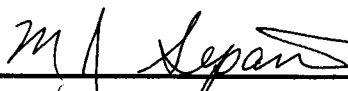


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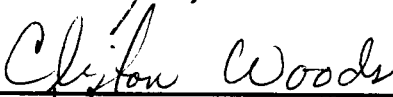
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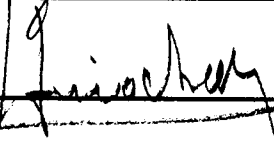


Dr. Michael J. Sepaniak, Major Professor

We have read this dissertation and
recommend its acceptance:







Accepted for the Council:



Associate Vice Chancellor
and Dean of The Graduate School

Optimization of Micellar Electrokinetic Capillary Chromatography with Solvent-Gradients

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

Alexander Craig Powell

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This dissertation is dedicated to my parents, Lt. Col. and Mrs. Fred J. Powell, Jr. (USAF, Ret.), whose love and support I have been so lucky to enjoy. I wish to also thank Dr. Michael J. Sepaniak, my research advisor, whose guidance and patience have been invaluable in my development of this work. I would also like to thank my many friends and coworkers at UTK, whose companionship has made my time here a memorable one.

Abstract

The development of instrumentation permitting the application of solvent-gradients to micellar electrokinetic capillary chromatography (MECC) is presented. A computational method is also described that is capable of predicting retention times of solutes separated in this manner. Theoretical predictions are based on capacity factor, electroosmotic flow rate, and micellar flow rate data obtained by the separation (under isocratic conditions) of a test mixture of fluorescently-labeled alkylamines and polyaromatic hydrocarbons using differing concentrations of organic modifier in the mobile phase. Separations are performed using continuous concave-, linear-, and convex-shaped gradients, as well as the demonstration of the possibility of generating more obscure gradient shapes. Resultant solute retention times are shown to correlate reasonably well with values obtained from the theoretical model. A computational method for optimization of solvent-gradient MECC separations is also presented, based on the modified simplex method. This computer program is then utilized to optimize the separation of a group of polyaromatic hydrocarbons (PAH's). The instrumentation and methodologies developed enhance the power of MECC as a separation technique by broadening its applicability to more complex systems.

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Chapter 1

Introduction

Although the process of electrophoresis can be traced as far back as 1809 when F.F. Reuss observed the migration of clay particles in an aqueous solution (1), its use as a technique for separating charged molecules (based on differing mobilities in an applied electrical field) was not described until the 1930's (2). Electrophoretic separations in capillaries was first demonstrated in 1967 by Hjerten (3). This method, labeled "free solution electrophoresis", employed a 3 mm i.d. (inner diameter) column. The "seminal" (4) or "landmark" (5) paper on the subject of capillary zone electrophoresis (CZE) is generally considered to be the work of Jorgenson and Lukacs (6), who demonstrated the high separation efficiency afforded by the use of small diameter capillaries ($< 100\ \mu\text{m}$ i.d.). A major source of band broadening in electrophoresis is due to Joule heating, a result of the ionic current generated when a large electrical field is applied. The high efficiency characteristic of CZE (compared to conventional electrophoresis) is attributed to the improved heat dissipation, per unit solution volume, that small diameter capillaries provide (i.e., the capillary wall serves as a heat sink for the solution inside; thus the smaller the capillary diameter, the larger the ratio of surface area to volume).

Along with the high efficiencies obtainable with CZE, the method has a number of other merits. The use of capillaries also results in very small injection volumes (on the order of nanoliters), making CZE an attractive separation method for cases where a limited amount of sample is available, such as biological microenvironments (i.e., single cells) (5). Likewise, the small volume flow rates associated with CZE mean lower consumption of potentially costly chemicals. The electroosmotic flow characteristic of CZE in fused silica capillaries (see discussion below) is usually powerful enough to

induce all solutes to elute at one end of the capillary, making detection easier. CZE is also marked by fast separations, with most taking only a few minutes.

However, since the primary separation mechanism of CZE is based on differences in the electrophoretic mobilities of ionic solutes, a major limitation of the technique is that it has little applicability for the separation of neutral solutes. To expand the scope of this method to include neutral solutes, Terabe and coworkers (7) introduced a surfactant, sodium dodecylsulfate (SDS), into the electrophoretic medium. This technique has been dubbed Micellar Electrokinetic Capillary Chromatography (MECC) (8).

Figure 1 illustrates the basic apparatus employed in MECC. It consists of a capillary, a high voltage power supply, two reservoirs containing mobile phase solution (comprising a buffer plus surfactant), and a detector. The typical capillary is composed of fused silica with an inner diameter of 25 - 75 μm .

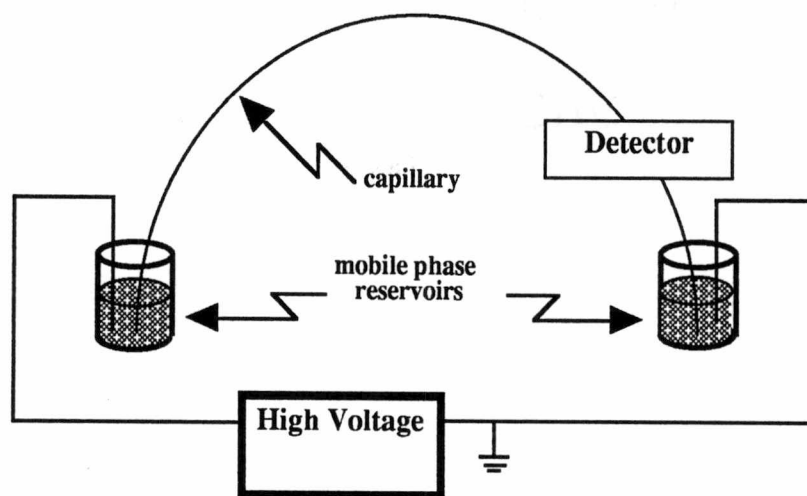


Figure 1. A generalized representation of the basic apparatus employed in MECC.

Surfactants are amphiphilic species, containing both hydrophobic and hydrophilic regions. They can be anions, cations, zwitterions, or even polar neutral molecules. In most cases the surfactant contains a polar and/or ionic head group attached to a nonpolar,

hydrocarbon tail. In the appropriate solvent, surfactants can aggregate to form colloidal-sized assemblies such as vesicles and micelles (9). Although there is some dispute as to the exact form of micelles (9), they are generally depicted as spherical aggregates. The makeup of the micelle and the micellization process is dependent on experimental conditions such as temperature, solvent type, solution additives, etc. Normal micelles (hydrophobic tails forming the interior of the micelle, polar head groups comprising the surface of the micelle) undergo rapid formation when the solution concentration of surfactant is greater than the critical micelle concentration (CMC); the process involves a dynamic equilibrium:



where N is referred to as the aggregation number. For surfactants used in MECC, CMC's range from 0.20 mM for Triton X-100 to 136.0 mM for Sodium Octylsulfate (9).

It should be noted that the actual numbers of surfactants making up individual micelles in solution constitute a Gaussian distribution; therefore, the aggregation number is defined as the average number of surfactant monomers per micelle present in a micellar solution (10). Most micelles have aggregation numbers in the 40 - 120 range (9). Solutions containing mixtures of surfactants can form "mixed" micelles (11). At concentrations very near the CMC, it is likely that "small" micelles are present. As the concentration of surfactant is increased appreciably beyond the CMC, N-mer micelle formation predominates, while the free monomer concentration remains at the CMC value. Surfactants with (or experimental conditions that produce) high CMCs are undesirable in MECC since the resulting large concentration of free, charged monomer results in an increased current and Joule heating that must be dissipated by the capillary.

Changes in experimental conditions can have dramatic effects on micellar properties. While pressure is generally not a factor, temperature changes, whether due to

ambient fluctuations or Joule heating, influence the CMC. For ionic surfactants, CMCs generally exhibit a minimum value in the 20 - 30 °C range (9). If high ionic strength buffers are employed, capillary temperatures in MECC can be considerably greater than this and furthermore, transverse temperature gradients can be created in the capillary, degrading efficiency. The addition of electrolytes to aqueous micelle systems usually results in an increase in aggregation number and a reduction in CMC (12). Organic solvents also affect micelle formation. Small alcohols enhance micelle formation at very low v/v %, but inhibit micelle formation at higher concentrations. Solvents that hydrogen bond with water such as acetonitrile also increase the CMC, even at moderate v/v % values (9). This organic solvent effect is an important consideration in MECC since these solvents are commonly used as organic modifiers to alter retention in reversed phase LC. Moreover, reductions in micelle concentration due to the addition of these solvents can result in serious reductions in efficiency (see discussion below).

Solutes can associate with normal micelles by a variety of mechanisms (9). Depending on the specifics of the solute/surfactant system, association can involve different regions of the micelle. Nonpolar to moderately polar solutes interact with the hydrophobic core of the micelle, based on dispersive and/or induced dipole interactions. Polar solutes can associate with the surface of micelles (i.e., the polar or charged surfactant head groups) via dipole-dipole interactions; amphiphilic solutes (organic compounds containing acid or base functionalities) tend to align themselves to interact dispersively with the hydrophobic core and electrostatically with the head groups of the micelles. Strong coulombic interactions (attractive or repulsive depending on solute charge) can be involved when charged solutes associate with charged head groups at the surface of the micelle.

Thus, the micelles constitute a secondary phase within the capillary's solution, allowing partitioning of solutes between the aqueous "mobile phase" (i.e., the buffer solution) and the surfactant-formed micellar phase.

Theory

As mentioned above, fused silica capillaries filled with a buffer solution experience "electroosmotic flow" when an electric field is applied (13). Electroosmotic flow is induced due to the "sorption" of ions onto the surface of the capillary wall (See Figure 2). Under normal aqueous conditions, this involves negatively charged functional groups on the surface of the capillary (such as silanol groups) and specifically adsorbed cations from the bulk solution. This layer is often referred to as the compact, Helmholtz, or Stern layer (14). Other ions are limited by how close they can approach the surface of the capillary by their solvent cage. This distribution of solvated ions from their closest approach to the surface to the bulk solution in the capillary comprises the diffuse layer. This discontinuity in charge distribution creates an electric double layer potential between the capillary wall and the bulk solution in the capillary.

When an electric field is applied, a *zeta potential* (ζ) is generated across the double layer due to the tendency of solvated ions to migrate within the field while adsorbed ions form a stagnant layer on the capillary surface. The magnitude of the zeta potential is therefore sensitive to changes in surface charge density, surface potential, and ionic strength of the medium (1). This potential difference has the net effect of causing the migration of buffer solution in the capillary or "electroosmotic flow". The electroosmotic flow rate, v_{eo} , is defined mathematically as

$$v_{eo} = \frac{-\epsilon \zeta_c E}{\eta} \quad (1)$$

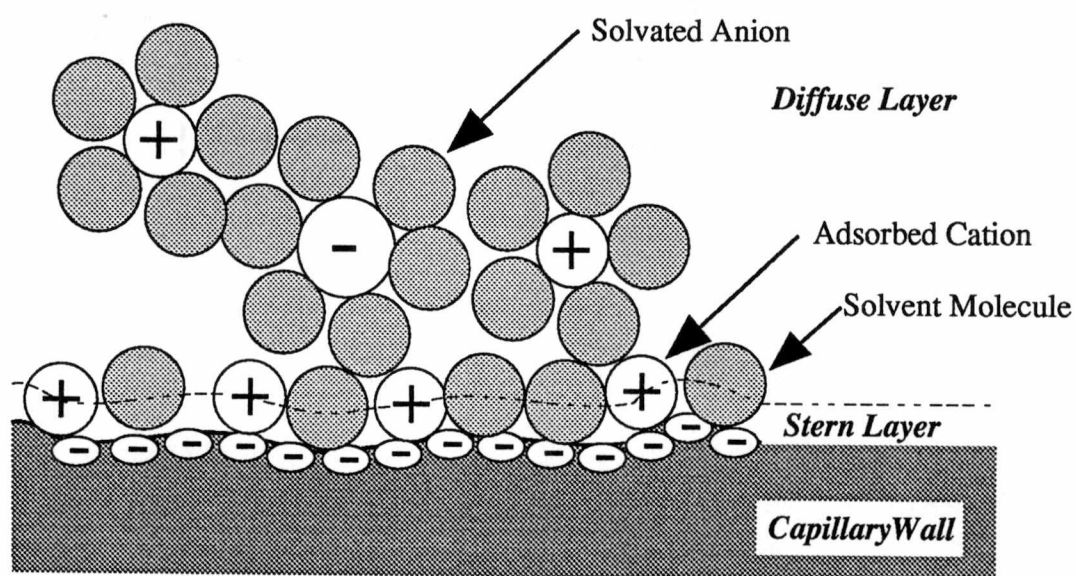


Figure 2. A schematic representation of ionic distribution at the capillary /solution interface.

where ϵ is the permittivity of the mobile phase, ζ_c is the zeta potential for the column wall, η is the viscosity of the mobile phase, and E is the electric field strength (15). Thus, the magnitude and direction of electroosmotic flow is governed by the sign and magnitude of the zeta potential. Under normal buffer conditions, electroosmotic flow is toward the cathode. As far as the effect of surfactant concentration on v_{eo} , Terabe et al. (15) demonstrated that changes in SDS concentration resulted in no significant deviation in v_{eo} .

As mentioned above, the micelles generally have a charged surface and thus possess an electrophoretic velocity, $v_{e,m}$, expressed mathematically as

$$v_{e,m} = \frac{2 \epsilon \zeta_m}{3 \eta} f(\kappa a) E \quad (2)$$

In this equation, ϵ , η , E are as above, ζ_m is the zeta potential for the micelles, and $f(\kappa a)$ is a factor that exhibits a functional dependence on the micelle shape and size (15). Note the difference in sign between the two expressions. The result is that micelles formed from anionic surfactants, such as SDS, possess an electrophoretic velocity that opposes electroosmotic flow (See Figure 3). The micelles are also affected by the electroosmotic flow and consequently possess a net velocity, v_M , which is a sum of the two vector quantities

$$v_M = v_{eo} + v_{e,m} \quad (3)$$

Under normal conditions $v_{eo} > -v_{e,m}$; consequently, the micellar phase moves toward the cathodic end of the capillary but at a slower rate than the surrounding aqueous solution. Thus, two distinct phases exist in the capillary, both moving toward the outlet of the capillary but at *different velocities*. Differential solute-micelle partitioning (see discussion above), provides a retention mechanism by which solutes can be separated. The band velocity, v_s , of a neutral solute will therefore have a value such that $v_M \leq v_s \leq v_{eo}$.

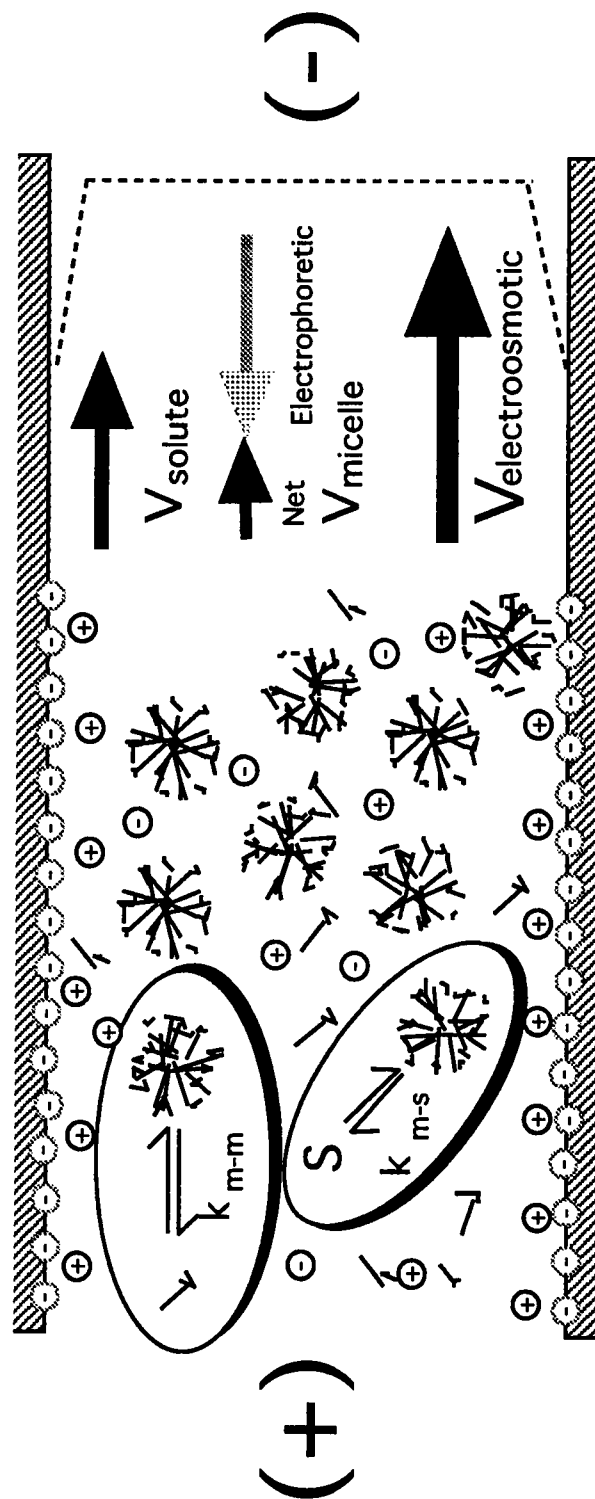


Figure 3. Depiction of flow dynamics in MECC showing velocity vectors for electroosmotic flow [$V_{\text{electroosmotic}}$ or v_{eo}], micellar velocity [both its electrophoretic ($v_{e,m}$) and net (v_M) vectors], and solute velocity [V_{solute}]. Also depicted are solute-micelle association and surfactant monomer-Nmer dynamics.

In contrast to conventional chromatography, the secondary phase (micelles) elutes from the column. Consequently, even solutes that totally associate with the secondary (i.e., micellar) phase are eluted. For this reason, the micellar phase is often referred to as a “pseudo-stationary” phase in MECC.

Chromatographic Factors

Capacity Factor and Resolution.

This “pseudo-stationary” phase nature of the micellar phase leads to a limited elution range for MECC. This elution range is often expressed as an elution range parameter, t_0/t_m , which is the ratio of the elution (or migration) time of a totally unretained component to the elution time of the micellar phase. For the most commonly used MECC system (SDS in phosphate/borate buffer) this ratio typically has a value between 0.2 and 0.5. For conventional liquid chromatography (LC), such a ratio would have a value of zero since t_m would be infinitely large. This limited elution range can result in poor peak capacity, relative to conventional LC, and, as discussed below, places constraints on optimum retention characteristics.

A limited elution range has a number of ramifications that alter some of the fundamental equations of conventional LC. For example, in conventional LC a solute's capacity factor (the ratio of the amount of solute present in the stationary phase to that in the mobile phase), k' , is expressed as (16):

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

where t_R is the solute's retention time. For MECC, the k' expression becomes (7):

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

Note that as t_m approaches infinity, the parenthetical expression approaches unity, and the expression becomes equivalent to the conventional LC equation.

The phase ratio ($V_{\text{micelle}}/V_{\text{mobile}}$) for a 0.05 M SDS solution is greater than 10^{-2} . This relatively large phase ratio, and the instability of micellar systems in mobile phases with large organic content (see discussion above), often results in prohibitively large capacity factors for nonpolar solutes (i.e., nonpolar solutes completely associate with the micelle and are not separated). The use of nontraditional surfactants (17) and unusual methods of imparting organic character to the mobile phase (18) have improved this situation somewhat.

The limited elution range of MECC also affects the expression for resolution, R_s (7):

$$R_s = \frac{N^{1/2}}{4} \left(\frac{\alpha - 1}{\alpha} \right) \left(\frac{k'_2}{1 + k'_2} \right) \left(\frac{1 - t_o/t_m}{1 + (t_o/t_m) k'_1} \right) \quad (6)$$

where N is the number of theoretical plates, k'_1 and k'_2 are the capacity factors for two solutes, and α is the selectivity factor (the ratio k'_2/k'_1). As above, if t_m has a value of infinity the resolution equation becomes the same as in the conventional case. For conventional chromatography, R_s increases with increasing k' ; however, with MECC the presence of the fourth factor in the R_s expression alters the relationship such that the product of the last two parenthetical factors, and thus R_s , decreases at large k' values and is optimum when k' is between one and five (15). The optimum value of k' is dependent on the breadth of the elution window. As the values of v_{eo} and $v_{e,m}$ approach the same magnitude (but opposite in sign), the elution window is extended and the optimum k' is extended (15, 19).

As mentioned previously, the limited elution range influences the peak capacity, n , of the technique (20):

$$n = 1 + \frac{N^{1/2}}{4} \ln \frac{t_n}{t_1} \quad (7)$$

where t_n is the retention time of the n th component ($t_n \leq t_m$ in MECC) and t_1 is the retention time of the earliest eluting component ($t_1 \geq t_0$ in MECC). If no effort is made to extend the elution range, the retention parameter (t_0/t_m) is approximately 0.3 (21). Assuming $N = 10^5$, this corresponds to a peak capacity of less than 100. While this is reasonably large, it assumes evenly distributed peaks. Moreover, it is much less than can be obtained for $N = 10^5$ using conventional techniques that do not exhibit a limited elution range.

Since R_s as a function of k' experiences a maximum in MECC, it follows that reduction of the late eluting components' large k' values would improve their resolution. As seen in Eq. 5, an increase in t_m (relative to t_R) would serve to decrease k' values. Thus by manipulating experimental parameters for the separation (as discussed below), the elution range can be extended and solute k' values reduced.

One method of reducing the zeta potential at the column wall/solution interface is to modify or change the wall surface, which in turn affects the surface charge density and surface potential. This can be accomplished in a number of ways. For example, reduction in the pH of the mobile phase (19, 22) can serve to "titrate" the silanol sites on the capillary surface, reducing ζ_c . In particular, Otsuka and Terabe (19) demonstrated that at solution pH's below approximately five, v_{eo} is reduced to a value less than $v_{e,m}$, causing the micellar phase to reverse its migration direction. A side effect of such a technique is that the acid/base functionalities of injected solutes can be altered, significantly changing their retention characteristics (17). An alternative method is to silanate the capillary's fused silica surface with a silanating agent such as trimethylsilane, TMS, (21) which reacts with the silanol sites on the capillary surface. Such a wall treatment produced a significant increase in t_m . Lux and coworkers (23) have also applied polymer coatings of

polymethylsiloxane, PMS, and polyethylene glycol, PEG, to the capillary wall, demonstrating the ability to either increase (with PMS) or decrease (with PEG) electroosmotic flow rates. Jorgenson and coworkers demonstrated that Teflon capillaries exhibit smaller ζ_c for CZE separations, at the expense of band dispersion, due to the coupling of a detection flow cell, and thermal problems, due to the poor heat dissipation of Teflon.

A solute's k' can also be considered as the product of the thermodynamic partition coefficient, K , and the phase ratio, β .

$$k' = \beta K \quad (8)$$

Therefore, in reversed phase LC, the introduction of an organic solvent to the mobile phase produces readily calculable effects on solute k' values based on the change in K . Because of the nature of the phases involved, MECC mimics reversed phase LC in retention characteristics; however, the situation is complicated by the fact that the presence of organic solvent affects β as well as K . This is because organic solvents affect the CMC and thus the micellar volume. Moreover, organic additives affect the viscosity and dielectric constants of the mobile phase and ζ_c , the net result being a reduction in the electroosmotic and electrophoretic flow rates (24).

Initial experiments with mixed organic-aqueous mobile phases have involved adding 2-propanol, methanol, or acetonitrile to the mobile phase (11, 21, 25-28). Particularly noteworthy is Bushey and Jorgenson's successful separation of dansylated methylamine and dansylated methyl- d^3 -amine using 20% methanol (27). These bulk additions of organic modifier to the mobile phase have produced significant reductions in k' for late eluting components, but at the expense of extremely long analysis times (i.e., extremely large elution windows were observed). This time factor was particularly prob-

lematic with alcohol organic modifiers, which strongly interact with the capillary wall and dramatically alter v_{eo} .

Selectivity

Organic modifiers have also exhibited the ability to affect selectivity. Gorse *et al.* (26) demonstrated that methanol and acetonitrile can favorably alter separation selectivity between the solutes toluene and nitrobenzene and between pyridine and phenol; in fact, the two solvents were shown to have differing influences on selectivity depending on solute polarity. Liu and coworkers have also shown beneficial changes in selectivity by using methanol and tetrahydrofuran (29). Moreover, Sepaniak and coworkers demonstrated a reversal in elution order for substituted phenolic compounds using an acetonitrile step gradient (28). Other mobile phase additives, such as tetraalkylammonium salts (30) and divalent metal cations of copper, zinc, and magnesium (31) also influence selectivity.

Selectivity can also be beneficially altered by employing a micellar system that enhances differences in micelle-solute association. This process of altering the pseudo-stationary phase is analogous to changing the stationary phase in HPLC. The process is much simpler and easier for MECC, involving only a rinsing of the capillary and then the pumping of the new micellar solution into the capillary. Surfactants such as sodium decyl sulfate (STS), dodecyltrimethylammonium chloride (DTAC), and cetyltrimethylammonium chloride (CTAC) have also been tested as pseudo-stationary phases (32). In particular, CTAC, a cationic surfactant, displayed differing selectivity from SDS, an anionic surfactant. Terabe *et al.* have also employed an "inclusion complex" mechanism, using an ionized form of β -cyclodextrin, to separate a number of organic isomers (18). Mixed micellar solutions, containing SDS and sodium octyl sulfate (SOS), have also demonstrated greater selectivity in the separation of catechols (33).

Furthermore, certain bile salts, a class of biological surfactants, have been used in MECC, demonstrating not only a general reduction in k' values due to their relatively polar nature but the possibility of chiral separations (17, 34-36). Dobashi and coworkers have likewise applied an enantioselective surfactant, N-dodecanoyl-L-valinate, to the separation of amino acid enantiomers, both as part of a mixed micellar system (with SDS (37)) and by itself (38). In combination, the successes of these various MECC systems indicate the versatility of the technique.

Efficiency.

As mentioned above, a hallmark of MECC is the high efficiency observed for separations. Compared to the typical theoretical plates of 5,000 to 25,000 exhibited by HPLC, the efficiency of 50,000 to 500,000 routinely reported for MECC is phenomenal (5). This increased efficiency is directly related to the relatively flat profile characteristic of electroosmotic flow, compared to the parabolic flow profile of conventional LC (39). This "plug-like" flow profile is expected when the capillary i.d. is greater than seven times the thickness of the double-layer, a condition easily met given that the double-layer thickness is estimated to range between 3 - 300 nm for an aqueous, binary electrolyte solution over the concentration range of 10^{-2} to 10^{-6} M (5). Comparable high efficiencies in LC require the use of extremely narrow-bore capillaries (5 - 10 μm i.d.), wherein the distance across the flow profile is much smaller, relative to the diffusion paths of the solutes, allowing an "averaging out effect" for the solutes since they quickly experience all the different flow rates (40). This use of such small diameter capillaries (called Open Capillary Liquid Chromatography or OCLC) puts rigid limitations on detection, injection, and column fabrication (8, 41). Thus MECC benefits, relative to OCLC, in that capillaries on the order of 25 to 100 μm i.d. may be used, resulting in more lax instrumental restrictions.

Ideally, the primary contributor to band broadening in CZE is axial (or longitudinal) diffusion (41). For MECC, the situation is more complicated, due to the partitioning of solutes between the micellar phase and mobile phase. In addition to axial diffusion (H_a), other contributions to plate height in MECC include micelle polydispersity (H_p), resistance to mass transfer (H_{mt}), and thermal gradients (H_t). The theoretical plate height (H_T) can be considered to be the sum of these:

$$H_T = H_a + H_p + H_{mt} + H_t \quad (9)$$

Axial diffusion can significantly contribute to H_T in MECC. As in CZE, the degree of contribution is directly related to the diffusion coefficient of the solute in the mobile phase. Furthermore, the presence of the micellar phase results in H_a having a dependency on the effective diffusion coefficient in the micellar phase. This effect is usually negligible because solutes with large k' values, while spending a lot of time in the slow-diffusing micellar phase, also have a longer residence time in the column, resulting in more opportunity for axial diffusion. As in conventional chromatography, the contribution of H_a to H_T is reduced for solutes with small diffusion coefficients and fast flow rates (i.e., high operating voltages).

Micelle polydispersity is a result of the dynamic equilibrium involved in micelle formation. The consequence of this constant exchange of monomers between the micelles and bulk solution is that the sizes of individual micelles are in flux. The coefficient of variation (in monomeric units per aggregate) for SDS micelles is reported to be 20 % (10), suggesting a substantial degree of polydispersity. Surfactants exhibiting a high degree of micelle polydispersity are undesirable as pseudo-stationary phases because the micelle's electrophoretic mobility is dependent on the size and shape of the micelle (See Eq. 2). Logically, a wide range of micelle sizes could result in a range of micellar velocities; solutes interacting with these micelles would then experience band broaden-

ing. This effect can be reduced by increasing the monomer-micelle exchange rate, thereby "averaging-out" the differences in micelle sizes. The exchange rate, and thus H_p , is usually controlled by two experimental parameters: micellar concentration and operating temperature. Increasing the formal concentration of surfactant (and thus the micellar concentration) increases the number of micelles and decreases the distance between micelles allowing for faster exchange rates. Likewise, increasing the temperature of the system increases the exchange rate and improves efficiency (11). Caution must be observed in that significant increases in temperature can increase the H_t term (See below).

Mass transfer effects for MECC can be due to resistance in the pseudo-stationary or the mobile phase. The resistance to mass transfer in the pseudo-stationary phase is analogous to the conventional LC stationary phase term. This term is usually negligible due to the small cross sections of most micelles (approximately 40 Å for SDS) and the rapid exchange kinetics of nonpolar solutes with the interior of the micelles (a result of the weak dispersive interactions involved). Polar or charged solutes which can associate with the micelle by stronger electrostatic interactions can exhibit slower solute-micelle kinetics, resulting in band broadening (8).

Resistance to mass transfer in the mobile phase has two contributing factors: intermicelle resistance and intracolumn resistance. The significance of intermicelle resistance to mass transfer is the result of slow diffusion between micelles. For MECC this term is reduced via the very small distance between micelles (12), their uniform shape, and their even dispersal within the column (due to coulombic repulsion between micelles). It is also for these reasons that the effect of eddy diffusion is neglected. Intracolumn resistance to mass transfer, important in OCLC, is a consequence of the parabolic flow profile: solute molecules close to the wall move more slowly than those at

the center of the capillary. The flat flow profile customary for MECC reduces this term. However, at high applied voltages it has been observed (12) that plate height has a dependency on capillary diameter (as in OCLC).

Thermal effects in MECC are directly related to Joule heating. At high operating voltages radial thermal gradients are generated, with the solution at the center of the capillary experiencing a higher temperature than that close to the walls, a consequence of the capillary walls serving as the heat sink for the system (42). The presence of a radial thermal gradient has a number of ramifications: (a) electrophoretic mobility is temperature dependent, thus charged species at the center of the capillary would move faster than those at the walls (a parabolic flow profile, see discussion above); (b) since CMC increases with temperature, micellar concentration would be lower in the center of the capillary, resulting a phase ratio gradient; and (c) increased solution temperatures change micelle-monomer kinetics as well as solute diffusion rates.

In essence, MECC is a highly efficient method, but, as with any technique, there are certain trade-offs in experimental parameters. Surfactant concentrations should be high enough to minimize micelle polydispersity effects while low enough to avoid high currents and the resultant Joule heating. Additionally, column diameter should be low enough to minimize intracolumn resistance to mass transfer and improve heat dissipation while large enough to allow convenient detection, injection, and capillary production. Likewise an operating voltage must be employed that is high enough to minimize axial diffusion while not significantly affecting the other terms.

Summary / Statement of Problem

MECC, although a relatively new separation methodology, has successfully been applied in numerous analyses. As seen in Table 1, separations employing MECC have been used in the analysis of compounds of environmental interest, pharmaceuticals, and

nucleic acids, as well as neurotransmitters and amino acids. This diverse array of analytes suggests, along with the advances in selectivity and detectability, the utility and further potential of MECC.

Certainly, a major hallmark of electrophoretic/electrokinetic techniques is the high efficiency that has been demonstrated. This high efficiency, coupled with the chromatographic partitioning afforded by the micellar pseudo-stationary phase, results in a separation technique that can separate both charged and neutral compounds. The low injection volume (on the order of nanoliters) also contributes to its applicability to biological separations that necessitate the use of small sample volumes. In fact, CZE has been applied to the cytoplasmic analysis for a single nerve cell (57).

While the examples of separations in Table 1 illustrate the potential of MECC to extend the utility of electrokinetic separation techniques beyond those possible with conventional CZE, MECC is currently suitable only for compounds of at least a moderately hydrophilic nature. In addition, the limited elution range of MECC renders the technique inappropriate for extremely complex samples. A number of methods have been employed to address these problems. One of the more successful has been the addition of organic solvents to the mobile phase, with the results being an enhancement in mobile phase solubility of moderately hydrophobic compounds, the possibilities of unique separation selectivities, and an extension of elution range. A further refinement of this type of mobile phase modification is the use of solvent-gradients to effectively "target" specific groups of solutes which are difficult to separate. The application of these solvent-gradients in a random fashion, although potentially useful, is somewhat limited in scope. Therefore, a means of optimizing MECC separations is needed that would be able to ascertain the best solvent-gradient for a specific separation problem.

Table 1. Separations of interest using MECC.

Analyte	Surfactant(s)	Detection	Remarks	Reference(s)
<i><u>Environmental Interest</u></i>				
Aflatoxins	SDS ¹	laser-based fluorescence [Ar ⁺ (488 nm)]	10 % acetonitrile in the mobile phase improves separation	(28)
Phenolic Compounds	SDS	UV-absorbance (270 nm)		(7)
"	SDS	UV-absorbance (220 nm)		(43)
"	SDS	UV-absorbance (254 nm)	Step-gradient from 0% to 15% acetonitrile to help resolution	(28)
<i><u>Biological Interest</u></i>				
B-Vitamins	SDS	laser-based fluorescence		(44)
"	SDS	UV-absorbance (254 nm)		(45)
"	SDS	laser-based fluorescence [Ar ⁺ (488nm) and HeCd (325 nm)]		(46)
"	SDS	UV-absorbance (210 nm)	Tetraalkylammonium salts used to improve separation.	(30)
"	SDS	UV-absorbance (210 nm)	pH effect on separation	(47)
Antipyretic analgesic	SDS	UV-absorbance (214 nm)		(48)
Antibiotics	SDS	UV-absorbance (210 nm)	Cephalosporin antibiotics; Tetraalkylammonium salts used to improve separation.	(30)

Table 1. (continued)

Analyte	Surfactant(s)	Detection	Remarks	Reference(s)
Antibiotics	SDS, LMT ²	UV-absorbance (210 nm with SDS, 220 nm with LMT)	Penicillin and cephalosporin antibiotics	(49)
Chiral drugs	Bile salts	UV-absorbance (210 nm or 220 nm)	Sodium cholate and derivatives used for chiral separation of diltiazem, trimetoquinol, and carboline isomeric drugs.	(35)
Cold medicine	Bile salts	UV-absorbance (210 nm or 220 nm)	Sodium cholate and derivatives used for chiral separation.	(36)
Amino acids	SDS, DTAB ³	UV-absorbance (260 nm)	Phenylthiohydantoin-amino acid derivatives separated.	(50)
"	SDS	UV-absorbance (254 nm)	Dinitrophenylhydrazine-amino acids separated.	(46)
"	Bile Salts	UV-absorbance (210 nm or 220 nm)	Sodium cholate and derivatives used for chiral separation of racemic dansylated-amino acids.	(51)
"	SDS and SDVal ⁴	UV-absorbance (254 nm)	A mixed micellar phase of SDS and SDVal was used to separate the N-(3,5-dinitrobenzoyl) O-isopropyl ester derivatives of enantiomeric amino acid pairs.	(37)
"	SDVal	UV-absorbance (230 nm or 260 nm)	N-(3,5-dinitrobenzoyl) O-isopropyl ester derivatives of enantiomeric amino acid pairs were separated.	(38)
"	SDS	Fluorescence [Excitation: 365 nm, Detection: 418 cutoff filter]	Mixture of o-Phthalaldehyde derivatized amino acids separated in the presence of 15% methanol, 1% tetrahydrofuran.	(29)
Catechols	SDS	Off-column amperometric		(52), (53)
"	SDS and SOS ⁵	Off-column amperometric	A mixed micellar solution of SDS and SOS demonstrated improved selectivity and efficiency.	(33)

Table 1. (continued)

Analyte	Surfactant(s)	Detection	Remarks	Reference(s)
Bases, Nucleosides, Oligonucleotides	SDS	UV-absorbance (260 nm)	Addition of divalent metals (Cu (II), Zn (II), Mg (II)) improved separation of oligonucleotides.	(31)
Nucleosides, Nucleotides	SDS	UV-absorbance (256 nm)		(54)
"		UV-absorbance (? nm)		(55)
Nucleosides	SDS, DTAB, CTAB ⁶	UV-absorbance (254 nm)	An anionic surfactant (SDS) and two cationic surfactants (DTAB and CTAB) were each used to separate phosphorylated nucleoside mixture.	(56)

¹ sodium dodecylsulfate; ² sodium N-lauroyl-N-methyltaurate; ³ dodecyltrimethyl-ammonium bromide;

⁴ sodium N-decanoyl-L-valinate; ⁵ Sodium octyl sulfate; ⁶ hexadecyltrimethyl-ammonium bromide

The purpose of the work presented here is to expand the applicability of MECC by addressing these limitations mentioned above. This was accomplished first by the development of instrumentation for the delivery of solvent gradients to the MECC column. A computational model was subsequently developed to predict retention times of solutes separated using Solvent-Gradient MECC. This model was then coupled with an algorithm to search for the "best" separation, resulting in a computer program capable of suggesting a solvent-gradient that would provide an optimal separation.

Chapter 2

Instrumentation and Experimental Methods

Reagents

The alkylamines employed in separations were obtained from Alltech Assoc. The polynuclear aromatic hydrocarbons, the fluorescent label, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl), and the surfactant, sodium dodecylsulfate (99% pure) were acquired from Sigma Chemical Co. HPLC-grade acetonitrile (CH₃CN), the organic solvent used in the gradients, was secured from Mallinckrodt. The aflatoxins B₁, G₁, and G₂, and polyaromatic hydrocarbons (PAH's) were purchased from Aldrich Chemicals. The additional mobile phase components, dibasic sodium phosphate and sodium borate, were obtained from Fisher Scientific.

Stock solutions of the derivatized alkylamines were prepared by combining an excess of NBD-Cl with a 10⁻³ M solution of the amines in ethanol (absolute) (58). After five minutes, the reaction mixture was diluted by a factor of 100 with mobile phase and then refrigerated in aluminum foil-covered containers until needed. The analysis mixture was prepared by pipeting 100 µl of each stock solution into a 10 ml volumetric flask and diluting to the mark with mobile phase.

Detection

On-column, laser-based fluorescent detection of the NBD-labeled alkylamines was performed using a Liconix Model 4230 NB He-Cd laser (325nm, 7 mW or 442 nm, 30 mW) for excitation. Fluorescence emission was isolated at 450 nm (for the aflatoxins) or 525 nm (for the derivatized amines) using an appropriate bandpass interference filter in combination with an Instruments SA Model H-10 monochromator (bandpass approxi-

mately 10 nm), processed with an RCA Model 1P28 photomultiplier tube and a Pacific Precision Instruments Model 8376-20 photometer, and displayed with a strip-chart recorder. On-column UV absorbance (254 and 210 nm) of the PAH's was performed with the Linear Model 204 absorbance detector, a commercial instrument.

Experimental Apparatuses and Procedure

General.

Fused-silica capillary (70 -100 cm in length, 50 μm i.d.) was supplied by Polymicro Technologies and used without chemical modification. Electrokinetic separations were performed by applying 18 - 24 kV potential across the capillary with a Hipotronics Model 840A high-voltage power supply. For gradient work, the power supply was operated in the reversed (or negative) polarity mode, such that the outlet of the column was at -24 kV and the inlet of the column was at ground potential. Such a configuration was necessitated by the problem of the high voltage grounding through the pumping solution and the pumps when the inlet reservoir was the "hot" end. Likewise, the outlet reservoir was electrically isolated by securing it, along with the detector, to a 1.5 in. thick slab of plexiglass tapped with 1/4"-20 holes (the same as on most optical benches).

First Generation Gradient Mixer.

The continuous solvent-gradients were produced by using an in-house built conical mixing chamber (with a micro stirring bar) as the inlet reservoir and two stepping motor syringe pumps to deliver solution and withdraw solution from the reservoir (See Figures 4 and 5). The solvent-gradient experienced at the inlet of the capillary can then be expressed by the exponential gradient equation (59):

$$\%_{\text{res}} = \left[1 - \left(\frac{V^0}{V^0 + \Delta R \cdot t} \right)^{\frac{R_{\text{in}}}{\Delta R}} \right] (\%_{\text{in}}) \quad (10)$$

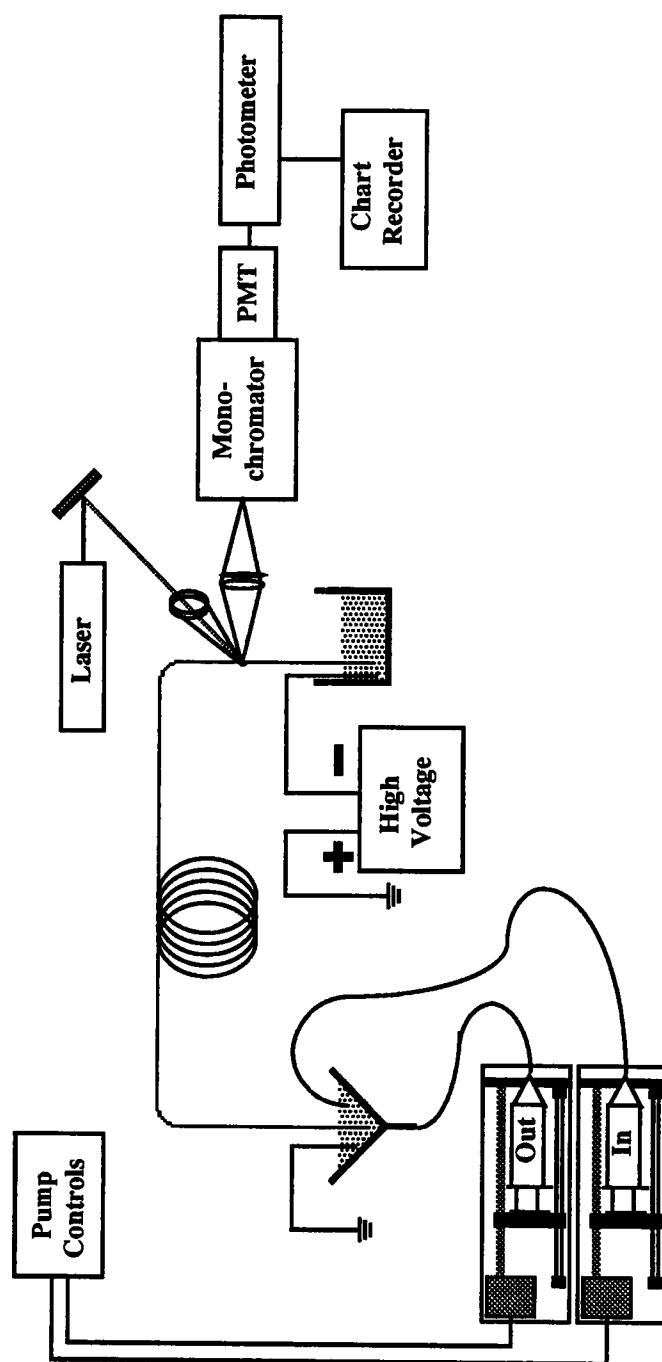


Figure 4. A schematic representation of the first generation gradient mixer.

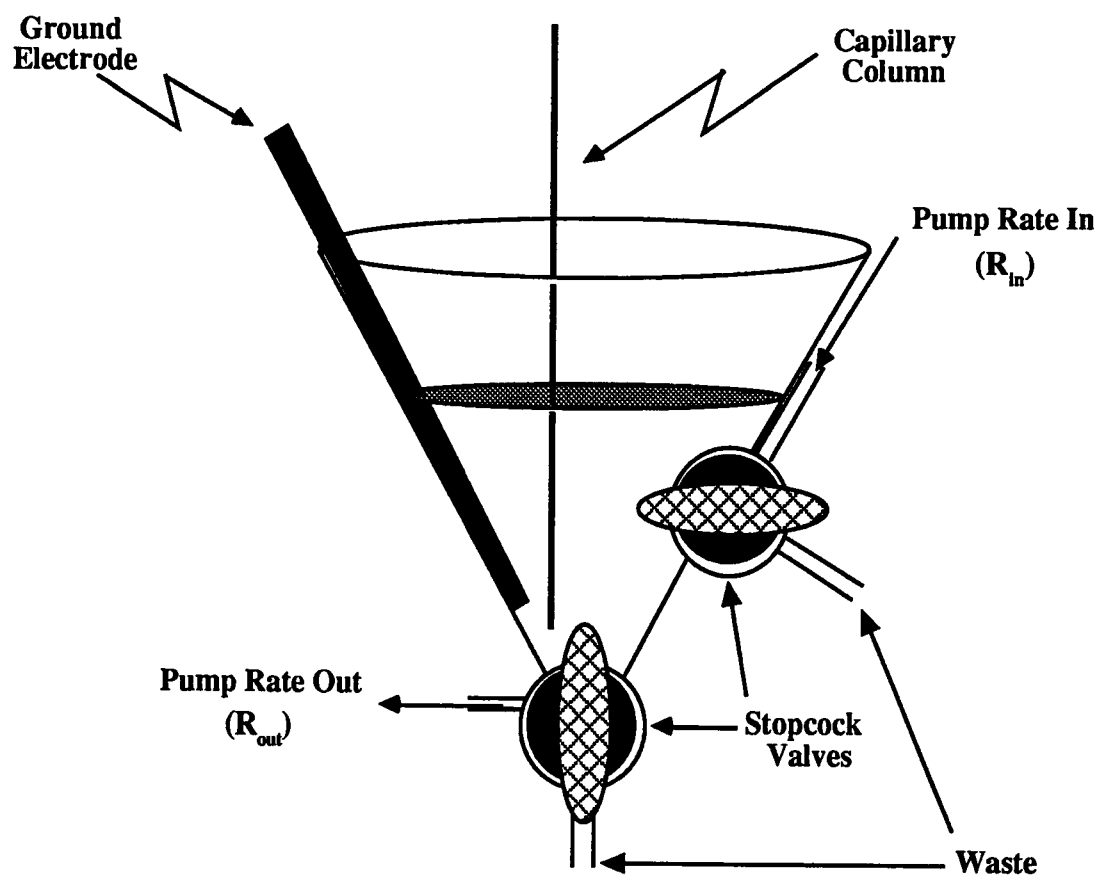


Figure 5. A closeup view of the conical mixing chamber used in the first generation gradient mixer.

where $\%_{\text{res}}$ is the % organic modifier in the reservoir at time t , V^0 is the initial volume of mobile phase in the inlet reservoir, $\%_{\text{in}}$ is the % organic modifier in the solution being pumped into the reservoir, and ΔR is the difference between the pump rate of solution into the reservoir (R_{in}) and the pump rate of mixed solution out of the reservoir (R_{out}). This equation assumes the initial mobile phase does not contain organic solvent. A linear gradient is generated when $R_{\text{out}} = 2R_{\text{in}}$, the slope of which is determined by ΔR and V^0 . Concave and convex gradients are formed when R_{out} is greater than and less than $2 R_{\text{in}}$, respectively. The slope, duration, and final $\%_{\text{res}}$ for the gradient are limited by factors such as the volume capacity of the inlet reservoir, the available range of pump rates, and the solubility limit of the buffer in mixed organic-mobile phase solutions. By manipulation of the four parameters (V^0 , $\%_{\text{in}}$, R_{in} , and R_{out}), an infinite number of gradient profiles can be generated. Figure 6 illustrates the gradients employed in this work.

The isocratic separations [0%, 5%, 10%, 15%, 17.5%, and 20% CH_3CN (v/v) in the mobile phase] were performed by rinsing the column for two minutes with the next step in CH_3CN concentration (using a pressurized syringe), electrokinetic pumping for two minutes, and then hydrostatically injecting (26) the sample. The solvent-gradients were electrokinetically delivered to the column via electroosmotic flow, a result of the applied potential across the capillary. The inlet of the capillary was placed at the bottom of the conical reservoir, as close to the micro stirring bar as possible, to insure a fully-mixed solution was electrokinetically drawn into the column. Between gradients, the column was rinsed with a buffer solution (10^{-2} M disodium phosphate, 6×10^{-3} M sodium borate). Intermittently, a normal (no organic solvent in the mobile phase) separation was performed to guarantee that the column behaved uniformly.

To provide a reasonable estimate for the relationship between the solvent gradient produced at the inlet reservoir and v_{∞} , an experiment was devised based on splitting the

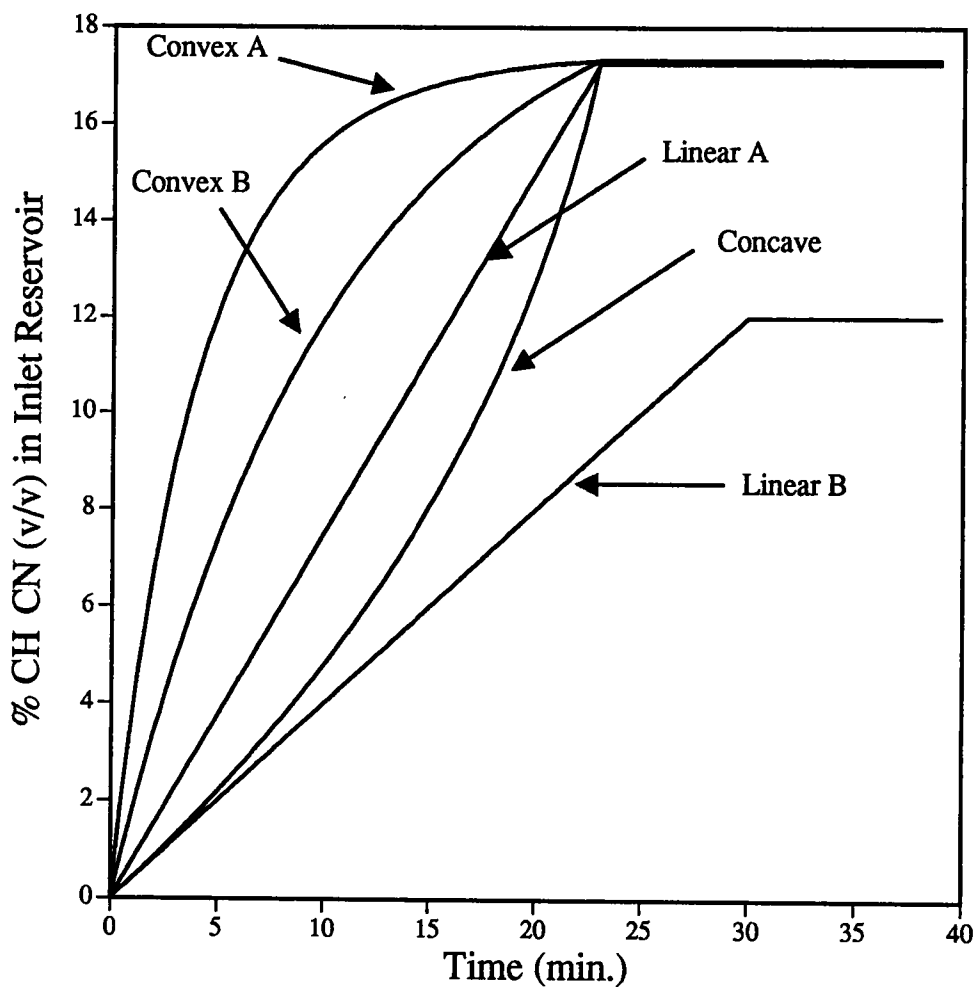


Figure 6. Profiles of acetonitrile concentration gradients (v/v%) generated in the inlet reservoir with the first generation gradient mixer.

laser output into two parallel beams (separated by ≈ 1.5 cm) which were individually focused onto the capillary column, near its outlet. This spatial separation produced two peaks whenever a fluorescently-labeled solute eluted from the column. By performing multiple injections of NBD-propylamine during the course of the gradient (with no surfactant present in the mobile phase solution), the temporal displacement of the two peaks as each band eluted provided a measure of v_{∞} at that particular moment in time (see Chapter 3 for further discussion). Please refer to Figure 7 for an illustration of the instrumental arrangement.

The theoretical calculations were performed on a Macintosh SE/30 personal computer (Apple Computer Inc.). Least-squares fit for experimental data was generated using Deltagraph 1.5 (DeltaPoint, Inc.). The spreadsheet program Excel 2.2 (Microsoft Corporation) was used for all computational work.

Second Generation Gradient Mixer.

The second generation gradient mixer was based on the same two worm-drive pumps used in the first generation device. The new design involved a much smaller mixing volume (≈ 45 μ L) and a tangential flow design to induce mixing of the two solutions. Figures 8 and 9 offer a schematic representation and an expanded view of the apparatus. This new design did not produce continuous gradients but rather a number of gradient steps (each one 30 seconds in duration). An IBM PC, running a BASIC program with LabSoft Drivers, was used to control an ISAAC 91-I (Cyborg) interface, which in turn controlled the syringe pumps by transmitting measured pulses to the pumps' stepping motors. By varying the pulse rates to the two pumps, different mixing ratios could be delivered to the capillary. The total flow rate (i.e., the sum of flow from the two pumps) was set such that at least 60 μ l of solution passed through the mixing chamber during each step, guaranteeing a complete flushing during each step. The actual mixing

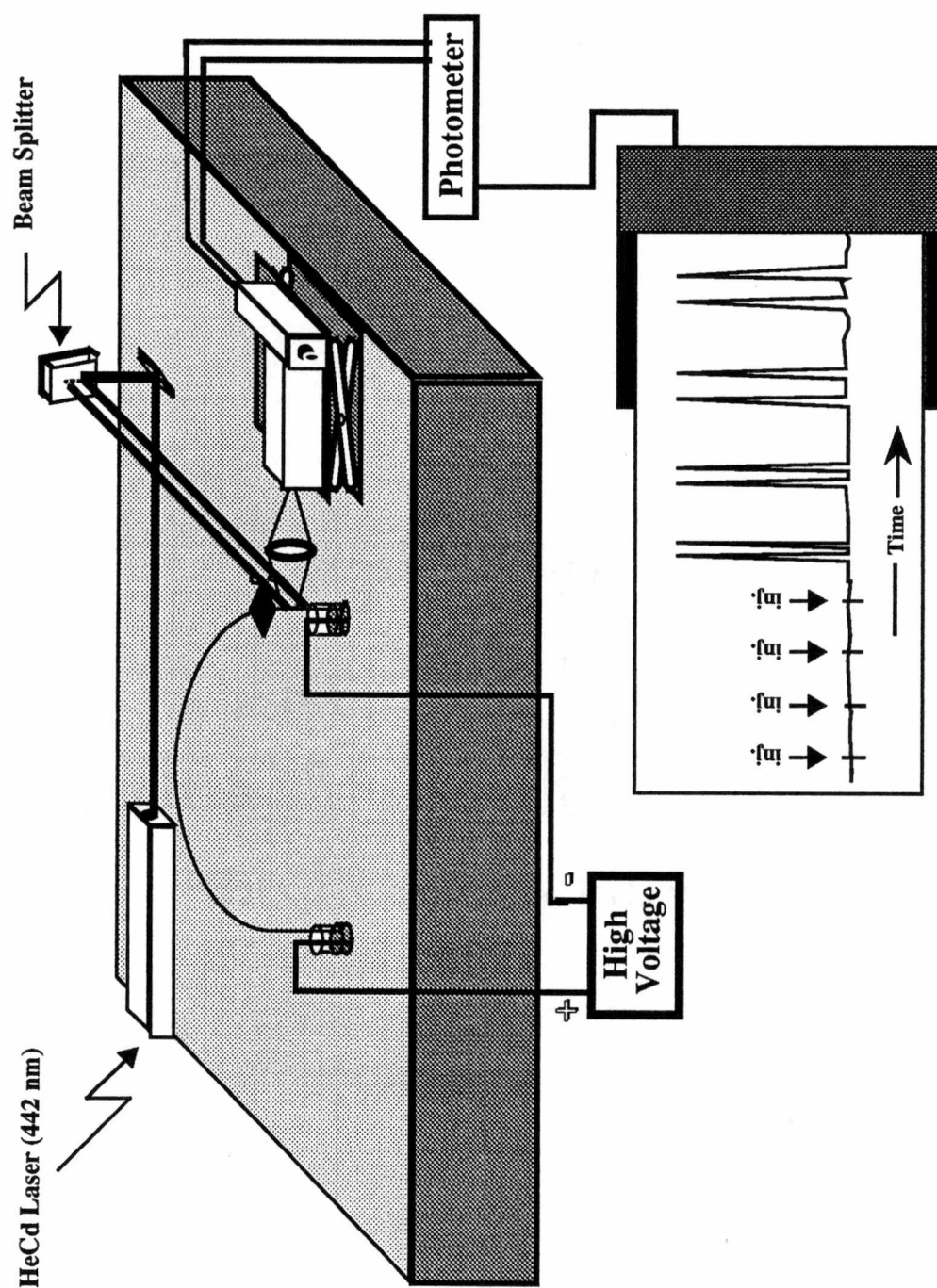


Figure 7. Illustration of the instrumental arrangement for the double-beam experiment used to measure V_{EO} .

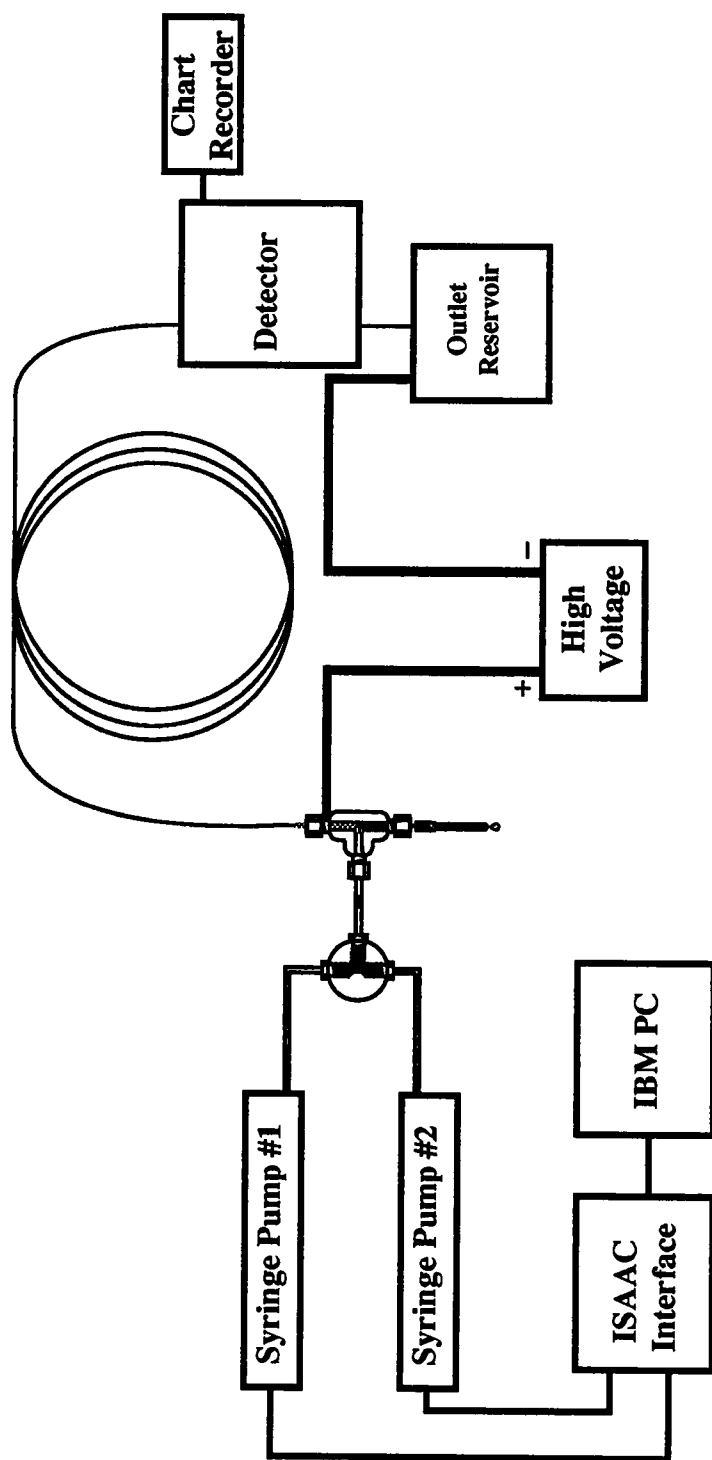


Figure 8. A schematic representation of the second generation gradient mixer.

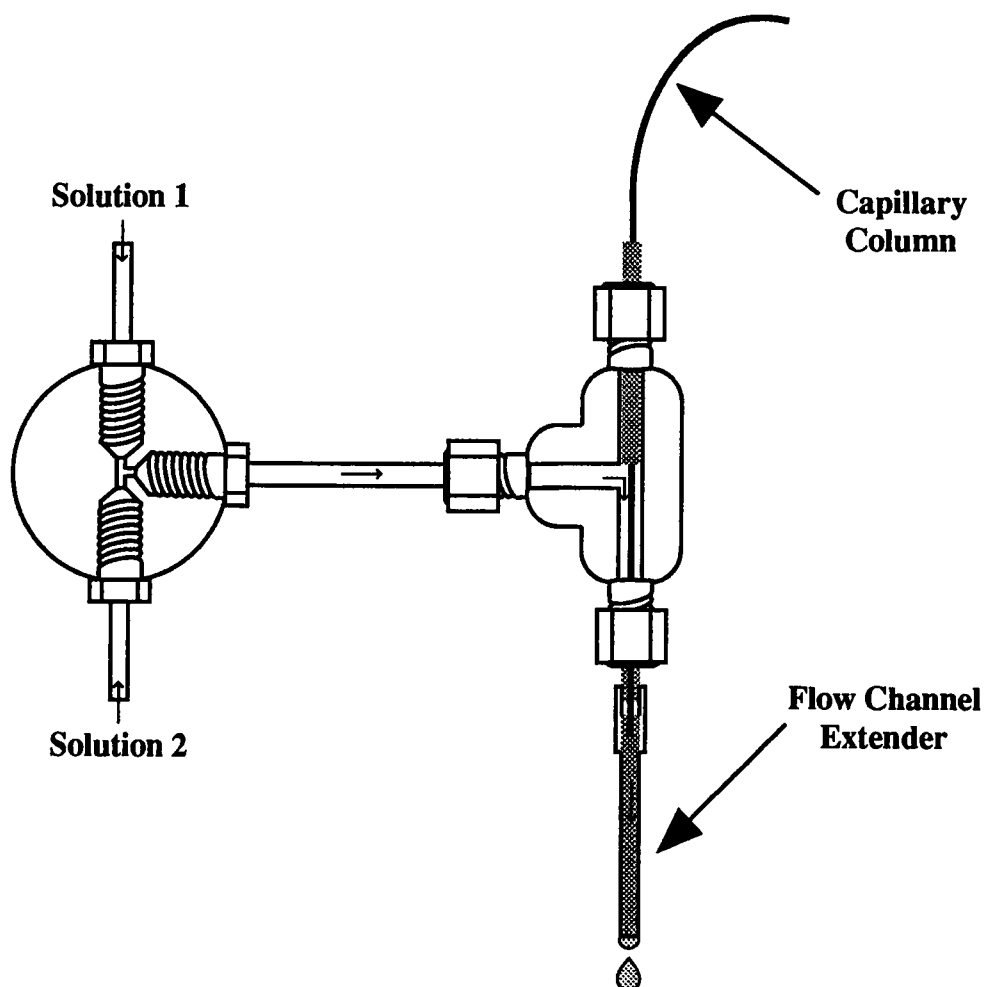


Figure 9. A closeup view of the tangential mixing chamber used in the second generation gradient mixer.

chamber was constructed by joining two LC fittings: a low dead volume tee to a 16 gauge-bore mixing tee (Swagelock). The capillary extended beyond the tee and was sheathed inside a length of tubing (the "flow channel extender" in Figure 9). This extension was used for the dual purpose of promoting mixing of the two solutions and to allow easy hydrodynamic injection of solution onto the column by just removing the extender (see below).

Initially, the BASIC program calculated the gradient shape based on a fifth order polynomial (%Solution 2 as a function of time) input by the user. Later, the program was modified to calculate gradient shape based on the exponential gradient equation:

$$\%_{\text{res}} = \left(\frac{t}{t_G}\right)^{\kappa} \%_G \quad (11)$$

where $\%_{\text{res}}$ and t are as above in eq. (10), t_G is the total time of the gradient run, κ is a factor related to the curvature of the gradient, $\%_G$ is the final concentration of the mobile phase modifier (60). When κ is less than one, a convex gradient results; when κ is greater than one, a concave gradient results. A linear gradient is produced when κ equals one. This alteration was made to more easily integrate the program with the simplex optimization employed, by way of limiting the number of variables involved in the calculations.

The injection procedure with the second generation gradient mixer was more complex in that the entire system was attached to the capillary. The procedure was as follows:

1. The column was rinsed for 3 min. with 0.01 M NaOH.
2. The column was rinsed for 3 min. with mobile phase solution.
3. The flow channel extender was attached to the mixer and the assembly lowered to a position level with the outlet of the column.

4. The two pumps were each turned on for 20 s to flush the mixing chamber.
5. The gradient mixer assembly was raised 21 cm, the flow channel extender removed, and the exposed capillary tip placed in the barrel of a 1 cc syringe containing sample solution. The capillary tip was then rinsed with deionized water and placed in a microcentrifuge tube containing mobile phase.
6. The assembly was then lowered and Pump #1, containing pure mobile phase, was allowed to run for 15 s to guarantee the mixing chamber contained only pure mobile.
7. The high voltage was then turned on.

The isocratic separations were performed in the same manner except after Step 2 above, the capillary was filled with the appropriate solution by electrokinetic pumping (18 kV) for 15 - 60 min., depending on the %CH₃CN. General information on hydrodynamic injection can be found in ref. 46.

Once again, theoretical calculations were performed on a Macintosh SE/30 personal computer (Apple Computer Inc.). Least-squares fit for experimental data was generated using Deltagraph 1.5 (DeltaPoint, Inc.). Microsoft QuickBASIC was used for all computational work.

Chapter 3

The Use of Organic Solvents to Modify MECC Mobile Phases and the First Generation Gradient Mixer

Mobile Phase Modification in MECC.

While the examples of separations in Table 1 illustrate the utility of MECC to extend the applications of electrokinetic separation techniques beyond those possible with conventional CZE, the full potential of MECC in biological and environmental analysis has not been realized because (a) the technique has a limited elution range which limits resolution and peak capacity and (b) hydrophobic compounds all partition completely into the micelles (i.e., capacity factors are very large) and are not resolved. As mentioned in Chapter 1, a number of methods have been employed to address this problem. One of the more successful has been the addition of organic solvents to the mobile phase, with the results being an enhancement in mobile phase solubility of moderately hydrophobic compounds (thereby improving their resolution), the possibilities of unique separation selectivities, and an extension of elution range.

Figure 10 illustrates the improved resolving power of an organically modified mobile phase for the separation of aflatoxins. As seen from the figure, the aflatoxins have very similar structures and are fairly hydrophobic. Note that the second and third aflatoxins to elute, G_1 and B_2 , respectively, are not baseline resolved (Figure 9.A) when the mobile phase is purely aqueous. Markers for t_0 and t_m were not included in this aflatoxin sample so capacity factors and the elution range parameter (t_0/t_m) can not be calculated. However, previous work with this system indicates that k 's are very large (i.e., they are eluting near t_m). When the mobile phase contains 10 % (v/v) acetonitrile,

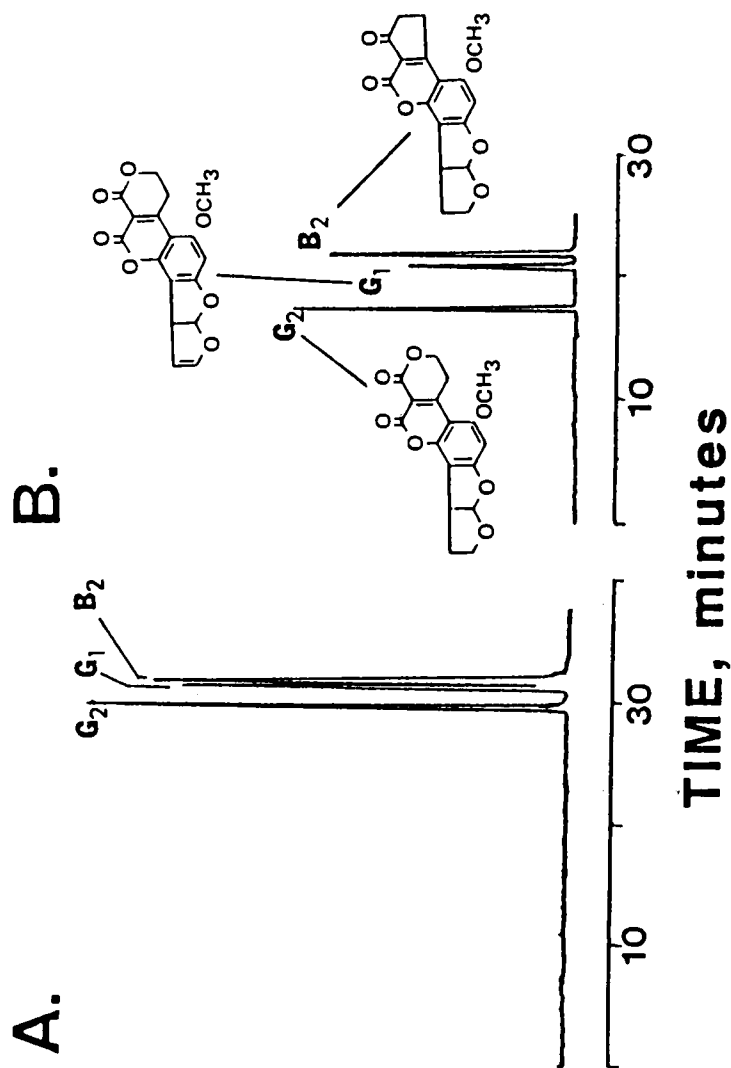


Figure 10. Separation of aflatoxins, G₂, G₁, and B₂. For A, the mobile phase consisted of only 0.10 M SDS and 0.02 M Na₂HPO₄. For B, the mobile phase also contained 10% (v/v) acetonitrile. The fused silica capillary used was 50 μ m i.d. x 65 cm. Fluorescence detection was employed, with the He-Cd laser (325 nm) for excitation.

the k' 's are reduced to more optimum values. Under these conditions the aflatoxins are baseline resolved with a reduction in separation time (Figure 10.B).

Organically modified MECC is not, however, "cure-all": the addition of organic solvents to MECC mobile phases can have detrimental effects. Balchunas and Sepaniak reported the addition of 20 % 2-propanol to the mobile phase increased t_m from 30 min. to 26 h (11). A solvent gradient was therefore a logical choice to provide a suitable compromise between the divergent effects of improved separations but prohibitively long analysis times.

Gradient Elution and the First Generation Gradient Mixer

As mentioned in Chapter 1, the capacity factor of a solute can be mathematically defined as the product of the thermodynamic partition coefficient, K , and the phase ratio, β (See Eq. 8). Therefore, in conventional reversed-phase LC, the introduction of an organic solvent to the mobile phase, in the form of a solvent-gradient, produces readily calculable effects on solute k' values based on experimentally determined K values. In MECC, such calculations are complicated by the fact that the presence of organic solvent affects β as well as K . Recall that β is the ratio of the micellar phase volume to the mobile phase volume; organic solvents affect the CMC and thus the micellar volume. Moreover, organic additives affect the viscosity and dielectric constants of the mobile phase and the zeta potential at the capillary surface; consequently, organic solvents in the mobile phase also affect the electroosmotic and electrophoretic flow rates.

A simple approach to generating solvent gradients in MECC is to alter the composition of the solution in the inlet reservoir during the course of the separation. Since reversed-phase elution characteristics are generally observed in MECC, this would normally involve increasing the organic content of the mobile phase during the separation. Initial gradient experiments by Balchunas and Sepaniak (11) involved the use of a

solvent step-wise gradient (wherein the voltage was turned off long enough for the inlet reservoir solution to be replaced with a new solution containing a higher concentration of organic solvent). This type of separation exhibited a marked effect on the k' values of late eluting components without the aforementioned excessive increase in elution range. Continuous solvent-gradients were not possible due to the fact that the inlet reservoir was the “hot” end of the capillary and electrical shorting often occurred.

The application of true, continuous gradients involved certain modifications to the usual instrumental design (Please see Chapter 2 for specifics), including the replacement of the inlet reservoir with a gradient mixer (See Figures 8 and 9). The implementation of calibrated syringe pumps and Eq. 10 made it possible to easily calculate the composition of the solution in the inlet reservoir. The expression was also validated experimentally by doping the mobile phase with a fluorophore and monitoring the fluorescence intensity at the inlet of the capillary for various shaped gradients (See Figure 11). However, it should be noted that the equation can only be used to directly calculate the *inlet* mobile phase composition. Under the conditions employed to generate solvent gradients in this work, there is a several percent difference in volume % organic solvent across the length of the column. Additionally, as the organic modifier content of the solution in the column increases, electroosmotic flow decreases; the rate of uptake of solution from the mixer therefore decreases. Thus, while the velocity of a neutral solute band, v_s , in MECC can be expressed mathematically as (46):

$$v_s = \frac{1}{1 + k'} (k' v_M + v_{eo}) \quad (12)$$

the actual value of v_s can only be calculated by knowing the values of the parameters in the equation for the mobile phase composition in the region of the band (which cannot be calculated directly from Eq. 10).

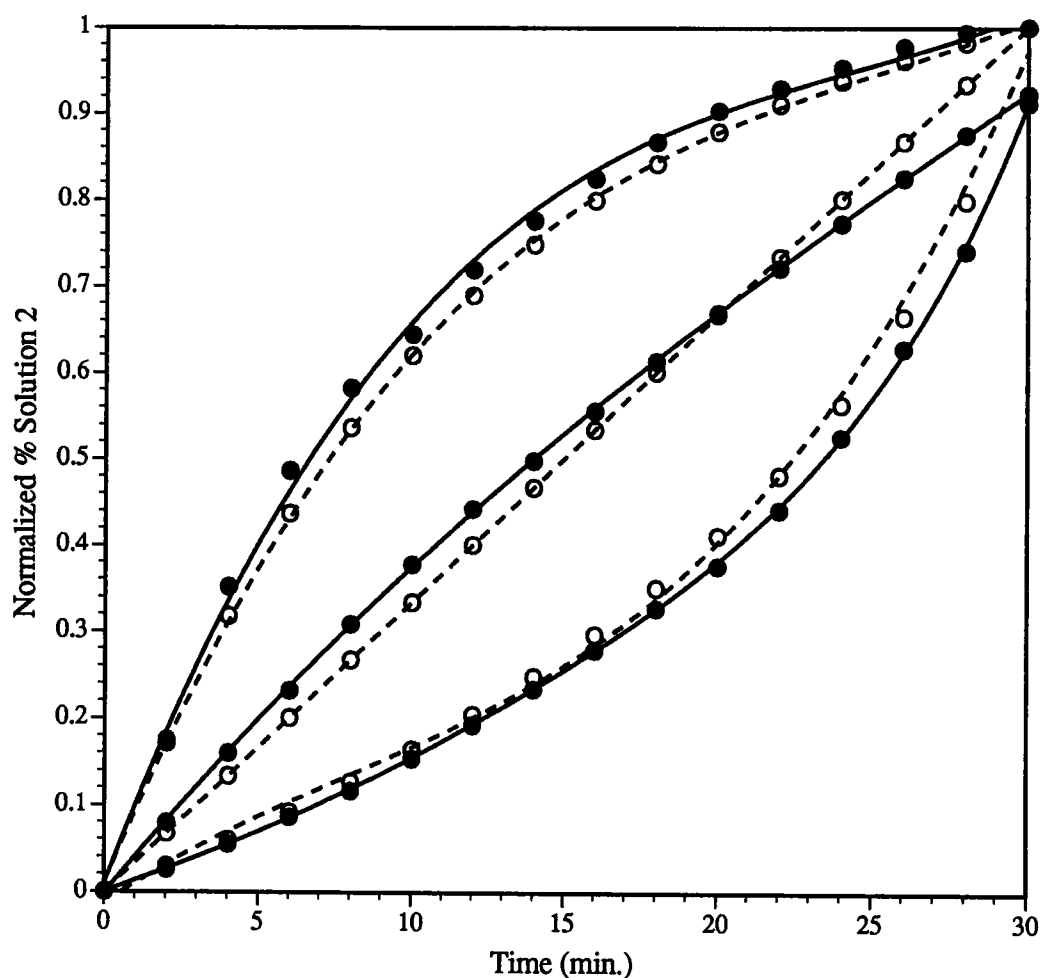


Figure 11. A comparison of the actual and theoretical gradients generated in the first generation gradient mixer. The solid lines refer to a normalized fluorescence signal of solution flowing from the gradient mixer; the dashed lines correspond to the calculated values, based on Eq. 10.

The initial evaluation of this solvent delivery system was performed on a homologous series of fluorescently-labelled n-alkylamines, ranging from n-propylamine to n-dodecylamine, using a variety of linear-, concave-, and convex-shaped acetonitrile gradients (See Figure 6). Acetonitrile was chosen because it does not cause large changes in flow rate and, hence, produces only a moderate extension of the elution range. As far as general trends in the separation of these test compounds is concerned, the separations seem to indicate that a linear gradient is best for the separation of early-eluting components, a convex gradient provides the best separation of intermediate-eluting components (the earlier the elution within this group the steeper the initial slope of the gradient necessary for separation), and a concave gradient affords the best separation of late-eluting components (when compared to a linear gradient with the same initial slope). The effects of several gradient profiles on the chromatographic separations for the test compounds and three unidentified impurities (most likely by-products from the derivitization) are shown in Figure 12, and the conditions for the gradients listed in Table 2. This particular instrumental design has also been used to create pH gradients (61).

Table 3 compares the separation efficiencies of the different gradients for n-pentylamine and n-octylamine. Given the usually high efficiencies possible with MECC, all the values are rather low; this is partially due to the difficulty in ascertaining peak widths, given their narrow character. Nonetheless, information concerning trends in efficiency is certainly evident. For n-pentylamine, a middle-to-late eluter, the most efficient separation is afforded with the Linear A gradient. Although the Linear B gradient does offer an improved efficiency over no gradient, it is rather small. Note also that as the "severity" of the gradient increases from Convex B to Convex A the efficiency drops off (as with the Concave gradient). This is consistent with earlier work (11) which demonstrated a decrease in efficiency at high organic solvent concentrations (see Chapter

Figure 12. The effect of solvent gradients on MECC separations. In particular, the separation of NBD-derivatized (a) n-propylamine, (b) n-butylamine, (c) n-pentylamine, (d) n-hexylamine, (e) n-heptylamine, (f) n-octylamine, (g) n-decylamine, (h) n-dodecylamine, and three impurities (I) using a mobile phase consisting of 0.01 M Na_2HPO_4 , 0.006 M $\text{Na}_2\text{B}_4\text{O}_7$, 0.05 M SDS with (A) no organic solvent; (B) the Linear A gradient; (C) the Linear B gradient; (D) the Convex B gradient; (E) the Convex A gradient; and (F) the Concave Gradient. A 50 μm x 80 cm column was used, along with fluorescence detection (He-Cd, 442 nm).

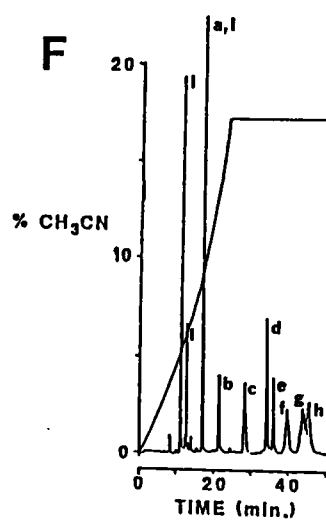
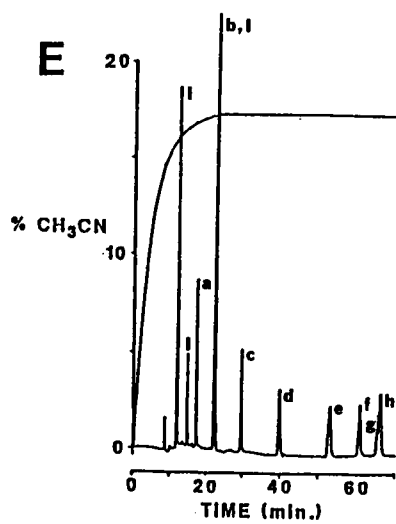
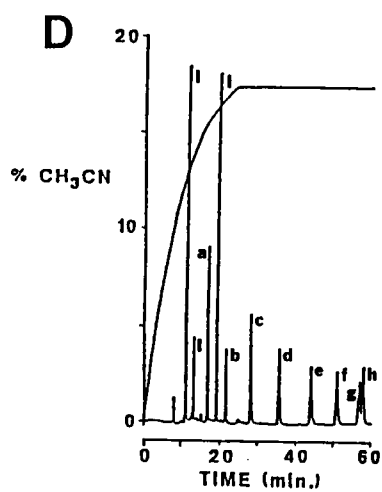
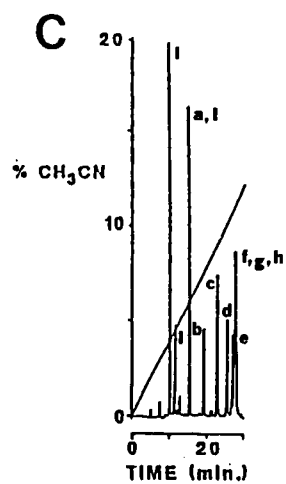
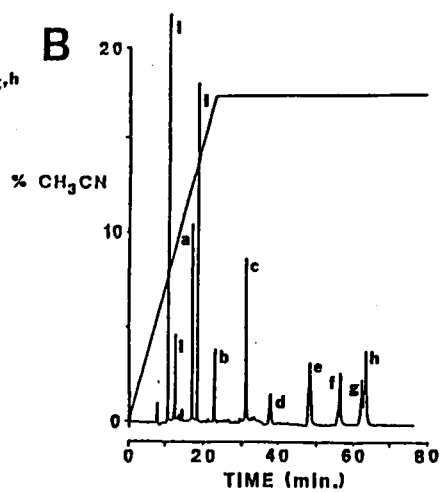
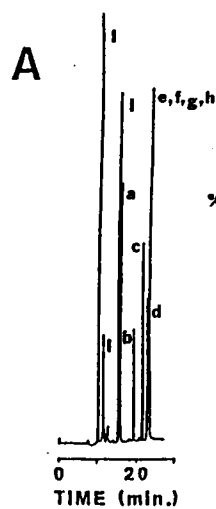


Table 2. Parameters for the solvent gradients demonstrated in Figure 12.

Gradient	V° (ml)	R_{in} (ml/min.)	R_{out} (ml/min.)	Duration (min.)	Final % CH₃CN
Linear A	6.0	0.22	0.44	23	17.5
Linear B	10.0	0.20	0.40	30	12.0
Convex B	4.0	0.38	0.35	23	17.5
Convex A	1.0	0.25	0.23	23	17.5
Concave	9.0	0.10	0.45	23	17.5

Table 3. Efficiency data (number of theoretical plates, N) for n-pentylamine and n-octylamine (peaks c and f, respectively) from the gradient separations in Figure 12.

Gradient	n-pentylamine (c)	n-octylamine (f)
No Gradient	29,000	n/a
Linear A	68,000	27,000
Linear B	36,000	n/a
Convex B	53,000	44,000
Convex A	23,000	44,000
Concave	9,300	10,000

2). For n-octylamine, a late eluting component (not even resolved without organic modifier), the best efficiency is demonstrated with the Convex gradients. This would seem to follow from the need for more extreme conditions to separate the more hydrophobic species.

A Computational Method for Predicting Retention Times with Solvent-Gradient MECC

The aim of solvent-gradient MECC is to provide a mechanism to increase the separating power of MECC (by optimizing solute k' values) without greatly increasing the analysis time. As stated earlier, the gradient mixer offers the possibility of an infinite number of gradients. A method is therefore required to optimize a solvent-gradient MECC separation without resorting to the time consuming process of trial and error to assign the correct gradient. The first step in the development of such a method has to be a way to calculate solute retention times for a particular gradient.

With conventional gradient elution LC, calculations of solute retention times can be based on Eq. 8 and a derivative of the particular gradient equation (such as Eq. 10 or 11) where the concentration of organic modifier at a particular point in the column (x) could be calculated by substituting t minus a delay factor [equal to x divided by the flow rate (a constant)] in place of t . The situation is more complex for MECC since the gradient experienced at different points along the column will not be the same because, as mentioned above, the presence of organic solvents in the mobile phase affects the electroosmotic flow rate, which is the basis of the electrokinetic pumping that moves solution through the column. This perturbation of the solvent gradient in the column serves to further complicate calculations since, as seen in Eq. 12, the solute band velocity, v_s , is a function of v_{eo} , v_M , and k' , all of which are changing during the gradient.

Certain assumptions and preliminary determinations were required in order to perform calculations in this work. First, a relationship between the gradient in the inlet reservoir and that in the capillary had to be determined. Since the solvent-gradient across the capillary was dependent on v_{eo} , which in turn was a function of the gradient, such a relationship was not readily apparent. To provide a reasonable estimate for the relationship between the inlet reservoir gradient and v_{eo} , an experiment was devised based on splitting the laser output into two parallel beams (separated by ≈ 1.5 cm) which were individually focused onto the capillary column, near its outlet (see Chapter 2 and Figure 7 for details). This v_{eo} data was then compared to the known % CH₃CN in the capillary (by assuming the % CH₃CN calculated by Eq. 10 at the time of an injection would be the % CH₃CN at the outlet at the time of elution). For a linear gradient (same slope as Linear A), a reasonable correlation between the theoretical values and the experimental results (See Figure 13) involved assuming that v_{eo} was a function of the average of the organic solvent concentrations at the inlet and outlet of the capillary (position 0 cm and 80 cm, respectively, in the matrix). The data points corresponding to the empty circles in Figure 13 were obtained by plotting the measured net v_{eo} (using the double-beam arrangement) at time, T, versus the average of the %CH₃CN in the inlet reservoir at time, T, and the %CH₃CN at the outlet of the column (assumed to be the %CH₃CN in the inlet reservoir at the time of t_0 marker injection). Additionally, v_M was assumed to be analogous to dodecylamine's band velocity (i.e., $k' = \infty$); a function relating dodecylamine's v_s to the % CH₃CN in the mobile phase was derived from the isocratic data. Although not determined experimentally, the function was again assumed to follow the average of the % CH₃CN at the inlet and outlet of the column.

The theoretical computations were based on an iterative method wherein the column was considered to be a number of individual segments each experiencing a

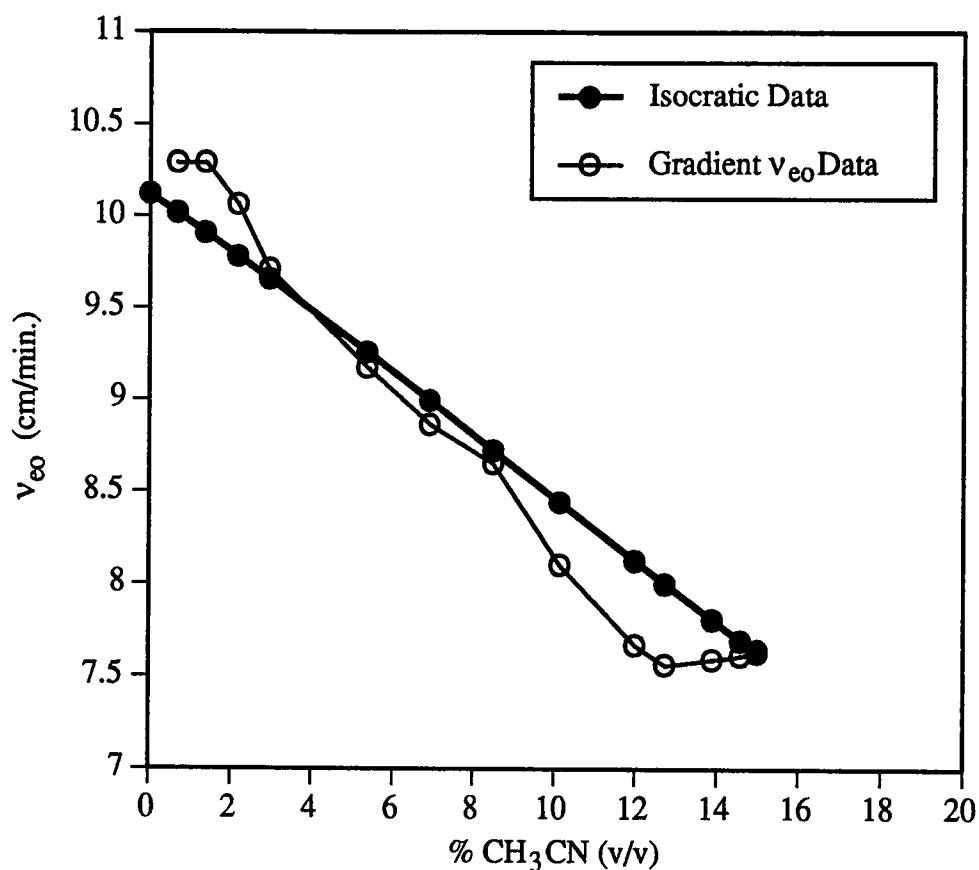


Figure 13. Comparison of the experimentally determined electroosmotic flow rate, v_{eo} , over the course of a gradient versus estimated values. The filled circles correspond to v_{eo} measured under isocratic conditions; the empty circles refer to the v_{eo} monitored over the course of the gradient (using the double-beam experiment) and plotted based on the average of the CH_3CN concentrations at the inlet and outlet of the column (see text for details).

temporal CH₃CN gradient. A time vs. column position matrix [40 min. (160 rows) X 80 cm (40 columns)] was thus generated, wherein each element corresponded to the CH₃CN concentration for a particular point in time and position in the column. Subsequently, each solute's band velocity within a column segment was calculated based on Eq. 12 and v_{eo} , v_M , and k' values determined from isocratic runs. The k' value for each solute was based on a computationally determined polynomial function relating k' to % CH₃CN (derived from the solute's experimentally determined, isocratic mobile phase data), solved using the % CH₃CN at the appropriate time/position in the column. The calculated band velocity was then used to determine the time necessary for the band to reach the next column position in the matrix. The acetonitrile concentration at the new position in the column was then interpolated from the time values in the matrix and a new band velocity was calculated. This procedure was repeated until the band reached a position value corresponding to the detection zone near the capillary outlet.

The test mixture of NBD-labeled alkylamines was formulated to contain two early eluting components (NBD-propanediolamine and NBD-ethanolamine), two components that eluted in the middle of the elution range (NBD-propylamine and NBD-iso-propylamine), and two late eluting components (NBD-octylamine and NBD-dodecylamine). This criterion for the mixture was used in order to test the retention time prediction abilities of the model over the entire elution range. Using the isocratic data, a functional relationship between k' and %CH₃CN was determined for each solute. Each solute's function, along with the %CH₃CN matrix (a 3-dimensional plot illustrating the matrix for a linear gradient is shown in Figure 14), was then used to calculate its retention time. This was accomplished by summing the transit times for each capillary segment based on v_s data calculated with Eq. 12. The results of these calculations can be seen in

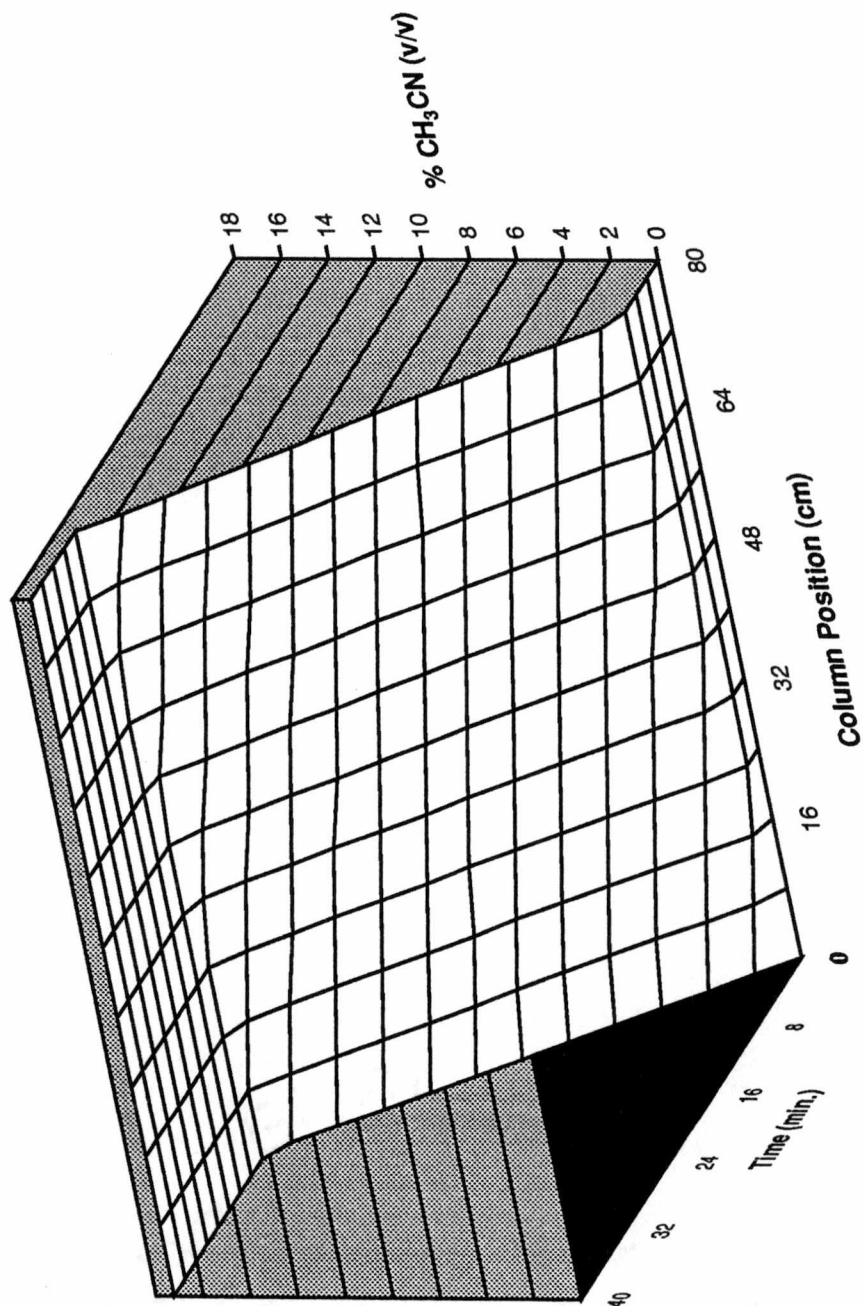


Figure 14. Plot of the temporal/spatial/concentration matrix used to calculate retention times for the linear A gradient.

Table 4 and a graphic comparison of theoretical and experimental retention times for gradient Linear A is presented in Figure 15.

As can be seen from Table 4, the method of retention time calculation correctly determined elution order of all components, even the impurity, x (probably a fluorescent labelling by-product), whose relative position changed depending on the gradient. Considering that solute retention times for our gradients have a % RSD of approximately 5 % ($N = 8$), there was also excellent correspondence for the early eluting components between the theoretical and experimental values. Notice especially the similarities between the retention times of NBD-propanediolamine and NBD-ethanolamine for the Linear B and the Concave gradients; this certainly follows, given that the initial slopes for the two gradient profiles are quite similar (see Figure 6). Linear B is also distinctive in that the retention times calculated for all seven components were larger than the experimental values. This may be related to the fact that in the experimental case, all of the components eluted before the gradient had reached its maximum value. The Linear A and Concave gradients seemed to have the most solutes to fall within the retention time reproducibility of the gradient. This probably results from the fact that some of the initial model assumptions were validated using only the Linear A gradient (the Concave gradient is also the most similar to Linear A). As the gradients become steeper, the deviation from expected values is larger, especially for the Convex A; however, the Convex A has a relatively evenly dispersed deviation (note that the highest percentage deviation for Convex A is 11.9 %, whereas the others deviate as much as 27.9 %). These trends in the gradients indicate that the model functions best (at least for the middle- and early-eluting components) when applied to gradients of intermediate extremity. They also suggest that the effect of the solvent on the micellar phase velocity (v_M) is over-compensated for at low concentrations (e.g., Linear B) and undercompensated for at high concentrations (see

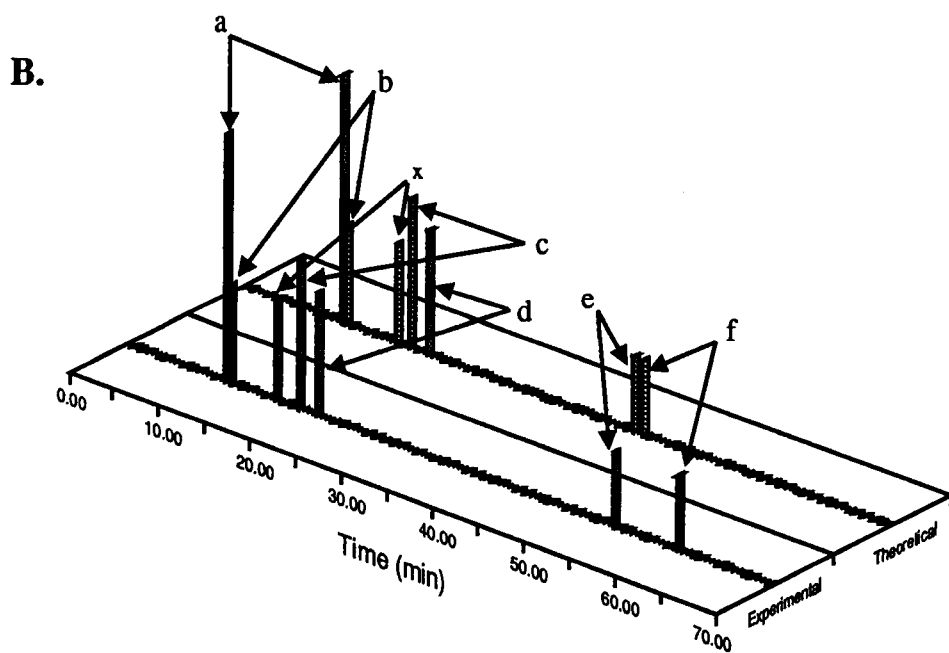
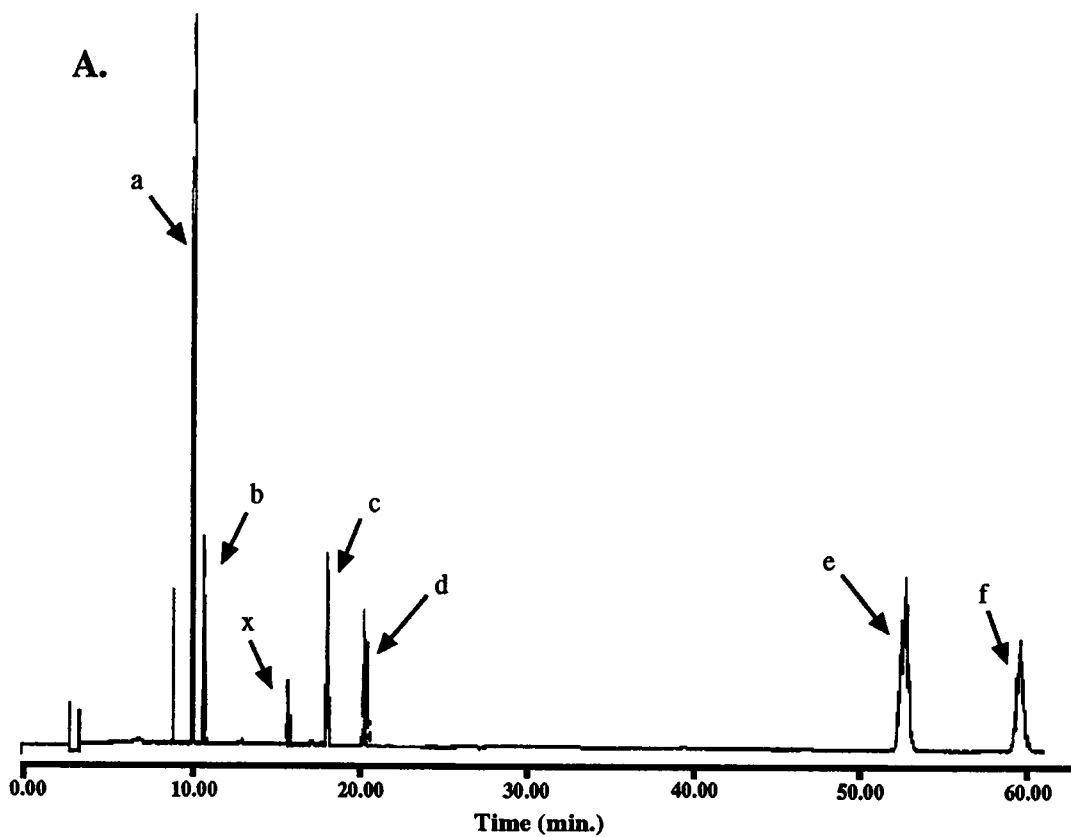
Table 4. Comparison of experimental and calculated solute retention times (in minutes) for (A) linear, (B) convex, and (C) concave gradients.

A. Component	Linear A			Linear B		
	Exp.	Calc.	% Dev.	Exp.	Calc.	% Dev.
NBD-propanediolamine	10.29	10.53	2.3%	10.02	10.43	4.2%
NBD-ethanolamine	10.91	11.06	1.4%	10.64	11.03	3.6%
NBD-n-propylamine	18.35	18.06	-1.6%	16.52	17.67	7.0%
NBD-isopropylamine	20.57	20.07	-2.4%	17.84	19.42	8.9%
NBD-octylamine	53.16	42.66	-19.8%	24.22	30.48	25.9%
NBD-dodecylamine	60.10	43.35	-27.9%	24.22	30.57	26.2%
x (impurity)	15.98	16.73	4.7%	14.39	15.83	10.0%

B. Component	Convex A			Convex B		
	Exp.	Calc.	% Dev.	Exp.	Calc.	% Dev.
NBD-propanediolamine	11.22	11.45	2.0%	10.04	10.90	8.6%
NBD-ethanolamine	12.15	11.71	-3.6%	10.70	11.28	5.4%
NBD-n-propylamine	16.96	17.79	4.9%	16.11	18.13	12.5%
NBD-isopropylamine	17.62	19.72	11.9%	20.06	20.14	0.4%
NBD-octylamine	61.63	57.15	-7.3%	50.80	50.50	-0.6%
NBD-dodecylamine	66.55	58.62	-11.9%	62.86	51.61	-17.9%
x (impurity)	18.35	20.14	9.8%	18.59	18.22	-2.0%

C. Component	Concave		
	Exp.	Calc.	% Dev.
NBD-propanediolamine	10.01	10.43	4.2%
NBD-ethanolamine	10.65	11.03	3.5%
NBD-n-propylamine	16.97	17.83	5.1%
NBD-isopropylamine	18.70	19.72	5.5%
NBD-octylamine	38.53	37.22	-3.4%
NBD-dodecylamine	38.53	37.61	-2.4%
x (impurity)	14.57	15.99	9.8%

Figure 15. A sample separation and a graphic comparison of theoretical and experimental retention times for the Linear A acetonitrile solvent-gradient. A) Solvent-gradient separation of NBD-derivatized: (a) propandiolamine, (b) ethanolamine, (c) n-propylamine, (d) isopropylamine, (e) n-octylamine, (f) n-dodecylamine, and (x) an impurity. Initial mobile phase conditions and detection were the same as in Figure 12. B) The graphic comparison of theoretical (computed from model) and experimental retention times for the Linear A gradient.



the large percent deviation for NBD-dodecylamine in all the other gradients). The even distribution of percent deviation for Convex A is most likely due to a situation where, as the gradient becomes more extreme, the separation approaches an isocratic-like state.

As stated previously, the reason for implementing a solvent-gradient is to improve the resolving power of MECC. To gauge the model's ability to predict peak resolution (an important criterion in ascertaining the best gradient for a particular separation) the data in Table 4 was used to calculate the "percent peak separation" of neighboring peaks (the difference in retention times divided by the average retention time). Table 5, which compares these values for the different gradients, illustrates that the model "mimics" peak separation best for less extreme gradients. Notice the correlation between the experimental and calculated values for Linear A, Linear B, and Concave. For comparison, the experimental resolutions for NBD-propylamine and NBD-isopropylamine were, respectively, 6.1, 6.9, and 7.5 for the Linear B, Concave, and Linear A gradients. Although these resolution values are larger than necessary, they do follow the same trend as the percent peak separation values. Of course, the use of percent peak separation for comparison disregards the effect of efficiency on resolution as well as analysis-time considerations. Subsequent application of this technique for gradient assignment will consider these criteria as well.

The fact that the theoretical expectations for solute retention times almost all fall short for the late eluting components seems to indicate some error in predicting the net micellar phase velocity (v_M); an error exacerbated at higher CH_3CN concentrations. Based on our earlier work, the model assumed that the presence of organic solvent in the mobile phase had a negligible effect on the electrophoretic mobility of the micellar phase ($v_{e,m}$). Therefore, assuming $v_{e,m}$ is constant, changes in v_M due to the presence of organic modifier should follow changes in v_{e0} (since v_{e0} is the only other component

Table 5. Comparison between experimental and calculated values of percent peak separation for different gradients.

Gradient	NBD-propanediolamine +		NBD-n-propylamine +		NBD-octylamine +	
	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
NBD-ethanolamine						
Linear A	5.80%	4.92%	11.37%	10.49%	12.25%	1.61%
Linear B	6.05%	5.55%	7.72%	9.43%	0.00%	0.27%
Convex A	7.95%	2.26%	3.81%	10.30%	7.68%	2.54%
Convex B	6.36%	3.42%	21.85%	10.52%	21.22%	2.17%
Concave	6.20%	5.54%	9.68%	10.03%	0.00%	1.03%

contributing to v_M) Thus v_M should only be dependent on the assumptions concerning how v_{e0} changes with CH_3CN concentration. However, if the micellar electrophoretic mobility is significantly affected by the presence of CH_3CN , its dependence may not mimic that of v_{e0} . If the functions determining the affect of organic modifier on v_{e0} and $v_{e,m}$ differ significantly, it is feasible that at higher concentrations of organic modifier (where these effects would be more pronounced) a divergence could occur between the expected and actual value for v_M . Such a divergence would affect the late eluting components more strongly since they spend significantly more time in the micellar phase. It is also possible that NBD-dodecylamine may not be a good enough indicator of v_M .

Although there is clearly a need to refine the computational model, the described method shows promise in being able to optimize a solvent-gradient MECC separation without resorting to the time consuming process of trial and error to assign the correct gradient. The next step in this work is to design a system that will employ this computational model and, with a minimal number of isocratic separations, analyze the isocratic data and decide on the best gradient to separate specified components.

Chapter 4

The Second Generation Gradient Mixer and the Optimization of Solvent-Gradient Separations

General Improvements.

The second generation gradient mixer described in Chapter 2 (See Figures 8 and 9) offers several advantages over the first generation mixer. Chief among these advantages is the increased reproducibility possible with this system. With the conical mixing chamber employed in the first generation device, the initial volume, V^0 , was administered to the mixer with a pipet; depending on the gradient, this volume varied from 1 to 10 mL. With the tangential mixing design employed with the second generation mixer, there was no need to deliver this varied amount of solution. Furthermore, with the first generation mixer, the final volume in the mixing chamber varied from approximately 10 mL down to a fraction of a mL. Any slight variance in solution flow or length of time the pumps were operating could have drastic effects on the final concentration of mobile phase modifier in the inlet reservoir, depending on the volume of solution present in the mixer. The enclosed nature of the second generation mixer reduced the possibility of evaporation of volatile solvents, a potentially significant factor, given that the solution surface area exposed to the atmosphere with the conical mixing chamber changed over the course of a gradient. The first generation apparatus also required manual termination of the pumping at the end of a gradient; the computer controlled nature of the second generation device allowed for unattended gradient generation of a more reproducible time length. The net result was an improvement in %RSD from approximately 5% ($n = 8$) for the first generation to 2% ($n=10$) for the second generation apparatus (see Table 6).

Table 6. Retention time reproducibility using the Second Generation Gradient Mixer. A linear gradient (%res = 1.67 t; t = 0 → 15 min.) was performed using a 0.1 M Msodium cholate mobile phase with phosphate/borate buffer the same as in Figure 12.

Run #	Retention Times (min.)										Mean	s	%RSD
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10			
t ₀	6.11	6.17	6.19	6.12	6.07	6.16	6.04	6.21	6.13	6.20	6.14	0.06	0.92%
Acridine	11.95	11.61	11.43	11.28	11.62	11.52	11.58	11.53	11.90	11.72	11.61	0.20	1.74%
x (impurity)	14.31	13.93	13.93	13.83	14.21	13.99	13.72	14.08	14.48	14.25	14.07	0.24	1.68%
Aminochrysene	16.12	15.65	16.03	15.71	16.02	15.89	15.39	16.03	16.45	16.30	15.96	0.31	1.96%
Benzo[a]pyrene	18.01	17.23	18.19	17.49	17.82	17.82	17.56	18.18	18.35	18.50	17.92	0.41	2.26%

The computer controlled nature of the second generation device allowed for more diverse gradient shapes. The gradients for the first generation mixer were limited to one-to-one functions (i.e., continuously increasing or decreasing). Since the mixer for the second generation device was actually a mini-step gradient generator, any gradient shape was possible. This capability can be of particular importance in MECC given the constraints of a limited elution range. Figure 16 demonstrates the usefulness of such an ability for the separation of a mixture of PAH's. Note that the center gradient, a non one-to-one function, was able to baseline resolve the latter three components in almost half the time of the linear gradient.

The major limitation to the second generation apparatus was the need for 30 second steps in gradient concentration. Smaller steps would be preferable to more closely mimic a continuous mixer; however, the step width was a function of the maximum pump rate possible with the syringe pumps.

The Revised Computational Model.

As mentioned in Chapter 2, the original model for retention time prediction was run using a spreadsheet program (Microsoft Excel). The "macro scripting" environment allowed for the tailoring of the Excel interface to include specific menubar choices and "dialog boxes" to prompt the user for the calculations. Such an interface provided a very "user-friendly" environment for predicting retention times; however, the generation of the matrices for each gradient required approximately two hours each. Such a time consuming process was obviously too long for use in an optimization scheme. Believing that a good deal of the computing time was being wasted maintaining the interface, Excel was replaced with the programming language QuickBASIC, a derivative of the BASIC language.

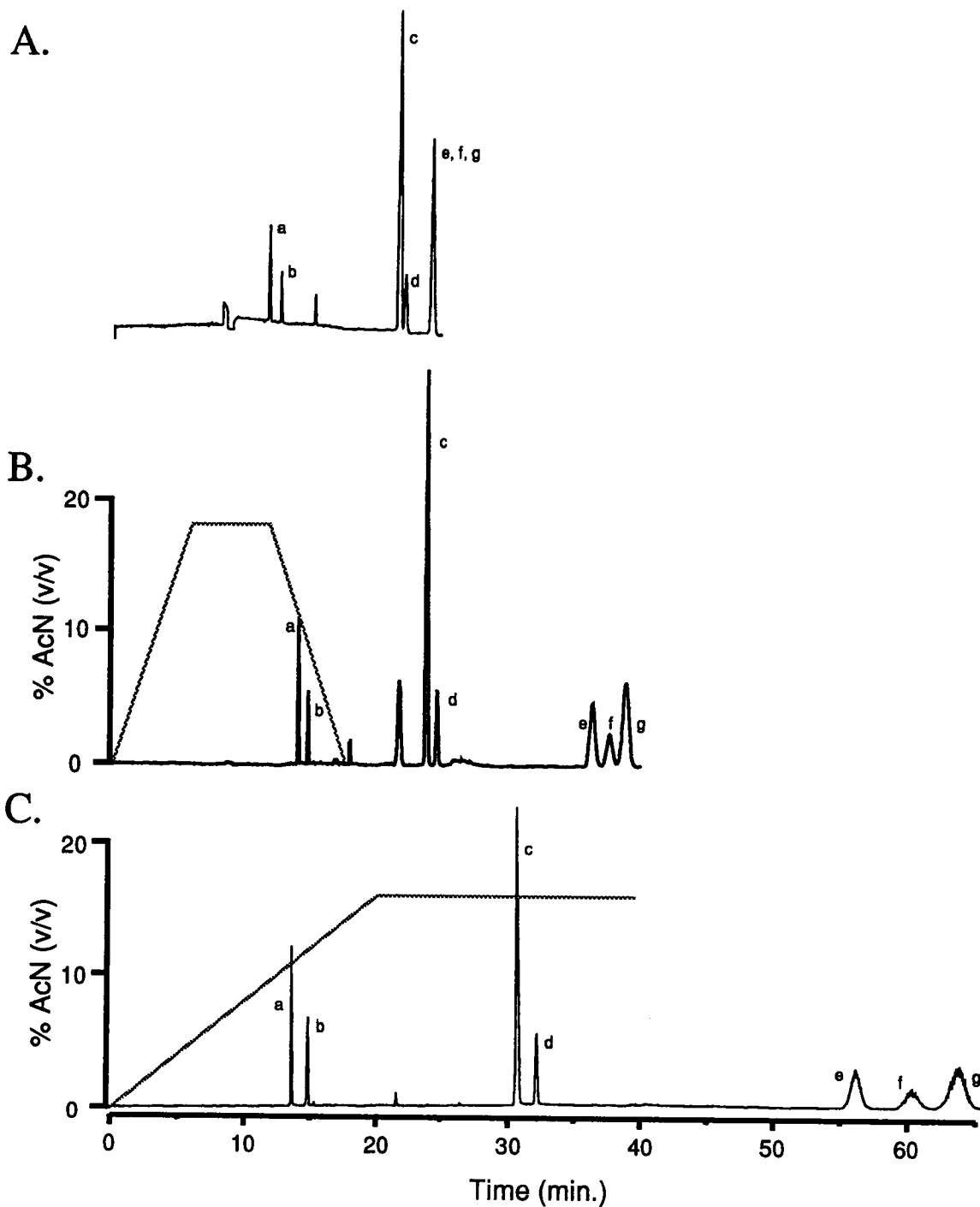


Figure 16. Comparison of separations employing (A) no gradient; (B) a dual-ramp gradient; and (C) a linear gradient. The test mixture was composed of NBD-derivatized (a) propandiolamine, (b) ethanolamine, (c) isobutylamine, (d) n-butylamine, (e) n-heptylamine, (f) n-octylamine, and (g) n-tetradecylamine. Conditions the same as in Figure 12, except column length of 65 cm.

The method of calculation for the model was also altered. The Excel version was based on a matrix mapped in the spatial domain of discrete points along the column and the time necessary for a solute to migrate from one position to another. The newer method involved the time domain in the sense that what was generated by the program was a successive number of solvent plugs the lengths of which were based on electroosmotic flow at discrete points in time. Calculations were then based on solute band migration distance during each time step, as a function of the solvent plug the band resided in during the time period. Retention times were then calculated by summing the number of time steps necessary for the band to reach a distance corresponding to the end of the column. The resulting QuickBASIC program was able to calculate retention times for a given gradient in approximately *10 seconds*, a marked improvement over the Excel version.

Table 7 contains a comparison of experimental data to calculated retention times for a number of sample gradients. Although direct comparison is not possible between this data and Table 3 since different experimental conditions were used (i.e., different capillaries, different solutions, etc.), in general, a significant improvement in retention time prediction was demonstrated. Note specifically the improvements with the early eluters for the Convex B and Concave gradients.

Solvent-Gradient Optimization.

The BASIC program employed for optimization of the gradients (See Appendix) was adapted from Berridge's example of a "Variable Size Simplex Optimization" (62). This modified simplex algorithm (63) allows for expansion and contraction of the step, as well as reflection. A two dimensional simplex was employed wherein κ and t_G (refer to Eq. 11) were varied.

Table 7. Comparison of experimental and calculated retention times for selected gradients using the revised computational program.

Convex B			
	Retention Time (min.)	Calc.	%Dev.
NBD-Propandiolamine	13.57	13.34	-1.71%
NBD-Ethanolamine	14.30	13.80	-3.50%
NBD-Propylamine	20.10	19.83	-1.35%
NBD-Isopropylamine	21.81	21.93	0.57%
NBD-Isobutylamine	26.31	27.48	4.46%
NBD-Butylamine	27.86	29.49	5.84%
NBD-Quadradecylamine	51.56		
Concave			
	Retention Time (min.)	Calc.	%Dev.
NBD-Propandiolamine	12.25	12.29	0.32%
NBD-Ethanolamine	13.16	12.93	-1.78%
NBD-Propylamine	20.49	20.36	-0.65%
NBD-Isopropylamine	22.26	22.35	0.41%
NBD-Isobutylamine	25.62	26.42	3.11%
NBD-Butylamine	26.41	27.8	5.26%
NBD-Quadradecylamine	32.42		
%res = 1.33 t (t = 0 → 15 min.)			
	Retention Time (min.)	Calc.	%Dev.
NBD-Propandiolamine	13.49	12.9	-4.40%
NBD-Ethanolamine	14.40	13.47	-6.47%
NBD-Propylamine	21.52	20.25	-5.90%
NBD-Isopropylamine	23.31	22.35	-4.13%
NBD-Isobutylamine	26.85	26.94	0.35%
NBD-Butylamine	27.87	28.41	1.94%
NBD-Quadradecylamine	50.54		

The simplex method simply involves considering the differing combinations of experimental parameters as a contour map, where the height of a given coordinate is related to its merit as an experimental result, relative to other combinations of parameters. Initially, $n + 1$ experiments are carried out, where n is the number of experimental parameters varied (in this case: two). Experimental results are compared, and the least optimal set of parameters is rejected in favor of a new set whose values correspond to a point that corresponds to the apex of a reflected triangle whose plane of reflection is the two sets of retained parameters. This new experiment is then run and its result compared to the others and either retained (in which case the process is repeated by now rejecting one of the other two points in the same manner) or rejected (in which case an expansion or contraction of the triangle is then used to arrive at a new set of parameters). The process is repeated until an optimum condition is reached.

The "experiments" called for with the simplex were carried out in a simulated fashion by the computer using the retention time prediction model and isocratic data. Some criterion was necessary, however, for assessing the relative merit of one separation over another. A Chromatographic Retention Factor, or CRF (62, 64, 65), was formulated to judge the differences in gradient separations:

$$\text{CRF} = \frac{N \sum_{i=1}^N \left(\frac{1}{t_{i+1} - t_i} + \frac{1}{t_i - t_{i-1}} \right)^{-1}}{t_M} \quad (13)$$

where N is the number of solute bands of interest, t_i is the retention time of the i^{th} solute band, t_{i+1} and t_{i-1} are the retention times of the neighboring bands, and t_M is the retention time of the micellar phase. Since the computational model for predicting retention times does not include the ability to calculate peak resolution (a complex factor for MECC), the

best measure of separation optimization was judged to be relative peak separation. The t_M factor was included so that the CRF would tend to favor shorter analysis times.

Subsequent gradient work was performed with a mixture of polyaromatic hydrocarbons. The switch from the test mixtures of derivatized amines was made to demonstrate the technique's separating ability in a more "realistic" situation. The switch to UV detection was made for the same reason. Unfortunately, this resulted in a decrease in sensitivity and an increase in baseline noise. The rhythmic nature of the noise seemed to correspond to the pumping of solution through the mixing chamber, possibly a refractive index change in the mobile phase solution. This rhythmic noise may have been invisible with laser-fluorescence detection due to its inherent selectivity.

Table 8 lists data for an optimization of PAH's in a bile salt (sodium cholate) mobile phase. Note that in this case, the favored gradient (highest CRF value) was a concave gradient of approximately 17 min with final %CH₃CN of 17.5%. This relatively mild gradient was probably a result of the easily separated nature of the mixture. Figure 17 provides an illustration of the simplex process by plotting the data from Table 8. The program initially ran A, B, and C. A was deemed the least desirable and was discarded. A new set of parameters, D, are generated by reflecting A across BC. The parameters corresponding to D were tested and passed; therefore, B was discarded next because it then had the lowest CRF value. Point E was generated in the same manner as D, and the process continued until a maximum was reached; in this case, the conditions associated with M.

A more difficult separation of PAH's was devised using a slightly different mixture (see Table 9 for the components) and an SDS mobile phase. With the same starting points as described in Table 8, an optimum CRF of 2.46 was calculated for a convex gradient (k of 0.425) of 3 minutes (i.e. a very steep gradient was necessary to

Table 8. Sample data employing optimization program. Same experimental conditions as in Table 6.

	A	B	C	D	E
t_G	12.00	10.00	15.00	13.00	18.00
κ	0.75	1.00	1.10	1.35	1.45
t_0	7.41 min.	7.40 min.	7.32 min.	7.31 min.	7.27 min.
Acridine	15.78	15.83	15.83	15.89	15.80
Naphthalene	16.29	16.34	16.78	16.84	16.88
x (impurity)	20.43	20.36	20.41	20.40	20.27
Aminochrysene	22.93	22.87	22.91	22.86	22.78
1-Aminopyrene	23.94	23.90	23.84	23.80	23.41
Chrysene	27.35	27.34	26.86	26.87	26.36
Benzo[a]pyrene	28.83	28.83	27.94	27.98	27.29
CRF	2.44	2.45	2.64	2.64	2.53

	F	G	H	I	J
t_G	16.00	18.00	19.00	16.00	18.00
κ	1.34	1.09	1.33	1.16	1.27
t_0	7.29 min.	7.30 min.	7.27 min.	7.30 min.	7.28 min.
Acridine	15.84	15.78	15.78	15.82	15.79
Naphthalene	16.86	16.78	16.85	16.81	16.83
x (impurity)	20.36	20.34	20.26	20.39	20.31
Aminochrysene	22.85	22.86	22.77	22.89	22.82
1-Aminopyrene	23.75	23.77	23.40	23.80	23.46
Chrysene	26.49	26.50	26.35	26.80	26.42
Benzo[a]pyrene	27.50	27.49	27.28	27.84	27.38
CRF	2.67	2.66	2.53	2.64	2.53

	K	L	M	N	O
t_G	16.50	17.50	16.75	14.75	17.20
κ	1.18	1.24	1.20	1.45	1.18
t_0	7.30 min.	7.29 min.	7.29 min.	7.28 min.	7.29 min.
Acridine	15.81	15.80	15.81	15.88	15.80
Naphthalene	16.82	16.83	16.82	16.89	16.81
x (impurity)	20.37	20.33	20.35	20.37	20.35
Aminochrysene	22.88	22.84	22.86	22.84	22.86
1-Aminopyrene	23.78	23.48	23.76	23.49	23.76
Chrysene	26.77	26.45	26.49	26.50	26.49
Benzo[a]pyrene	27.79	27.43	27.75	27.78	27.49
CRF	2.65	2.53	2.70	2.56	2.66

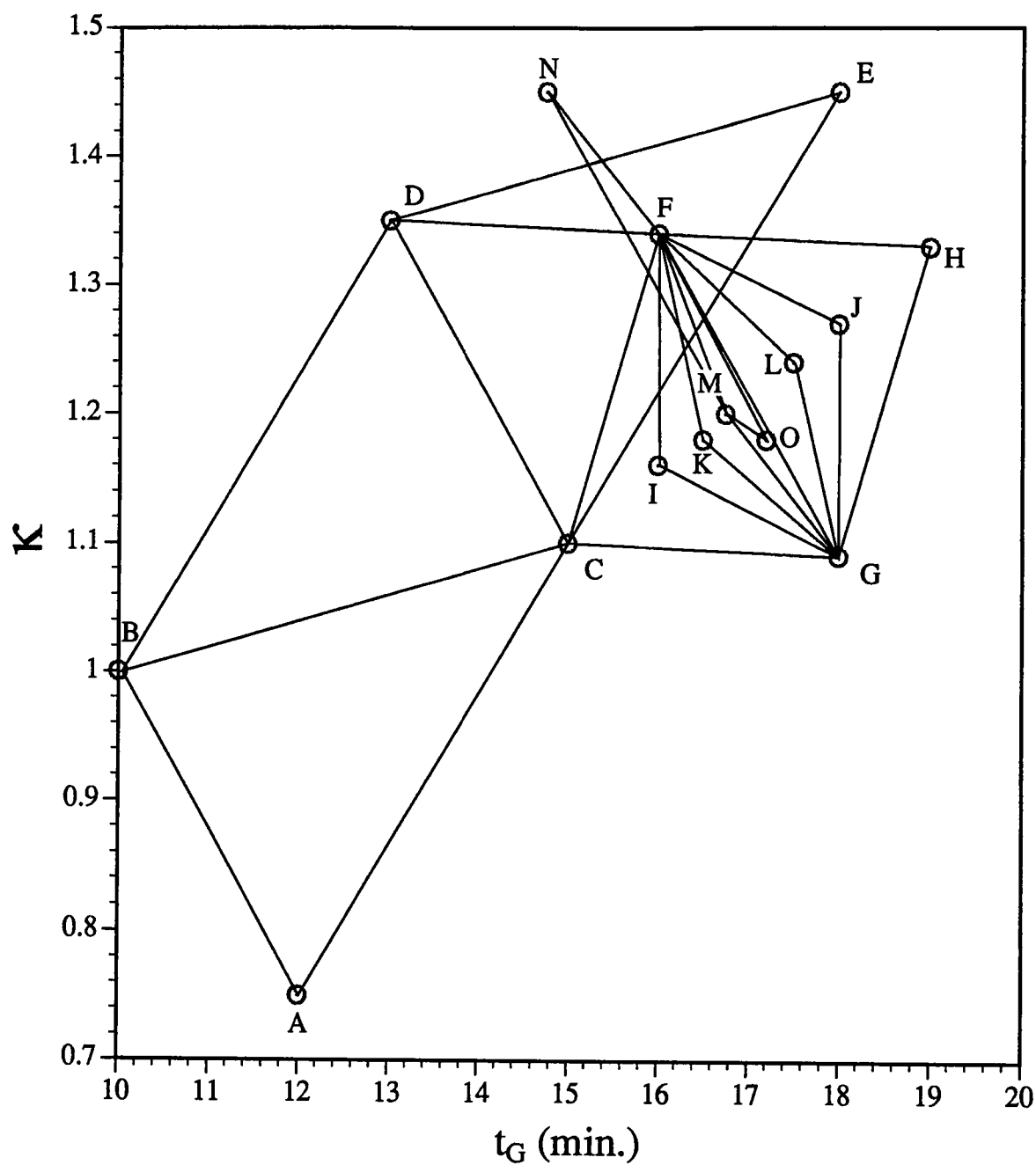


Figure 17. Sample plot of simplex data (Table 8) from optimization program.

Table 9. Comparison of calculated and experimental results for best and worst gradients, as judged by computational model.

t_G	25.00		6.25	
κ	2.00		0.13	
	Calculated	Experimental	Calculated	Experimental
t_0	3.75 min.	3.94 min.	3.97 min.	4.13 min.
Benzyl Alcohol	4.49	4.72	4.86	5.22
Benzene	5.31	5.39	6.26	6.25
Naphthalene	9.31	9.90	11.29	10.70
Dinitronaphthalene	9.50	10.34	13.93	10.95
Pyrene	10.45	11.47	40.92	34.38
Chrysene	10.46	11.67	48.00	39.51
Benzo[a]pyrene	10.46	11.67	51.80	43.60
CRF	1.90	1.99	3.74	2.67

resolve the final three components). The optimization was then attempted again with a higher final concentration of acetonitrile of 20%. Table 9 lists the gradients assigned the high and low CRF values for this optimization along with experimental data for the actual separations. Note that experimental and calculated values for the low CRF gradient agree reasonably well, but there is a deviation for the indicated optimal gradient. This is probably an aberration due to the extreme nature of the gradient and the fact that the isocratic data used in the generation of the functions was from data taken two days before.

The net result of the modified simplex procedure is a quick method of arriving at a "ballpark" estimate for gradient separations. For complex samples, it might be suggested that a "real-time" simplex method involving actual separations might be useful to "tweak" the optimum. However, the computer model has demonstrated usefulness in narrowing the field and can distinguish between different separation needs, evidenced in the assignment of such different shaped gradients for the two different systems.

Chapter 5

Conclusion

To summarize, MECC is a separation method characterized by high efficiency and short analysis times. The method is, however, limited to hydrophilic species. One method of expanding its separating ability to hydrophobic species is by the introduction of organic solvents. This addition has a number of ramifications beyond just increasing the solubility of the analytes in the aqueous phase. One result is that prohibitively long analysis times often occur.

A method of compromise between the increased separation power and long analysis times is the application of solvent-gradients to the mobile phase so that the hydrophobic (late-eluting) components are preferentially affected by the organic solvent. Two "generations" of solvent-gradient delivery systems have been developed and described herein, each with relative success in terms of utility, reproducibility, and "ease of use." Their applicability to difficult separations proved a "hit or miss" proposition, in terms of finding the best gradient for a particular situation.

In an effort to in some way quantitate the effects of solvent-gradients on MECC separations, a computational method was proposed to ascertain the best gradient for a given separation problem. The first step in this process was the development of a mathematical model for the prediction of analyte retention times with solvent-gradient MECC. Such calculations were complicated, relative to conventional LC, in that organic solvent in the mobile phase affects not only the partition coefficient of a solute, but other separation parameters in MECC such as the phase ratio, electroosmotic flow, and the micellar flow velocity. This model proved reasonably sound, and a subsequent revision proved even more successful both with regard to accuracy and speed of calculation. The reten-

tion time prediction model was then coupled to a modified simplex algorithm, which employed a "chromatographic retention factor" to judge the relative merits of one solvent-gradient separation over another. The resulting program was capable of testing different gradients and then assessing which was best for a particular separation dilemma. This program demonstrated the ability to distinguish between different separation needs and assign appropriate gradients with good correlation.

The work presented here, therefore, presents a foundation for future applications of solvent-gradients to MECC. The second generation gradient mixer offers the opportunity to produce more unusual gradient shapes. The separation in Fig. 16 should be viewed as an indication of its possibilities. Tertiary gradient systems could also be utilized. For example, a pH gradient could be applied, concurrent with the organic modifier gradient, to offset the effects of diminished electroosmotic flow.

Reverse gradients are also a possibility, wherein the concentration of organic modifier in the mobile phase is decreased over time, rather than increased. This may actually be a closer parallel to the use of solvent-gradients in conventional LC, where organic solvent is added to increase the mobile phase solubility of late-eluting analytes, speeding up the movement of the analyte bands through the column. Increased organic modifier concentration in the MECC mobile phase has the opposite effect on band velocities since its presence decreases electroosmotic flow, as well as decreasing solute partition coefficients (See Chapter 1). A reverse solvent-gradient for MECC would therefore involve an initial high concentration of organic modifier in the mobile phase, with the effects of increasing the mobile phase solubility of hydrophobic analytes as well as decreasing electroosmotic flow; over time organic modifier concentration would be decreased, which would result in an increase in electroosmotic flow and faster band velocities for the same analytes.

Future work should also involve improvement in the computer assisted optimization. As mentioned in Chapter 4, the current program does not take into account peak resolution in assessing a separation; only retention times are considered. Although this worked well for the test mixtures, more complex systems will probably require a more discriminating method of appraisal that takes into account efficiency of separation. Perhaps a function relating the ratio of $w_{1/2}$ (peak width at half height) and t_R to organic modifier concentration could be generated for the analytes from isocratic data. (Computer-based data acquisition would be useful here.)

Improvements are also warranted for the gradient mixer itself. Although its performance has been improved, this solvent-gradient delivery system is far from perfect. Future designs should address the problems with "gradient noise" evident when using UV detection. This is probably an artifact of incomplete mixing, evidenced by changes in the refractive index of the mobile phase as the concentration of organic modifier changes. A faster pumping system would also be useful to improve the mixing of solutions and allow for smaller gradient steps.

In conclusion, a method has been presented to improve the separating power of MECC. This optimization of separations involves the coupling of a computer-controlled solvent-gradient generator with a computational method for assigning the best gradient for a particular separation problem.

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Appendix

Appendix

MECC Solvent-Gradient Optimization Program

DIM STATIC GradStep!(200,200) 'gradient matrix(time[min.],position[cm])

DIM STATIC kprime!(10,5)

DIM STATIC Solute\$(10)

DIM STATIC Veo!(200)

DIM STATIC Vm!(200)

DIM STATIC StepConc!(200)

DIM STATIC Outlet!(200)

DIM STATIC GradExt!(200)

DIM STATIC tR!(10)

DIM STATIC Kappa!(5)

DIM STATIC tG!(5)

DIM STATIC CRF!(5)

DIM STATIC Ret!(10)

DIM STATIC Strike%(10)

DEF FNFOP!(A,B,C,D,E,F,x) = A*x^5 + B*x^4 + C*x^3 + D*x^2 + E*x + F

TEXTFONT 2

TEXTSIZE 12

PRINT "This is a program to compute solute retention times for"

PRINT " Solvent-Gradient MECC."

Step1: PRINT "The first step involves declaring the relationship between "

PRINT " electroosmotic flow (Veo) as a function of %OM."

PRINT ""

PRINT "We assume that the relationship can be expressed by a fifth order"

PRINT " polynomial:"

PRINT " $V_{eo} = A * \%OM^5 + B * \%OM^4 + C * \%OM^3 + D * \%OM^2 + E * \%OM + F$ "

PRINT ""

INPUT " Enter the value for A: "; V5

INPUT " Enter the value for B: "; V4

INPUT " Enter the value for C: "; V3

INPUT " Enter the value for D: "; V2

INPUT " Enter the value for E: "; V1

INPUT " Enter the value for F: "; V0

PRINT ""

INPUT "Are these values correct? [Y for yes]"; Q2\$

IF Q2\$ <> "Y" THEN GOTO Step1

PRINT "Thank you"

Step2: PRINT ""

PRINT "Second, we must declare the relationship between micellar velocity"

PRINT " (V_m) As a function of $\%OM$."

PRINT ""

PRINT "We assume that the relationship can be expressed by a fifth order"

PRINT " polynomial:"

PRINT " $V_m = A * \%OM^5 + B * \%OM^4 + C * \%OM^3 + D * \%OM^2 + E * \%OM + F$ "

```

PRINT ""
INPUT "    Enter the value for A:";VM5
INPUT "    Enter the value for B:";VM4
INPUT "    Enter the value for C:";VM3
INPUT "    Enter the value for D:";VM2
INPUT "    Enter the value for E:";VM1
INPUT "    Enter the value for F:";VM0
PRINT ""
INPUT "Are these values correct? [Y for yes]";Q3$
IF Q3$<>"Y" THEN GOTO Step2
PRINT "Thank you"

```

Step3: PRINT ""

PRINT "The third step involves declaring the relationship between"

PRINT " solute capacity factors and % OM."

PRINT ""

PRINT "First, how many solutes are involved? (This program is limited"

PRINT " to a maximum of ten solutes.)"

INPUT SoluteMAX%

IF SoluteMAX%>10 THEN GOTO Step3

PRINT ""

PRINT "Once again, the function relating the capacity factor to %OM is"

PRINT " assumed to be a fifth order polynomial:"

PRINT " $k = A * \%OM^5 + B * \%OM^4 + C * \%OM^3 + D * \%OM^2 + E * \%OM + F$ "

PRINT ""

FOR S% = 1 TO SoluteMAX%

Check: PRINT "For solute";S%,""

INPUT " Enter the name of the solute:";Solute\$(S%)

INPUT " Enter the value for A:";kprime!(S%,5)

INPUT " Enter the value for B:";kprime!(S%,4)

INPUT " Enter the value for C:";kprime!(S%,3)

INPUT " Enter the value for D:";kprime!(S%,2)

INPUT " Enter the value for E:";kprime!(S%,1)

INPUT " Enter the value for F:";kprime!(S%,0)

PRINT ""

INPUT "Are these values correct? (Type Y, then RETURN if yes.);Q5\$

IF Q5\$ <> "Y" THEN GOTO Check

NEXT S%

LET Solute\$(0) = "to"

LET kprime!(0,5) = 0

LET kprime!(0,4) = 0

LET kprime!(0,3) = 0

LET kprime!(0,2) = 0

LET kprime!(0,1) = 0

LET kprime!(0,0) = 0

Step4:PRINT ""

PRINT "We shall assume that the solvent gradient can be expressed"

PRINT " mathematically as:"

PRINT " $\%OM = C_g * (t/tG)^K$ "

```

PRINT ""
PRINT "Cg is assumed to be 17.5 % (v/v)0!"
PRINT ""
INPUT "What is the length of the capillary column (in cm)";ColMAX!
PRINT "Is this value correct? Type Y for yes, then RETURN"
INPUT Q4$
IF Q4$ <> "Y" THEN GOTO Step4
PRINT ""

```

```

REM Variable Size Simplex Optimization
REM Version 4.4 by J.C. Berridge  January 1985

```

```

REM Define Variables
LET B1 = 1: REM Simplex Status

```

```

REM Main Simplex Program Start
LET VAR = 2: REM Number of variables in simplex
LET M = 10: REM M is the optimum value the simplex searches for
LET W6 = M: REM W6 used to track best vertex
LET Cg = 17.5

```

```

REM Input array of first experiments and results
PRINT ""
LET tG!(1) = 12

```

```

LET tG!(2) = 10
LET tG!(3) = 15
LET Kappa!(1) = .75
LET Kappa!(2) = 1
LET Kappa!(3) = 1.1
FOR P=1 TO 3
    PRINT "Run #:";P
    CALL RetentionCalc (P)
    CALL CRFCalc (P)
    IF CRF!(P) > CRF!(P-1) THEN W = M - CRF!(P)
NEXT P

```

Start:

```

LET B = 1: REM B is simplex expansion coefficient

```

Flop: GOSUB WorstV

```

LET W = W2

```

Flip: GOSUB ReflectedV

```

REM Check whether best vertex has been retained again

```

```

IF W8 < VAR+1 THEN GOTO Bypass

```

```

PRINT ""

```

```

PRINT "The best vertex has been retained in "VAR + 1" experiments and may be a
falsely high result."

```

PRINT "It is suggested that you recheck the starting parameters."

LET I = W9

LET W8 = 0

INPUT "Press return to continue";A\$

Bypass:

REM Print new variables

PRINT ""

PRINT "The next experiment will use "

LET I = VAR +2

PRINT "For variable tG: ";tG!(I)

PRINT "For variable K: ";Kappa!(I)

INPUT "Do you wish to quit? If so, type Q";Q6\$

IF Q6\$ = "Q" THEN GOTO Finish

IF B1 = 3 THEN LET B1 = 1

LET CRF!(I) = 1E+20

PRINT ""

INPUT "Are these valid conditions (Y/N)";A\$

IF LEFT\$(A\$,1) <> "N" THEN GOTO Again

PRINT "A very poor result has been entered"

LET W8 = W8-1: REM Don't count this as an experiment

LET CRF!(I) = -1000000!

GOTO DistanceCalc

Again: REM input next results

LET I = VAR + 2

LET B = 1

PRINT ""

CALL RetentionCalc (I)

CALL CRFCalc (I)

DistanceCalc: LET DIST = ABS(M-CRF!(VAR+2))

REM W6 is the best result so far

IF DIST < W6 THEN LET W6 = DIST

LET W8 = 0

REM Check whether best vertex retained again

IF DIST >= W3 THEN LET W8 = W8 + 1

IF B1 = 5 THEN GOTO Skip1

IF DIST >= W3 AND DIST < W4 AND B1 = 1 THEN GOTO Skip2

IF DIST >= W5 AND B1 = 1 THEN LET B = -.5

IF DIST < W5 AND DIST > W4 AND B1 = 1 THEN LET B = .5

IF B < 1 THEN LET B1 = 5

IF DIST < W3 THEN LET B1 = B1 + 1

IF DIST < W3 AND B1 = 2 THEN LET B = 2

IF DIST < W3 AND B1 = 3 THEN GOTO Skip1

IF B1 = 2 AND B = 1 THEN GOTO LastV

GOTO Flip

Skip1: LET B1 = 1

Skip2: LET tG!(W) = tG!(VAR+2)
LET Kappa!(W) = Kappa!(VAR+2)
LET CRF!(W) = CRF!(VAR+2)

GOTO Flop

WorstV:

LET W2 = 0

FOR K = 1 TO 2

LET X1 = 1

LET W1 = 0

LET W3 = 1E+20

LET J = VAR + 1

FOR I = 1 TO VAR + 1

IF I = W2 THEN GOTO Skip3

LET x = M - CRF!(I)

IF ABS(x) > X1 THEN

LET W1 = I

LET X1 = ABS(x)

END IF

REM W9 is pointer to best vertex

IF ABS(x) <= W3 THEN

LET W3 = ABS(x)

LET W9 = I

END IF

Skip3: NEXT I

IF W2 = 0 THEN

LET W5 = X1

LET W2 = W1

END IF

LET W4 = X1

NEXT K

RETURN

LastV:

LET tG!(W) = tG!(VAR+3)

LET Kappa!(W) = Kappa!(VAR+3)

LET CRF!(W) = CRF!(VAR+3)

LET B1 = 1

LET W9 = W

GOTO Flop

ReflectedV:

IF B $\diamond$ 2 THEN GOTO Skip4

LET tG!(VAR+3) = tG!(VAR+2)

LET Kappa!(VAR+3) = Kappa!(VAR+2)

LET CRF!(VAR+3) = CRF!(VAR+2)

Skip4: LET P0 = 0

LET P1 = 0

```

FOR I = 1 TO VAR+1
    IF I = W THEN GOTO Skip5
    LET P0 = P0 + tG!(I)
    LET P1 = P1 + Kappa!(I)
Skip5:
    NEXT I
    LET P0 = P0/VAR
    LET P1 = P1/VAR
    LET tG!(VAR+2) = P0 + B * (P0 - tG!(W))
    LET Kappa!(VAR+2) = P1 + B * (P1 - Kappa!(W))
RETURN
Finish:
END

```

```

SUB RetentionCalc (Numbr) STATIC
SHARED GradStep!(), kprime!(), Solute$, Veo!(), Vm!(), StepConc!(), Outlet!(), tR!(),
GradExt!()
SHARED V5,V4, V3, V2, V1, V0, VM5, VM4, VM3, VM2, VM1, VM0, ColMAX!,
SoluteMAX%
SHARED Kappa!(), tG!(), Cg

LET J2% = 0
LET OM0 = 0
LET StepConc!(0) = OM0

```

```

LET Veo!(0) = FNFOP!(V5,V4,V3,V2,V1,V0,OM0)
LET Vm!(0) = FNFOP!(VM5,VM4,VM3,VM2,VM1,VM0,OM0)
LET AVG! = OM0
PRINT ""
PRINT "RUN: ";Numbr

FOR T% = 1 TO 200
    LET GradStep!(T%,1) = Veo!(T%-1)/2
    FOR Z% = T% TO 2 STEP -1
        LET GradStep!(T%,Z%) = GradStep!(T%-1,Z%-1) + GradStep!(T%,1)
    NEXT Z%
    IF GradStep!(T%,T%) <= ColMAX! THEN
        LET Outlet!(T%) = OM0
    ELSE
        LET Count% = 0
        LET ColPen! = GradStep!(T%,T%)
        WHILE ColPen! > ColMAX!
            LET ColPen! = ColPen! - (Veo!(Count%)/2)
            LET Count% = Count% + 1
        WEND
        LET Outlet!(T%) = StepConc!(Count%)
    END IF
    IF T% <= (tG!(Numbr))*2 THEN
        LET StepConc!(T%) = Cg*((T%/2)/tG!(Numbr))^Kappa!(Numbr)
    ELSE

```

```

    LET StepConc!(T%) = StepConc!(T%-1)
END IF
LET AVG! = (StepConc!(T%) + Outlet!(T%))/2
LET Veo!(T%) = FNFOP!(V5,V4,V3,V2,V1,V0,AVG!)
LET Vm!(T%) = FNFOP!(VM5,VM4,VM3,VM2,VM1,VM0,AVG!)
NEXT T%

FOR U% = 0 TO SoluteMAX%
    LET K! =
    FNFOP!(kprime!(U%,5),kprime!(U%,4),kprime!(U%,3),kprime!(U%,2),kprime!(U%,1),kprime!(U%,0),StepConc!(0))
    LET Vb! = (1/(1+K!))*(K!*Vm!(0) + Veo!(0))
    LET Position! = Vb!/2
    FOR Time% = 1 TO 200
        LET Location% = 1
        WHILE GradStep!(Time%,Location%) < Position!
            LET Location% = Location% + 1
        WEND
        LET N% = Time% - Location% + 1
        LET K! =
        FNFOP!(kprime!(U%,5),kprime!(U%,4),kprime!(U%,3),kprime!(U%,2),kprime!(U%,1),kprime!(U%,0),StepConc!(N%))
        LET Vb! = (1/(1+K!))*(K!*Vm!(Time%) + Veo!(Time%))
        LET Position! = Position! + (Vb!/2)
        IF Position! > ColMAX! THEN GOTO Out
    NEXT Time%

```

```

Out: LET tR!(U%) = (Time% - ((Position! - ColMAX!)/Vb!))/2
PRINT ""
PRINT Solute$(U%); " has a retention time of "; tR!(U%); " min."
PRINT ""
NEXT U%
END SUB

```

```

SUB CRFCalc (Vertx) STATIC
SHARED tR!(), SoluteMAX%, CRF!()

```

```

LET SUM!=0
FOR K = 1 TO 10
    LET Strike%(K) = 0
NEXT K
FOR N = 1 TO SoluteMAX%
    LET Q = 1
    WHILE Strike%(Q) > 0
        LET Q = Q + 1
    WEND
    LET Tester! = tR!(Q)
    LET Counter = Q
    FOR J = 1 TO SoluteMAX%
        IF Strike%(J) = 0 AND tR!(J) < Tester! THEN
            LET Tester! = tR!(J)
            LET Counter = J
        
```

```

ELSE
END IF

NEXT J

LET Strike%(Counter) = 1

LET Ret!(N) = Tester!

NEXT N

LET Ret!(0) = tR!(0)

FOR Z = 1 TO SoluteMAX% -1

    LET SUM! =(2/((Ret!(Z+1)-Ret!(Z))^-1 + (Ret!(Z)-Ret!(Z-1))^-1)) + SUM!

NEXT Z

LET CRF!(Vertx) = (SoluteMAX%*SUM!)/Ret!(SoluteMAX%)

PRINT "CRF =";CRF!(Vertx)

END SUB

```

Vita

Alexander Craig Powell was born on the MacDill Air Force Base, Tampa, Florida on December 3, 1964. Since his father was a chaplain in the Air Force, he and his family moved around a great deal as he grew up. He lived in places as varied as England, Alabama, Thailand, California, and South Carolina, where he graduated from Chester Senior High School in June 1982. The following fall, he entered Presbyterian College, a small, liberal arts college in Clinton, South Carolina. In May of 1986, he graduated magna cum laude from Presbyterian with a Bachelor of Science degree, double-majoring in Chemistry and Mathematics. In August of 1986, he entered the graduate program at the University of Tennessee, Knoxville and in May 1993 received a Doctor of Philosophy degree in Chemistry.

He is presently employed as an Assistant Professor of Chemistry at his alma mater, Presbyterian College.