ELECTROKINETIC SEPARATIONS INVOLVING SURFACTANTS AND PROTEINS

by

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(ABSTRACT)

Methods for the analysis of surfactants and proteins by Capillary Electrophoresis (CE) were investigated. Several modifications of the system to achieve detection and separation of these analytes were examined. These modifications included buffer additives, sample additives and surface treatment and modification of the fused silica capillary.

For the analysis of anionic surfactants, the addition of an anionic UV absorbing compound to the buffer was investigated to achieve indirect detection of the non-absorbing surfactants. The effect on detection sensitivity and separation efficiency of the absorbing ion was examined. These parameters were affected by differences in the electrophoretic mobilities of the analytes in comparison to the absorbing ion. The use of organic modifiers was also investigated to minimize micelle formation of the surfactants which leads to zone spreading.

For the analysis of serum and urine proteins, the use of high pH buffers

was investigated to minimize solute/capillary surface interactions and achieve separation. At high pH's the proteins are negatively charged; therefore, they should be repelled by the negatively charged fused silica surface. To improve reproducibility of migration times of the proteins the addition of polyvinyl alcohol to the sample was also investigated. The polyvinyl alcohol improved reproducibility by reversibly covering the active sites on the capillary surface to minimize protein interactions. Migration time reproducibility was also improved by optimizing the capillary cleaning procedure. Lastly, the addition of methyl cellulose to the buffer to work as a dynamic molecular sieving medium was investigated to improve resolution.

Analyte/capillary surface interactions are a major limitation in CE especially for the analyses of proteins. The use of coated capillaries to eliminate these interactions has been widely investigated. However, reproducibility and degree of surface deactivation with these coating can be poor. In this work hydrothermal treatment of the fused silica capillary surface prior to deactivation was examined. Hydrothermal treatment was used to produce a homogenous surface prior to coating which leads to the production of more highly deactivated, reproducible columns. The effects of the treatment were studied by coating the surface with a silane and examining the influence of the coating on electroosmotic flow and analyte adsorption.

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CHAPTER I

INTRODUCTION AND HISTORICAL

1.1. Electrophoresis

Electrophoresis is the migration of electrically charged particles or ions in solution due to an applied electric field. Research in the area of electrophoresis began as early as 1791 when Faraday [1] presented his laws of electrolysis . Electrophoresis has since become one of the most widely used separation techniques for proteins and other biomolecules. In fact, over half of the publications in the field of biochemistry currently use some form of electrophoresis for sample analysis [1].

There are several modes of electrophoresis currently being employed [2]. These include moving boundary, isotachophoresis, zone (including zone with molecular sieving), and isoelectric focusing [3,4]. The use of two-dimensional electrophoresis (separation by one mode followed by a second mode in a direction perpendicular to the first) makes the technique even more powerful [5].

The first reproducible, widely used form of electrophoresis was introduced by Tiselius in 1930 in his thesis "The Moving Boundary Method of Studying the Electrophoresis of Proteins" [2]. Tiselius was building on the work of Svedberg

in ultracentrifugation of proteins [6]. However, ultracentrifugation had not allowed for complete separation of the protein molecules into discrete zones. Tiselius hope to achieve this result with moving boundary electrophoresis.

The experiment was performed in a quartz U-tube and detection was obtained by photography employing an ultraviolet light. The sample was placed as a long band between two buffer solutions inside the tube. An electric field was then applied causing the proteins to begin migrating. Migration direction and rate were determined by each protein's electrophoretic mobility. However, the boundaries of the zones were blurred due to the thermal convection caused by electrical heating in the solution [2]. Tiselius reduced the electrical heating problem by using a rectangular cell and cooling the system to 4° C [7]. In 1948, he won the Nobel Prize partly for his developments in moving boundary electrophoresis. However, because the zones are not completely resolved with this technique it is not widely employed today [5].

Moving boundary electrophoresis lead the way to the development of free zone electrophoresis. In this mode, the sample is introduced as a narrow zone or band, surrounded by buffer [5]. In truth, free zone electrophoresis differs from moving boundary electrophoresis only by how the sample is introduced. Upon application of an electric field, each zone begins to migrate based upon its mobility. Ideally, each zone should be completely separated or resolved from its neighboring zones. To achieve complete separation, the differences in the

mobilities of each of the samples components must be maximized and zone spreading must be minimized.

However, in free zone electrophoresis convection is a major problem even with very small electric fields (1 kV/m). The passage of current through the medium results in the production of joule heat. Dissipation of this heat can only take place at the wall of the electrophoresis chamber. This produces a temperature gradient where the medium is hotter in the center of the chamber and cooler at the walls. In turn, a density and viscosity gradient are also formed, leading to convection currents. These currents can easily destroy the separation by causing mixing of the zones. Therefore, the use of anticonvection stabilizers were investigated to make the technique more viable and reproducible.

The first breakthrough in anticonvective stabilizers was by Wieland and Fischer [8] and others [9,10] using filter-paper as a support for the analysis of serum. Thus, the invention of paper electrophoresis. The success of paper electrophoresis led to other stabilizers such as cellulose acetate, glass beads, and starch gels.

Gels of gelatin and agar had been used as supports since the introduction of the technique, but little progress was made with these until about 1955. Starch gels for electrophoresis of proteins were pioneered by Smithies [11]. Later, Hjerten [12] prepared granulated dextran gels for use in electrophoresis and

discovered the molecular sieving properties (commercially known as Sephadex). He later searched for a more reproducible, well defined gel where the pore size could be easily chosen for a particular separation. He and others [13] discovered that polyacrylamide gels could fulfill these requirements. , In 1961, Hjertèn introduced agarose gels, the neutral component of agar, for use in electrophoresis [14,15]. These stabilizers have the following advantages over free solution: 1) gels permit added separation based on molecular sieving [16]; 2) pH gradients can be formed when using gels; and 3) the stabilizers can be easily stained and manipulated allowing for easier handling and generation of a permanent record. Agarose and polyacrylamide gels are still popular and widely employed.

However, these stabilizers can introduce forms of zone spreading other than those caused by convection. One source of zone spreading can occur due to eddy migration caused by the non-uniformity of the pores (meaning, not all possible routes of migration through and around the stabilizer will yield the same net migration). Also, the high surface area of the porous stabilizers will create extensive sites for adsorptive interactions between the stabilizer and the analytes. Lastly, even with the anticonvective stabilizers a temperature gradient will form causing an increase in mobilities in the center of the chamber versus the walls. This will lead to skewed zones, where the center is migrating faster than the edges [17].

Even with the wide acceptance and use of electrophoresis by biochemists, chemists have until recently shown little interest in the separation technique. This may be attributed to the belief that the technique is time consuming, labor intensive and yields limited quantitative results. Also, chemists are much more accustomed to chromatographic techniques which have well developed instrumentation, including on-line detection and automated sample injection, and a wide range of established applications. However, with the introduction of High Performance Capillary Electrophoresis (HPCE) [18,19] the above limitations can be overcome.

HPCE was first introduced by Everaerts et. al. in 1979 [17]. Everaerts work was the first free zone electrophoresis performed in a capillary. The separation compartment was formed by a 0.2 mm id PTFE tube. A sixteen component sample was separated and detected with potential gradient detectors used in the conductance mode. Separation efficiencies were not exceptionally large, but, results appeared promising. In 1981, Jorgenson and Lukacs [18] expanded on the work of Everaerts using 75 μm id fused silica capillaries with much improved results in terms of efficiency.

HPCE allows for separation based on the same principle as conventional electrophoresis; that charged species will migrate in solution when placed in an electric field. However, separation is carried out in a fused silica capillary that serves as a micro-separation chamber. The fused silica capillaries, with typcial

lengths of 50 - 100 cm and internal diameters of 50 - 200 μm, are able to dissipate the joule heat produced when an electric field is applied. Heat dissipation is very efficient due to the large ratio of the surface area of the capillary to its volume. In fact, these small capillaries are so efficient that very high electric fields (up to 300V/cm) have been used without encountering the convection problems seen in conventional electrophoresis. Therefore, no anticonvective stabilizers are needed to perform HPCE. However, it is important to note that all the modes of conventional electrophoresis, not just zone electrophoresis, can be done by HPCE. This includes the use of gels to gain the added spearation mechanism of molecular sieving. The use of the high electric fields also allows for very rapid (less than 20 min), highly efficient separations (over 1 million theoretical plates).

1.2. Research Objectives

Since HPCE is such a young technique there are many possible new applications and areas of research . However, to prove that HPCE is a valuable separation technique it must compete with other well established techniques such as Gas or High Performance Liquid Chromatography (GC and HPLC). Since HPCE is best suited for ionic or ionizable compounds, this research will focus on the separation of two such classes of compounds, anionic surfactants and

proteins. These analytes have proven to be difficult to analyze by other separation techniques. As previously discussed there are several modes of electrophoresis that could be used for analysis. However, this work will focus on capillary zone electrophoresis (CZE) and modifications to this mode of electrophoresis to produce and optimize the separation of these analytes.

1.3. Capillary Zone Electrophoresis

Jorgenson and Luckacs [18] were the first to name the technique, Capillary Zone Electrophoresis, based on their work in 1981. Since its introduction, interest in the technique has grown dramatically. The interest can be seen by the number of publications involving the technique since its introduction (see Figure 1).

1.3.1. Theory of CZE

Separation in free zone electrophoresis is based on the electrophoretic mobility of the analytes. The analyte's mobility is dependent upon its charge and size. According to Coulomb's law, the force exerted on a charge in an electric field is:

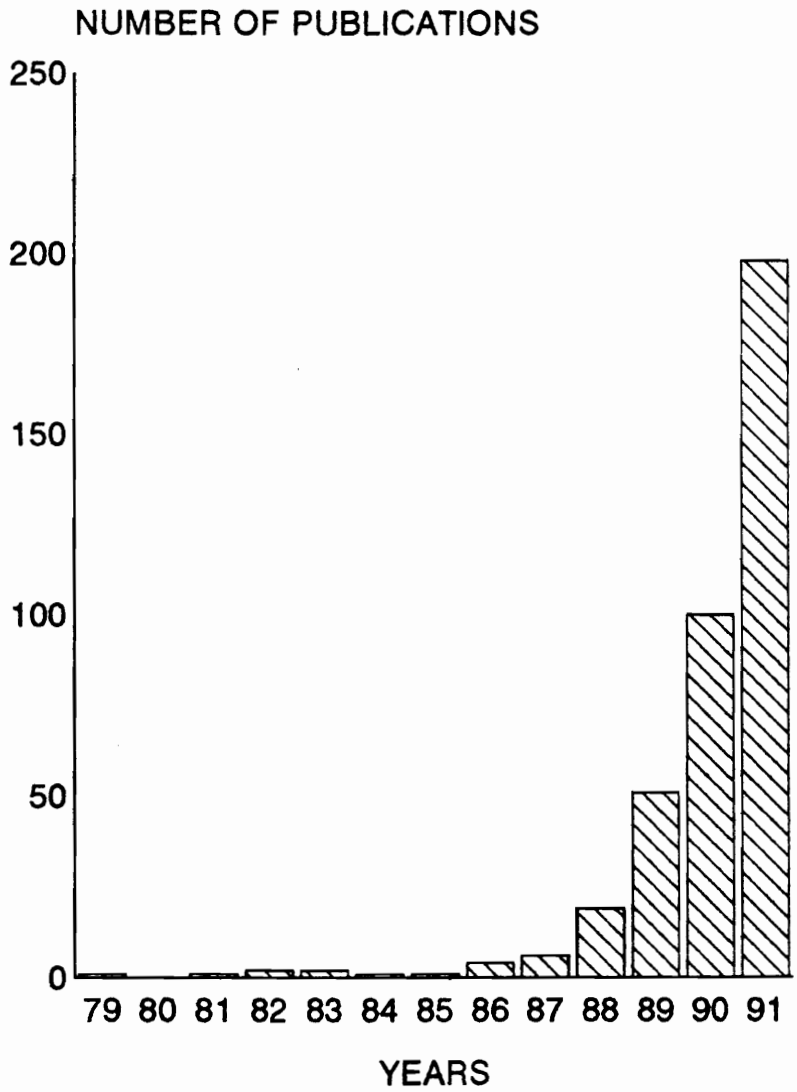

Figure 1. Number of publications in Capillary Zone Electrophoresis.

$$F = zeE \tag{1}$$

where z is the charge of the analyte, e is the charge of an electron, and E is the applied electric field. By Newton' s law the charged analyte should be accelerated toward the oppositely charged electrode with a velocity that increases at a constant rate. However, this only holds true if the analyte is in a vacuum. In a solution the motion of the ionic analyte will be slowed by a frictional drag. Because of this, the analyte will reach a terminal velocity (electrophoretic velocity), v_{el} [20]:

$$v_{el} = \frac{zeE}{f} \tag{2}$$

where f is the friction constant.

The frictional drag on the analyte is primarily a viscosity effect. Stokes' Law gives the friction constant (f) for a sphere or radius as:

$$f = 6\pi\eta r \tag{3}$$

where η is the viscosity of the solvent medium and (in the case of electrophoresis) r is the hydrodynamic radius of the solvated analyte.

Combining and rearranging equations 2 and 3, electrophoretic velocity can be expressed as:

$$v_{el} = \frac{zeE}{6\pi\eta r} \tag{4}$$

Therefore, cationic analytes will move towards the cathode based on their +z/r ratios. Conversely, the anionic analytes will progress towards the anode based on their -z/r ratios.

CZE differs from free zone electrophoresis based on the fact that the separation chamber is a fused silica tube. To understand the separation in CZE, the apparatus used will be briefly described here; however, it will be covered in more detail later when the instrumentation of this thesis is discussed. In CZE, each end of the fused silica capillary is placed in a buffer reservoir. Towards one end of the capillary a small amount of the protective polyimide coating is removed to form a window in the capillary. This window is placed in the detector and aligned with its optical path to form an on-column detection cell. The sample is introduced at the end of the capillary opposite the detector. With the two ends of the capillaries placed in the buffer reservoirs an electric field is applied. The end of the capillary where the sample is introduced is conventionally the anode and the detection end the cathode (Fig. 2).

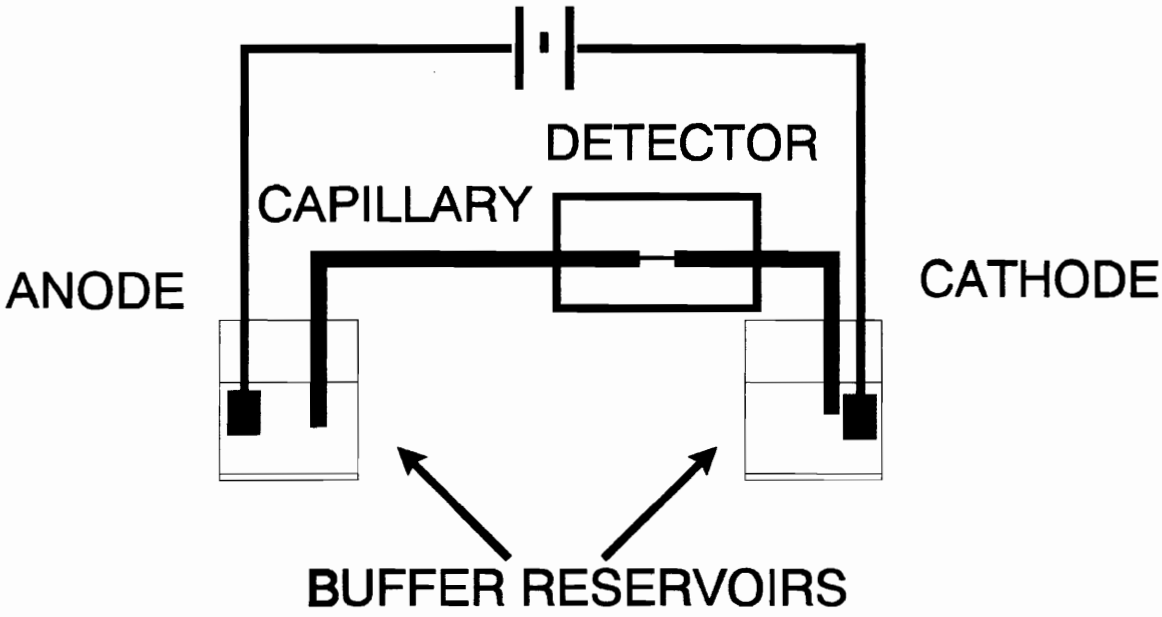

Figure 2. Schematic of CZE Apparatus

Based on this instrumental design the cationic analytes will migrate toward the detector and be detected. Furthermore, based on conventional electrophoresis the anionic species should migrate in the opposite direction away from the detector and never be seen. However, anions can also be moved through the capillary towards the cathode by a phenomenon known as electroosmotic flow [18]. Mathematically, electroosmotic velocity can be expressed by the following equation:

$$v_{eo} = \frac{-\varepsilon \zeta E}{4\pi\eta} \tag{5}$$

where ε is the dielectric constant of the buffer solution, ζ is the potential across the electric double layer [21]. Electroosmosis is the means of transport for the analytes and can be considered the pump in CZE. Electroosmotic flow arises due to the chemical nature of the surface of the fused silica capillary. When the capillary is filled with the aqueous buffer (above pH 3.0) the surface silanol groups are ionized. This forms a net negative charge on the surface. To counter balance this negative charge, an electrical double layer of counterions forms. This leads to the formation of a potential across the capillary /buffer interface. The structure of the electrical double layer was first described by Stern [22] in in 1924. The counterion double layer can be divided into two parts: 1) a layer of

strongly held counterions adsorbed close to the charged surface on fixed sites; and 2) a diffuse layer of counterions extending into solution. The two parts of the electrical double layer are separated by the Stern plane [22,23] (Fig. 3). When an electric field is applied across the capillary the solvated cations in the double layer migrate towards the cathode. As they migrate they drag along the bulk electrolyte solution via momentum transport. Therefore, the net velocity (v_{net}) of analyte under the influence of electroosmosis becomes:

$$v_{net} = v_{el} + v_{eo} \tag{6}$$

It can be seen that as long as the v_{eo} is larger than the v_{el} for anionic compounds they can be transported towards the cathode. From equation 6 the order of elution in CZE can determined as follows: 1) positive ions, 2) neutral molecules, and 3) negative ions (Fig. 4).

As mentioned earlier, CZE separations can generate up to 1 million theoretical plates. Electroosmotic flow is the primary reason for these highly efficient separations. When an electric field is applied across the capillary, the bulk electrolyte solution throughout the capillary begins moving instantaneously. This produces a flat flow profile versus laminar flow found in chromatography (Fig. 5). Equation 5 shows that there if no radial velocity dependence for electroosmotic flow. This holds true as long as the radius of the capillary is 10

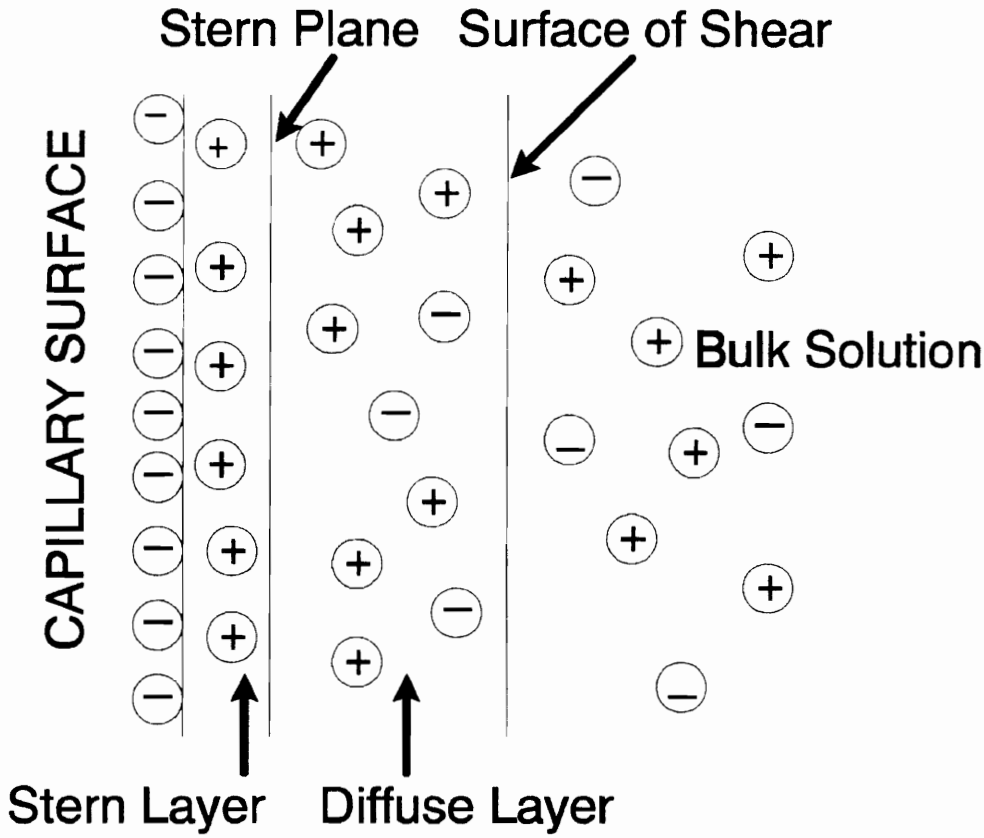

Figure 3. Schematic representation of the structure of the electric double layer according to Stern's theory.

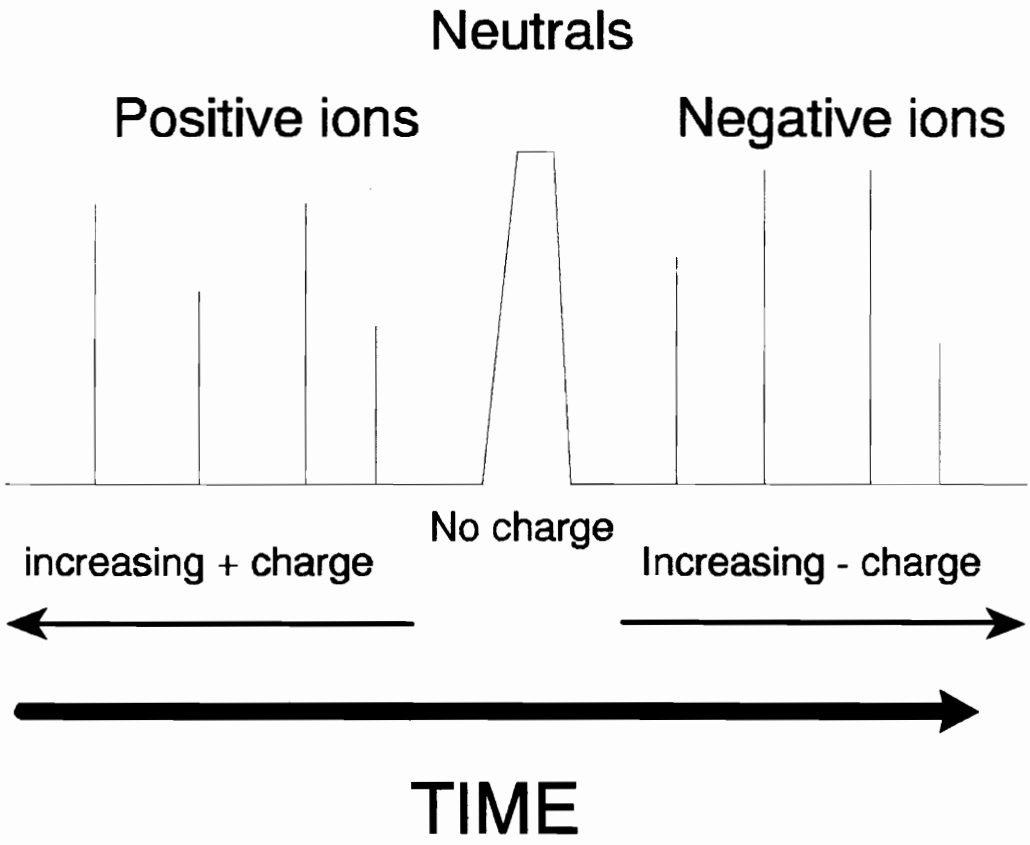

Figure 4. Order of elution of analytes in CZE.

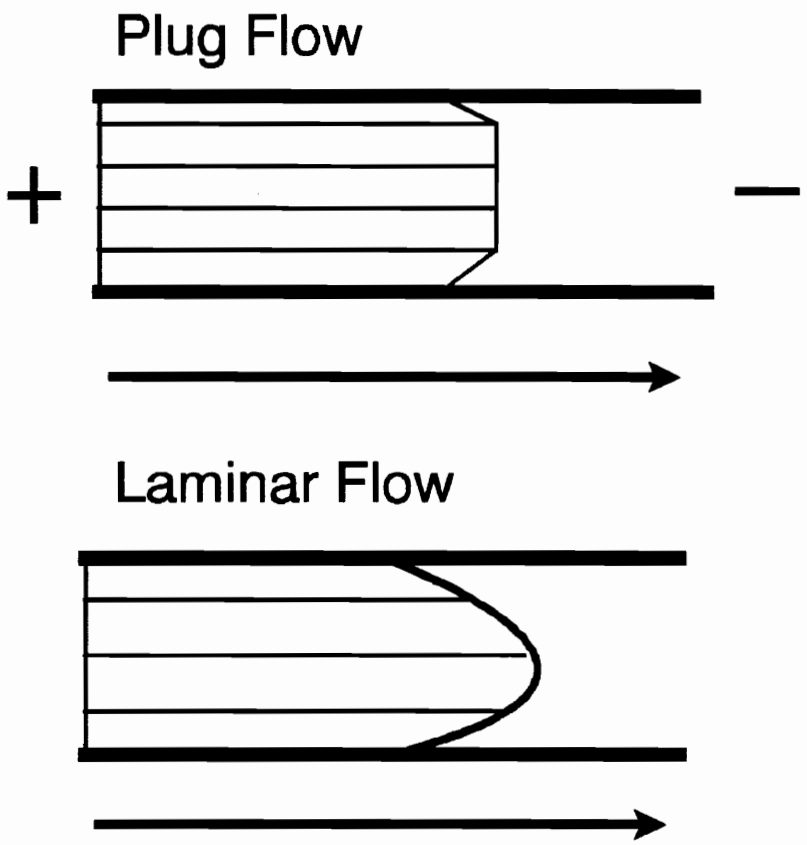

Figure 5. Flow profiles found in CZE and HPLC.

times the width of the double layer [24]. This is not a problem in CZE since typical capillary id's are 50-200 μm and typical double layers in aqueous solutions are 10 nm [24].

Since the technique and instrumentation are parallel to chromatography, Jorgenson [17] and Knox [24] borrow the concepts of migration time (retention times), theoretical plates, and resolution from chromatography. In CZE the migration time for a solute is given by the following equation:

$$t = \frac{L^2}{(\mu_{el} + \mu_{eo})V} \tag{7}$$

where L is the length of the capillary, μ_{el} is the solute's electrophoretic mobility (velocity in a unit electric field, $\mu_{el} = v_{el} E$), μ_{eo} is the electroosmotic flow (electroosmotic velocity in a unit electric field, $\mu_{eo} = v_{eo} E$), and V is the applied voltage. Jorgenson[17] also showed that with the plug flow profile in an open tube, efficiencies in CZE are limited only by longitudinal (axial) diffusion. Therefore, the separation efficiency can be expressed by the following:

$$N = \frac{(\mu + \mu_{eo})V}{2D} \tag{8}$$

where D is the solute's diffusion coefficient.

Equations 7 and 8 lead to some interesting predictions. First, optimal efficiencies can be obtained by employing high voltages. Second, length plays no role in separation efficiencies, but has an extreme influence over run times. Therefore, it would seem that optimal separations should be performed at high voltages in short capillaries. However, there is one factor that has been ignored. That is the production of joule heat when an electric field is applied to an aqueous buffer. The following equation shows the relationship of joule heat to voltage and current:

$$P = I^2R = VI \tag{9}$$

where P is joule heat, I is current, R is resistance and V is applied voltage. At higher voltages there will be more joule heat generated and as the capillary gets shorter there is less surface area to dissipate that heat. . Also, at constant voltage, more current is generated as the capillary gets shorter producing more joule heat. This leads to a temperature gradient across the capillary (where the solution in the center of the tube is hotter than solution at the walls). This temperature gradient results in a density gradient (leading to convective flow), producing a flow profile similar to laminar flow. Laminar flow leads to a loss in efficiency; therefore, there is a limit to how short the column can be when using

a particular applied voltage.

Equation 8 also implies that increases in electroosmotic flow will increase efficiencies. However, this is a misleading approach to enhance separation. Higher electroosmotic flows will yield high separation efficiencies in regards to the number of theoretical plates produced;but, it is actually detrimental to the resolution of the zones [19]. Since electroosmotic flow affects all substances in the same way, it does not aid in separation. The only effect of high electroosmotic flows is to flush all the solutes rapidly through the capillary. This will results in sharp zones, but, it does not allow enough time for complete resolution of those zones. Expanding on Giddings [25] approach, Jorgenson and Lukacs [19] derived the following equation for resolution in CZE:

$$R_s = 0.177\ (\mu_1 + \mu_2)\ [\frac{V}{D(\mu_{ave} + \mu_{eo})}]^{1/2} \qquad (10)$$

where R_s is the resolution, μ_1 and μ_2 are the electrophoretic mobilities of the two solutes, and μ_{ave} is the average of their mobilities. This equation shows that if electroosmotic flow is large the resolution between two zones will be poor. The equation also shows that if the average mobility and electroosmotic flow are equal but in opposite direction, maximum resolution would be obtained. Therefore, a way to optimize resolution is to try to balance the electroosmotic

flow against the average electrophoretic migration. However, the cost of this approach is increased analysis time.

1.3.2. Instrumentation for CZE

Since CZE's introduction many advances have been made in developing and improving instrumentation. The instrumentation used in CZE is fairly simple and straightforward. It consists of a high voltage power supply (0 - 30 kV), two buffer reservoirs (high voltage and ground), the capillary, a detector, and a data handling device (Fig. 6). This design was introduced by Jorgenson and Lukacs in their original work [19].

One important detail to notice when examining the instrumental design is that there is no apparent direct means for sample introduction. In chromatography there is an injection port or sample loop to introduce the sample into the system. In CZE, several methods of sample introduction have been investigated. Some hardware injectors including rotary valves [26], split-flow injectors [27,28,29], and microinjectors [30,31] have been developed; however, these are not widely used due to their complexity. Easier modes of injection include electrokinetic [17,19,32,33], hydrodynamic/hydrostatic [32-34] or pressure driven [35] (both positive and negative). They can be performed manually or automated and are available on most commercial systems.

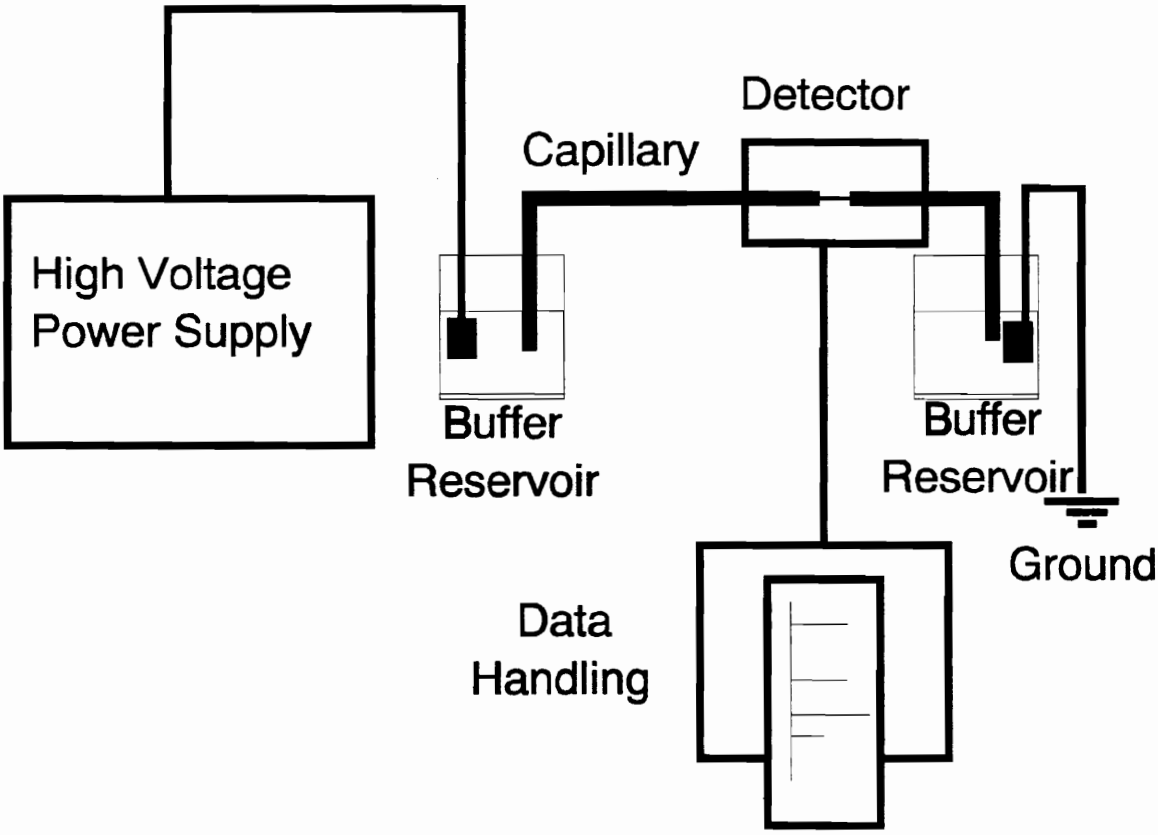

Figure 6. Schematic of instrumentation for CZE

Electrokinetic injection is performed by replacing the operating buffer reservoir at the high voltage end with a reservoir containing the sample. A small potential (1-5 kV) is then applied for a short period of time (1-10 s) causing the sample to migrate on to the head of the column due to the components electrophoretic mobilities and electroosmotic flow. Since electroosmotic flow is towards the cathode, injection is usually made at the anodic end (Fig. 7). The amount of sample introduced can be calculated by:

$$S = \frac{(\mu_{ep} + \mu_{eo})\pi r^2 V_i \, t_i}{L} \cdot C \qquad (11)$$

where S is the amount of sample injected, r is the radius of the capillary, t_i is the introduction time, V_i is the introduction voltage, and C is the sample concentration[32]. From the equation it can be seen that the amount of sampleintroduced can be controlled through variations in the introduction voltage and time. This injection technique is fairly reproducible (1-5% RSD) and simple to use. However, since many components have a different electrophoretic mobility there will be sample discrimination especially for small, negatively charged components.

Hydrostatic injection is also performed by replacing the buffer reservoir with a sample reservoir. But, instead of using electroosmotic flow and

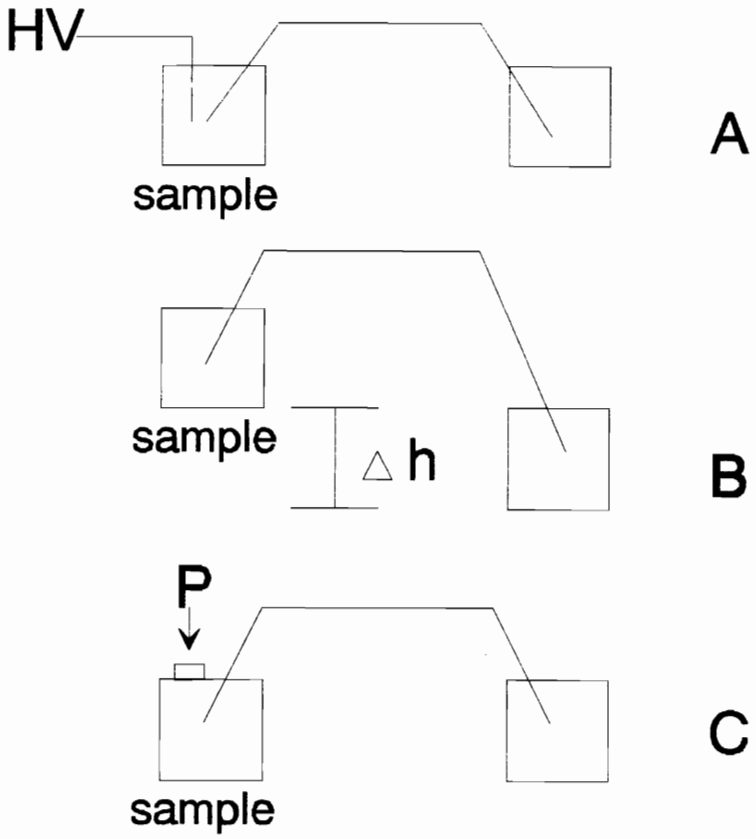

Figure 7. Injection Modes in CZE: A) Electrokinetic, B) Hydrostatic, and C) Pressure.

electrophoretic mobilities to transport the analytes onto the capillary head, hydrodynamic or gravity flow is used. With this mode of injection the sample reservoir is elevated a small distance (1 - 10 cm) for a short period of time (1 - 10 s) (Fig. 7). Due to gravitational flow, a small plug of sample will move onto the head of the column. The amount of sample that is introduced can be calculated by the following equation:

$$S = \frac{\rho g \pi r^4 \Delta h t_i}{8 \eta L} \tag{12}$$

where ρ is the density of the sample solution, g is the constant of gravitational acceleration, r is the capillary inner radius, Δh is the height difference, t_i is the introduction time, η is the viscosity of the sample solution and L is the column length. The quantity of sample that is introduced with hydrodynamic flow can be controlled by varying the time interval and height difference. In comparison to electrokinetic injection, there should be no discrimination because the quantity of sample introduced is independent of electrophoretic mobility. This technique is also simple to use and can be reproducible especially with an automated system. However, with larger id capillaries sample control is more difficult. It is also important to note, hydrodynamic flow can occur in the system if the buffer reservoir levels are not even. This can drastically affect the

reproducibility of migration times and flows.

Pressure injection is performed by replacing the buffer reservoir with a sample reservoir and using pressure (positive or negative) to force the sample onto the head of the column. With positive pressure, the pressure is applied at the head of the column where the sample reservoir is located. With negative pressure, a vacuum is pulled at the opposite end of the capillary and the sample is drawn into the capillary. This technique has fair reproduciblity (1-5% RSD), but it does require more complicated instrumentation than the other two injection modes previously described. It can also be difficult to control the sample flow when large columns are used.

All the previously described techniques are available on commercial systems. All systems come with electrokinetic injection and either hydrodynamic or pressure driven injection. Since the commercial instruments have automated injection systems, the reproducibility of these techniques are much better than on a "home built" systems (1-2% compared to 5% RSD). However, due to ease of use, electrokinetic and hydrodynamic flow are the most commonly employed modes with home built systems.

Once the sample is introduced into the column and the buffer reservoir placed in line, an electric field is applied. The high voltage power supply is used to maintain and control the electric field. The power supplies typical generate 0 - 30 kV. They are designed to maintain constant voltage and/or constant

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current (comparable to constant pressure and constant flow in chromatography). An added feature is the ability to reverse the polarity of the voltage, enabling an analysis to be performed with the cathode at the high voltage end and the anode at the ground end of the capillary. This allows separation to be performed when using coated or modified capillaries that eliminate or reverse electroosmotic flow.

The separation is carried out in a fused silica capillary with typical inside diameters of 50 - 200 μm [18-19]. Typical lengths for the capillaries are 30 - 100 cm. However, when short or 200 μm capillaries are used some type of cooling must be employed to control the joule heat. Also, by having temperature control over the capillary (thermostating) the reproducibility of electroosmotic flow, retention times and efficiencies will be improved. There are several means of cooling the capillary. However, the most widely accepted are forced air convection [36,37,38] and liquid coolant baths [35]. The surface of the capillary and controlling electroosmotic flow will be discussed in a later section.

Most of the major advances in instrumentation have come in the area of detection. Several modes of detection have successfully been employed in CZE. There are two major types of detection available: on-column techniques, such as UV [39,40,41] and fluorescence [42,43,44], and post column technique, such as conductivity [45,46,47], electrochemical [48,49,50], and mass spectrometry [51,52,53]. Other interesting modes of detection such as thermo-optical [54], raman

spectroscopic [55], radioisotopic [56,57] , chemiluminescence [58], and fluorescence microscopic [59] have also been investigated.

On column UV detection is the most widely employed detection mode in CZE and is available on all commercial systems. Typical concentration detection limits are in the order of 1×10^{-5} to 3×10^{-7} M [39,41] . These limits are relatively poor due to the small path of the capillary cell (50 - 100 μm). Many researchers have worked to improve these detection limits by increasing the path length [60] or concentrating the sample upon injection [61,62]. Also, UV detection is a selective detection mode. To be detected by UV, the analyte must contain a chromophore.

Fluorescence detection is the second most often employed detection mode in CZE and is also available on some commercial systems. Fluorescence detection yields improved concentration detection limits (10^{-7} M)[42] compared to UV. In fact, laser induced fluorescence, as introduced by Dovichi et. al. [63], has produced concentration detection limits of 10^{-10}-10^{-12} M for some compounds[64,65]. However, with added sensitivity comes added selectivity which limits the applicability of this mode of detection even more than UV. Detection by fluorescence requires a sample that fluoresces or can be derivatized to fluoresce.

The most desirable mode of detection would provide a universal response. Conductivity detection as introduced by Zare et. al. [45] provides this universal

response, but detection limits are still very high (10^{-7} M for strong conductive analytes and 10^{-5} M for poor conducting analytes) and the detector is not commercially available. Another promising universal detector is mass spectroscopy (MS). MS provides universal response and yields valuable structural information about the analyte. Because of the small flow rates (μl/min) used in CZE, the problems with solvent elimination encountered in HPLC/MS are minimized. Several interfaces have been used in CZE/MS including electrospray [51] and fast atom bombardment [52]. However, MS is a very expensive technique and CZE/MS is still in the early research stages.

1.3.2.1. Indirect Detection for CZE

As previously discussed, one of the major weak points in CZE is the lack of a sensitive and universal detection system. Indirect detection modes might be a relatively simple solution to this problem. Several indirect approaches have been successfully employed, including indirect UV absorbance [18, 66,67], indirect fluorescence [68,69,70], and indirect amperometric [71] detection. However, for this work only indirect UV absorbance detection will be discussed in detail.

Indirect photometric detection is based on the use of an absorbing co-ion as principal component of the background electrolyte; thus, creating a high background signal. The non-absorbing analytes are detected by the reduction in

the background absorbance as a charge displacement of the absorbing co-ion occurs. This charge displacement occurs in order to maintain charge neutrality in the zone. The charge on the analyte and co-ion must be of the same sign to obtain this charge displacement.

Foret et al. [67], discussed the selection of a suitable UV-absorbing background electrolyte and the relationship with electromigration dispersion. They found that the highest sensitivity is obtained for those analytes having an effective electrophoretic mobility close to the mobility of the absorbing background ion. As the mobilities of the analytes deviate from the mobility of the background the dynamic range of this detection mode is reduced to approximately one order of magnitude. Peak shape is also highly dependent on the matching of these mobilities. If the mobilities of the analytes deviate from the effective mobility of the absorbing background ion the peak shapes will be highly skewed (Fig. 8).

Quantitative analysis with indirect UV detection is dependent on the need for a one-to-one charge displacement in the analyte zone. If a one-to-one displacement does not occur a response factor must be calculated. The Kohlrausch theory [72] shows the changes in the concentration of the absorbing background ion, d[B], caused by an analyte concentration [A] can be calculated using the following equation:

$$R = \frac{\text{mobility of co-ion}}{\text{mobility of analyte}}$$

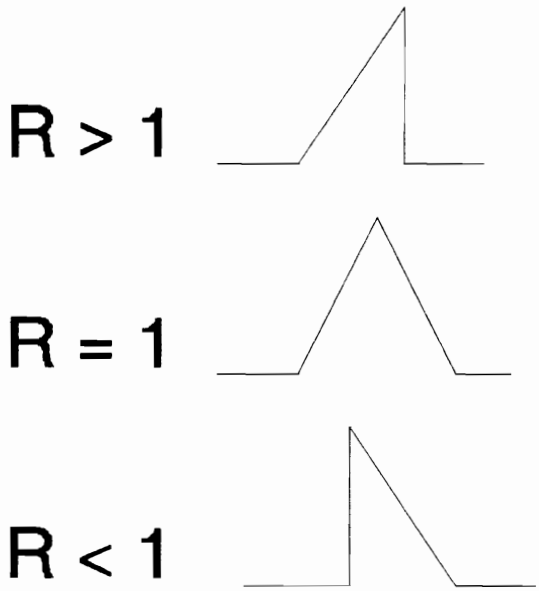

Figure 8. Effects of mobility matching on peak shape for indirect UV detection in CZE.

$$d[B] = -\frac{\mu(B)[(A) + \mu(C)]}{\mu(A)[(B) + \mu(C)]}[A] \quad (13)$$

where the μ terms are the effective electrophoretic mobility of the analyte (A), the UV-absorbing background ion (B), and the counter ion (C). From this equation it can be seen that a one-to-one charge displacement will only occur for analytes having the same mobility as the background ion. Response factors for other analytes must be calculated from accurately determined mobility data [73].

It can be seen that the selection of buffers for indirect UV detection in CZE is critical. Currently, most indirect detection is performed with monobuffers (one acid and one base). As discussed these monobuffers lead to a simple displacement between the analyte ions and the background co-ion. As also previously discussed, these systems have several shortcomings in relationship to the mobility match of the co-ion with the analyte ions. Wang and Hartwick [74], suggest overcoming these problems by employing a binary buffer system (two acids and one base). These binary systems can be made up of two absorbing species or an absorbing and non-absorbing species. The two absorbing ion buffer system will allow for detection of analytes with broad mobility ranges. The absorbing/non-absorbing buffer system will allow for better control over the separation. The non-absorbing ion can be employed to optimize the separation, allowing the absorbing analyte to be optimized for detection.

Indirect UV detection has some major shortcomings. However, these binary buffer systems make the technique feasible. Also, indirect UV is a simple alternative for the detection of analytes that do not contain a chromophore while using the same instrumentation employed by UV detection.

1.3.2.2. Capillary Surface Modification in CZE

In CZE, the capillary column is the heart of the separation. However, it can also be the Achilles' heel. To better understand this statement, the surface of the fused silica capillary must be discussed. The surface is comprised of active and inert silanol sites (Fig. 9). The active sites are where electroosmotic flow arises. However, these sites can also cause analyte adsorption due to hydrogen bonding of the analyte with the surface. This adsorption can be reversible leading to peak tailing and poor efficiencies or it can be non-reversible leading to loss of analyte (poor quantitation). Due to their hydrophobic nature, proteins, especially at low pH's, are prime candidates for analyte adsorption. Also, depending on the conditioning of the capillary column the number of active and inert silanol sites can vary with time leading to reproducibility problems with electroosmotic flow and migration times.

To solve these problems a great deal of research has been done to deactivate or modify the fused silica surface. There are two types of

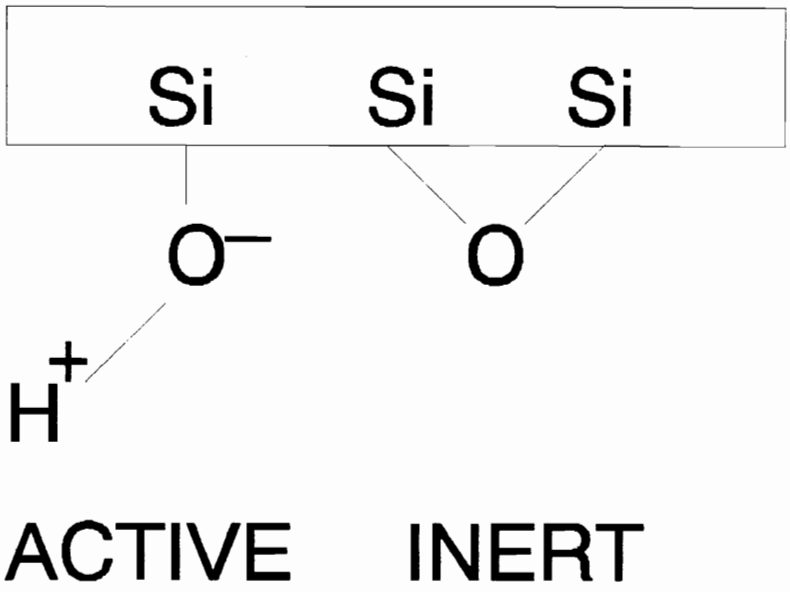

Figure 9. Surface of fused silica.

deactivation being investigated, permanent and dynamic. In permanent deactivation the deactivating compound is chemically bonded to the surface. In dynamic deactivation there is no chemical bond formed. The deactivating compound associates with the surface while it is in the system; but, it can be easily removed.

Since there is a great deal of research in fused silica surface modifications for both GC and HPLC many researchers began using similar modifications of the capillary for CZE. Hjertén [75] developed the first permanent deactivation that completely modified the surface allowing for no electroosmotic flow or analyte adsorption. This deactivation involves a silane linkage between the surface and a mono-molecular layer of polyacrylamide. These capillaries have proven to be well suited for low pH's analyses; however, at higher pH's the silane linkage is easily hydrolyzed. For the analysis of proteins, the problems of analyte interaction occur mainly at low pH's making these columns a useful complement to fused silica for protein analysis. Cobb et al. [76] improved on these columns by using a polyvinyl linkage between the surface and polyacrylamide. This improvement allows for the use of these columns up to pH 10.0.

Several other coatings have been developed for CZE of proteins including methyl cellulose [75], glycol [77], glycerol-glycidoxypropyl [78], poly(vinlypyrrolidinone) [78], polyethylene glycol (PEG) [79], maltose [80], aryl pentafluoro (AFP) groups [81], and polyethyleneimine (PEI) [82]. The goal of

these columns is to eliminate protein adsorption by covering the active sites and in turn eliminating electroosmotic flow. However, the PEI columns work under a different principle to eliminate analyte interaction. The PEI coating provides a positively charged surface and thus repels the similarly charged analytes (as well as reversing the electroosmotic flow). Eliminating analyte adsorption is a positive, however these columns also eliminate electroosmotic flow. The suppression of electroosmotic flow eliminates the ability for both positively and negatively charged species to be analyzed in the same run. Therefore, the development of hydrophylic silane phases that do not completely suppress electroosmotic flow have also been investigated [83,84].

The use of dynamic coatings is a much newer area of research. Dynamic coatings include the addition of zwitterions [85], cationic and non-ionic surfactants [86], polyacrylamide [87], and polyvinylalcohol [88] to the buffer. The solution, when flushed through the capillary allows the components to reversibly interact with the surface. The addition of zwitterions and cationic surfactants can be used to reverse the charge on the surface. Thus, the surface is positively charged which will repel basic proteins. Also, since the surface now has a net positive charge, electroosmotic flow will be reversed when using this type of deactivation. Linear polyacrylamide and polyvinylalcohol groups lay down and cover the surface active sites minimizing the surface/analyte interaction. Polyacrylamide and polyvinyl alcohol also add a potential secondary

means of separation, molecular sieving.

Both modes of deactivation have yielded promising results. For complete surface deactivation, chemically bonded surfaces are more efficient and reproducible. However, the dynamic deactivations are better for sample with dirty or complex matrices because it allows the regeneration of the surface between runs. Both modes are becoming widely used and are dependent on the separation.

1.3.2.3. Buffer Additives for CZE

To perform CZE the buffer must be an electrolyte to allow the establishment of an electric field across the capillary. The most commonly used buffers are sodium or potassium phosphate and borate. Typical concentrations are 1 mM to 100 mM. The concentration of the buffer is critical and will affect electroosmotic flow and joule heat. Higher concentrations decrease electroosmotic flows and increase the current producing joule heat. The pH of the buffer is also critical. The pH of the buffer affects the ionization of the surface and sample components. At high pH's, the surface is highly ionized producing large electroosmotic flows. Proteins will also have a net negative charge causing them to be repelled by the surface. At low pH's, the surface is poorly ionized which produces low electroosmotic flows. Proteins will also have

a net positive charge causing them to be attracted to the surface leading to adsorption.

There has been a great interest in buffer modifications to help effect the separation of neutral or hydrophobic molecules. In CZE, neutral molecules will not separate because they have no electrophoretic mobility. To gain the separation of neutral molecules, Terabe et al. [89,90] added an anionic surfactant, (dodecyl sulfate) above its critical micelle concentration, to the buffer. The micelles are formed with the polar head group forming an outer shell and the non-polar tails forming a hydrophobic core. This produces a pseudo stationary phase that allows for separations based on partitioning similar to reverse phase HPLC. This technique has been named micellar electrokinetic chromatography (MEKC). Selectivities of the micelles have been altered by the addition of tetraalkyl ammonium salts [91], methanol [92,93], metal ions [94], sodium octyl sulfate alcohols [95] and Brij 35 [96].

Separation of hydrophobic molecules has been improved by the addition of organic solvents to the buffer. Wahlbroeh and Jorgenson [97] and Fujiwara and Honda [98] showed that methanol and acetonitrile will improve the solubility of hydrophobic analytes, as well as improve the separation of positional isomers. Separation of enantiomers have been affected by the addition of chiral complexes (cylcodextrins) to the operating buffer [99,100,101,102]. Separations based on size exclusion have been shown with the addition entangled polymers

including methyl cellulose [103,104], linear polyacrylamide [87], and polyvinyl alcohol [88], to the buffer. The results of these additives allow for changes in selectivity in CZE.

1.4. Applications for CZE

Since its introduction CZE has been used for the analysis of many different analytes from small ions [70,105,106] to very large biomolecules [103,107]. Specific applications include amino acids and peptides [68,108,109,110], proteins [18,39,85], pharmaceuticals [111,112,113], and even the contents of a single cell [114]. This research focuses on applications involving both anionic surfactants and serum proteins.

1.4.1. Surfactants

Surfactants are one of the most widely utilized compounds in the chemical industry. They are most commonly used as emulsifiers and detergents in many different commercial products and chemical processes. When surfactants are placed in a chemical system at relatively low concentrations, they have the property of adsorbing onto the surface (an interface where one phase is a gas) or interface (a boundary between any two immiscible phases) of the

system. This adsorption markedly alters the surface or interfacial free energies [22,23].

The interfacial free energy is the minimum amount of work needed to create and sustain that interface. This free energy per unit area is what is measured to determine the surface or interfacial tension between the two phases. A surfactant is therefore a substance which when added to the system at relatively low concentrations drastically alters the amount of work required to sustain the interface. Surfactants are usually added to the system to reduce the work required. Thus, lowering the surface or interfacial tension of a system which will allow the chemical process to be more easily carried out [22,23].

The characteristic molecular structure of a surfactant is amphipathic. This means that the surfactant consist of two structural groups, a lyophobic group that has little attraction to the solvent and a lyophilic group which has a strong attraction to the solvent. In an aqueous system these two groups are referred to as hydrophobic and hydrophylic. The hydrophobic group is usually a long chain hydrocarbon residue. The hydrophylic group is a highly polar or ionic group. Surfactants are classified depending on the nature of their hydrophylic group as follows: 1) anionic 2) cationic 3) zwitterionic and 4) non-ionic. The type of hydrophilic group determines the surfactants chemical application due to their differing surface interactions [22,23]. This work focuses on the analysis of a specific class of anionic surfactants; therefore, only their use

and chemical structure will be discussed in detail.

The class of anionic surfactant studied in this work is ethoxylated alcohol sulfates (AEOS) which have the following general formula [22]:

$$C_xH_{2x+1}\text{-}(O\text{-}CH_2\text{-}CH_2)y\text{-}SO_4\text{-}M^+$$

where M^+ is sodium (Na^+) or ammonium (NH_4^+). Commercially available AEOS blends are mixtures of compounds varying both in alkyl chain lengths (x) and in the degree of ethoxylation (y). Therefore, they are relatively complex in the chemical make-up.

Since most natural surfaces are negatively charged, cationic surfactants will make the surface hydrophobic or water-repellent. Their positively charged hydrophilic group will orient towards the negative surface due to electrostatic attraction. Therefore, their hydrophobic tail is oriented away from the surface making the surface water repellent. Conversely, anionic surfactants, such as AEOS, will make the surface hydrophilic or water wettable. Therefore, AEOS's are often used in commercial cleaning products, such as, laundry or dishwashing detergent. They aid in cleaning by making the surface of the item more water wettable, in turn, making cleaning easier. However, if the surface is positively charged, an anionic surfactant will make the surface water-repellent. AEOS are also a good choice as detergents because they are highly water soluble due to

the ethoxylated portion of the compound [22,115].

Both the alkyl chain length and degree of ethoxylation are important factors in the performance of AEOS as a detergent. Therefore, it is highly desirable to develop a method of analysis. Due to the lack of a UV chromophore and low volatility, a direct method of chromatographic analysis has been elusive. The compounds may be desulfated and analyzed by Supercritical Fluid Chromatography [116] or Gas Chromatography [117]. The surfactants may also be derivatized for analysis be High Performance Liquid Chromatography [118]. All of the above techniques require a great deal of time and sample handling. Therefore, a more direct means of analysis should be sought. Since CE is a highly efficient separation technique, especially for ionic compounds [18] it could be well suited for anionic surfactants assuming that a suitable method of detection is developed. The absence of a UV chromophore can be overcome by employing indirect UV detection [66,67,119].

1.4.2. Serum Proteins and Kidney Function:

Since the introduction of moving boundry electrophoresis the analysis of serum proteins and globulins has been of interest. Tiselius first separated albumin and α-, β-, and γ - globulin in 1937 [7]. These proteins are of interest when found in the urine and serum of patients. The presence of these

compounds in the urine and serum can indicate possible kidney malfunction or damage.

By means of filtration, readsorption, and secretion the kidneys accomplish the following functions: 1) regulation of osmotic pressure of extracellular fluids which is accomplished by regulating the amount of water and sodium chloride excreted; 2) regulation of the electrolytic pattern of extracellular fluids which is accomplished by tubular readsorption and secretion; 3) excretion of metabolic waste such as urea, uric acid, creatinine and ammonia; 4) regulation of pH which is accomplished by controlling the excretion of hydrogen ions and electrolytes; 5) regulation of the volume of extracellular fluid to maintain constant blood volume; and 6) secretion of renin, an enzyme necessary to control blood pressure [120]. All of these functions are necessary for maintaining mormal bodily function.

The above functions are carried out in the functional units of the kidney, the nephrons. A nephron consists of a glomerus set inside a Bowman's capsule, its tubules, and its blood supply. Filtration occurs through the glomeruli capillary (Fig. 10). Approximately 5% of the glomeruli capillary surface is penetrated by pores with diameters of 70 to 100 A°. Readsorption and secretion of components takes place in the tubules [121].

Every 5 minutes the total volume of blood in the body passes through the kidneys with 20%-25% of the fluid in that blood being filtered by the glomuruli.

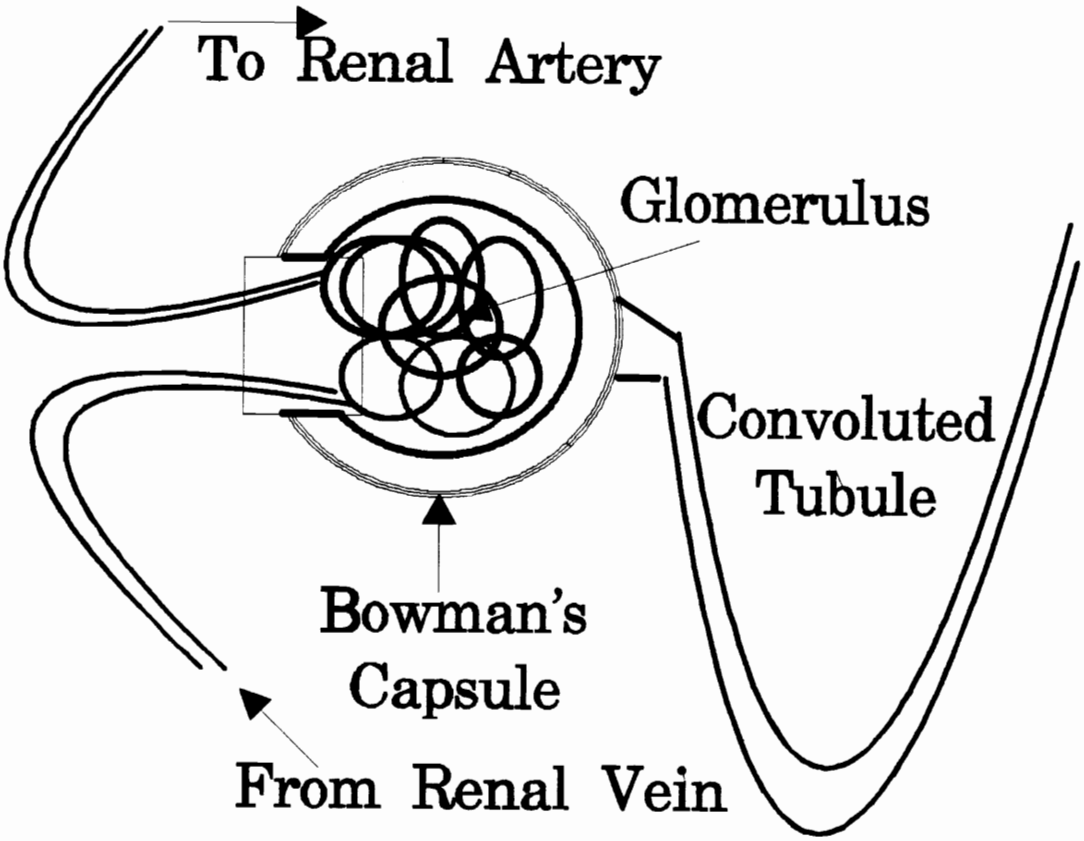

Figure 10. Schematic of a nephron.

Under normal condition the filtrate contains very little protein, indicating that the glomeruli is highly impermeable to large molecules, such as plasma proteins. Albumin, one of the smallest plasma proteins can be found in the filtrate but only in very small amounts (2mg/dL or less). This is a minute amount considering that a normal adult human must filter 3 g of albumin a day. The glomeruli is even less permeable to globulin proteins. However, smaller proteins, enzymes, and fragments of immunoglobulins can pass through the glomeruli with a fair amount of ease. The ability of these components to pass through the barrier is dependent on there size, shape, and rigidity. However, under normal conditions these components are readsorbed or secreted by the tubules as needed.[120,122]

Normal human urine contains small amounts of protein, with the most commonly found protein being albumin. A normal adult will excrete approximately 100 mg/1.5 L of urine/day. The excretion of proteins into the urine is called proteinuria. Abnormal proteinuria can occur when there is renal disease or dysfunction. However, there are other factors which can elevate proteinuria such as stress, fever, some drugs, and even strenuous exercise. Therefore, the sample collection and timing is very important when abnormal proteinuria is suspected.[120,121]

Proteinuria can be classified according to the pathophysiologic mechanism responsible for the condition (see Table 1). Many conditions and diseases can

Table I. Proteinuria Classified According to Pathophysiologic Mechanism Responsible [121]

I. Persistent Proteinuria

A. Increased glomerular permeability to plasma proteins

1. Damage to glomeruli
2. Loss of nephron mass

B. Decreased tubular readsorption of filtered proteins

C. Overflow proteinuria

D. Secretory proteinuria

E. Histuria

II. Postural proteinuria (increased protein levels when patient is in upright position)

III. Intermittent Proteinuria

A. Random findings: no known pahtologic cause

B. Nonrenal abnormalities

C. Renal diseases

D. Contamination of Urine

E. False positive due to interferences from drugs

cause proteinuria including diabetes and hypertension. Both of these conditions with time cause damage to the kidney affecting the glomuruli ability to retain larger proteins, especially with older subjects [120,121]. However, there are several other conditions that can cause proteinuria such as liver cirrhosis, inflammatory response and myeloma [123,124].

Currently, serum proteins are analyzed by agarose gel electrophoresis [125]. There are several major disadvantages to using this technique including: 1) the analysis is time consuming, taking up to 4 hrs for one analysis; 2) the separation is very inefficient resulting in poor resolution, especially with the globulin peaks (This is a problem because light chains of the globulins are obscured. These light chains yield important information about the cause of the proteinuria); and 3) a great deal of sample handling is required. This is of extreme concern due to the diseases that can be transmitted by contact with blood. Therefore, an analysis which would produce high resolution separations in reasonable time and use automated systems would be desirable. CZE should provide these options.

CHAPTER II

EXPERIMENTAL

2.1. Instrumentation

The instrumentation used for this work consisted of two systems. The first was built in-house using commercially available components according to the schematic previously described in Figure 6. In CZE, a fused silica capillary is placed between two buffer reservoirs (1 ml vials) and separation occurs when an electric field is applied. This electric field was established by connecting one of the reservoirs to a Spellman model CZE 1000R high voltage power supply (Spellman, Plainville, NY) and the other reservoir to ground. The electrical connection was established with a 30 kV electrical cable and two 4 - 5 cm long platinum electrodes (Aldrich Chemical Co., Milwaukee, WI). The reservoir at the high voltage end was placed inside a 18" x 18" x 18" plexiglass box wired with an interlock system for the protection of the operator. If the operator failed to manually turn off the power supply prior to opening the box, the interlock would shut the power supply down and ground the system. Hydrostatic injection was performed for all work with this system. On-column detection was performed with a Linear (Linear Instruments, Reno, NV) UVIS 200 detector. Data handling was done with a Hewlett-Packard model 3390A integrator

(Hewlett-Packard, Avondale, PA).

The second system was the commercially available Beckman P/ACE System 2100 (Beckman Instruments, Palo Alto, CA) equipped with a UV detector and autosampler. Injection was performed with positive pressure. Data handling was performed using an IBM PS2 (IBM,Wappingers Falls, NY) personal computer equipped with System Gold software (Beckman Instruments).

2.2. Experimental Conditions

The basis for operation in CZE was discussed in detail in section 1.4. However, certain conditions used throughout this work need to be discussed. They involve the conditioning of the fused silica capillary prior to and during use. To prepare the capillary for use requires the preparation of a detection window. The window is prepared by burning off approximately 0.5 cm of the protective polyimide coating on the fused silica capillary. The capillary is then placed into the detector cell, aligned, and tightened into place. After installation the capillary is conditioned by flushing successively with 1.0 N NaOH for 5 - 15 min (15 min for home built system and 5 min for Beckman system due to the pressure used to draw the solution through the capillary) Followed by 0.1 N NaOH for 5 - 10 min. Followed finally by HPLC grade water for 5 - 10 min. This preparation was suggested by Lauer and McManigill [126]. By washing the

capillary thoroughly, the surface should be stripped of any adsorbed ions which could lead to reproducibility problems with electroosmotic flow and analyte migration.

After the wash step, the capillary was filled with the operating buffer. The operating buffer was sonicated prior to use to minimize the formation of bubbles during the run. The buffer was also filtered through 0.2 μm syringe filters (Scientific Resources Inc., North Brunswick, NJ) prior to use to remove any particulate matter. After filling, the buffer in the capillary was prepared for analysis by applying a voltage across the capillary for approximately 5 min. This helped to stabilize the electroosmotic flow and baseline.

Cleaning between runs was performed when necessary. The need for cleaning the capillary between runs was dependent upon the samples being analyzed. Since surfactants and proteins tended to interact with the capillary walls, frequent cleaning was required. For the proteins, cleaning between every run was necessary to achieve reproducibility. Cleaning was performed by flushing successively with 1.0 N NaOH, followed by HPLC grade water, and then the operating buffer (all for 5 - 15 min). Cleaning was also necessary if the system was allowed to sit for an extended period of time, such as storage overnight.

2.2.1. Conditions for Analysis of Surfactants with Indirect UV Detection

Separation and detection in CZE/indirect UV requires a buffer that is both an electrolyte and chromophore. Since ammonium ethoxylated alcohol sulfates (AEOS) do not contain a chromophore, initial experiments were focused on finding a buffer chromophore for detection. Several possible chromophore were studied, including benzoic acid, salicylic acid, diethyl-phthalate, and potassium dichromate. These compounds were evaluated for separation efficiency and detection sensitivity. Also studied was a binary buffer system with sodium tetraborate as the electrolyte and potassium dichromate as the chromophore. Separations were performed in 100 μm id fused silica capillaries from 50 - 100 cm in length (J & W Scientific, Folsom, CA).

After preliminary evaluations, two buffer systems were selected for further study. One consisted of 1 mM potassium dichromate/1 mM sodium tetraborate/ 30 mM boric acid (all Aldrich Chemical Co.) in HPLC grade water (JT Baker, Phillipsburg, NJ). The other was 1 mM potassium dichromate/1 mM sodium tetraborate in 70/30 (v/v) HPLC grade water/acetonitrile (JT Baker), adjusted to a pH of 8.0 with boric acid (the addition of acetonitrile will be discussed in detail later). A mixture of surfactants consisting of decyl (C10), dodecyl (C12), and tetradecyl (C14)(all Aldrich Chemical Co.), which corresponds to unethoxylated AEOS, was prepared to concentrations of 1- 3 mg/ml. Samples of the various AEOS (Colgate-Palmolive, Piscataway, NJ) were also prepared to concentrations of 1-3 mg/ml in the operating buffer. Samples were filtered

through 0.2 μm syringe filters (Scientific Resources Inc.) prior to analysis to remove particulates.

All separations were performed in a 100 μm x 100 cm (80 cm to detector) capillary (J & W Scientific) using an operating voltage of + 10 kV. Sample introduction was performed hydrostatically at 2.0 cm for 10 seconds and indirect UV detection was effected at 265 nm. For this work the home built system was employed. Washing and filling of the capillaries was achieved by placing one end of the capillary in the appropriate solution and connecting the other end to house vacuum.

2.2.2. Conditions for Surface Modifications

2.2.2.1. Hydrothermal Treatment of the Fused Silica Tubing

As discussed previously there is a great deal of interest in developing reproducible coated capillaries (in terms of electroosmotic flow and analyte migration times) for use in CZE. Since reproducibility is dependent upon the number of active silanol sites on the surface of the capillary, conditioning the surface prior to coating needs to be investigated. Ogden and McNair [127] showed that hydrothermally treating the surface with acid prior to coating yielded more highly deactivated columns. In this work, this same principle was

investigated for CZE.

Applying a method based on the work of Ogden and McNair [127], the fused silica capillaries were prepared for hydrothermal treatment by successively flushing with deionized water for 10 min., methanol (JT Baker) for 15 min., and 20 % nitric acid (Aldrich Chemical Co.) for 20 min. The capillary was then filled with the 20 % nitric acid solution and the ends were sealed. The capillary was heated at 200° C for 10 hours. After heating, the capillary was flushed with a 1 % nitric acid solution for 1 hour. Flushing was followed by drying with nitrogen at 120° C for 1 hour. The ends of the capillary were heat sealed until used to avoid hydrolysis of the surface upon exposure to the atmosphere.

A second method of hydrothermal treatment was also evaluated. Since 0.1 N NaOH is used to clean and prepare the surface of the capillary prior to use, it was selected for evaluation as a hydrothermal treating agent. The capillary was prepared by flushing with deionized water and methanol as with the nitric acid. Instead of 20 % nitric acid solution, the capillary was flushed and filled with 0.1 N NaOH (Aldrich Chemical Co.) and the ends heat sealed. The tubing was heated at 200° C for 1, 3, 5, and 7 hours to determine the optimal process time. The capillary was then flushed with methanol and dried with nitrogen at 120° C for 1 hour. Again the ends were heat sealed until used.

For the nitric acid method, 15 meters of 100 μm fused silica capillary (J & W Scientific) were processed for use in coating studies. For the 0.1 N NaOH

method, 5 meters of 100 μm fused silica was prepared for each process time. The large quantity of tubing was processed to insure reproducibility in the coating studies.

2.2.2.2. Conditions for Silane Deactivation of Capillary Surface

Deactivation of the fused silica surface with a coating minimizes analyte adsorption interactions with the capillary wall in CZE. Surfactants and proteins tend to interact with the uncoated silanol sites on the surface yielding inefficient separations. This work focused on finding a simple, reproducible coating procedure for the deactivation of the fused silica surface. In GC and HPLC, columns are endcapped with small silanes to cover active sites. This idea was investigated for use in the preparation of columns for CZE. Also, the use of hydrothermally treated tubing prior to coating was investigated.

The fused silica tubing (both untreated and hydrothermally) was prepared for coating by successively flushing with 0.1 N NaOH for 10 min., deionized water for 10 min., methanol for 15 min., and silane solution for 20 min.. The capillary was then filled with the silane solution and the ends heat sealed. The column was heated at 300° C for 3 hours. After heating the capillary was flushed and dried with nitrogen at 120° C. The ends were heat sealed until the capillary was used.

Two silane solutions were evaluated in this work, trimethylchlorosilane (TCMS) and t-butyl-dimethylchlorosilane (both Aldrich Chemical Co.). The trimethyl solution was prepared with 1 ml of the silane and 5 ml of pyridine (Aldrich Chemical Co.) diluted to 25 ml in methanol (J.T. Baker). The t-butyl solution was prepared with 0.1 g of silane and 5 ml of pyridine diluted to 25 ml in methanol.

2.2.2.3. Conditions for Evaluation of the Surface Deactivation

To evaluate the efficiency of the surface deactivation in this work, electroosmotic flow was studied. Electroosmotic flow is directly proportional to the number of active silanol site on the capillary surface. If surface deactivation is obtained by coating the active sites, electroosmotic flow will be reduced or eliminated. Another factor that can be used to evaluate surface deactivation is analyte adsorption. Since proteins, especially at low pH's, tend to interact with the active site on the surface their separation efficiencies and peak shape can be investigated to evaluate surface deactivation.

This work focuses on the evaluation of the untreated and hydrothermally treated silane coated capillaries. All capillaries were 100 μm x 70 cm (effective length 50 cm)(J & W Scientific). Three columns were prepared and investigated for each coating condition, as well as, the uncoated tubing used as controls.

Uncoated columns were prepared for used as previously described. Coated columns were prepared for use by flushing with methanol for 20 min. and then filling with operating buffer.

A 2 % solution of acetone (J.T.Baker) in run buffer was used as a neutral marker for the measurement of electroosmotic flow. Analyses were performed with an electric field of 215 V/cm. UV detection was effected at 254 nm. The operating buffer was 10 mM sodium formate (Aldrich Chemical Co.), pH 4.5.

Based on the results of the electroosmotic flow study, the hydrothermally treated t-butyl columns were investigated for analyte adsorption. Two proteins, trypsinogen and β-lactoglobulin (both Sigma Chemical) were used to study the deactivation efficiencies in terms of analyte adsorption. These proteins have previously been shown to be difficult to separate at low pH. Separations were performed in a 100 μm x 55 cm (length to detector = 35 cm) capillary with an electric field of 273 V/cm. The operating buffer was 10 mM sodium formate, pH 4.0. Detection was performed at 200 nm.

A final study performed with these coated capillaries was an investigation of the lifetime of the coatings at varying pH's. The electroosmotic flow was studied for 10 runs at pH's of 4.5 (10 mM sodium formate buffer), 7.4 (10 mM sodium phosphate buffer), and 8.6(10 mM sodium tetraborate buffer) using TMCS columns (all Aldrich Chemical Company).

2.2.3. Experimental Conditions for Proteins in Rat Urine

Diabetes can lead to proteinuria. With time the glomeruli are damaged and loses it's ability to filter proteins. The amount of albumin in the urine can be tracked to determine the degree of damage to the kidney. In this work the separation of proteins and detection of albumin in rat urine was investigated. The urine samples were collected from 3 rats in various stages of diabetes: 1) normal rat; 2) rat with diabetes for 3 weeks; and 3) rat with diabetes for 6 weeks. Samples were obtained from University of Virginia Medical School (Charlottesville, VA). Approximately 100 μl of sample was collected for each rat.

Separations were performed on the Beckman P/ACE system with a 75μm x 57 cm (effective length 50 cm) fused silica capillary (J & W Scientific) at + 10 kV. Injection was performed at low pressure for 1.0 seconds. There was no sample preparation performed on the urine; therefore, the column was cleaned with 1.0 N NaOH between runs to improve reproducibility. The run buffer was 10 mM sodium tetraborate, pH 10.0(Aldrich Chemical Co.). Detection was performed at 200 and 280 nm. A standard of rat albumin (Sigma Chemicals) was prepared at 2.5 mg/ml in the operating buffer to confirm the migration time of albumin in the urine sample.

2.2.4. Conditions for Separation of Serum Proteins

The amount of albumin and globulins in serum yields important information about kidney dysfunction and it's cause. For this work, a separation of normal and abnormal human serum control (Beckman Biochemicals, Palo Alto, CA) was used to investigate the use of CZE for their analysis. Several conditions, including buffer and sample additives, were investigated to obtain an efficient, reproducible separation.

Results were compared to the analysis of the serum proteins by agarose gel electrophoresis. The gel electrophoresis was performed using an agarose universal electrophoresis film 470100 (Corning Chemicals, Corning, NY). Separation was performed with a barbital buffer (Corning), pH 8.6 at + 300 V. Detection was performed by staining with Amido Black 10B Colorimetric stain (Corning) and scanning with an optical densitometer. The agarose gel separations were performed at the Veteran's Hospital in Salem, VA.

CZE separations were performed on the Beckman P/ACE system using a 100 μm x 57 cm (effective length of 50 cm) capillary (J & W Scientific) at + 20 kV. The run buffer was 20 mM sodium tetraborate at pH 10.0 (Aldrich Chemicals Co.). Samples were injected at low pressure for 1 - 3 seconds. Detection was performed at 200 nm.

Two conditions for sample preparation were investigated. Samples were diluted 1:20 in 1 mM boric acid, pH 4.0(Aldrich Chemical Co.). Also the addition of 0.2 % polyvinyl alcohol (Aldrich Chemical Co.) to the boric acid was

investigated to improve reproducibility of migration times. The use of buffer additives were also investigated to improve separation and reproducibility. In the 10 mM sodium tetraborate buffer, 0.2 % methyl cellulose (MW of 82,000) was dissolved to act as a molecular sieving medium (all Aldrich Chemical Co.). Due to foaming problems, the methyl cellulose solution must be sonicated under vacuum to minimize bubble formation.

Lastly, the sodium hydroxide rinse time between runs was investigated to improve reproducibility of migration times. Five runs at each rinse time were performed with rinse times of 1, 2, 3, 4, 5, and 10 mins. between runs. The capillaries were rinsed with 1.0 N NaOH.

CHAPTER III

RESULTS AND DISCUSSION

3.1. Analysis of Surfactants with Indirect UV Detection

The goal of this research was to develop a simple, reproducible method for the characterization of AEOS surfactants. Characterization of these surfactants (for quality control) is necessary to insure that the product in which they are used functions properly. AEOS consist of a mixture of alkyl chain lengths with varying degrees of ethoxylation. The proportion of one chain length to the other affects how the surfactant functions; therefore, this ratio must be precisely determined. As previously discussed, there is currently no direct means for analysis of AEOS surfactants. CZE should allow for the separation of these charged species without sample preparation or derivitization.

Detection of other ionic surfactants by direct UV detection at 190-200 nm has been reported [128]. However, through this research it was found to be inappropriate for the detection of AEOS. Consequently several buffers were evaluated for indirect UV detection. To match the electrophoretic mobilities of the co-ion with the electrophoretic mobilities of the AEOS components, large

anions such as phthalates, benzoate and salicylate were evaluated with and without the presence of varying amounts of tetraborate/boric acid. Dichromate, which has been shown to be useful for the indirect analysis of small anions [119], was also investigated.

When using indirect detection, the co-ion must be selected to obtain optimal detection sensitivity. However, when the co-ion is also used as the electrolyte (as in the case of the phthalate, benzoate and salicylate), it's concentration must also be optimized to obtain the best separation. This research found that when trying to optimize for separation, the concentration of the co-ion was too high causing the detector to be saturated with signal. When optimizing for detection, poor separations and poor signal to noise ratios were obtained.

Therefore, a binary buffer system was investigated. A binary buffer system consists of an absorbing co-ion to be used for detection and a second non-absorbing co-ion to be used for separation. A comparison of the various absorbing anions indicated that dichromate/tetraborate provided the best signal to noise ratio and peak symmetry.

Two relatively simple sample mixtures were prepared for an initial evaluation of the dichromate based buffer for the analysis of AEOS. These were selected to elucidate the influence of alkyl chain length and degree of ethoxylation on migration behavior. The first sample mixture consisted of decyl,

dodecyl and tetradecyl sulfates to mimic the unethoxylated AEOS. This sample should indicate if migration is influenced by alkyl chain length. The second sample contained a C10 3EO sulfate (C10 alkyl chain and 3 degrees of ethoxylation). This sample should show if migration is influenced by degrees of ethoxylation.

Separations achieved with an aqueous buffer containing 1 mM potassium dichromate/1 mM sodium tetraborate/30 mM boric acid (Fig. 11) indicates that elution is in order of both decreasing degree of ethoxylation and decreasing alkyl chain length. Such behavior is consistent with the larger charge-to-mass ratios of smaller species: ie. smaller species have larger electrophoretic mobilities to counter the electroosmotic flow and, therefore, migrate with a slower net velocity.

While adequate separation of both sample mixtures was achieved using the conditions of Fig 11, two primary concerns were noted. Due to a large difference in the concentration of the C10 3EO sample components, it was difficult to adjust sample concentration to a level in which detection of minor components was adequate and peak shapes for major components were symmetrical. Efforts to improve sensitivity by increasing sample concentration resulted in reduced resolution as a result of peak broadening. Efforts to improve peak shape by increasing sodium tetraborate concentration resulted in decreased sensitivity. Secondly, it was clear that a complex elution pattern with significant

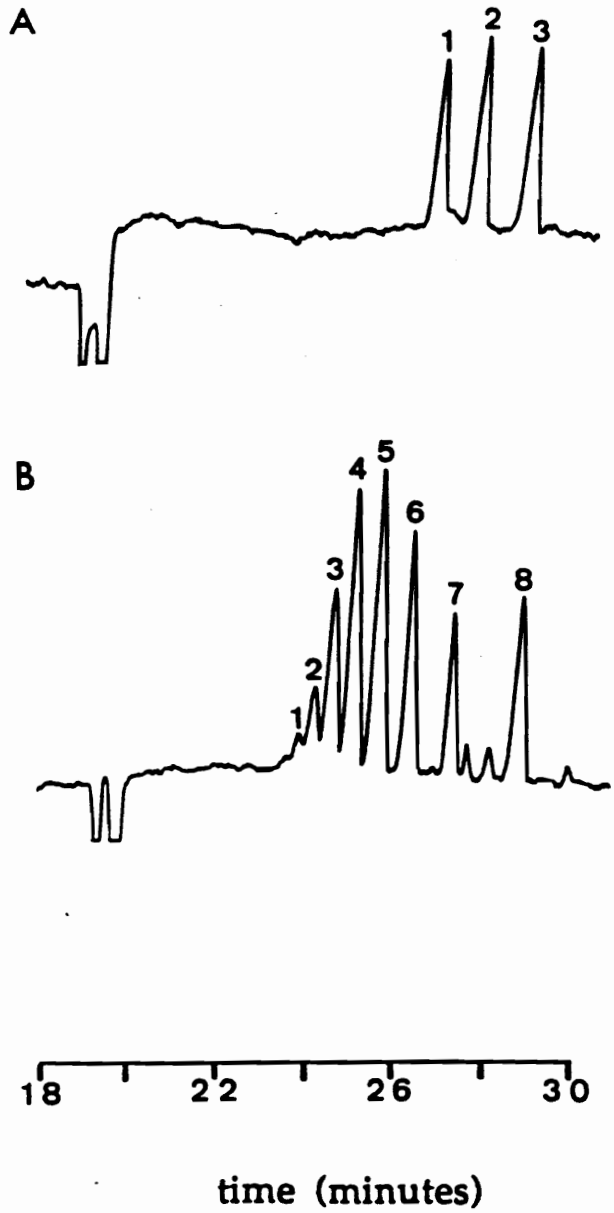

Figure 11. Separation of (A) tetradecyl (1), dodecyl (2), and decyl (3) sulfates and (B) C10 3EO ethoxylated alcohol sulfates - Peak identities (1) C10 7EO, (2) C10 6EO, (3) C10 5EO, (4) C10 4EO, (5) C10 3EO, (6) C10 2EO, (7) C10 1EO, and (8) C10 0EO. Conditions: 100 μm x 100 cm fused silica capillary (l = 80 cm); + 10 kV operating voltage; 1 mM potassium dichromate/1 mM sodium tetraborate/30 mM boric acid buffer; sample introduction by siphoning for 10 sec. at 2 cm; indirect UV detection at 265 nm.

peak overlap would occur if ethoxylates with mixed carbon chain lengths were analyzed.

The problems associated with sensitivity and separation of more complex mixtures may be solved by using a detector with superior signal to noise characteristics. This detector would allow for a dilution of the sample; therefore, yielding better resolution. Alternatively, better resolution may be obtained by modifying the buffer to improve selectivity. If excess resolution is achieved using the latter approach, sample concentration can be increased to improve sensitivity [129].

One concern working with surfactant concentrations of 1- 3 mg/ml was possible molecular interactions between the individual surfactant molecules. The main concern was the formation of micelles. Table II shows the critical micelle concentrations (CMC) in water for some of the surfactants of interest. It shows that most of the surfactants of interest have CMC values below the concentrations of the samples. Therefore, micelle formation was occurring.

Micelle formation will drastically alter the migration of the surfactants, leading to zone distortion. The formation of micelles is one of the major causes for poor resolution in these separations. A way to avoid micelles is to lower the sample concentrations; however, this was not possible due to the lack of sensitivity. An alternative method to avoid micelle formation was to add a modifier to the buffer that would disrupt the molecular interactions. By adding

Table II. Critical micelle concentrations (CMC) for selected surfactants [22].

SURFACTANT	CMC (M)	CMC (mg/ml)
Decyl Sulfate	4.3×10^{-2}	10.5
Dodecyl Sulfate	1.2×10^{-2}	3.3
Tetradecyl Sulfate	2.5×10^{-4}	0.08
C10 1EO	5.5×10^{-3}	1.6
C12 1EO	3.9×10^{-3}	1.2
C12 2EO	2.9×10^{-3}	1.0
C12 3EO	2.0×10^{-3}	0.8
C12 4EO	1.3×10^{-3}	0.6

an organic modifier to the buffer, solubility of the surfactants would improve. This improvement in solubility should increase the CMC of the surfactants and minimize the interactions.

Several modifiers were investigated, including methanol, isopropyl alcohol (IPA), and acetonitrile. After evaluation of the above modifiers it was shown that methanol and IPA (in concentrations of 10% - 50% (v/v)) yielded little to no improvement in resolution. However, an improvement in resolution (Fig. 12) was observed when the buffer was prepared with 30% (v/v) acetonitrile. As documented previously, acetonitrile reduces electroosmotic flow [130]. As a result, analysis time, as well as resolution was increased. It was found that 30% acetonitrile yielded the best resolution. When adding less than 30% acetonitrile little to no improvement was seen. With the addition of 40% - 50% acetonitrile no improvement in resolution (versus 30%) was seen; however, analysis time was drastically increased.

Neodol 23-2A, which is a mixture of C12 and C13 alkyl groups with an average degree of ethoxylation of 2 was separated by both alkyl chain length and degree of ethoxylation using the modified buffer (Fig. 13). This separation was not possible without the acetonitrile. This separation indicates an excellent potential for precise characterization (quality control) of known raw materials. However, the migration times of the C12 unethoxylated sulfate in Figs. 13A and 12 do not correspond to each other. Such irreproducibility currently prevents

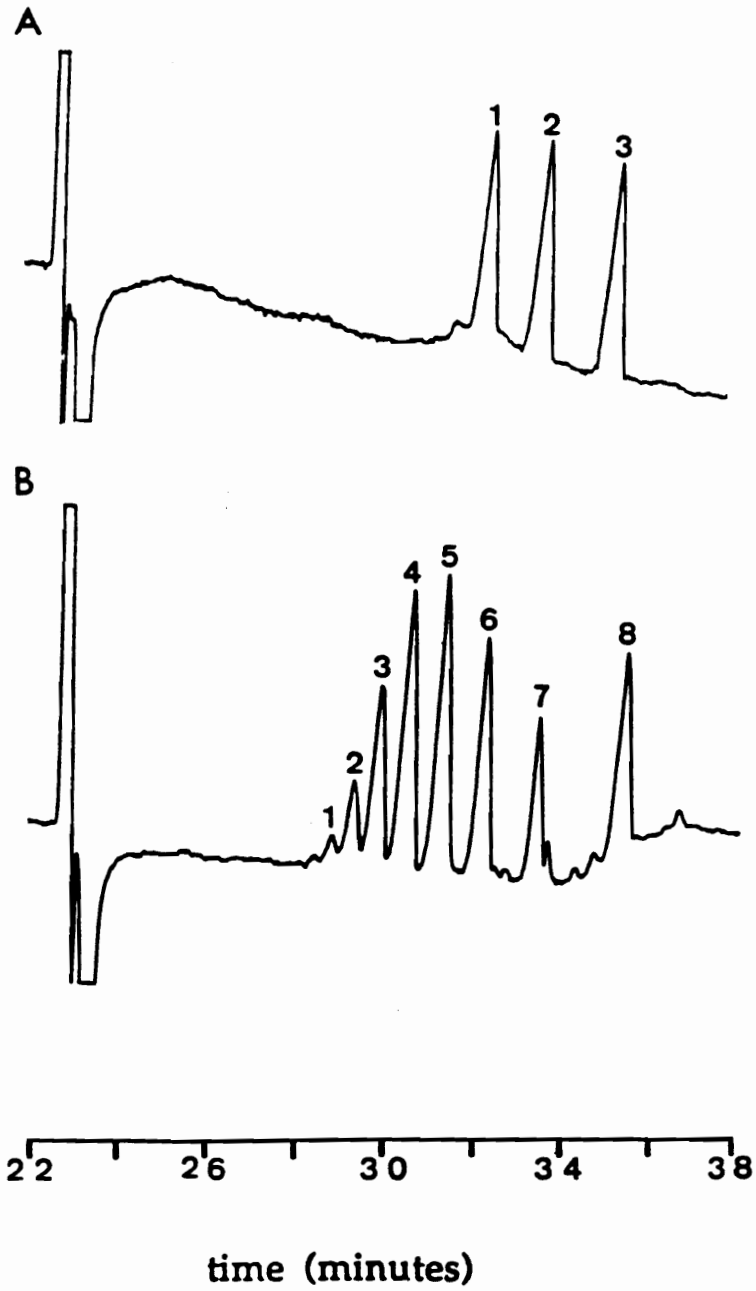

Figure 12. Separation of (A) tetradecyl, dodecyl, and decyl sulfates and (B) C10 3EO ethoxylated alcohol sulfate using 1 mM potassium dichromate/1 mM sodium tetraborate in 70/30 (v/v) HPLC grade water/acetonitrile adjusted to pH 8.0 with boric acid. Other conditions and component identification are as in Fig. 11.

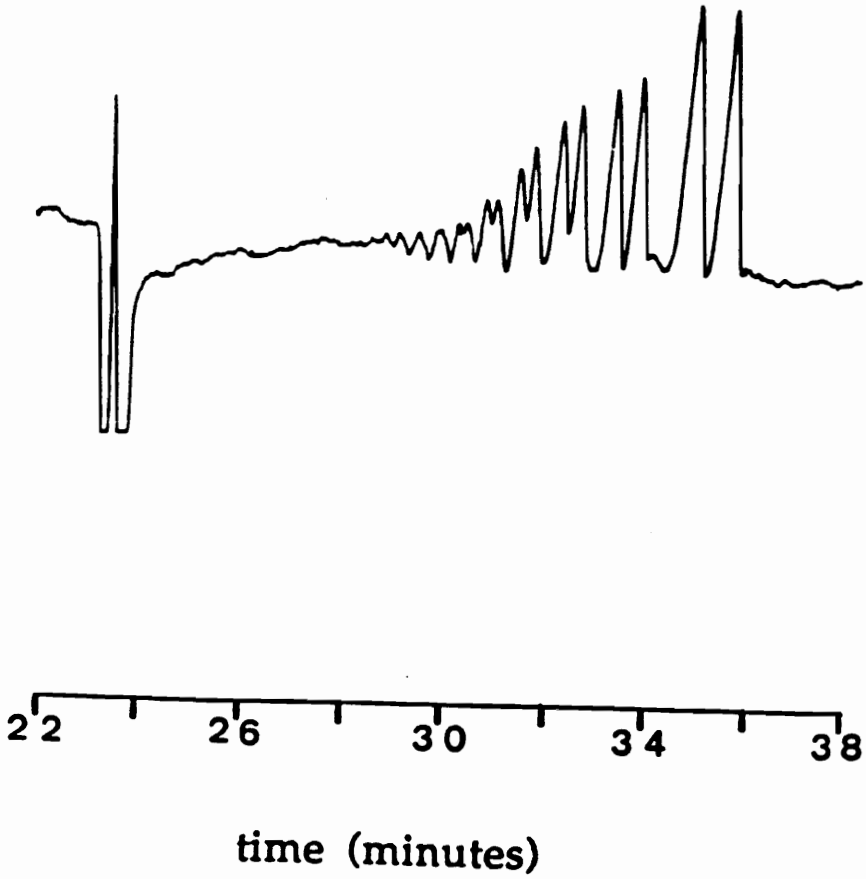

Figure 13. Separation of Neodol 23-2A. Conditions are as in Fig. 12.

the method from being used for routine qualitative control of raw materials. This non-reproducibility is due to the rinsing process of the capillary between runs. This research was done on a "home built" system; therefore rinse volumes and times were difficult to control. This caused the surface of the fused silica not to be regenerated reproducibly between runs. This led to reproducibilities of migration times for the AEOS surfactants of 5- 10 % RSD for 5 runs. However, this work was repeated by another group using a commercial system where the rinse times could be better controlled. Lovern and Rasmussen's work has yielded 0.8% RSD values for the alkyl sulfates and 2.0% RSD values for the ethoxylated alcohols for migration times [131].

However, an additional problem was encountered for mixtures containing other chain length combinations. For example, in a C12/C14 1EO mixture (Fig. 14) there is some overlap between the C14 0EO and the C12 1EO. An overlap is also seen between peaks with higher degrees of ethoxylation. This problem is related to the lack of resolution due to the high sample concentrations. The only way to improve or solve this problem is to investigate other buffer systems.

For a practical application, the method was applied to the analysis of a commercial dishwashing detergent. As shown in Fig. 15A, the AEOS distribution is separated from this formulation without interferences. The AEOS mixture can be identified as a bimodal distribution. The major AEOS constituent is similar to the Neodol 23-2A (Fig 15B), although slightly shorter migration times are

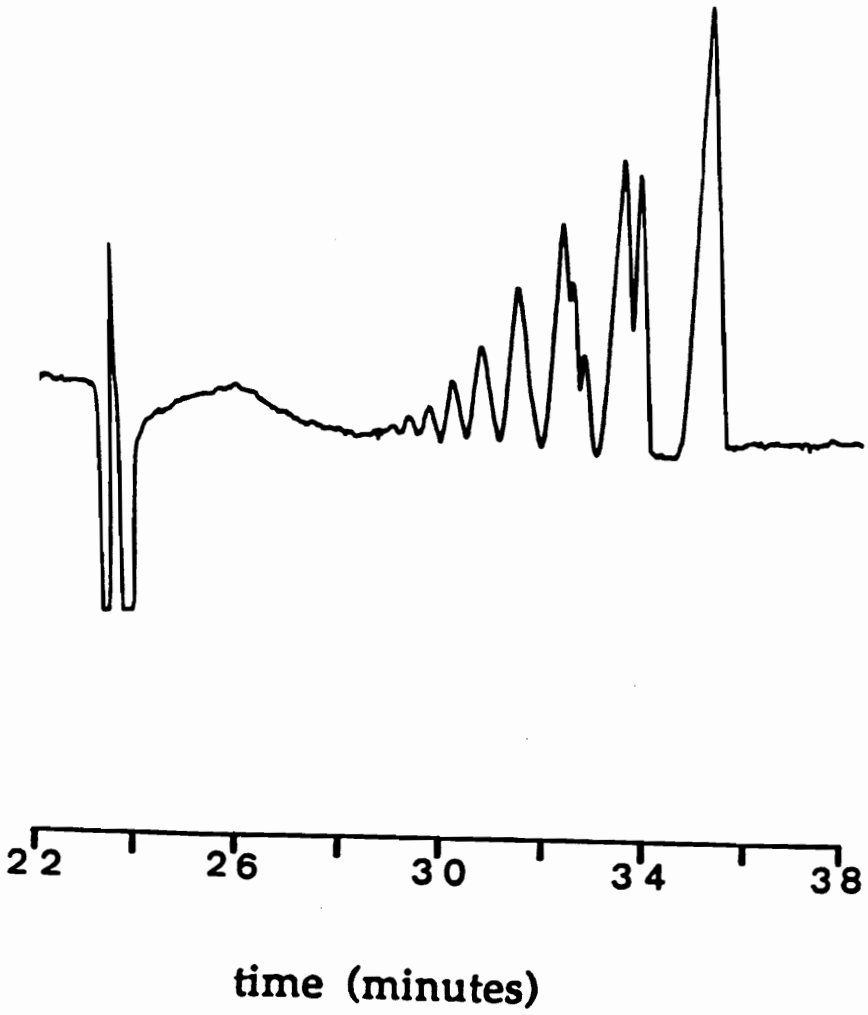

Figure 14. Separation of a C12/C14/1EO blend. Conditions are as in Fig. 12.

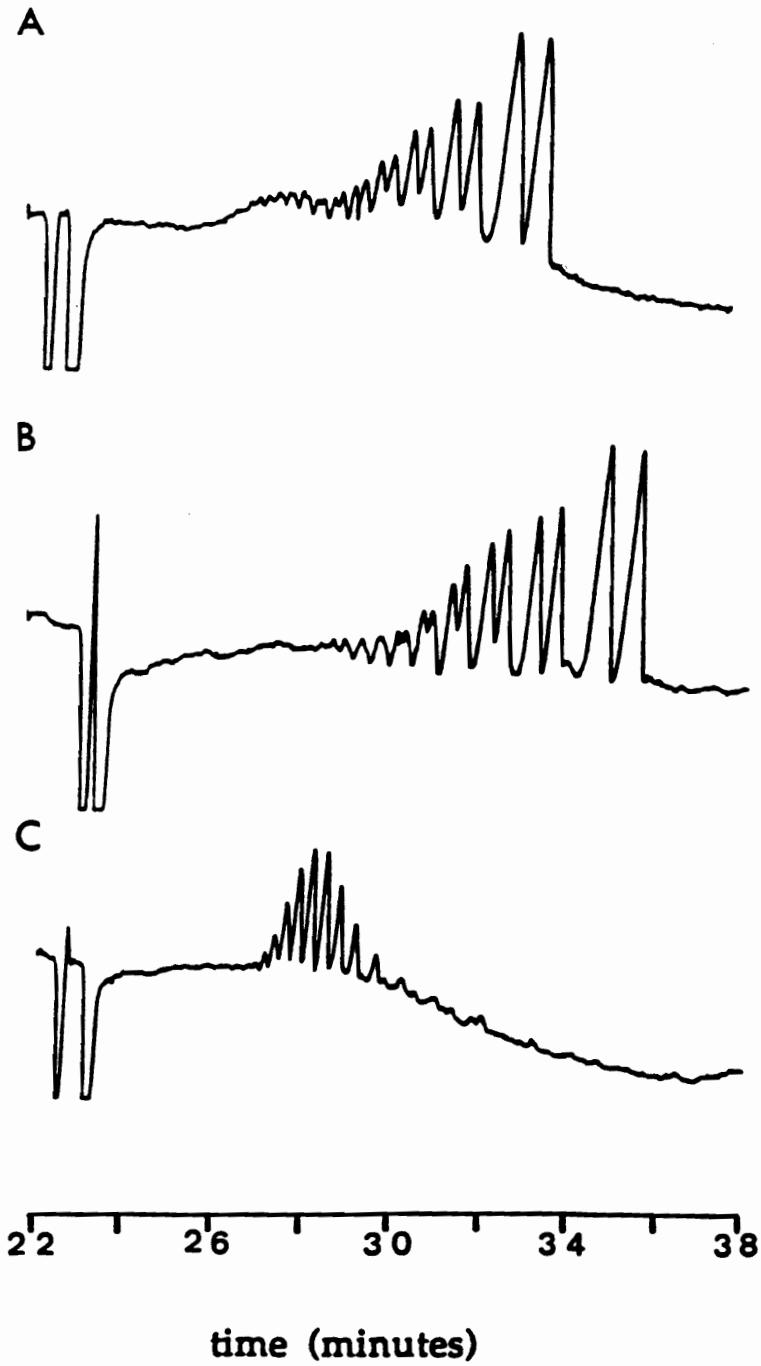

Figure 15. Separation of (A) a commercial dishwashing formulation, (B) Neodol 23-2A, and (C) a 6.5EO blend. Conditions are as in Fig. 12.

observed. A second lesser distribution which is similar to a 6.5 EO (Fig. 15C) is also apparent. It is unclear whether the migration time dissimilarity between the Neodol 23-2A and the major component in the dishwashing detergent is a result of irreproducibility or is due to the presence of longer alkyl chain lengths. Notably, the migration time of the shortest unethoxylated alcohol in Fig. 15A is similar to the migration time of C12 OEO in Fig. 12A.

One underlying theme to this research is the lack of resolution and sensitivity due to the buffer system. Indirect UV detection has shown detection limits of 10^{-5} to 10^{-7} M [67]. In this research the detection limits were only 10^{-3} to 10^{-4} M. To determine the possible reason for this lack of resolution and sensitivity the theory of indirect UV detection must be discussed.

For a UV detector the measured absorbance A can be determined from Beer's Law [132]:

$$A = \varepsilon c l \tag{14}$$

where ε is the molar absorption coefficient, c is the analyte concentration, and l is the effective path length in the detector. With indirect UV detection the capillary is filled with the absorbing carrier electrolyte AB (consisting of a co-ion A and a counter-ion B) at a concentration c_A [133]. Absorbance for the carrier

electrolyte becomes:

$$A^{C} = (\varepsilon_{A} + \varepsilon_{B})c_{A}^{C}l \tag{15}$$

For the sample zone the absorbance will be:

$$A^{S} = (\varepsilon_{A} + \varepsilon_{B})c_{A}^{S}l + (\varepsilon_{B} + \varepsilon_{i})c_{i}^{S}l \tag{16}$$

where ε_A, ε_B and ε_i are the molar absorption coefficients of the co-ion, counter-ion, and sample analyte respectively. The superscripts C and S refer to the composition of the pure carrier AB zone and the sample zone, respectively. Therefore, the UV signal of a sample zone will be:

$$A = A^{C} - A^{S} \tag{17}$$

As a mixture of component i and the carrier electrolyte AB pass through the capillary the following equation becomes valid [133]:

(18)

$$c_A^c = c_A^s + c_i^s k_i$$

where:

$$k_i = \frac{m_i + m_B}{m_A + m_B} \cdot \frac{m_A}{m_i} \tag{19}$$

where m_A, m_B, and m_i are the electrophoretic mobilities of the co-ion, counter-ion, and analyte, respectively. Therefore, the UV signal of a sample zone becomes:

$$A = c_i^s l\ [(\varepsilon_A + \varepsilon_B)k_i - (\varepsilon_i + \varepsilon_B)] \tag{20}$$

For non-UV-absorbing counter-ions and sample ions, applying indirect UV detection with UV-absorbing co-ions, the UV signal is proportional to $c_i k_i$ [133]. Optimal peak shape and sensitivity occur when the mobility of the analyte matches the mobility of the co-ion. Dichromate has a much higher electrophoretic mobility (based on it's charge to mass ratio) compared to the AEOS surfactants, resulting in peak distortions as shown in Fig. 8. These peak distortions lead to poor resolution between the peaks. Also, as the difference in mobilities of the analytes compared to the co-ion increase the response

decreases, reducing the sensitivity.

A second factor leading to the lack of sensitivity is the process of charge displacement of the buffer co-ion with the analyte ion to maintain charge neutrality in the sample zone. Since dichromate has a net -2 charge, it requires two surfactant molecules to replace one dichromate molecule. Theoretically, this should decrease the sensitivity by 50%. Therefore, a better choice of a buffer co-ion would be a molecule that had a net -1 charge. Also, since a binary buffer was used for this work (dichromate/tetraborate) there will be a decrease in sensitivity due to displacement competition between the dichromate and the tetraborate with the analytes. If the analyte replaces a non-absorbing borate molecule no change in signal will be measured; thus a reduction in sensitivity occurs.

3.2. Evaluation of Silane Bonded Capillaries for CZE

By deactivating the surface of the fused silica capillary two major factors of the separation are effected. They are the reduction of electroosmotic flow and analyte adsorption. In turn, these factors should improve resolution (R) and efficiency (N). The ultimate goal of deactivation is to cover as many of the active silanol sites on the surface as possible. The number of silanol sites can be directly related to the electroosmotic flow. Therefore, electroosmotic flow can

be measured to determine the extent and reproducibility of deactivation. The main goal of this research was to find a simple, effective, reproducible way to deactivate the capillary.

3.2.1. Effects of Hydrothermal Treatment on the Surface of the Capillary

One of the major problems with deactivated capillaries in CZE has been the effectiveness and reproducibility of the deactivation process. Ogden and McNair [127] showed that by hydrothermally treating the fused silica prior to coating, more deactivated columns for use in GC and SFC were produced. The number of silanol sites on the surface is dependent on how the tubing was stored prior to coating. With time the silanol sites will be hydrolyzed due to exposure to air and moisture. Therefore, the number of active sites on the surface is constantly changing with time. By hydrothermally treating the surface, all of the available silanol sites should be activated prior to coating producing a homogeneous surface. Therefore, when the column is coated all of the silanol sites will be covered and yield more deactivated, reproducible columns. In this research, the same idea was applied to bonded columns for use in CZE.

In this work, two types of hydrothermal treatment were evaluated: 1) a 20% nitric acid solution [127] and 2) a 0.1 N NaOH solution. Using the work of

Ogden and McNair [127], a piece of tubing was filled with the 20% nitric acid solution and heated at 200° C for 10 hrs.. If the hydrothermal treatment worked there should be an increase in electroosmotic flow when compared to an untreated piece of tubing. Electroosmotic flow was calculated using the following equation:

$$\mu_{eo} = \frac{\pi r^2 d}{t_m} \tag{21}$$

where μ_{eo} is the electroosmotic flow, r is the radius of the capillary, d is the distance to the detector, and t_m is the migration time of the neutral marker. This increase is due to the activation of the hydrolyzed silanol sites on the surface. Fig. 16 shows the results of hydrothermal treatment on the electroosmotic flow. With all three buffers an increase in electroosmotic flow was seen for the hydrothermally treated tubing. To determine if this increase was statistically significant a t-test was performed to compare the means of the measured electroosmotic flows. First a pooled standard deviation was calculated using the following equation:

$$s^2 = [(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2]/(n_1 + n_2 - 2) \tag{22}$$

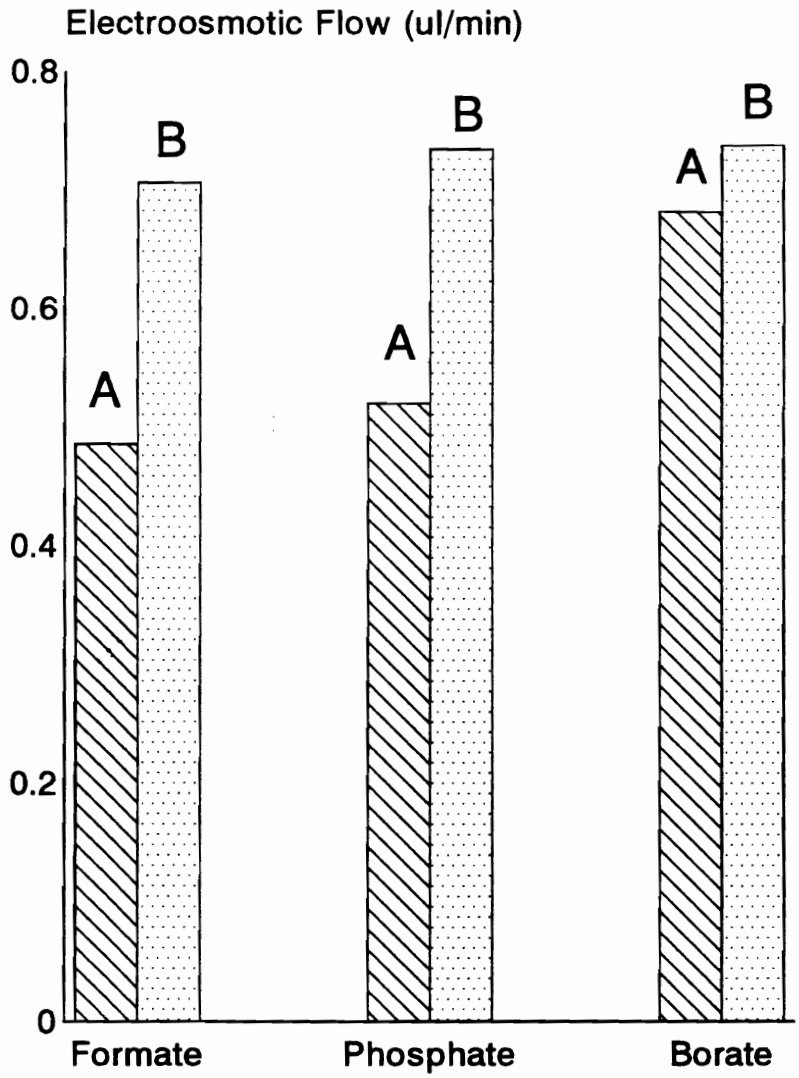

Figure 16. Effect of hydrothermal treatment of the fused silica capillary with 20% nitric acid solution on electroosmotic flow. Where (A) is the untreated capillary and (B) the hydrothermally treated capillary. Electroosmotic flow measured with 2% acetone solution in run buffer -- (1) 10 mM sodium formate buffer, pH 4.5, (2) 10 mM sodium phosphate buffer, pH 7.4, and (3) 10 mM sodium tetraborate buffer, pH 8.6. (n = 5 for each buffer)

where s is the pooled standard deviation, n_1 and n_2 are the numbers of runs for sample 1 and 2, respectively, and s_1 and s_2 are the standard deviations for sample 1 and 2, respectively. Using the pooled standard deviation, the t value was calculated by the following equation:

$$t = (\overline{x_1} - \overline{x_2})/s\sqrt{(1/n_1 + 1/n_2)} \tag{23}$$

where x_1 and x_2 are the means for sample 1 and 2, respectively, and t has $n_1 + n_2 - 2$ degrees of freedom [134]. At a 95% confidence interval (CI), all hydrothermally treated capillaries showed a statistically higher electroosmotic flow then the untreated tubing (Table III). Therefore, the hydrothermal treatment with 20% nitric acid was successful in activing the surface silanol groups.

The second mode of hydrothermal treatment, the use of 0.1 N NaOH, was investigated because it is the solution used to prepare and clean the capillaries between runs in order to obtain a uniform surface. Therefore, it is also a promising candidate to activate silanol groups hydrothermally. Also, the nitric acid treatment takes 10 hr. and it was hoped that the 0.1 N NaOH would require a shorter heating time. Since NaOH has not previously been investigated as a hydrothermal treating agent the temperature and time of heating had to be

Table III. Statistical comparison of μeo for hydrothermally treated fused silica (at a 95% CI).

	ELECTROOSMOTIC FLOW (ul/min)	T CALC.	T TABLE
Formate Buffer: (υ = 13)			
Untreated	0.486 +/- 0.007	71	2.1
Treated	0.707 +/- 0.005		
Phosphate Buffer: (υ = 18)			
Untreated	0.520 +/- 0.012	55	2.2
Treated	0.735 +/- 0.004		
Tetraborate Buffer: (υ = 13)			
Untreated	0.683 +/- 0.003	17	2.1
Treated	0.739 +/- 0.007		

υ = degrees of freedom

investigated. To maintain some constant with the nitric acid treatment it was decided that the heating temperature for the NaOH would also be 200° C. Several pieces of fused silica tubing (all from the same batch) were filled with the solution and heated for 1, 3, 5, and 7 hrs. Using the formate buffer, the electroosmotic flow was measured for each heating time. Fig. 17 shows that heating for 3 hrs at 200° C yields the highest electroosmotic flow (ie. the most active surface). After 3 hrs. there is a slight decrease in the electroosmotic flow which is most likely due to surface etching caused by the NaOH.

The 0.1 N NaOH yielded the same degree of surface activation as the 20% nitric acid solution. Hhowever, reproducibility in migrations times was poor; therefore, the nitric acid treatment was used in all future work. The next step of the research was to coat the activated tubing to determine if hydrothermally treating the surface improves the deactivation.

3.2.2. Evaluation of Hydrothermal Treatment with Silane Bonding

The second stage of this research was to find a simple, reproducible way to deactivate the surface of the fused silica capillary. It was decided to investigate the use of small silanes as deactivation agents. Two silanes were selected for this research, trimethylchlorosilane and t-butyl-dimethylchlorosilane. TMCS has been used in both GC and HPLC as an endcapping reagent. The small

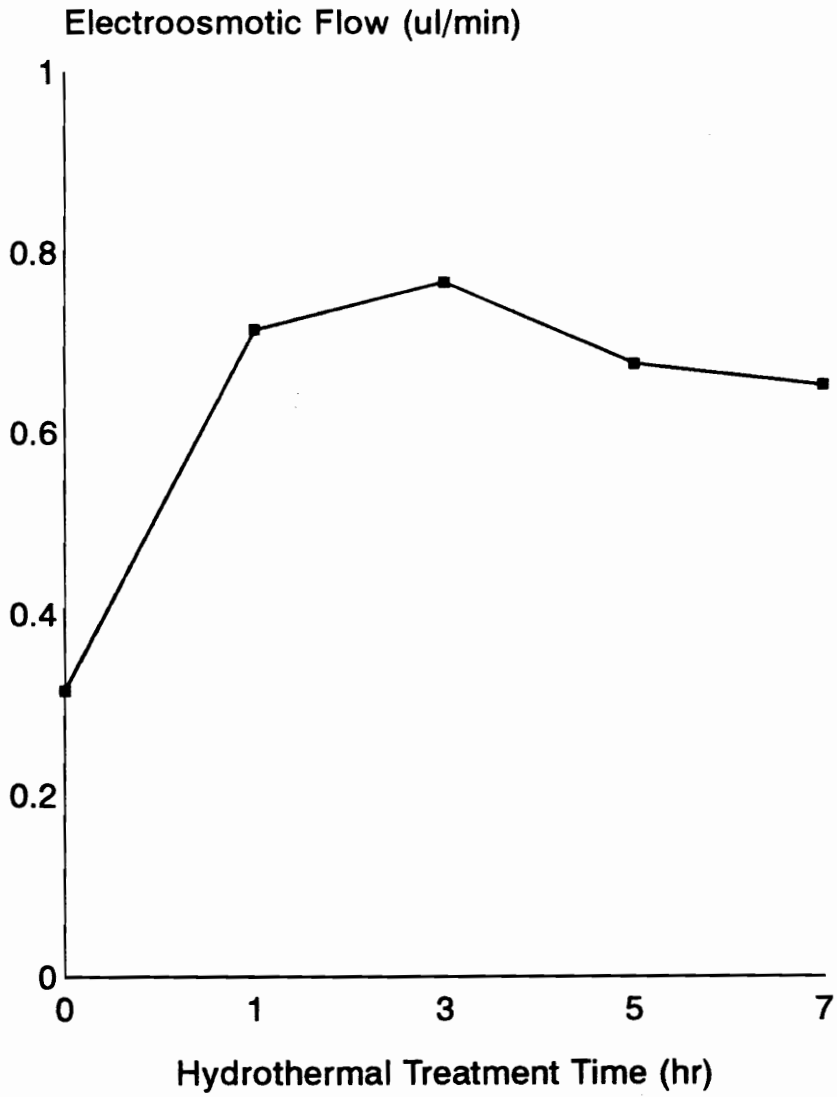

Figure 17. Evaluation of optimal heating time for 0.1 N NaOH for hydrothermal treatment of the fused silica capillary. Electroosmotic flow measured with 2% acetone solution in 10 mM sodium formate buffer, pH 4.5. (n = 5 for each time)

TMCS can cover active silanol sites that large silanes may not be able to reach due to steric hinderence. The t-butyl silane was selected to study the use of a bulky functional group on the surface coverage. By using a bulky functional group not all the active sites may need to be chemical bonded to eliminate interactions. The t-butyl group may be able to cover active groups by steric hindrance.

Fig. 18 shows a comparison of the electroosmotic flows in formate buffer for the 20% nitric acid treated capillaries bonded with TMCS and t-butyl. It also shows the comparison of electroosmotic flows for untreated and hydrothermally treated tubing upon deactivation with the two silanes. Both silanes showed a larger reduction in electroosmotic flow for the hydrothermally treated tubing. This can be attributed to the production of a uniform surface produced by the hydrothermal treatment. A t-test was performed to determine if there was a statistical difference between the deactivation of the hydrothermally treated and untreated capillary. Table IV shows that at a 95% CI there is a statistically significant reduction in electroosmotic flow for the hydrothermally treated fused silica for both the TMCS and t-butyl silane.

Fig. 18 also shows that the bulkier t-butyl group produced better deactivation then the TMCS with both the hydrothermally treated and untreated tubing. This indicates that not all of the sites with the t-butyl silanes are being chemically bonded. Some deactivation is due to steric hindrance of the sites by

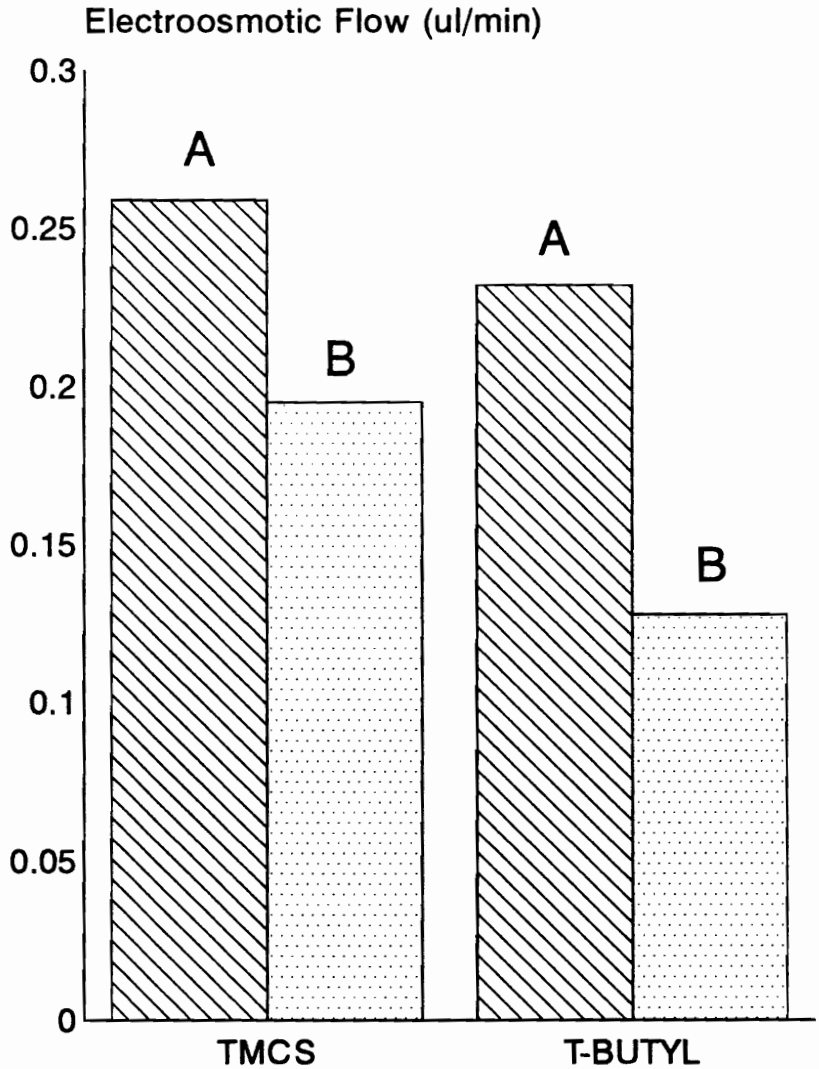

Figure 18. Effect of hydrothermal treatment with 20% nitric acid and steric hinderance of bonding group on surface deactivation. Where (A) is the untreated capillary and (B) is the hydrothermally treated capillary. Electroosmotic flow measured with 2% acetone solution in 10 mM sodium formate buffer, pH 4.5. (n = 5 for each condition)

Table IV. Statistical comparison of deactivation for hydrothermally treated vs. untreated fused silica (at a 95% CI).

	ELECTROOSMOTIC FLOW (ul/min)	t-CALC	t-Table
TMCS: (υ = 20)			
Untreated	0.259 +/- 0.007	17.3	2.09
Treated	0.195 +/- 0.009		
T-BUTYL: (υ = 28)			
Untreated	0.232 +/- 0.018	15.0	2.04
Treated	0.128 +/- 0.020		

υ = degrees of freedom

the t-butyl group. At a 95% CI there is also a significant statistical difference inthe best choice for a surface deactivation is produced using a combination of chemical bonding and steric hinderence.

A second type of hydrothermal treatment was also studied, 0.1 N NaOH. Since the t-butyl coating produced the best deactivation it was selected for evaluation of the 0.1 N NaOH treated columns. Fused silica tubing the deactivation using the t-butyl silane versus the TMCS (Table V). Therefore, hydrothermally treated with NaOH showed better deactivation versus untreated tubing by generating a lower electroosmotic flow (0.149 ul/min vs. 0.232 ul/min.). However, the 20% nitric acid treatment yielded a lower electroosmotic flow and better deactivation then the 0.1 N NaOH (0.149 ul/min vs. 0.128 ul/min). The difference is caused by a greater amount of surface etching produced by the 0.1 N NaOH. Fused silica chemically dissolves at pH's higher then 7.0 [135]. Therefore, some of the silanol sites activated by the NaOH were down in surface grooves caused by the etching. Theses sites could not be deactivated due to the inability of the silane to diffuse into the grooves and bond with the sites.

3.2.3. Evaluation of Analyte Adsorption with Deactivated Capillaries

A reduction in electroosmotic flow indicates that the surface silanol sites

Table V. Statistical comparison of TMCS vs. t-Butyl silane (at a 95% CI).

	ELECTROOSMOTIC FLOW (ul/min)	t-CALC.	t-TABLE
UNTREATED: (υ = 21)			
TMCS	0.259 +/- 0.007	4.1	2.1
T-BUTYL	0.232 +/- 0.018		
TREATED: (υ = 26)			
TMCS	0.195 +/- 0.009	11.4	2.0
T-BUTYL	0.128 +/- 0.020		

υ = degrees of freedom

have been deactivated. However, that is only one goal of coating a capillary foruse in CZE. A more important goal is the minimization of analyte adsorption. In this work two proteins, trypsinogen and β-lactoglobulin, were used to evaluate the reduction of analyte adsorption.

These two proteins have been difficult to separate by CZE at low pH's due to their basic nature. Trypsinogen has a pI value of 9.4 and β-lactoglobulin has a pI value of 5.4 [136]. When placed in a solution with a pH lower then their isoelectric point (pI) values they are net positively charged. Therefore, in CZE at low pH's they interact with the negatively charged fused silica surface. These interactions lead to poor peak shape and loss of sample. Fig. 19 compares the analysis of these two proteins in formate buffer, pH 4.0, in an uncoated column and a hydrothermally treated t-butyl deactivated column. In the uncoated column, analyte adsorption leads to terrible peak shape. It should be mentioned that the intergrator attenuation is a mean factor of 4 lower for the uncoated capillary. At the higher attenuation the β-lactoglobulin peak could not be detected in the uncoated capillary due to irreversible adsorption. Peak shape and detection limits were greatly improved in the coated capillary. Over 150,000 theoretical plates were generated for the trypsinogen peak with the coated capillary compared to 5,000 with the uncoated capillary.

3.2.4. Evaluation of the Lifetimes of Silane Bonded Capillaries

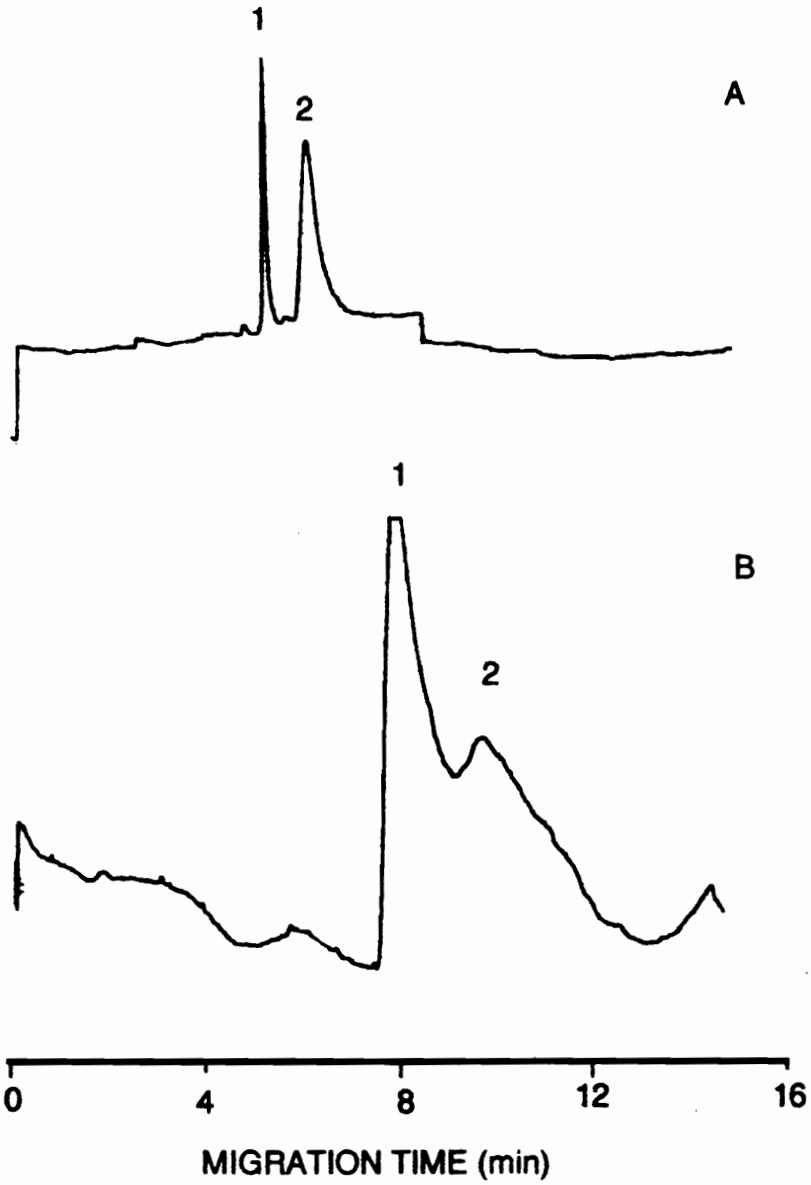

Figure 19. Comparison of the separation of (1) trypsinogen and (2) β-lactoglobulin with a (A) hydrothermally treated (20% nitric acid) t-butyl bonded capillary and (B) an uncoated fused silica capillary. Conditions: 100 μm x 55 cm (l = 35 cm) capillary; 273 V/cm electric field; 10 mM sodium formate buffer, pH 4.0; UV detection at 200 nm.

The last factor that must be addressed is lifetime of these silane bonded capillaries. For a coated column, it must be determined under what conditions these columns are stable. The major factor in CZE that affects coated capillaries is the wide range of pH values used for separations. At high pH values (above 7.0) it was found that these silane based coating are extremely unstable (Fig. 20). After 10 runs at pH 7.0 all of the silane phase is stripped off the surface. The instability is caused by base hydrolysis of the silanol sites at the high pH's. However, at low pH values (below 5.0) these columns have been shown to be very stable (up to 100 runs). Since proteins interaction with the surface occurs at low pH's, these columns are very well suited for protein analyses. Capillary coating is usually not needed when analyzing proteins at high pH values because the proteins have a net negative charge and are repelled by the surface.

3.3. Analysis of Serum and Urine Proteins

3.3.1. Analysis of Albumin in Rat Urine

In the latter stages of diabetes the kidney begins to lose it's ability to retain larger molecules due to damage of the glomeruli. Diabetes is induced into laboratory rats to study the stages of the disease and effectiveness of various treatments. Currently, samples are analyzed by reverse phase HPLC. Sample

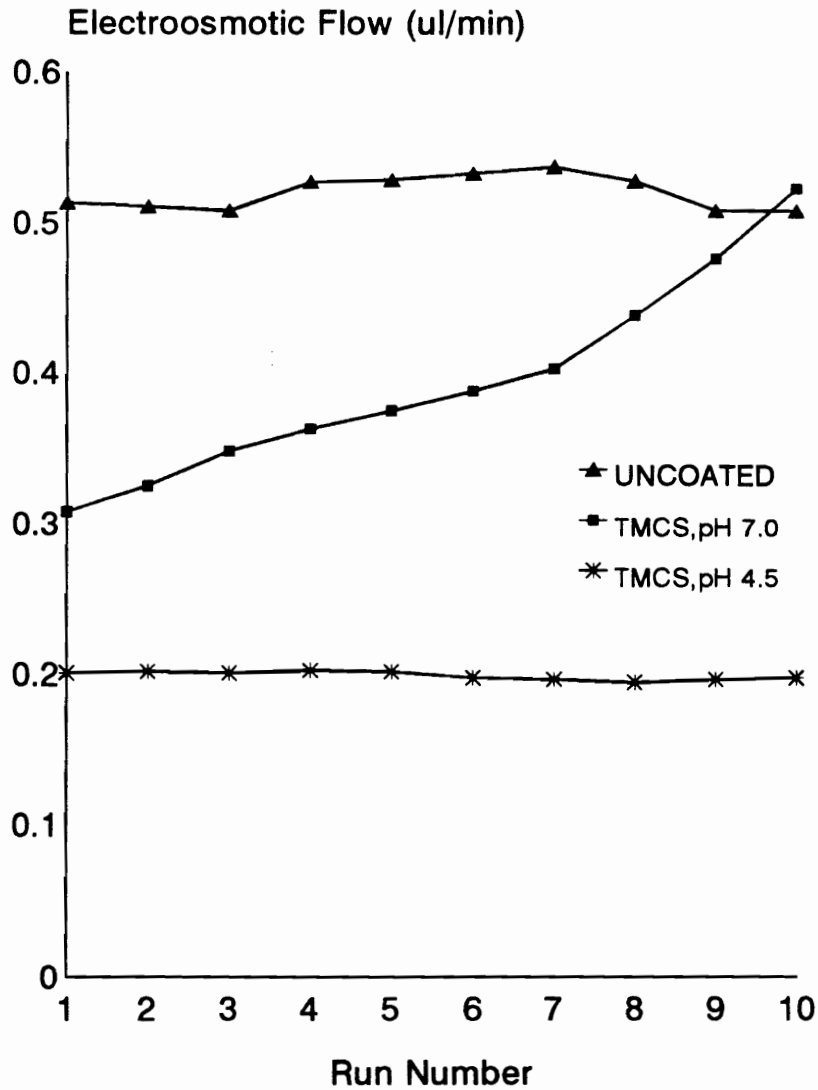

Figure 20. Effects of pH on the lifetime of silane coating. Electroosmotic flow measured with 2% acetone solution in 10 mM sodium phosphate buffer, pH 7.0.

injection volumes of 10μl are used for these analyses. Since the rats generate approximately 50 - 100 μl of sample per day, HPLC is a very sample limited technique for this analysis. The interest in converting to CZE is due to the small sample requirements. In CZE, typical injection volumes are 1- 10 nl; therefore, sample limitation would not be a problem. Another advantage of CZE, is that no sample clean-up or preparation is needed to analyze these samples. Urine is a very dirty sample that quickly limits the lifetime of HPLC columns. With CZE, the surface can be easily regenerated between runs by washing with sodium hydroxide.

In this research, urine samples were collected for three rats to evaluate the use of CZE for the analysis of albumin. To determine the best separation and detection conditions, a standard of rat albumin was prepared and analyzed. The best detection limit (0.02mg/ml) for the standard was found at 200 nm. However, there was a concern that when the sample was analyzed there would be a great deal of interference at that wavelength due to the complexity of the urine matrix. Aromatic proteins tend to show a secondary UV maximum at 280 nm; therefore, the standards and samples were also analyzed at this wavelength. Also, to keep the separation conditions as simple as possible no coated capillaries were employed. Instead a borate buffer with a pH of 10.0 was employed to minimize the solute/surface interactions.

Fig. 21 shows the separation of the urine of a normal rat. A very small

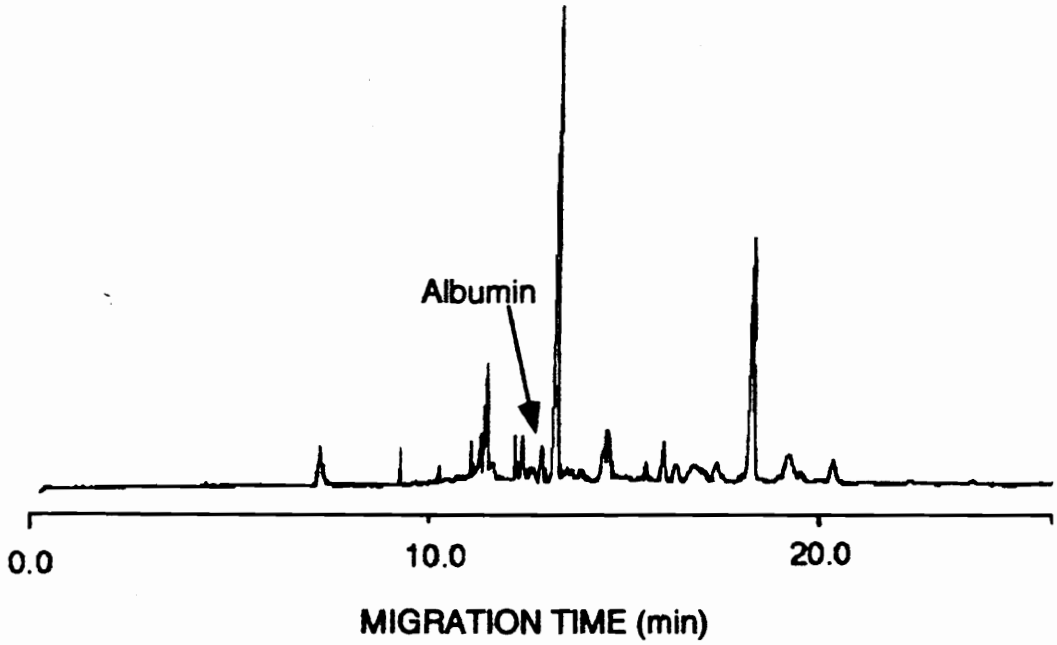

Figure 21. Separation of urine proteins for a normal rat (rat 1). Conditions: 100 μm x 57 cm (l = 50 cm) fused silica capillary; + 15 kV operating voltage; 20 mM sodium tetraborate buffer, pH 10.0; sample introduction with low pressure for 1 sec.; UV detection at 280 nm.

peak for albumin can be seen at 12.89 min.. Fig. 22 shows the separation of the urine of a rat that has been placed in the severe, latter stages of the disease. A large albumin peak (approximately 5 mg) can be seen at 12.77 min.. Separation efficiencies were approximately 200,000 theoretical plates for all analyses. The albumin peaks were identified by spiking with the standard.

The main concern with using CZE was the reproducibility of the migration times, especially with the urine matrix. To maximize reproducibility, the capillary column was flushed with sodium hydroxide between every run to recondition the surface. Table VI shows the reproducibility of the migration times for the rat albumin standard and the albumin in sample rat 3. Both the standard and the sample generated approximately 2.0% RSD values for migration times. This reproducibility is comparable to other separation done by CZE.

Preliminary qualitative results for CZE match the results obtained by HPLC. However, the main advantage with CZE was the ability to inject pure urine onto the capillary. The same capillary was used for all the analyses in this work (approximately 100 runs). An HPLC column used for these samples last about the same amount of time when a guard column is employed. The difference, to replace a CZE column costs approximately $10 versus $500 for an HPLC column. Also, approximately 20 runs were performed on each sample to optimize separation and identify the albumin peak. With HPLC, on average 2-5

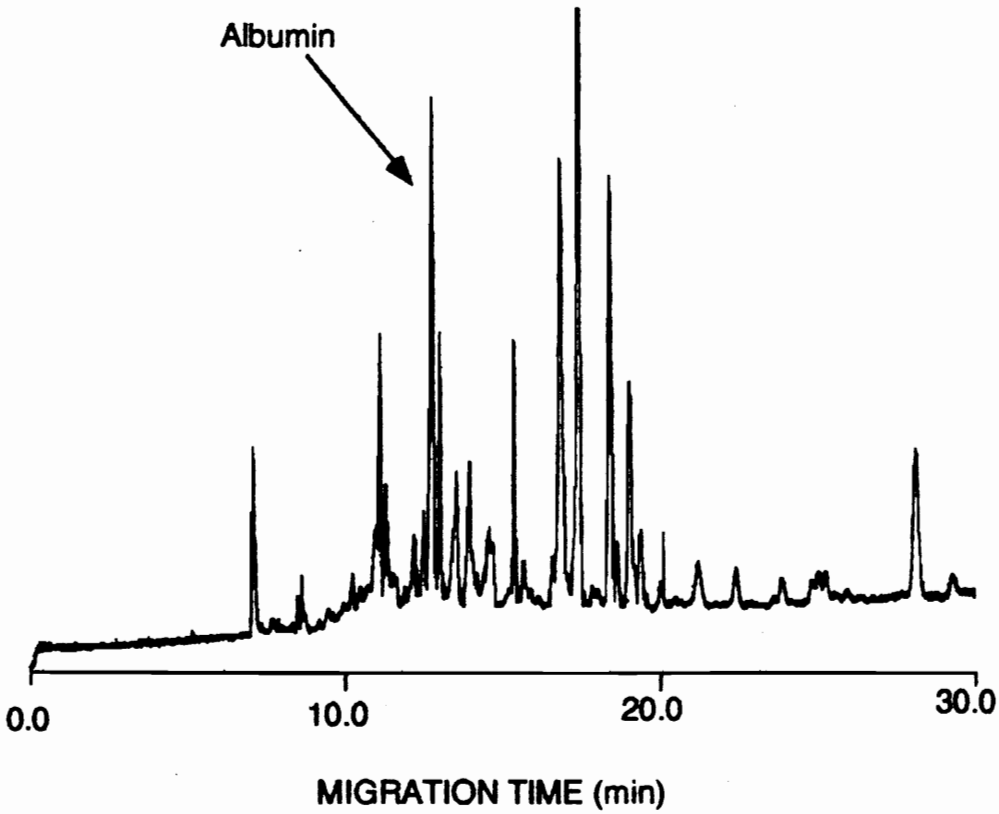

Figure 22. Separation of urine proteins for abnormal rat (rat 2). Conditions are as in Fig. 21.

Table VI. Reproducibility of migration times for albumin in rat samples.

	MIGRATION TIMES FOR ALBUMIN (min)	
	STANDARD ALBUMIN	URINE ALBUMIN
	12.91	12.77
	12.64	12.68
	12.51	13.18
	12.66	12.60
	13.19	12.87
	12.82	12.58
	12.30	13.15
mean	12.72	12.83
+/- std. dev.	0.29	0.25
% RSD	2.3	2.0

runs can be done per sample.

Due to the small quantity of standards and samples very little quantitation was performed on these samples. However, one point of quantitation was discovered. Low limits (less then 0.5 mg) of albumin were difficult to detect in the urine samples due to interferences from the matrix. This was not a major concern since the researchers are interested in levels of 1 mg or more. Also, the darker, more concentrated urine sample (rat 3) contained a great deal more interference in regards to the number of peaks.

3.3.2. Analysis of Serum Proteins

Albumin is also the marker of interest for studying kidney dysfunction in humans. However, there are smaller, less intense immunoglobulins that can give information on the cause of kidney malfunction. Currently, analysis of proteins in human serum is performed by agarose gel electrophoresis (Fig. 23). This test yields quantitative information about albumin content and total immunoglobulin content. However, it has poor efficiency and detection limits. Also, the immunoglobulins can have structural isomers that cannot be separated and detected with agarose gel electrophoresis. Therefore, a more expensive, time consuming test must be performed to identify these peaks. If a patient has a history of disease that leads to kidney dysfunction, this second test would not be

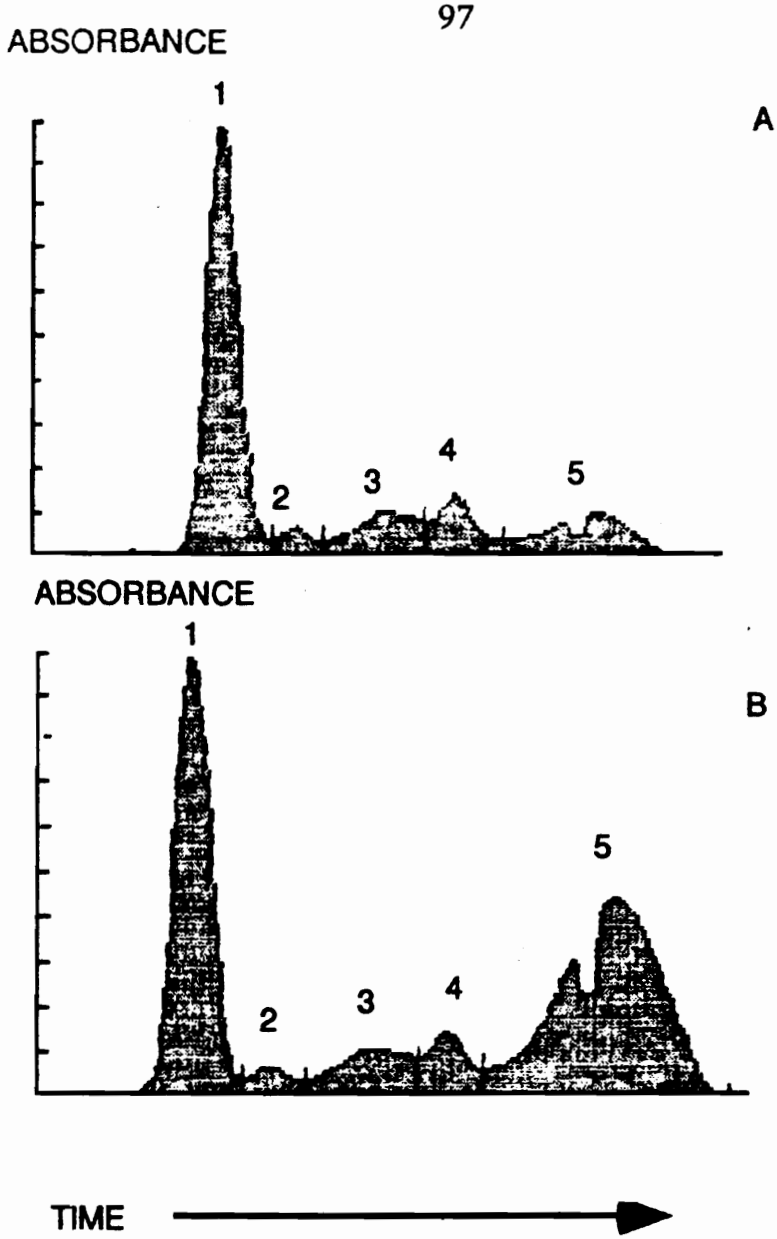

Figure 23. Separation of human serum controls by agarose gel electrophoresis; (A) normal control and (B) abnormal control. Peak elution order is (1) albumin, (2) α_1-globulin, (3) α_2-globulin, (4) β-globulin, and (5) γ-globulin. Conditions: Corning agarose universal electrophoresis film 470100; +300 V operating voltage; barbital buffer, pH 8.6; sample 6μl; detection stain with black 10B colorimetric stain.

performed and the proper diagnosis may not be made. Also, analysis time for gel electrophoreis is approximately 3 hrs. and a great deal of sample handling and manipulation is required. Therefore, an alternative method would be to employ CZE for the analysis.

3.3.2.1. Separation of Serum Proteins by CZE

Fig. 24 shows the separation of normal and abnormal serum samples by CZE. Peaks are not as efficient as previously seen in CZE. This lack of efficiency is due to interactions of the proteins with the silanol sites on the capillary wall and hydrophobic interactions between the proteins and immunoglobulins. A coated capillary was not used because a simple, reproducible technique was being sought. Even though a high buffer pH of 10.0 was employed, the hydrophobic nature of the proteins allows for these interactions. However, separation and detection is better than gel electrophoresis. Detection limits are approximately 20 times better with CZE than gel electrophoresis. This sensitivity allows the prealbumin peak to be detected which can not be seen with the gel electrophoresis.

The main concern with CZE is the reproducibility of migration times to insure qualitative identification of the immunoglobulin peaks. Two approaches were taken to improve the reproducibility. The first approach was to prepare

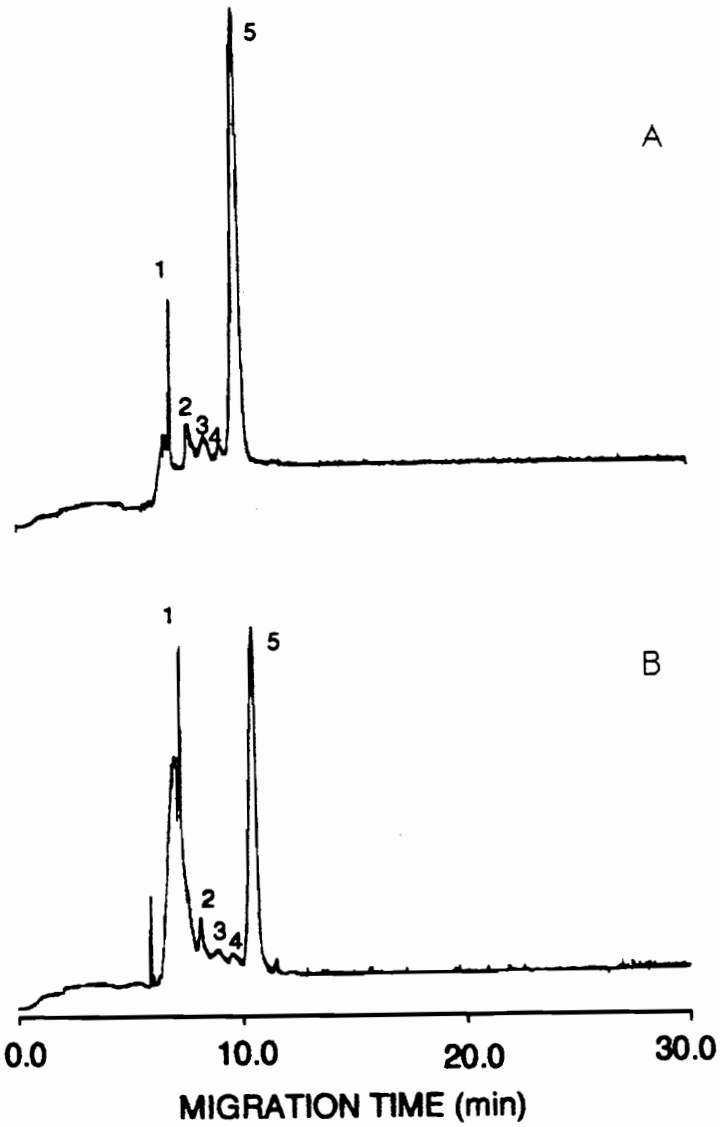

Figure 24. Separation of human serum controls by CZE; (A) normal control and (B) abnormal control. Peak elution order is (1) γ-globulin, (2) β-globulin, (3) α_2-globulin, (4) α_1-globulin, and (5) albumin. Conditions: 100 μm x 57 cm (l = 50 cm) fused silica capillary; + 20 kV operating voltage; 20 mM sodium tetraborate buffer, pH 10.0; sample diluted 1:20 in 1mM boric acid, pH 4.0; sample introduction with low pressure for 1.5 sec.; UV detection at 200 nm.

the sample with an additive. This additive will migrate through the capillary ahead of the sample. As it passes through the capillary it temporarily modifies the surface to minimize the analyte/surface interactions for the later eluting peaks. Gordon et. al. [123] have shown promising results with the addition of ethylene glycol for the separation of proteins. In this research, a 0.2% solution of polyvinyl alcohol was used to dilute the sample. The polyvinyl alcohol was selected based on its use as a dynamic coating material. As the alcohol passes through the capillary the long chains cover the active silanol sites minimizing solute/wall interaction. The polyvinyl alcohol does not interfere with the separation of the sample peaks (Fig. 25). Reproducibility in migration times of the albumin without the alcohol was 1.8% RSD under the best conditions. Reproducibility with the alcohol was 1.5% RSD.

A second factor affecting the reproducibility was the rinse time with sodium hydroxide between runs. A sufficient rinse time must be employed to unsure that the surface is sufficiently cleaned and reconditioned for the next analysis. Fig. 26 shows the reproducibility of migration times for albumin (based on 5 runs) using varying sodium hydroxide rinse times. If the selected rinse time was not sufficient, reproducibilities in migration times high as 11.3% were seen. A rinse time of 10 min. with 0.1 N NaOH yielded a reproducibility of migration time of 1.8%. The use of polyvinyl alcohol with a 2 min. rinse time improved these results to 1.5%.

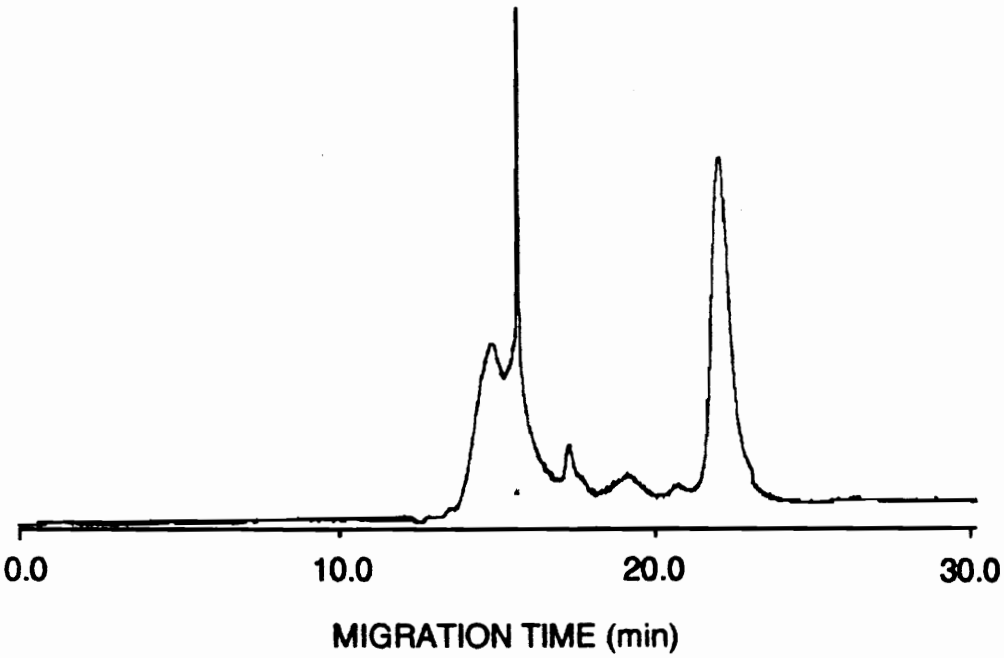

Figure 25. Separation of abnormal human serum control with sample dilution of 1:20 in 0.2% polyvinyl alcohol in 1 mM boric acid, pH 4.0. Operating voltage is + 15 kV ; all other conditions the same as Fig. 24.

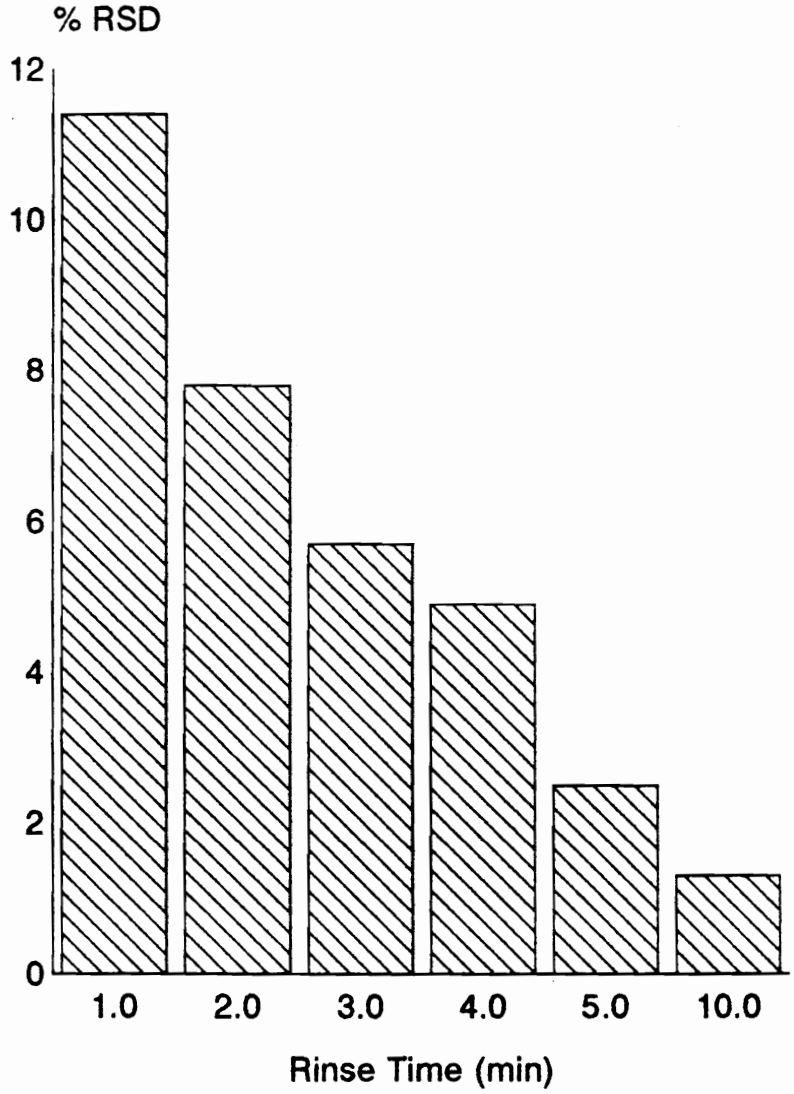

Figure 26. Effect of 0.1 N NaOH rinse time on the reproducibility of the migration time of albumin for abnormal control. Conditions are as in Fig. 24. (n = 5 for each rinse time)

3.3.2.2. Analysis of Serum Proteins with Methyl Cellulose as a Buffer Additive

Since the analysis of protein content is currently performed by gel electrophoresis it would be wise to consider using capillary gel electrophoresis as the mode of separation. However, to form gels inside capillaries reproducibly has been a difficult task [137]. An alternative to a permanent gel is to dissolve a component in the buffer to work as a dynamic gel. These dynamic gels add molecular sieving as a secondary mode of separation; however, unlike fixed gels they are continuously regenerated. Low concentrations of methyl cellulose (0.2 - 0.6% w/v) have been shown to produce promising results for the analysis of DNA fragments [103,104]. In this research a 0.2% w/v solution of methyl cellulose prepared in 20 mM sodium tetraborate buffer was investigated to determine it's effect on the separation of the abnormal serum control. Fig. 27 show the separation of the abnormal serum with the 0.2% w/v methyl cellulose using the same conditions previously employed. Resolution is improved between the albumin and the g-globulin peak. However, with this improvement in resolution also came a drastic increase in analysis time and baseline noise. The baseline noise was due to bubble formation of the buffer. The methyl cellulose had a strong tendency to foam. Another difficulty with using methyl cellulose was the viscosity of the buffer solution. The high viscosity caused a drastic increase in

fill time for the capillary. With the borate buffer alone it took approximately 0.5 min. to fill the capillary. With the methyl cellulose buffer it took 20 min. to completely fill the capillary. This fill time also added to the analysis time. Also, if the capillary was not properly or completey filled large fluctuations in current caused the system to shut down. The capillary then had to be flushed and refilled to insure that all the air was displaced from the capillary by the solution.

CHAPTER IV

CONCLUSION

CZE has been proven to be an exceptionally efficient mode of separation for the analyses of ionic and ionizable compounds, such as surfactants and proteins. Ionic species have proven difficult to separate by other separation modes without derivatization or chemical modification of the charged functional group. CZE however, allows for these separations to be performed without modification to the compound. However, modifications to the CZE system may be needed to achieve detection and separation.

Ammonium ethoxylated alcohols are an excellent candidate for analyses by CZE based on their anionic nature. With the addition of potassium dichromate to the buffer as a absorbing ion, detection is possible by indirect UV of these non-absorbing analytes. Potassium dichromate should not be the best choice as a co-ion based on its large difference in electrophoretic mobility compared to the surfactants: however, upon investigation it yielded the most promising results. However, there is a skewing of the peaks due to this mismatch in mobilities that leads to a reduction in resolution.

The other major cause of lower efficiencies and poor resolution is the concentrations of the surfactant samples. Due to poor detection limits, sample concentrations were above the CMC's of the surfactants which leads to the

formation of micelles during analysis. Since micelle formation is a very dynamic process, both free surfactants and micelles are being separation during the run. This formation of micelles changes the mobilities of the surfactants which leads to zone spreading. These interactions were minimized by the addition of acetonitrile to the buffer. The acetonitrile provides a more hydrophobic environment, improving the solubility of the surfactants and minimizing micelle formation. However, even with the addition of the organic solvent, not all of the molecular interactions will be eliminated. The only way to completely eliminate these interactions is to work at lower sample concentrations.

The poor detection limits seen with the dichromate/tetraborate buffer can be attributed to two major factors. First dichromate has a net -2 charge which requires two net -1 charged surfactants to replace it in the sample zone. This should theoretically reduce the detection limits by half. Also, by using a binary buffer system (needed to obtain separation) the non-absorbing tetraborate also competes for displacement with the surfactants. If a non-absorbing borate ion is displace by the surfactants no change in signal will be register. To obtain the best detection limits, a co-ion with an electrophoretic mobility similar to the analyte and that can provide both detection and separation at a reasonable concentration should be sought.

Indirect UV has provided detection of AEOS to allow for their analyses by

CZE. However, the method of detection is very sensitive to small changes is the experimental parameters. Therefore, it is not a very robust method at this time.

Another separation that has proven difficult by other techniques is that of proteins found in urine and serum. Based on their ionizable nature these proteins are also well suited for analysis by CZE. Another important factor that makes CZE a good choice for these analyses is based on the complicated nature of the matrices. With CZE, the capillary can be flushed with a strong solvent between runs to remove any components of the dirty matrix that might have adsorbed to the walls and could cause interferences in later runs. In other words, each run is begun with a clean, uniform surface. This type of clean-up is not possible with other separation techniques because columns would be destroyed. The strength of the cleansing solvent as well as the time of the cleaning process is very important in maintaining separation reproducibilities. As seen with the serum proteins, reproducibilities of migration times were between 1.8-11% depending on the rinse time.

Even though proteins, based on their chemical nature, are well suited for analyses by CZE they have proven difficult to analyze with the technique. The main difficulty is the sample interaction with the active silanol sites on the fused silica surface. These interactions can lead to reversible (poor peak shape) or irreversible adsorption (loss of sample). However, there are several ways these interactions can be minimized. The easiest way is to work with a buffer at a

high pH. At high pH's, the proteins should have a net negative charge which will cause them to be repelled by the surface. The high efficiencies seen in the analyses of rat urine show the feasibility of the method. Another reasonably easy way to minimize these interaction is with sample additives. By adding polyvinyl alcohol to the sample the surface active sites are reversibly covered as the polymer passes through the capillary minimizing surface interaction with the later eluting protein peaks.

To obtain reproducible separation of the serum proteins all of these factors had to be employed. However, efficiencies in these separations were still relatively poor compared to other analyses by CZE. The low efficiencies are probably due hydrophobic interactions between the proteins and immunoglobulins. However, compared to the current mode of analyses, agarose gel electrophoresis, CZE is a more efficient, less labor intensive means of analysis. Sample analysis times went from 3-4 hrs. to 20 minutes. Also, with automation available in CZE less sample handling is required minimize the time the analyst is exposed to the potentially hazardous sample.

A more labor intensive means of eliminating the analyte/surface interactions involves permanent deactivation of the capillary surface. However, as previously discussed, coated capillaries for use in CZE have had problems with deactivation and reproducibilities. The main reason for these problems is the treatment or lack of treatment to the surface prior to coating. It has been

known for many years that improve deactivation and coating of silica for use in chromatography is achieved by hydrothermally treating the surface prior to deactivation. However, many researcher felt this treatment was not necessary due to the very limited number of silanol sites on the limited surface area of the capillaries used in CZE.

However, this research shows that upon hydrothermal treatment, the number of silanol sites available drastically increases. In fact the electroosmotic flow is almost doubled in the treated capillaries compared to the untreated. By activating these sites prior to coating a homogenous surface is produced leading to more uniform and reproducible deactivation. Without this treatment, surface deactivation can very dramatically depending on the history of the fused silica tubing used. The results of this can be seen in the comparison of the untreated versus treated tubing deactivated with the silanes. The electroosmotic flow is almost 1/3 lower in the treated capillaries.

Electroosmotic flow is not completely eliminated with these deactivated columns, however. This indicates that not all of the active sites were covered with the coating. However, the improve deactivation with the bulkier t-butyl group indicates that not all the sites have to be chemically bonded to be deactivated. Steric hinderance by the t-butyl group aids in deactivation.

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