} is the concentration of the standard, t_i is the length of time of the injection, and t_r is the retention time of the analyte. There are two conditions which must be met for Equation 10 to be valid. The analyte must be dissolved in the mobile phase and the applied voltage during the injection must be equal to that of the voltage applied during the separation process. Since injection precision is limited by the ability to reproduce the injection time, it is possible to improve precision by injecting for longer

Table 6

B₆ Vitamer Calibration Plot Data

Vitamer	L. O. D. ¹	R. S. D. ²	Corr. Coeff.
PN	750 pg	3.8 %	0.9999
PM	500 pg	5.3 %	0.9993
PL	150 pg	6.2 %	1.0000
PI	800 fg	2.3 %	0.9999
PMP	180 pg	5.8 %	0.9999
PLP	900 pg	6.0 %	0.9998

1. L. O. D. = limit of detection (3 x S/N)

2. R. S. D. = relative standard deviation

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P.
J. Chromatogr. Sci. 1986, 24, 347-351.

times, at voltages less than that used for the separation (see Chapter III). However, the nonlinear relationship between applied voltage and retention times, which is often observed in our work, would make it difficult to determine amounts injected if the separation and injection voltages differ.

PI is the vitamer with the lowest limit of detection due to its large fluorescence quantum efficiency. PLP has the highest detection limit. There are two reason for this. First, PLP has the poorest quantum efficiency. This may be overcome through derivatization (67, 69, 79). Secondly, the emission maximum for the PLP is 462 nm at the pH employed for the separation. At the emission wavelength monitored in this work, 430 nm, the intensity is only 40% of the maximum. Similarly for the PN the emission maximum is at 400 nm and the intensity is approximately 20% of the maximum intensity. At the pH of the micellar solution the excitation maximum for PN is at 350 nm, whereas for the other vitamers is between 320 and 330 nm under these conditions.

Table 6 also shows the reproducibility of injections. These range from 2.3-6.2% relative standard deviation. Each of the serial dilutions used in determining the calibration plots is done in triplicate. The relative standard deviation was determined by a pooling of the data obtained in the serial dilutions. The reproducibility for retention times is also quite good. Retention times will decrease over a period of time (approximately a week). This is probably due to a concentrating of the micellar solution from evaporation. With this concentrating of the mobile phase the current increases, which results in a faster

flow rate and, therefore, a decrease in the retention time for a particular solute. Each day a standard solution was run to check the retention time of each vitamer.

Quantitation of Pyridoxic Acid in Urine

Vitamin B₆ was ingested as pyridoxine hydrochloride. Quantities of pyridoxine, taken in the form of commercial vitamin supplement tablets, were 3 mg and 100 mg. Morning urine was collected 10 hours after ingestion. The urine was filtered through a five micron frit prior to extraction. A 50 mL aliquot of urine was extracted three times with 100 mL of ethyl acetate. The aqueous layer was mixed with an equal portion of the mobile phase and analyzed for pyridoxic acid. All calibration standards are prepared in the mobile phase.

Chromatograms of spiked and unspiked human urine are shown in Figure 22. Figure 22A represents the urine sample chromatogram obtained following the ingestion of 3 mg of vitamin B₆ as pyridoxine hydrochloride and extracted as previously described. The peak corresponding to PI is shown. The concentration of PI is approximately 1 ppm, as determined by standard addition. This corresponds to 0.22 mg of PI in the total volume of urine. The conditions of the separation are the same as for Figure 21.

Despite the discrimination afforded through fluorescence detection and the clean-up procedures employed, an interferent, which is not completely resolved from PI, is observed in Figure 22. The presence of the interferent introduces a source of error in the determination of

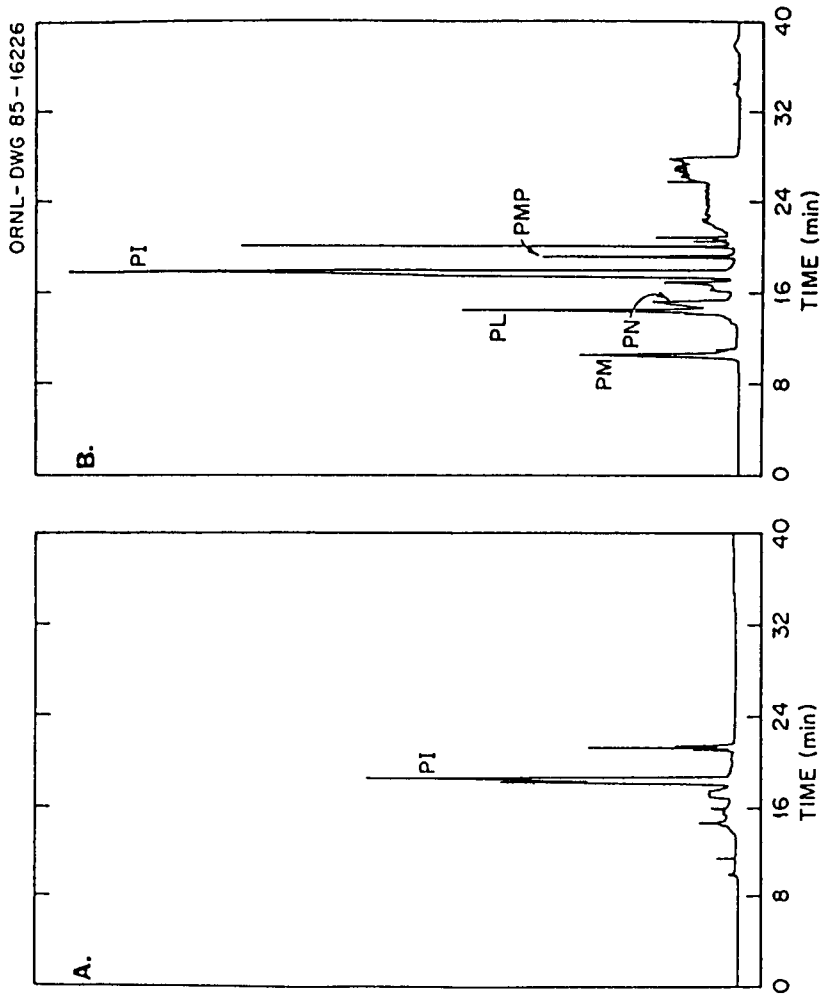


Figure 22. MECC chromatograms of (A) human urine and (B) spiked human urine. The ingested quantity of pyridoxine was 3 mg, yielding a PI concentration of 1 ppm. The conditions are the same as for Figure 21.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. J. Chromatogr. Sci. 1986, 24, 347-351.

the concentration of PI. Quantitation was based on peak heights which are measured from the chromatographic baseline established by the combination of the PI and the interferent. For a precise determination of the amount of PI present, separation conditions would have to be altered to completely resolve these two peaks. Changing the voltage or lengthening the column could provide the resolution necessary to completely separate the two peaks.

Figure 23 is an MECC chromatogram of human urine with the ingestion of 100 mg of pyridoxine 10 hours prior to the collection of the morning urine. This sample is a 1:200 dilution of the extracted urine in the mobile phase. The concentration of PI is 140 ppm and this corresponds to 40 mg of PI in the total volume of urine.

In summary, micellar electrokinetic capillary chromatography is well suited for analyses of biological samples, where its excellent efficiency and compatibility with charged and neutral solutes can be important. Utilizing fluorescence detection, spectroscopic and chromatographic selectivity are possible. This technique can complement conventional HPLC and should provide an alternative to HPLC for difficult separations.

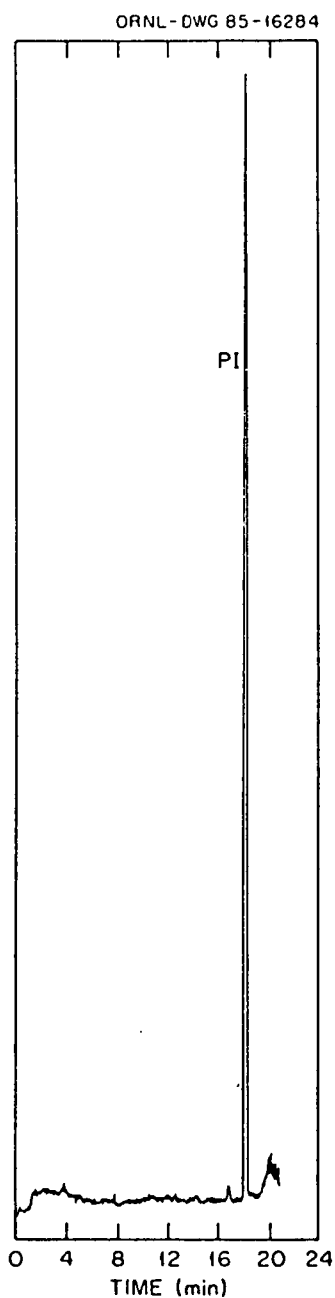


Figure 23. MECC chromatogram of 200-fold dilution of extracted human urine. The ingested quantity of pyridoxine was 100 mg, yielding a PI concentration of 140 ppm. The conditions are the same as for Figure 21.

Source: Burton, D. E.; Sepaniak, M. J.; Maskarinec, M. P. J. Chromatogr. Sci. 1986, 24, 347-351.

CHAPTER VI

CONCLUSIONS

Significant contributions have been made to the field of liquid chromatography and have been presented in this dissertation. The thrust of this dissertation has been in three areas. First, the experimental parameters effecting the chromatographic efficiency observed in micellar electrokinetic capillary chromatography (MECC) were investigated. Second, the utility of different surfactant systems was examined. Third, the first utilization of MECC for an actual analysis was presented.

In this dissertation, the effects of the injection process on the chromatographic efficiency in MECC were discussed. The principles of electroinjection were examined in detail. It was shown that improper injections procedures can degrade efficiency by as much as 50-fold. The contributions to the observed height equivalent to a theoretical plate by the column parameters was also discussed. The experimental parameters in MECC were evaluated in terms corresponding to those of conventional liquid chromatography. The concentration of the surfactant in the mobile phase was found to be directly related to the observed efficiency. The adverse effects of the joule heating of the mobile phase, a condition unique to MECC, was found to be an important source of band broadening.

Various surfactant systems were examined for their use in MECC. In general, the sodium dodecyl sulfate micellar system was found to be the system which could be most universally applied. The isomers, 1-nitronaphthalene and 2-nitronaphthalene, were partially resolved with the sodium dodecyl sulfate micelle system. The cationic surfactants, dodecyltrimethylammonium chloride and cetyltrimethylammonium chloride were found to have superior solubilizing characteristics, however, were not as useful as the sodium dodecyl sulfate system. Sodium decyl sulfate was investigated in an attempt to extend the elution range in MECC. Unfortunately, the sodium decyl sulfate system did exhibit sufficient reproducibility to be considered useful.

Pyridoxine, vitamin B₆, and its metabolites have traditionally presented a formidable challenge to the separation scientist. MECC was employed to separate these B₆ vitamers. To demonstrate the applicability of MECC for "real" analyses, pyridoxic acid, the major metabolite of vitamin B₆ excreted in urine, was quantitated in human urine. The limit of detection for pyridoxic acid with this technique was shown to be 0.9 pg injected.

The future of MECC is at present in the hands of the many researchers involved in its development. Many problems remain to be addressed. The limited elution range and overcoming the dependence of resolution on the capacity factors of the solutes are two important problems yet to be conquered. The actual mechanisms involved in the partitioning of solutes with the micelles still require much research

to deduce. The high efficiencies obtained in MECC are a compelling justification for continued research.

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APPENDIX

LIST OF ABBREVIATIONS

C_{std}	Concentration of the analyte solution
CMC	Critical micelle concentration
CTAB	Cetyltrimethylammonium bromide
CTAC	Cetyltrimethylammonium chloride
CZE	Capillary zone electrophoresis
D	Diffusion coefficient of a solute in the mobile phase
DTAC	Dodecyltrimethylammonium chloride
E	Applied voltage
fg	Femtogram, 1×10^{-15} g
H	Height equivalent to a theoretical plate
H_A	Plate height due to axial diffusion
$H_{M,c}$	Plate height due to the resistance to mass transfer in the mobile phase (intracolumn)
$H_{M,m}$	Plate height due to the resistance to mass transfer in the mobile phase (intermicelle)
H_S	Plate height due to the resistance to mass transfer in the pseudo stationary (micelle) phase
H_T	Plate height due to thermal effects
HPLC	High performance liquid chromatography
L_I	Length of the injection plug
L.O.D	Limit of detection
LC	Liquid chromatography

MECC	Micellar electrokinetic capillary chromatography
N	Number of theoretical plates
nL	Nanoliter, 1×10^{-9} L
nm	Nanometer, 1×10^{-9} m
OTLC	Open-tubular liquid chromatography
PAH	Polyaromatic hydrocarbon
pg	Picogram, 1×10^{-12} g
PL	Pyridoxal
PLP	Pyridoxal-5-phosphate
PM	Pyridoxamine
PMCLC	Packed micro-column liquid chromatography
PMP	Pyridoxamine-5-phosphate
PN	Pyridoxine
R	Retention ratio (fraction of a solute not solubilized by the micelle)
R.S.D.	Relative standard deviation
SDS	Sodium dodecyl sulfate
STS	Sodium decyl sulfate
t_i	Length of time of the injection process
t_m	Retention time of a solute completely solubilized by the micelles (sudan III)
t_o	Retention time of a solute not solubilized by the micelles (methanol)
t_r	Retention time of the solute
u	Observed mobility of a solute in CZE

u_{el}	Electrophoretic mobility of a solute in CZE
u_{osm}	Electroosmotic mobility of a solute in CZE
uv	Ultraviolet
V_c	Column volume
V_{eo}	Velocity of the electroosmotic flow
V_m	Net velocity of the micelles
$V_{m,e}$	Electrophoretic velocity of the micelles
V_s	Velocity of the solute
μA	Microampere, 1×10^{-6} A
μL	Microliter, 1×10^{-6} L
μm	Micrometer, 1×10^{-6} m

VITA

Dewey Edward Burton was born in Nashville, Tennessee, on January 14, 1959. His family moved to Memphis, Tennessee, in 1961, while his father attended dental school at The University of Tennessee Center for the Health Sciences. He graduated from Germantown High School in June 1977. The author enrolled at Rhodes College (formerly Southwestern at Memphis) in September 1977 and graduated with a Bachelor of Science degree in June 1981. While there, he majored in chemistry. The author was employed as a laboratory technician by the City of Memphis at the North Memphis Waste Water Treatment Plant from March 1979 through September 1980, where he gained an appreciation for the applications of analytical chemistry. In September 1981, he entered the graduate program at The University of Tennessee, Knoxville. He received the Doctor of Philosophy degree with a major in analytical chemistry in March 1987. After graduation the author will be employed by the Procter & Gamble Company in Cincinnati, Ohio.