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**Analysis of Selected Proteins using Capillary Zone Electrophoresis
with Absorbance and Mass Spectrometric Detection**

by

Teresa M. Loiselle, B.Sc.

**A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment for the degree of**

Master of Science

**Ottawa-Carleton Chemistry Institute
Department of Chemistry
Carleton University
Ottawa, Ontario**

September 8, 1995

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**ANALYSIS OF SELECTED PROTEINS USING
CAPILLARY ZONE ELECTROPHORESIS WITH ABSORBANCE
AND MASS SPECTROMETRIC DETECTION**

submitted by **Teresa M. Loisel, B.Sc.**
in partial fulfilment of the requirements for
the degree of Master of Science

Thesis Supervisor

Chair, Department of Chemistry

Carleton University
September 7, 1995

Abstract

Protein ions in solution were analysed by means of capillary zone electrophoresis (CZE) with on-column absorbance detection and on-line mass spectrometric detection. Proteins were analysed as either cations or anions via modification of the buffer pH while retaining a positive electric field gradient. Since CZE and electrospray mass spectrometry are tools for examining ionic analytes in solution, it was hypothesized that coupling of the two might provide additional information not available individually. In the conceptual stages, it was thought that it might be possible to separate individual protein ions; however, the protonation/deprotonation equilibria among the charge states were too fast, resulting in ions migrating through the column in one band. In contrast, different conformations of proteins have much slower equilibria resulting in the conformations being 'frozen-in-time' within the time frame of a CZE separation. It was possible to separate the conformational isomers.

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Glossary

CE	Capillary Electrophoresis
CZE	Capillary Zone Electrophoresis
CITP	Capillary Isotachophoresis
CIEF	Capillary Isoelectric Focussing
CGE	Capillary Gel Electrophoresis
MEKC	Micellar Electrokinetic Capillary Chromatography
CEC	Capillary Electrochromatography
MS	Mass Spectrometry
ES	Electrospray
ES-MS	Electrospray Mass Spectrometry
CZE-MS	Capillary Zone Electrophoresis Mass Spectrometry
HPLC	High Performance Liquid Chromatography
PAGE	Polyacrylamide Gel Electrophoresis
CTAB	Cetyltrimethylammonium bromide
UV	Ultra-Violet
FD	Field Desorption
PD	Plasma Desorption
FAB	Fast Atom Bombardment
PEG	Polyethylene Glycol
ID	Inner Diameter
EOF	Electroosmotic Flow
m/z	mass to charge
μ_{eo}	electroosmotic mobility
$\mu_{ep,i}$	electrophoretic mobility of component i
v_{eo}	electroosmotic velocity
$v_{ep,i}$	electrophoretic velocity of component i
t_i	injection time

V_i	injection voltage
t	separation time
V	separation voltage
L	length of column
L_{det}	length to detector
ζ	zeta potential
η	viscosity
E	electric field gradient
D	diffusion coefficient
R	resolution
N	efficiency, number of theoretical plates
C	concentration
r	radius of the column
M_r	molecular mass of protein
M_a	molecular mass of adduct carrying charge

Chapter 1- Introduction

1.1 Introduction

Capillary electrophoresis (CE) is a group of relatively new techniques which provide efficient separation of charged species in solution. Before the development of CE, analysis of proteins was time consuming, requiring complicated procedures such as polyacrylamide gel electrophoresis (PAGE). In the following sections, CE, specifically capillary zone electrophoresis (CZE) is discussed including the concepts of analyte migration and electroosmotic flow. As well, UV absorption and MS detection are investigated for their suitability as detectors for various protein species in solution. While the absorption detector is most common for CZE, MS provides the added advantage of unequivocal identification of species based on their mass to charge (m/z) ratios. MS is fast and sensitive. The transfer of the macroions from the solution phase, necessary for CZE analysis, to the gas phase, necessary for MS analysis, is the key step in an on-line interfacing of CZE with MS. Electrospray (ES) facilitates this ion transfer from the solution to the gas phase. Highly charged intact macromolecules are formed in lieu of the complicated fragmentation patterns common to many other ionization methods. However, the electrospray interface for CZE-MS is more complicated than the requirements for UV absorption detection. These aspects of CZE-MS are discussed in detail in the following pages.

1.1.1 Electrophoresis

Electrophoresis, first observed by Lodge in 1886, involves the migration of charged species in an externally applied electric field. Species that have been analyzed successfully include both inorganic and organic species, such as ionic salts, organic acids, amino acids, peptides, proteins and nucleic acids (1,2,3,4). Unfortunately, the analysis of larger species, *e.g.* proteins, has been plagued by difficulties both in their separation and in the identification of the components after separation. In the early days of electrophoresis, analysis was carried out in glass U-tubes several centimetres in diameter. This type of electrophoresis was encumbered with several problems, such as relatively large sample volume requirements, excessive Joule heating (causing convective mixing), low resolution, low detection sensitivity, and difficult staining procedures for detection (5). At that time, the only method available to circumvent the problem of convective mixing was the use of stabilizing media, such as agar, polyacrylamide gel, silica gel, paper or glass wool. Until recently, the best technique to analyze proteins was polyacrylamide gel electrophoresis (PAGE) which involved the movement of proteins through a gel. Separation was governed by both protein charge and size. Later, as small diameter capillary columns became available, a superior method for the analysis of proteins in solution was developed that minimized temperature gradients, decreased sample volume requirements, increased resolution, and simplified detection.

Since capillary columns of inner diameter (ID) $5\mu\text{m}$ and larger first became commercially available, a new branch of electrophoresis, capillary electrophoresis (CE), emerged. Not surprisingly, the growth of CE has paralleled many of the major technological advances in chromatography with respect to capillary column manufacturing and coating techniques. Most of the problems associated with conventional electrophoretic analyses have been eliminated or minimized while new problems, such as protein adsorption onto the capillary column and electroosmotic flow control, have been generated. With the use of small ID columns, joule heating is reduced due to a larger surface area to volume ratio providing efficient cooling of the buffer solution during electrophoresis. Consequently, temperature gradients in the capillary leading to band broadening and loss of efficiency are minimized. Resolution and sensitivity are now limited only by diffusion. Because sample size requirements are in the pL to nL range, large volumes of solvent are not consumed and wasted (1). Low-volume samples no longer present difficulties in analysis. Also, on-column absorbance and fluorescence detectors are used to simplify detection. Most recently, on-line MS detection has been reported. As a result, there is now a widespread, renewed interest in the electrophoretic technique and its potential capabilities.

Capillary electrophoresis is simpler, faster and more sensitive than conventional techniques for protein analysis such as slab gels and large-tube electrophoresis; its resolution is often better than that of high performance liquid chromatography (HPLC). There have been several comprehensive reviews of CE and

its applications in recent years (1,6,7).

1.1.2 Modes of Capillary Electrophoresis

The heart of CE is a capillary whose two ends are typically dipped into buffer or electrolyte reservoirs; a detector and a high voltage power supply constitute the remaining components as illustrated in Figure 1. Capillary electrophoresis can be tailored to separate a given set of analytes due to the availability of several operating modes. Each mode uses similar and relatively simple instrumentation. Within each mode there is also the possibility of selective separation through the selection of various buffer systems and additives. The main differences among the CE modes lie in the components contained within the capillary. The column may contain gels, chromatographic packings or coatings. The CE separation modes include:

1. Capillary Zone Electrophoresis (CZE)
2. Capillary Isoelectric Focusing (CIEF)
3. Capillary Isotachopheresis (CITP)
4. Capillary Gel Electrophoresis (CGE)
5. Micellar Electrokinetic Capillary Chromatography (MEKC)
6. Capillary Electrochromatography (CEC)

Figure 1. Schematic of Capillary Electrophoresis Apparatus.

The buffer systems may be either continuous or discontinuous (1). In a continuous system, the capillary is filled with a single background electrolyte solution during the entire separation. With the composition of the background electrolyte constant, the electric field gradient and the mobilities of ions also remain constant throughout the analysis. As a result, migration velocities are constant for each ionic species. CZE, MEKC, CGE, and CEC are all examples of this type of separation. In a discontinuous system, the background electrolyte composition varies across the capillary. As the composition of the electrolyte is changed during the analysis, the electric field gradient and species mobilities may vary within the analysis. An example of this type of separation is CITP and CIEF.

Because capillary zone electrophoresis (CZE) is the simplest and most rugged of the available modes, it is the most common form of CE in use at present. In CZE a sample solution is injected into the head of the capillary and the ions are induced to migrate through the capillary in response to an applied electric field through a continuous background electrolyte. Each species has a mobility which reflects many intrinsic factors including its charge, size, and solvation geometry as well as many external factors such as buffer concentration and pH. The effective mobility of each species is a composite of the electroosmotic flow of the buffer solution and the electrophoretic mobility of the ion. The final result is that each species migrates through the capillary with a distinct, and hopefully, different migration velocity to separate into a discrete zone. As these zones reach the detector, they are monitored

to determine sample composition and component concentrations.

The other five forms of CE have specific applications, but are less commonly used. In CIEF the analytes are separated on the basis of their isoelectric points by the construction of a pH gradient across the capillary. When the pI of an analyte equals the pH of the buffer solution, migration ceases. This mode of CE is useful if the analytes have a range of isoelectric points. In order to detect the analytes a second mobilization step is required to move the analyte zones through the capillary to the detector. Zone broadening must not occur during this stage or the advantages of analyte focussing are lost. CITP is performed in a discontinuous buffer system and the analyte species reside between the leading and tailing electrolytes. In CGE separation is based on solute size. The analytes migrate through the pores in a gel-filled capillary. This technique is essentially capillary PAGE. MEKC is very similar to CZE except a surfactant at a concentration greater than its critical micelle concentration is added to the buffer. Micelles are formed within the solution phase. The analytes partition themselves between the free solution phase and the micelles. This mode allows both charged and neutral species to be resolved. In CEC the capillary contains a chromatographic packing. Because the buffer solution is in contact with the packing, the distribution of analytes is similar to that in column chromatography. Electroosmotic flow arises from the charges on all the surfaces, including both the column wall and the surface of the packing material, consequently the plug-like flow characteristic of CZE (discussed in section 1.1.3.1) is less

apparent.

1.1.3 Migration in Capillary Zone Electrophoresis

Models based on fundamental principles, such as the model by Compton *et al.* using the Debye-Huckel-Henry theory, have been proposed in an attempt to predict ion mobility within the capillary column under given conditions (8). At present these models give only approximations as the processes occurring in the column are complex. Also, modelling requires the calculation of charge and size information concerning the analyte. This is time consuming and introduces a degree of approximation because the size of all analytes cannot be reported as a simple hydration sphere as many are not spherical. For simpler molecules, these models can accurately predict ion mobilities in capillary zone electrophoresis. The following simple explanation of CZE provides a basic understanding of the electrophoretic process. Because proteins can adopt conformations of varying compactness and shape, all mobilities were determined from experimentation. It was assumed that there are no analyte/column wall, or buffer/column wall interactions. As stated previously, migration in CZE is a composite of the electroosmotic flow of the buffer and the electrophoretic mobility of the analyte.

1.1.3.1 Electroosmotic Migration

Electroosmotic flow (EOF) in the capillary column originates from an electric double layer formed on the inner wall of the capillary. When the fused silica column wall is in contact with a solution above a pH of approximately 2, an appreciable number of the silanol groups on the inner surface of the column are negatively charged. This stationary layer of negative charge induces a layer of positive charge, which may extend several nanometres into the buffer solution. The positive layer consists of an immobile or Stern layer as well as a diffuse, mobile layer of cations. The potential across the double layer is termed the zeta potential ζ (in Volts). The zeta potential can be calculated from theory by using the following equation:

$$\zeta = \frac{\mu_{eo}\eta}{\epsilon\epsilon_0} \quad (1)$$

where η is the viscosity of the electrolyte ($\text{kg s}^{-1} \text{m}^{-1}$), ϵ is the permittivity of the buffer, ϵ_0 is the permittivity constant, and μ_{eo} is the coefficient of electroosmotic flow ($\text{m}^2 \text{V}^{-1} \text{s}^{-1}$). In the presence of an applied electric field, the positive layer becomes mobile and migrates toward the cathode guided by the electric field. The EOF velocity is given by the equation:

$$v = \frac{\epsilon\epsilon_0}{\eta} E\zeta = \mu_{eo}E \quad (2)$$

where E is the electric field gradient ($V\ m^{-1}$). From experiment the electroosmotic mobility can be measured using the following equation where t is the migration time of a neutral marker carried through the column by EOF only, L is the total length of the column and L_{det} is the length of the column to the detector.

$$\mu_{eo} = \frac{LL_{det}}{Vt} \quad (3)$$

Because the double layer is only nanometres thick, the EOF is viewed as originating at the wall of the capillary. Since the cations are solvated, the migration of these ions en masse drags the bulk solution toward the cathode. In the absence of a pumping system to force the electrolyte and sample through the capillary, there is also an absence of the usual, parabolic flow profile. In its place is the flat, plug-like flow profile characteristic of capillary electrophoresis.

As the EOF decreases, the residence time of a positive analyte in the capillary is increased thus allowing more time for separation. For a negative ion, its residence time is dependent on the difference between the electroosmotic velocity toward the cathode and its electrophoretic velocity towards the anode. If the electroosmotic velocity is too low, negative ion cannot pass through the column to be detected at the cathodic end. A large EOF allowed anions to be analyzed under a positive applied

electric field. Electroosmotic flow may be advantageous in some CE systems such as MEKC and CZE, but, in general, disadvantageous in others such as CGE, CITP, and CIEF and CEC.

Control of electroosmotic flow is the key to turning CZE into a reproducible, valuable analytical technique. In untreated fused-silica columns, the electroosmotic flow is often not reproducible over a series of analyses. Even under careful control of experimental parameters, minor variations between analyses, such as local effects on the capillary surface, can produce significant variations in retention time and zone length. Controlling the electroosmotic flow should be performed in such a way that adsorption of cationic species onto the negatively charged fused silica wall is minimized.

Several methods to control electroosmotic flow have been reported. Direct manipulation of the electroosmotic flow was performed by Wu *et. al.* by using a radially applied electric field to fix the zeta potential (9). However, this does not reduce cation adsorption. A self-regulating dynamic control of the electroosmotic flow has recently been proposed by Chang and Yeung (10). This is performed by establishing a gradient across the capillary of the surfactant cetyltrimethylammonium bromide (CTAB). The gradient remains stationary except for small shifts to counteract small changes in surface charge due to the sample matrix. The use of high ionic strength buffers has been shown to reduce cation adsorption, but does little to combat

the problem of variations in electroosmotic flow between and during analysis. The simplest and most effective method to reduce cationic adsorption and stabilize the electroosmotic flow is to coat the capillary column wall with a neutral compound. This reduces EOF at low pH and almost completely eliminates cationic protein adsorption (11, 12, 13). Many of these types of columns are now commercially available. This method is most suitable for CZE with detection by MS since high ionic strength buffers are not tolerated by MS.

1.1.3.2 Electrophoretic Migration

The effective mobilities exhibited by analyte zones as they migrate through the capillary are a composite of both the electroosmotic flow of the buffer and the electrophoretic mobilities of the analytes. The values of the electroosmotic and electrophoretic mobilities can be found experimentally by monitoring migration times. In general, the mobility values are related to the charge, size, molecular weight and conformation of the ions in solution. Because the direction of motion for cations under an positive electric field is towards the cathode, their velocities will be augmented by the electroosmotic flow while the velocities of anions will be reduced. Consequently, anions typically exhibit higher resolution because of their longer retention times. As long as the magnitude of the electroosmotic flow towards the cathode is greater than that of the electrophoretic movement of anions towards the anode, the elution order at the detector positioned at the cathodic end under a positive

electric field gradient, E , is cations, followed by bulk elution of all uncharged species, and finally anions. Thus, in theory, all charged species can be analyzed in a single run. Uncharged species are not separated because they possess no electrophoretic mobility. The migration velocity of a charged species in the presence of electroosmotic flow is given by:

$$v = \mu E = \frac{(\mu_{eo} + \mu_{ep}) V}{L} \quad (4)$$

where V is the applied voltage, L is the total length of the capillary, and μ_{ep} is the electrophoretic mobility of the analyte. The migration time of each species is given by:

$$t = \frac{L^2}{(\mu_{eo} + \mu_{ep}) V} \quad (5)$$

Because capillary electrophoresis is in many aspects similar to column chromatography, many definitions in CE are borrowed from chromatography. The number of theoretical plates, N , reflecting efficiency, is calculated as:

$$N = \frac{(\mu_{eo} + \mu_{ep}) V}{2D} \quad (6)$$

where D is the diffusion coefficient of the analyte. The resolution, R may be calculated from the following equation.

$$R = \frac{(\mu_{ep,1} - \mu_{ep,2})}{4(2)^{0.5}} \left[\frac{V}{LD(\mu_{ep,ave} + \mu_{eo})} \right]^{\frac{1}{2}} \quad (7)$$

where $\mu_{ep,1}$ is the electrophoretic mobility of component 1, $\mu_{ep,2}$ is the electrophoretic mobility of component 2, and $\mu_{ep,ave}$ is the average electrophoretic mobility of components 1 and 2. Because anions remain in the column longer and achieve better resolution at the expense of N , their actual efficiency may be lower than that given in the simple theory.

1.1.4 Injection Methods

Several methods may be used to introduce a sample plug into the head of the capillary prior to CE analysis. The most popular of these methods are electrokinetic and hydrodynamic injections (14). Electrokinetic injection requires that the injection end of a buffer-filled capillary be dipped into the sample solution and a voltage applied for a finite period of time. Electrokinetic injection causes preferential injection of more electrophoretically mobile species. In effect, rapid electrophoresis is being performed during the injection time. Hydrodynamic injection uses gravity, suction or pressure to place a sample plug into the head of the buffer-filled capillary

column. Hydrodynamic injection seems to be a more complicated injection procedure in that it requires mechanical movements of the capillary. It does not lend itself as easily to automation as the electrokinetic injection method.

In electrokinetic injection the high voltage electrode and the capillary injection end are placed in the sample solution and a voltage is applied across the capillary. Sample enters the capillary by both electrophoretic migration and electroosmotic flow (14). The length of the injection plug, l , is given in terms of the injection time, t_i

$$l = (v_{ep} + v_{eo}) t_i = (\mu_{ep} + \mu_{eo}) \frac{V_i}{L} t_i \quad (8)$$

where v_{ep} refers to the electrophoretic velocity of the analyte, v_{eo} refers to the electroosmotic velocity of the buffer. The length of the injection plug is analyte dependent. In general, the volume of the injected plug is 1 to 50 nL depending on the column diameter. The amount of sample injected into the column, n , is calculated as a function of the sample concentration, C .

$$n = \pi r^2 l C = (\mu_{ep} + \mu_{eo}) \pi r^2 V_i t_i \frac{C}{L} \quad (9)$$

where r is the radius of the column and V_i is the applied voltage during injection.

This equation shows that the amount injected into the column can be controlled by the injection time and voltage. The sample zone injected must be a suitable length and concentration such that deleterious effects (*e.g.* localized Joule heating, band broadening, and local variations in voltage) do not occur in the analysis. In the extreme case, subsequent electrophoresis may be jeopardized by the formation of air bubbles if Joule heating is excessive.

Not only does electrokinetic injection show preferential injection of the more mobile species, but the amount injected may vary if the conductivities of the sample solution and the buffer do not equally match. Sampling bias by electrokinetic injection was investigated by Huang *et. al.* (15) who defined the bias factor, b , for a two species system as:

$$b = \frac{(\mu_{ep,1} + \mu_{eo})}{(\mu_{ep,2} + \mu_{eo})} = \frac{t_{m,2}}{t_{m,1}} \quad (10)$$

where t_m is the migration time of the first (1) and second (2) species through the column. If the electrophoretic mobilities are not identical, but the electroosmotic mobility, μ_{eo} , is much greater than the electrophoretic mobilities of the analytes, no sampling bias occurs ($b \approx 1$). If b does not equal one then sampling bias occurs and must be taken into account when calculating the absolute amount injected.

A related effect, sample stacking, may occur during injection if the sample is

in a solution of lower ionic strength than the buffer (16). Because less concentrated solutions experience a higher electric field and hence greater mobility, the sample components enter the column and migration slows when the sample-buffer interface is reached. There is an absolute limit to sample stacking in that at the boundary there is an increase in electroosmotic pressure. At the extreme the high ionic strength at the boundary may lead to laminar flow and peak broadening at high analyte concentration. This technique is most advantageous when the sample solution has an ionic strength one to two orders of magnitude below that of the buffer. Injection time is increased by a factor of 5 to 10 to gain maximum benefit.

A second method, on-column transient isotachophoretic preconcentration, has been used by different groups to further lower the detection limits for CZE (17,18). CITP preconcentration is most suitable for samples that are dissolved in more complex sample matrices than simply water or diluted buffer. In CITP the sample is deposited between a leading electrolyte having greater mobility than the sample ions, and a terminating electrolyte having a lower mobility than the sample ions. The electric field gradient over each of the sample zones is inversely proportional to the conductivity of each zone, which is in turn dependent on the mobility and concentration of the sample ions. The result is a series of adjacent bands separated on the basis of their concentrations and mobilities. Highly mobile ions are conductive which results in decreased field strength and hence electrophoretic velocity. Less mobile species generate greater E and exhibit greater velocity. After a time an

equilibrium is reached where each species' velocity is normalized. All zones are well defined. Throughout this CITP step, concentration or focussing has occurred. If a band diffuses into another, it is quickly returned to its band by the encountered field strength. Quantitative information is found in the zone length rather than the height or area. As separation continues, the leading and tailing electrolyte boundary disintegrates. The bands migrate into the background electrolyte and finish separation as CZE zones.

1.1.5 Detection Methods

The efficiency and resolution in CE is dependent on the sensitivity and response time of the detection system. Fortunately, the flow of zones in capillary electrophoresis is relatively slow as compared to some of the other CE modes so that very fast detection is not required to obtain good results. The most common detectors are fluorescence (both laser induced and lamp based) and UV absorbance detectors (3, 19, 20). Detection limits have been lowering over the last few years and it is now possible to reach attomol and even lower on-column detection limits. High sensitivities have allowed the analysis of the contents of single cells by CE (21). These detectors must be equipped with a flow through cell for on-column detection. The difficulty with the universality of the two above methods is that they require specific activity of the analyte. Sample species must either absorb strongly in the UV region or exhibit fluorescent behaviour. Labelling with proper chromophores has

been performed (22). Labelling can be avoided by indirect measurement of the sample zones in which the buffer is the optically active species (20) and the analyte signal is seen as a drop in the 'background' response of the detector.

A less common but potentially more powerful detector is the mass spectrometer. In order for the ions to enter the MS they must be transferred to the gas phase from the solution phase. Two ionization methods by which this can be accomplished are electrospray ionization or fast atom bombardment (1). Electrospray has proven to be a simple, quantitative technique that provides sensitive detection of proteins in solution. Before this "ionization" technique was developed, the analysis of large molecules such as proteins by MS was difficult. Other ionization techniques typically fragment the protein ions extensively into unrecognizable pieces. Because the ions exist pre-formed in solution, a soft desorption technique such as electrospray is all that is required. For the experiments of this thesis work, an absorption detector was used in all CZE experiments; when needed, the MS was placed in series with and downstream from the absorption detector. Electrospray mass spectrometry (ES-MS) is discussed in detail in the following section.

1.2 History of ES-MS

MS is arguably the most information-rich detection system presently available to analytical chemists. Several requirements must be fulfilled in order for the analyte

to be detected by MS. The sample ions must survive the ionization step and remain stable over a long enough interval to be transmitted through the instrument. The limiting factor in the analysis of many species is the availability of a suitable ionization method. For conventional methods like electron impact, the ionization efficiency typically decreases with increasing molecular weight. Each mass spectrometer has a working mass range into which the analyte must fall in order to be detected. Until the development of such techniques as field desorption (FD), plasma desorption (PD), fast atom bombardment (FAB) and other soft ionization desorption techniques, large, thermally-labile species such as proteins were not amenable to MS. A recently discovered desorption technique is electrospray and it has been successfully applied to the analysis of both positively and negatively charged proteins. Electrospray induces the desorption of ions, already present in solution, into the gas phase by means of a high electric field. Intact protein ions are observed with charges reflecting those on the molecules in the solution phase. Because MS monitors ions via their mass-to-charge ratios, the mass range of quadrupole mass spectrometers, typically 1000 to 3000 m/z units, is 'extended' by a factor equal to the charge on the ions.

As previously mentioned, electrospray involves desorption of charged species from a liquid surface by the application of a high electric field. The technique was first described by Dole *et. al.* in the 1960's. The experiment involved spraying a polystyrene solution through a needle tip held at a high potential (23). Gas phase ions

that were formed migrated through a nozzle, skimmer and a set of lenses to reach a pair of grids opening into a Faraday cage. Upon passing through the grids, the ions generated a current. As the potential on the grids was increased, there was a threshold beyond which the observed current decreased because ions were being repelled. The threshold potential was called the stopping voltage (V_r) and was used to calculate the mass to charge (m/z) ratio of the polystyrene ions. In the calculation, an assumption was made that the migrating charged species were molecular and not fragment ions. The m/z ratios were calculated according to the following equation:

$$\frac{1}{2}mv^2 = qV_r \quad (11)$$

where m is the molecular mass of the ions, q is the charge on the ions, and v is the velocity of the macroion in the beam (equal to the gas velocity). The polystyrene ions were calculated to be of mass 140,000 Da.

Once it was established that the electrospray technique could produce large molecular ions, it was only a matter of time before this phenomenon was explored as a source of ions for MS. A similar spray technique, so-called 'ion evaporation', was invented in the late 1970's by Thompson and Iribarne (24). A solution containing ions was nebulized and the droplets produced were passed by an induction needle. Small ions were desorbed into the gas phase after much solvent evaporated from the

droplets. A quadrupole mass spectrometer equipped with an atmospheric pressure ionization interface was used to detect the ions formed. The ions seen were solvated clusters of simple ions dissolved. Unfortunately, no multiply charged ions were observed.

The observation of multiply charged ions in electrospray MS was first made by Fenn and co-workers in the mid 1980's when they were analyzing soluble polymers and proteins (25, 26, 27, 28). Several poly(ethylene glycols) (PEG) were dissolved in water-methanol solutions and electrosprayed. Multiply charged ions containing up to +23 charges due to the addition of Na^+ to PEG were observed showing that large, multiply-charged, molecular ions could be produced by ES and detected by MS. Proteins prepared in solutions similar to those of PEG resulted in ES-MS spectra containing a series of peaks. Fenn postulated that the peaks were of the same analyte but charged to different extent thus rendering the analyte a series of mass-to-charge (m/z) ratios. Based on the assumption that each adjacent pair of peaks were separated by one charge and that charging was due to protonation, algorithms were proposed to determine the protein's average molecular mass and to deconvolute the multiple peaks on the m/z scale spectrum to a single peak on the mass scale. In this way, the molecular mass of the analytes can simply be read from the deconvolution spectrum.

1.2.1 Theory and Mechanics of ES

Several models of electrospray have been proposed for the electrospray phenomenon. The ion-evaporation model is well accepted by many; however, many components of the theory lack experimental verification. In this model, the evaporation of solvent from the droplets formed during ES has been assumed to play a key role in effecting ion desorption from charged droplets to the gas phase (29). The conventional view of the ES process has a droplet emerging from the ES probe charged by the high electric field. As the solvent evaporates, the coulombic repulsion between the droplet and the bulk liquid overcomes the surface tension. Following the electric field gradient, the droplets drift toward the MS orifice. During flight, solvent evaporation may induce droplet fragmentation through Rayleigh fission or subdivision to lessen increasing Coulombic repulsion as a result of decreasing droplet size. Ion desorption occurs when the Coulombic repulsion exceeds ion solvation energy.

Recently, experimental results that were difficult to reconcile with the above model have been accumulating. They suggest strongly that ion desorption occurs earlier rather than later in the ES process, and most likely at the ES probe tip. A support for this model is found in a study of dilute solutions (less than 0.2%) of acetic acid and propionic acid (30). Upon evaporation, the pH of aqueous solutions of acetic acid and propionic acid change in opposite directions; the pH of a 0.2% acetic acid solution decreases from pH 2.9 while the pH of a 0.2% propionic acid solution increases from pH 2.9. If solvent evaporation were a prerequisite to ion desorption, shifts in the mass spectral profiles of proteins would result because proteins desorbing

from a lower pH acetic acid solution should show a greater degree of ionization than proteins desorbing from a higher pH propionic acid solution. As a matter of fact, minimal differences were found in the spectral profiles of the same proteins in 0.2% acetic acid and propionic acid solutions. This suggested that the proteins were desorbed from solutions of identical pH. In other words, ion desorption took place from a solution in which the conditions were not different from those of the initial solution; that is to say, in a solution where minimal solvent evaporation had taken place.

This finding corroborates previous ones in which the only significant effect of temperature (25°C to 91°C) on 0.2% acetic acid solutions of proteins was the observation of thermal denaturation of some proteins. For proteins that do not undergo conformational changes in this temperature range, no significant differences in the ES-MS spectra were discernible (31). This same lack of correlation between source temperature and ES response was observed in a later study involving aqueous solutions of metal salts. According to the ion-evaporation model, a rise in source temperature, which enhances solvent evaporation, is expected to be accompanied by an increase in ES response. This increase was not observed.

A third set of experimental evidence contradicting the ion-evaporation model involves a comparison of protein ions in ethylene glycol solutions to those in aqueous solutions (32). The evaporation of water is much more extensive than that of ethylene

glycol at any given temperature. Under conditions where a water droplet would shrink by approximately 30%, an ethylene glycol droplet shrinks negligibly. Computer modelling showed that droplets, which were initially hot, cooled rapidly due to evaporation and heat loss to ambient air, and subsequently evaporate much like droplets initially at room temperature. For the peptide, gramicidin s, ion abundances in ethylene glycol solutions were approximately 200 times lower than those in aqueous solutions under room temperature operating conditions. Heating the electrospray probe to 100 °C decreased the viscosity of the solution that it contained. At that temperature, the gramicidin s response differences between the use of water and that of ethylene glycol as solvent decreased to approximately a factor of 40 times. That is to say, the abundances in ethylene glycol, relative to those in water, were orders of magnitude too large to be accounted for using the conventional, ion-evaporation theory. It was speculated that decreasing the viscosity of ethylene glycol by raising the source temperature, increased the velocity of the ions drifting toward the ethylene glycol/air interface thereby increasing the total number of analyte ions desorbed at the Taylor cone during ES.

Even though the details of the ES process is still under debate, the mechanics of the process are straightforward. The solution to be electrosprayed is delivered to the capillary by means of a syringe pump. The flow rate may vary between 1 and 20 $\mu\text{L}/\text{min}$. The voltage applied to the capillary is usually in the range of 2 to 4 kV. With a positive applied voltage, the positive ions in solution migrate to the surface of

the liquid at the open end of the capillary. With a reversal of the polarity of the applied potential, negative ions can be coaxed to the surface of the liquid to become desorbed. Due to the high applied electric field at the capillary tip, the electrostatic pressure on the surface forces the liquid into a cone shaped spray known as the Taylor cone (33).

Charged droplets are ejected from the Taylor cone when electrostatic repulsion overcomes surface tension. These droplets drift toward the MS orifice plate following the electric field lines. At some point during this series of events ion desorption occurs. The exact mechanism is the subject of much controversy as previously outlined. There is a small current generated as a result of the ions drifting from the capillary to the orifice of the MS. The stability of this current reflects the ES stability. The ES stability is dependent on many factors such as liquid flow rate, viscosity and surface tension of the liquid as well as its dielectric constant and conductivity. It has been suggested that a conductivity of $\leq 10^{-5} \Omega^{-1}$ is required to produce a stable electrospray at 25°C. The corresponding aqueous electrolyte concentration is $\leq 10^{-4}$ M (34). Liquids with higher surface tensions require higher voltages (*e.g.* approximately 3.5 kV for water versus approximately 2.8 kV for methanol in our typical ES-MS set-up). The flow rate is also important. Low flow rates are required with high conductivity samples. Too high a flow rate can lead to formation of larger droplets and eventually an irregular flow (pulsation). The electrospray current will increase steadily with increasing applied voltage to a point

where significant corona discharge occurs (≥ 5 kV at a 1 cm capillary-interface distance). At this point, current is relatively high and erratic and the ES response is greatly depressed.

1.2.2 Electrospray as an Interfacing Technique

There are several features of CZE which are inconsistent with the requirements for ES-MS. In order to facilitate the interfacing of the two techniques some modifications were introduced. Smith and co-workers realized that the cathodic end of the capillary need not be placed in a buffer solution contained in a grounded buffer reservoir, but can terminate in an electrospray probe, which acts as the cathode for CZE (35). The interfacing method must be compatible with low CZE flow rates and must not introduce pressure-driven laminar flow which promotes mixing and degrades separation quality. The electroosmotic flow should be in the direction of the mass spectrometer or the sheath liquid may be drawn into the capillary possibly causing problems. If there are to be any advantages of using MS in place of other detection techniques, MS detection limits must produce better detection limits than the UV on-column picomol level.

Two mass spectrometric ionization techniques, electrospray (ES) and fast atom bombardment (FAB) meet the requirement of low flow rate in capillary electrophoresis. ES is preferred because it introduces fewer matrix effects as

compared to FAB. By simple modification of a typical ES probe, Smith and co-workers developed the first on-line CZE-MS instrument (35). Since the introduction of this first probe, there have been at least three other probe designs introduced. A review containing a discussion of the designs was published by Smith *et. al.* (36). In the original design, the electrical connection at the cathodic end was made by depositing a film of silver metal onto the fused silica column. The metallized surface served two purposes - acted as the cathode for CZE and served as the ES source by applying a voltage of 3 to 6 kV. This design had limited performance because flow rates were well below the optimum for ES. The lifetime of the metallized surface was only a few days. Analysis was limited to non-aqueous, low conductivity buffers (36). The sheath flow design eliminated most of the problems with low flow rates (36). In this design, a coaxial flow of organic solvent (1 to 5 $\mu\text{L}/\text{min}$) served to establish electrical contact with the CZE effluent. There was negligible mixing at the capillary tip. Henion and co-workers added another variation; using a liquid junction to establish electrical contact with the capillary and provide the make-up flow of buffer (37). The potential for loss of separation efficiency due to mixing and band-broadening was greater here because of the liquid junction. The fourth design was proposed by Jorgenson; it was a sheathless design which used a microspray approach. The ion source operated in a vacuum in a manner similar to the electrohydrodynamic ion sources for MS (36).

At least a dozen or more laboratories are presently involved in developing a

better interface for CZE-MS. The advantages of MS over other detectors include its high specificity, high sensitivity, and high information content. Retention time, which sometimes varies because of surface modification or degradation or a complex sample matrix, is no longer as important for identification purposes.

1.3 Proteins

In general, protein analysis has inherently been difficult due to the large size, high charge and complex conformation of the biopolymer. Proteins are composed of α -amino acid sub-units. There are only 20 common α -amino acids (Table 1). The amino acids are composed of a basic amino group, an acidic carboxylate group, a hydrogen atom and a characteristic side chain group all attached to a central carbon atom. The zwitterionic form of the α -amino acid is shown below.

The identity of the R side chain defines each amino acid. The names of the amino acids, their one and three letter codes, and the pK_a values for the ionizable groups are given in Table 1. A protein is composed of a specific sequence of amino acids that are linked via amide bonds. An amide bond is formed when the carboxylate group of the preceding amino acid reacts with the amino group of the amino acid that follows, resulting in the formation of an amide and concomitant elimination of a water

molecule.

The ionizable side chain functional groups, such as -NH_2 , -COOH , -SH and -OH , may gain or lose a proton, allowing the protein to assume a net positive or negative charge. The degree of ionization for each side chain functional group, the amino terminus and the carboxylate terminus is dependent on several factors including solution pH, the protein conformation, and the positioning of the group in the 3-dimensional structure of the protein. Because a protein has a large number of ionizable groups, typically, it exists in solution in a number of ionic states that are in equilibrium with one another.

For example, in Figure 2 at pH 7.8 there are 3 states of the amino acid, histidine, present in solution. This is a model of the protein with an amino and carboxylate terminus and one ionizable side chain. The most abundant species is the uncharged state with an appreciable amount of the +1 and -1 states present. For proteins, the amino acids are linked by amide bonds and contain an amino terminus and carboxylate terminus. The number of possible charge states is more numerous due to the fact that the proteins usually contain a number of amino acids with ionizable side chain functional groups. Most proteins are cationic in an acid solution of pH 3 and anionic in a basic solution of pH 10.

Proteins exhibit several orders of structure. The primary structure is defined

Amino Acid Name	Codes		pK _a of groups		
	1	3	α -COOH	α -NH ₂	R
Alanine	A	Ala	2.35	9.87	-
Cysteine	C	Cys	1.86	10.34	8.35
Aspartic Acid	D	Asp	1.99	10.00	3.90
Glutamic Acid	E	Glu	2.13	9.95	4.32
Phenylalanine	F	Phe	2.58	9.24	-
Glycine	G	Gly	2.35	9.78	-
Histidine	H	His	1.81	9.15	6.05
Isoleucine	I	Ile	2.32	9.76	-
Lysine	K	Lys	2.16	9.20	10.80
Leucine	L	Leu	2.33	9.74	-
Methionine	M	Mrt	2.17	9.27	-
Asparagine	N	Asn	2.02	8.80	-
Proline	P	Pro	1.95	10.64	-
Glutamine	Q	Gln	2.17	9.13	-
Arginine	R	Arg	1.82	8.99	13.20
Serine	S	Ser	2.19	9.44	-
Threonine	T	Thr	2.09	9.10	-
Valine	V	Val	2.29	9.72	-
Tryptophan	W	Trp	2.43	9.44	-
Tyrosine	Y	Tyr	2.20	9.11	10.07

Table 1. Twenty common α -amino acids, 1) single letter code, 3) three letter code, and pK_a's of ionizable groups (41).

Figure 2. Charge state of histidine as a function of solution pH.

by the linear sequence of amino acids and the location of disulphide bonds (Cys-Cys). The secondary structure arises from the spatial arrangement of amino acids that are relatively close to one another in the linear sequence. Examples of secondary structure are the α -helix and the β -pleated sheet. Associations between amino acids that are relatively far apart in the linear sequence give rise to the tertiary structure of proteins. Quaternary structure is defined as the associations between two or more subunits which constitute the complete protein. If a protein retains its molecular integrity, the primary structure will not change. However, the secondary, tertiary and quaternary structures can be modified by changes in pH, temperature, solvent composition and/or ionic strength. A protein in its physiologically active state is referred to as being in its native conformation. Any changes in the secondary or tertiary structure result in denaturation.

The degree of ionization of a protein has a direct result on the conformation of that protein. The charge state of a protein depends not only on the nature of the side chain residues, but also on the immediate electrostatic and chemical environments of those residues. Table 1 shows that only seven amino acids have ionizable side chains; these plus the amino terminus and the carboxylate terminus constitute the ionizable components of a protein. Basic residues, arginine, lysine, histidine and the amino terminus, contribute to the total number of positive charges, while acidic residues, aspartic acid, glutamic acid and the carboxyl terminus, contribute to the total number of negative charges on a protein. The overall charge is the difference between the

two. From this information, it is obvious that most proteins will assume a net positive state in acidic solutions ($< \text{pH } 4$) below their isoelectric point and a net negative state in basic solutions ($> \text{pH } 9$) above their isoelectric point.

Figure 2 shows the abundances of the various charged states of the amino acid, histidine, as a function of pH. At many pH values there are two or more possible charge states in equilibrium. In the mass spectrum of a protein, a similar phenomenon manifests itself as an "ion envelope" having a series of peaks due to ions whose neighbours differ by one unit of charge. With proteins, the number of charge states in solution is much greater than that with a simple amino acid. An algorithm was proposed by Fenn *et al.* (38) and Covey *et al.* (39) based on the assumptions that the adjacent peaks differ by one unit of charge, the charging is due to proton attachment, and the molecular integrity of the protein is retained *i.e.* no covalent bonds are broken. The peak at position $(m/z)_2$, carrying a charge of z_2 , is described by the following equation:

$$(m/z)_2 = \frac{(M_r + M_a z_2)}{z_2} \quad (12)$$

where M_r is the molecular mass of the protein and M_a is that of the adduct carrying the charge ($M_a = 1$ for a proton). When the protein carries z_1 charges, where z_1 equals $z_2 + j$ and j equals 1 for the adjacent peak on the m/z side, it produces a peak at $(m/z)_1$

(where $(m/z)_2 > (m/z)_1$) given by the following equations:

$$(m/z)_1 = (M_r + M_a z_1) / z_1 \quad (13)$$

$$(m/z)_1 = (M_r + M_a (z_2 + j)) / (z_2 + j) \quad (14)$$

Solving equations 12 and 14 for charge,

$$z_2 = j \left(\frac{(m/z)_1 - M_a}{(m/z)_2 - (m/z)_1} \right) \quad (15)$$

Taking the nearest integer of z_2 , the molecular mass of the protein may be calculated as:

$$M_r = ((m/z)_2 - M_a) z_2 \quad (16)$$

In a spectrum with n peaks, there are $(n-1)$ pairs of adjacent peaks thus providing $(n-1)$ estimates of M_r . The accuracy of M_r as compared to sequence information is usually between 0.1% to 0.005%, which is about two to three orders of magnitude better than that of gel electrophoresis. It is also about one order of magnitude better than what is feasible on a time-of-flight mass spectrometer using laser desorption or plasma

desorption methods.

1.4 Objectives

The basic objectives of the following research were to assemble a working CZE and CZE-MS apparatus for probing and analyzing protein species in solution. Since both CZE and electrospray MS are tools for examining and manipulating ionic analytes in solution, coupling the two might offer additional information on solution species that neither technique, on its own, can offer. Separations were performed in both acidic and basic buffer systems. Because little research has been performed on CZE-MS of protein anions, CZE-MS of protein anions was explored and studied. The methods of sample stacking and on-column CITP preconcentration were also explored for increasing CZE sensitivity while keeping experimental conditions simple.

Chapter 2 - Experimental

2.1 Capillary Zone Electrophoresis

The components of a CZE system with an on-column absorbance detector are shown in Figure 1 (p.5). This system was assembled in-house from components and instruments already present in the laboratory. A Glassman high voltage power supply capable of delivering up to ± 60 kV was used to supply a voltage of ± 10 to 30 kV to a platinum electrode. The platinum electrode was immersed in a buffer solution contained in a plastic centrifuge vial acting as the buffer reservoir. Both the electrode and the vial were mounted on a plexiglass panel and contained within a plexiglass box. This box was equipped with an interlock system on the door to protect the operator from contact with high voltages. Extending from the high voltage buffer reservoir to the grounded reservoir was a fused silica capillary of 50 μm ID. Over the course of the research project two types of fused silica capillary columns were used. The first type was uncoated fused silica used to analyze small, charged compounds such as acridine and 8-hydroxyquinoline. The second type was polyethylene glycol coated (PEG) fused silica supplied by J & W Scientific (California) which was used to analyze proteins.

8-hydroxyquinoline and acridine were first used to investigate some of the parameters relevant to CZE analysis. Because on-column adsorption is minimal for smaller compounds separation was performed on an uncoated fused silica column. Some problems, such as unreproducible electroosmotic flow, were encountered with

these columns. For proteins, CZE is typically performed at low pH (<4). At these pH's the proteins are cationic species as they are in a solution of pH below their isoelectric point (pI). Upon extension of CZE to protein analysis, adsorption of the cationic proteins onto the fused silica column resulted in non-reproducible migration times and electroosmotic flow and very poor peak shapes. In most cases, no discernable peak migrated through the column. These problems were solved when the PEG coated column was employed.

2.1.1 Acidic Buffer Systems

At low pH, general operating conditions for CZE analysis included electrokinetic injection for 5 s at 20 kV and a running voltage of 15 to 20 kV until all analytes were eluted from the column. The buffer solutions included 10 mM ammonium acetate/acetic acid (pH=3.8) and 10 mM ammonium formate/formic acid (pH=3.5). Preliminary experiments were performed using simple positively charged compounds, acridine and 8-hydroxyquinoline. In these experiments a neutral marker such as mesityl oxide was used to monitor the electroosmotic mobility as its magnitude can be quite large in uncoated columns. For the analysis of positively charged proteins, similar buffer solutions were used. A neutral marker was not included in the protein experiments as the PEG coating minimized the EOF at this pH. The magnitude of the electrophoretic mobility was 10 to 100 times lower than the mobility of the cations. A neutral marker did not elute from the column before a

period of 1 hour had passed while most cations eluted in 15 min.

To wash the column, a 0.01 M solution of NaOH was pumped, by means of a liquid chromatographic pump, through the column for 5 min at a flow rate of 50 $\mu\text{L}/\text{min}$, followed by rinsing with the appropriate buffer for half an hour at a flow rate of 50 $\mu\text{L}/\text{min}$. As many as five CZE runs could be carried out before there was a noticeable change in electroosmotic mobility or analyte peak shape, which signalled the need to wash the column. Overnight, the column was filled with water.

2.1.2 Basic Buffer Systems

At high pH ($\text{pH} > 10$), general operating conditions included injection for 5 s at 15 kV and analysis at 15 kV. At pH's greater than approximately 6, the PEG coating no longer minimized the EOF. When necessary, a neutral marker, such as mesityl oxide, was used to determine the electroosmotic mobility. In general, two buffer systems were used. The first was a 10 mM ammonium chloride/ammonium hydroxide buffer in water (pH 7.2 to 9.6). This system allowed for the separation of two peptides, gramicidin s and bradykinin, as cations. The second was a solution of 0.40 % methylamine in water (pH 11.3). With this buffer system, the chosen proteins, pepsin, ubiquitin and myoglobin, were separated as anions.

2.2 Absorption Detection

Detection was performed on-column by means of a Waters Model 450 Variable Wavelength detector. The original flow-through cell was removed and replaced with a home-made mounting block capable of holding the narrow bore fused silica capillary. The mounting block was fabricated from a piece of plexiglass of dimensions 18 mm by 18 mm by 7 mm. A hole was drilled through the centre of the block along its short axis providing the detection window. The CZE capillary was inserted into a second hole drilled perpendicular to the first through the long axis of this block. Two razor blades were fixed in place on either side of the long axis of the capillary to block radiation that had not passed through the capillary.

A fused silica capillary column typically has a polyimide coating on its exterior to provide tensile strength and retard hydrolysis. Before inserting the uncoated capillary into the mounting block, a section of the polyimide coating was removed by either burning it in a flame followed by careful scraping of the charred portion or scraping of it directly with a razor blade to provide a detection window. The bonded phase columns were purchased with the detection window already in place. In general, detection was performed at 254 nm for the small organic species and 200 nm for protein compounds. The electropherograms were recorded on a Fisher Recordall Series 5000 chart recorder.

2.3 MS Detection

For some protein analyses, UV detection was complemented by MS detection. This was performed 10 to 20 cm downstream from the absorption detector. The MS used was a TAGA 6000E (SCIEX, Thornhill, Ontario, Canada) triple quadrupole MS. Figure 3 details the design of the TAGA 6000E. This instrument has an upper mass limit of m/z 1400. This limit was adequate for most protein ions produced by electrospray ionization as they were highly charged (*i.e.* large z). The ions were desorbed from solution at atmospheric pressure and sampled through a small diameter orifice (100 μm) into the mass analyzer region which was held at a pressure of 10^{-6} Torr. To maintain such a pressure difference, the TAGA was equipped with a single-stage cryopumping system. Since there was a large pressure differential across the orifice, supersonic-jet expansion as well as cooling of the gas and sampled ions promoted the formation of clusters between ions and residual water and organic vapours. To minimize cluster formation and ease identification, a counter flow of nitrogen (Linde high purity nitrogen, 45 psi) exiting into the ion source acted as a barrier for solvent vapours and neutral gases. A small percent of the nitrogen gas was drawn into the lens region where it served as a collision gas to decluster solvated ions.

In all experiments single stage mass spectrometry was used, meaning the first quadrupole, Q_1 , scanned the appropriate m/z range. Typical lens and quadrupole voltages are recorded in Table 2 for detection of positive and negative ions.

Figure 3. Schematic representation of ion optics of TAGA 6000E.

	Positive Mode (Volts)	Negative Mode (Volts)
OR	+40	-40
Q0	+32	-32
Q1	+30	-30
L7	-250	+250
Q2	0	0
L9	0	0
Q3	0	0
FP	+50	-50
MU	-5000 or -3300 ^a	+3300

Table 2. Typical ion optics voltages used in positive and negative ion detection. See Figure 3 for the ion optics codes.

^a The lower voltage was used after a new electron multiplier was installed.

2.3.1 Sheath Flow Electrospray Design

For the TAGA 6000 MS to be used as a detector for CZE, the corona discharge assembly was removed and replaced with a laboratory-built interface. The electrospray ionization interface for low pH CZE-MS, as shown in Figure 4, was a concentric design using a sheath flow of liquid to augment the flow rate from the nL/min range in CZE to the required $\mu\text{L}/\text{min}$ necessary for electrospray. The capillary was threaded through a length of 24 gauge stainless steel tubing. The sheath liquid was continuously infused into the annular space between the stainless steel tube and the CZE capillary column using a syringe pump, Harvard Apparatus Model 22. The sheath liquid also served to establish electrical contact between the column effluent and the conducting stainless steel probe tip. The sheath flow could also be used to modify the buffer composition exiting the column to one conducive to ion formation. The capillary protruded from the stainless steel probe tip by approximately 1 mm. The optimum position of the probe tip was found to be 1 to 2 cm from the interface plate at an angle of approximately 30° . With this design, positive ion detection was successfully performed.

In the positive ion detection mode, the electrospray probe was floated above ground at a typical voltage of 3 to 4.5 kV via a $50\text{ M}\Omega$ current-limiting resistor in series with a dual polarity high voltage power supply (Tennelec Model TC950). The

Figure 4 Electrospray ionization interface for CZE-MS.

current was monitored with a laboratory built microammeter and was typically between 9 to 12 μA . The probe tip was electrically isolated using plastic clamps and polytetrafluoroethylene (Teflon) tubing for sheath delivery. In the positive ion mode, success was found with this design using a sheath liquid of equal parts of water and methanol plus 0.02% to 2% acetic acid. Typical sheath flow rates were between 1 and 5 $\mu\text{L}/\text{min}$. Unfortunately, this interface design was unsuitable for the analysis of protein ions in the negative ion mode. A stable spray was nearly impossible to sustain because a corona discharge seemed to occur at the onset of electrospray.

2.3.2 Pneumatically Assisted Electrospray Design

Analyzing ions in the negative detection mode required modification of the previous design to allow for a sheath gas flow. A third concentric, 1/16" stainless steel tube was added. A sheath flow of air was passed through the annular space between this tube and the liquid sheath tube at the minimum flow rate required to sustain a stable spray. This interface design, shown in Figure 5, utilized pneumatically assisted electrospray (also called 'ionspray') and had been known to increase the stability of the electrospray as well as delay the onset of corona discharge, especially in the negative ion mode (40). It is believed that oxygen in the air scavenged electrons and suppressed corona discharge. Detection of anionic species was accomplished with the use of this pneumatically assisted electrospray probe.

Figure 5. Pneumatically assisted electrospray ionization interface design for CZE-MS.

2.4 Chemicals

All proteins used in this work were purchased from Sigma Chemical and used without further purification. Table 3 lists the proteins used, their molecular masses calculated from sequence data and their pI's. Protein samples were prepared at concentrations of 0.5 to 187 μ M in water or dilute buffer for analysis in the positive mode. In general, the ionic strength of the samples was lower than that of the buffer in the column, which allowed sample stacking to occur. In later experiments, CITP was performed prior to CZE separation to sharpen peaks and increase sensitivity. When analyzing negative ions at high pH, protein solutions were prepared in dilute buffer solutions. All solvents were reagent grade or better and were used without further purification. A list of these chemicals and their suppliers are given in Table 4. Conditions for all experimental results are outlined in Chapter 3 as the results are presented.

Protein	Source	Mass (Da) ^a	pI ^d
Bradykinin	peptide	1035	12.5
Gramicidin s	Bacillus Brevis	1214	11.0
Cytochrome c	Equine skeletal	12360 ^b	9.99
Myoglobin	Equine muscle	16954 ^c	8.04
Pepsin	Porcine stomach mucosa	34695	3.03
Ubiquitin	Bovine red blood cells	8567	7.58

Table 3. Proteins used during this thesis along with their calculated molecular masses and pI values.

^a reference 41

^b heme group attached

^c heme group detached

^d calculated using General Protein/Mass Analysis for Windows, Lighthouse Data, 1992-94.

Chemical	Supplier
Methanol	Anachemia
Ammonium acetate	Anachemia
Ammonium formate	J.T.Baker Chemical Co.
Acetic acid	Anachemia
Formic acid	Anachemia
Methylamine	Anachemia
Ammonia	Aldrich
Ammonium chloride	Anachemia
Piperidine	BDH Chemicals
Diethylamine	BDH Chemicals
Sodium Hydroxide	Anachemia
Morpholine	Aldrich

Table 4. Chemicals used during this thesis and their suppliers.

Chapter 3 - Results and Discussion

3.1 CZE of Simple Ionic Compounds

As the CZE apparatus was newly assembled for this thesis work, preliminary experimentation was carried out that involved the analysis of simple, charged species, acridine and 8-hydroxyquinoline, under various CZE conditions with absorbance detection. Upon establishing a properly operating CZE system, the more complicated analysis of proteins was begun. First, cationic proteins in low pH buffer systems were investigated. This is the simplest and the most common type of CZE analysis of proteins performed to date. These results include both absorbance and MS detection, in tandem. Following this, CZE analysis of cationic proteins in high pH buffers was performed. The final and most exciting group of experiments performed is CZE of anionic proteins in high pH buffers.

Acridine and 8-hydroxyquinoline can accommodate a maximum of one proton each in solution. A simple background electrolyte, composed of methanol, water and acetic acid with various concentrations of chloride salts added was used. Parameters such as the effects of both cation size and concentration of the background electrolyte on electroosmotic mobility and electrophoretic mobilities of the analytes were investigated.

The analyses were carried out with an uncoated fused silica column of inner diameter (ID) 50 μm . The column length was 59.9 cm; however, the length from the injection end to the detection point was only 45.2 cm. The background electrolyte solvent

was a mixture composed of 49.9% deionized water, 49.9% methanol, and 0.2% acetic acid by volume. Various electrolytes such as LiCl, NaCl, and KCl at concentrations extending over the range 1×10^{-4} to 1×10^{-1} M were added to constitute the running buffer. The analytes were prepared in a solvent composition of 94.99% deionized water, 4.99% methanol, 0.02% acetic acid by volume and 1×10^{-6} M of the appropriate electrolyte. Samples prepared in solutions of 10 to 100 times diluted buffer have been shown to give better separations, with regard to peak shape (1). Unless otherwise noted, all experiments were performed with the concentrations of the analytes at 1×10^{-4} M. Injection was performed electrokinetically for 5 s at an electric field strength of 334 V/cm. Separation was performed at 334 V/cm. An example of the results obtained from such a separation is shown in Figure 6. Mesityl oxide was added to the sample solution at a concentration of 0.05% to serve as a neutral marker used to estimate EOF.

Figure 6. Example of separation of 1×10^{-4} M of A) acridine and B) 8-hydroxyquinoline prepared in 4.99% methanol, 94.99% water and 0.02% acetic acid. Injection was performed electokinetically for 5 s at 334 V/cm into a 50 μ m ID column with background electrolyte of 1×10^{-4} M KCl, 49.9% methanol, 49.9% water, and 0.2% acetic acid. Neutral marker was C) mesityl oxide. Separation was performed at 334 V/cm.

3.1.1 Manipulation of Electroosmotic Flow

As stated earlier, electroosmotic flow is inherent in CZE systems and can have significant effects on separations especially in uncoated fused silica columns. For this reason, the electroosmotic mobility was monitored in relation to both the electrolyte concentration (Figure 7) and cation size, as reflected in its hydration volume (Figure 8). A neutral marker was not necessary in this case because the injection plug produced a dip in the baseline absorption. The solvent in the injection plug was carried through the column under the influence of EOF and its migration time was monitored by the absorbance detector to determine electroosmotic mobility.

From Figure 7, it can be seen that the magnitude of the electroosmotic mobility decreases as the electrolyte concentration increases. This finding is supported by the results of other researchers (1, 43). The electroosmotic mobility was calculated using the following equation:

$$\mu_{eo} = \frac{LL_{det}}{Vt} \quad (3)$$

where L is the total length of the column, L_{det} is the length of the column to the detector, V is the running voltage, and t is the retention time of a neutral species.

Figure 7. Electroosmotic mobility as a function of the logarithm of the buffer concentration of A) KCl and B) LiCl in a mixture of 49.9% methanol, 49.9% water and 0.2% acetic acid. The electric field gradient was 334 V/cm.

Figure 8. Electroosmotic mobility as a function of the buffer electrolyte cation size at an electric field strength of 334 V/cm.

According to equation 1, the velocity of the electroosmotic flow is directly proportional to the magnitude of the zeta potential. In the more concentrated background electrolytes, it would seem that the higher ionic strength does not allow the double layer to extend as far into the buffer solution. For a constant surface charge density σ , the zeta potential is directly proportional to the thickness of the double layer. A reduction of this thickness leads to a smaller zeta potential and a lower electroosmotic mobility. The difficulty in precise control of the electroosmotic mobility is evident in the lack of linearity for the two plots. This was consistent with the observation that the electroosmotic mobility in uncoated columns fluctuates from run to run even using identical running buffer.

Figure 8 shows the dependence of the electroosmotic mobility on the buffer cation size as reflected in the Stokes hydration volume (44). Larger cations decrease the zeta potential in the column. Larger cations may be more effective in screening the surface charge and result in a more compact double layer that leads to a smaller zeta potential.

Both these parameters have been used to reduce the adsorption of proteins onto the column with some success. Increasing the concentration of the buffer cations produces an enhanced competition between the cations and the protein species for access to the negative charge sites. However, the very high electrolyte concentrations in some separations are not suitable for ES-MS. As well, Joule heating becomes a problem at high electrolyte concentrations due to excessive current ($> 50 \mu\text{A}$). Salts having cations

of larger size show some selectivity in discouraging protein adsorption; however, these effects are small and are seen only at high electrolyte concentrations. These remedies are not applicable for minimizing electroosmotic mobility or cation adsorption in CZE-MS. because MS detection requires that the electrolyte concentration in the buffer solution be no greater than 1×10^{-3} M, which is too low for the effects to be significant.

At low electrolyte concentrations, a large electroosmotic mobility can decrease the quality of separation of cations analyzed under a positive CZE electric field gradient. Their electrophoretic mobility is augmented by the electroosmotic mobility, thereby decreasing their migration time and resolution. When analyzing cations, the electroosmotic mobility should be reduced as much as possible. A solution is to use a coated capillary column which minimizes electroosmotic mobility at low pH. This will be discussed in detail, later (Section 3.2). When analyzing anions under the same positive electric field gradient, the electroosmotic mobility must be greater than the electrophoretic mobility of the anions toward the injection or anodic end of the capillary. Fortunately, the coated column chosen was also suitable for anionic protein analysis because at high pH, the electroosmotic mobility became appreciable and is sufficiently high to drag the protein anions toward the cathodic end of the capillary for detection.

3.1.2 Relative Velocities

The relative velocity between two analytes through the capillary reflects their degree of separation or resolution. All other parameters being equal, larger values correspond to better resolution between peaks in the electropherogram. The calculation of relative velocity values was performed according to the following equation:

$$v_{rel} = \frac{(\mu_{ep,1} - \mu_{ep,2})}{(\mu_{ep,ave} + \mu_{eo})} \quad (17)$$

From Figure 9, the apparent conclusion is that the analytes experienced better separation at higher background electrolyte concentrations. Again the effect of electrolyte size on separation was more pronounced at higher concentrations as shown by the divergence of these plots. As shown by the intersection of the two plots, this effect seemed to diminish at low concentrations ($< 5.5 \times 10^{-5} \text{M}$). The absolute migration times for the analytes also increased. The lower electroosmotic mobility at high concentrations allowed the analytes to remain in the column longer under the influence of the separation voltage. The peak shapes for the analytes were good at all buffer concentrations. There was little or no adsorption and no Joule heating was apparent.

The role the cation size played in the efficiency of separation is illustrated in Figure 10. The better degree of separation at larger hydration volumes illustrates the

Figure 9. Relative velocity of the two analytes, acridine and 8-hydroxyquinoline, as a function of the logarithm of the concentration of the electrolyte in the buffer for A) KCl and B) LiCl. The electric field strength was 334 V/cm.

Figure 10. Relative velocity of the analytes, acridine and 8-hydroxyquinoline, as a function of the buffer electrolyte cation hydration volume,

effect of decreased EOF at larger cation hydration volumes. As the EOF decreased, the analytes spent a longer time in the column mainly under the influence of the electrophoretic electric field thereby increasing the degree of resolution.

3.2 Protein Analysis

Upon extension of CZE analysis to protein species, a more complex capillary/buffer system was needed. The pH of the background electrolyte was the main factor determining the charge state of the proteins. At a pH below the pI of a protein, the protein has a net positive charge in solution. By altering the pH of the buffer, different charge states of proteins may result. By increasing the pH of the buffer above the pI of the protein, it was possible to analyze the proteins as anions. As the pI's of proteins vary over a range of 1 to 13, varying the pH of the electrolyte can have dramatic effects on the protein's relative mobilities. Because separation was pH dependent, the background electrolyte chosen was a buffer effective at the chosen pH. This eliminated any localized variation in pH which might decrease the efficiency of separation by increasing zone/band broadening.

The first attempt to analyze cationic proteins by CZE in an uncoated column with absorbance detection was unsuccessful. As the chosen proteins, cytochrome c and myoglobin, were positively charged in an acidic solution and the capillary wall was negatively charged, there was a severe problem of adsorption that prevented the proteins from eluting. For the simple peptide, gramicidin s, adsorption was not as severe. However, after several injections it became evident that the migration times were non-reproducible and the peak shapes remained poor.

A preferred method to decrease protein adsorption was to use a coated column in which the negatively charged silanol groups are covered. Fortunately, J&W Scientific was able to supply several experimental polyethylene glycol (PEG) coated columns. The PEG coated columns were found to exhibit better long-term stability than other neutral hydrophilic coatings (13).

3.3.1 Proteins in Low pH Buffers

Separation of proteins in their cationic states was successfully performed with the use of the PEG coated columns. Several buffer systems were investigated including phosphate ($\text{NaH}_2\text{PO}_4/\text{H}_3\text{PO}_4$), acetate ($\text{CH}_3\text{COONa}/\text{CH}_3\text{COOH}$), and formate ($\text{HCOONH}_4/\text{HCOOH}$). All systems gave similar results with respect to peak shapes, migration time, and minimal EOF in the PEG columns. The formate buffer was chosen for subsequent cationic CZE and CZE-MS experiments for the major reason that the formate salt was the most volatile of the three and was therefore least likely to cause electrospray-related problems.

Figure 11 is a typical electropherogram of the separation of three common proteins, cytochrome c, ubiquitin and myoglobin, and the peptide, gramicidin s, by low pH CZE with absorbance detection. The order of migration of the analytes was

Figure 11. Absorbance detection of CZE separation of A) cytochrome c ($9.05 \mu\text{M}$), B) ubiquitin ($15.35 \mu\text{M}$), C) myoglobin ($18.89 \mu\text{M}$) and D) gramicidin s ($27.48 \mu\text{M}$) prepared in 8 mM $\text{pH}=2.85$ formate buffer. Column was a PEG coated, $50 \mu\text{m}$ ID capillary, filled with 10 mM formate buffer. Injection was performed electrokinetically for 5 s at 205 V/cm . Analysis was performed at 205 V/cm .

cytochrome c, ubiquitin, myoglobin and, finally, gramicidin s. In general, the isoelectric points of the proteins might be used as a rough guide to migration order provided all other parameters such as charge and size are equal. At pH=2.85, the protein with the highest pI had the largest percentage of its ions in their more highly charged states; this meant that the electrophoretic mobility was also likely to be the largest. Cytochrome c has a pI of approximately 10, myoglobin 8, and ubiquitin 7.5. Gramicidin s (pI ~ 11) has a small charge/size which was likely responsible for its late migration. A more exact determining factor for elution order was the charge to size ratios of the protein species.

Figure 12 shows a CZE separation of cytochrome c and gramicidin s using conditions that were identical to those used in Figure 11, except for a smaller electric field strength. MS data were also obtained for this separation in the form of a total ion current electropherogram (TIE). Figure 13a shows the TIE for the separation. It seems that the sensitivity displayed in the TIE was lower than that in absorbance detection. However, the TIE contained much more chemical information. As the migration times were known from the absorbance detector, the time window where the analytes were expected in the TIE were also known. By averaging the scans 48 to 52 for cytochrome c and 121 to 126 for gramicidin s, typical-looking electrospray mass spectra of cytochrome c and gramicidin s were readily discernible in Figure 13b and 13c, respectively.

Figure 12. Absorbance detection of CZE separation of A) cytochrome c ($9.05 \mu\text{M}$) and D) gramicidin s ($15.34 \mu\text{M}$) prepared in 8 mM, pH 2.85 formate buffer. Column was a PEG coated, 50 μm ID column filled with 10 mM formate buffer. Injection was performed electrokinetically for 5 s at 158 V/cm. Analysis was performed at 158 V/cm.

Figure 13. a) Total ion current electropherogram (TIE) of CZE separation shown in Figure 12. Electrospray voltage was 4.6 kV.
 b) Electrospray mass spectrum of cytochrome c, calculated from the average of scans 48 to 52 in TIE.
 c) Electrospray mass spectrum of gramicidin s, calculated from the average of scans 121 to 126 in TIE.

The above results were obtained using the sheath flow design electrospray probe. The sheath liquid used was a mixture of 79.9% methanol and 19.9% water with 0.2% acetic acid. A voltage of 4.6 kV was applied to the probe tip. The CZE electric field gradient remained at 158 V/cm. Each TIE was acquired by scanning the TAGA 6000E mass analyzer from a m/z ratio of 500 to 1250 at a rate of 1 m/z unit every 10 ms. The time for each scan was therefore 7.5 s.

In order to identify the analytes, the intensities of the specific protein ions were extracted from the respective scans and plotted in a reconstructed ion electropherogram (RIE) as shown in Figure 14a. The resulting sensitivity enhancement is obvious. By extracting single ions from the TIE the identity of the peaks were again confirmed. By extracting $m/z=824$, one of the ions of cytochrome c, the protein is identified easily in Figure 14b. The peptide, gramicidin s had its characteristic ion at $m/z=571$ as shown in Figure 14c.

In the data reported in Figure 13a, the peak in the TIE for cytochrome c is less than well defined. The reason for the poor signal/noise ratio was that the scan range was extended down to $m/z=500$ for the measurement of gramicidin s. For cytochrome c a range of m/z 700 to 1250 would have been optimum. Unfortunately, the range 500 to 700 amu had a fairly high intensity background. By removing this range from the scans, a much more typical TIE of cytochrome c was obtained, as shown in Figure 15.

Figure 14. a) Reconstructed ion electropherogram (RIE) of the CZE separation shown in Figure 12.

b) Extract of ion, $m/z = 824$ (cytochrome c)

c) Extract of ion, $m/z = 571$ (gramicidin s)

Figure 15. a) TIE of CZE analysis of cytochrome c (8 μM) in a pH 2.9, 10 mM formate buffer. Electric field strength was 343 V/cm. b) Electropray mass spectrum calculated from scan 143. c) Electropray mass spectrum calculated from the average of scans 132 to 153.

In the conceptual stage of this work, it was thought that there would be a small but finite chance that the different charge states of a protein might be resolvable by CZE. Unfortunately, that has not been proven to be the case. The most likely reason is that the protein charge states were in chemical equilibrium with one another, and protonation/deprotonation reactions are typically very fast. As the more highly charged ions advanced relative to the less highly charged in the capillary, separation of the two groups resulted deviation from equilibrium which would quickly cause deprotonation of the more highly charged ions and protonation of the less highly charged ions to preserve the equilibrium. That is to say, because the protein ions were not 'frozen' in their charge states, there physical separation by CZE was therefore impossible.

3.2.2 Peptides in High pH Buffers

As the pH of the background electrolyte was increased from 7.0 to 9.6, the electroosmotic mobility increased dramatically. The most likely cause of this increase was ionization of the glycol groups of PEG at high pH's. While the pKa of this ionization reaction is relatively high (pKa ~ 14), the sheer number of the hydroxyl groups could allow the increasing electroosmotic mobility to become noticeable at pH's well below the pKa. A less important contribution to the increased electroosmotic mobility is that the PEG coating is unlikely to be complete; meaning that there are a small number of exposed silanol groups. Increased ionization of the hydroxyl groups on PEG and the residual silanol groups made the EOF appreciable even in the PEG-coated column when a high pH buffer was used. Two short peptides, gramicidin s and bradykinin, were used to model the effects the buffer pH on electroosmotic mobility as well as on electrophoretic mobilities. Peptide migration times were recorded using absorption detection at 200 nm.

In Figure 16a, the electroosmotic mobility is plotted as a function of the pH of the background electrolyte, which was prepared using 10 mM ammonium chloride in water and adjusted to the appropriate pH using ammonium hydroxide. The electroosmotic flow was determined using a neutral marker, mesityl oxide that was added to the sample solutions at a concentration of 0.05%. The electroosmotic mobility showed a maximum at approximately pH 8.5. As stated above, the increase in electrophoretic mobility with

Figure 16. a) Electroosmotic mobility as a function of pH in a 10 mM ammonium buffer. b) Electrophoretic mobility of gramicidin s as a function of pH in a 10 mM ammonium buffer. Samples were prepared in buffer and injected for 5 s at 334 V/cm. Analysis was performed at 334 V/cm. c) Electrophoretic mobility of bradykinin as a function of pH in a 10 mM ammonium buffer. Samples were prepared in buffer and injected for 5 s at 334 V/cm. Analysis was performed at 334 V/cm.

increasing pH was due to increasing ionization of the glycol or silanol groups. Above pH 8.5 the electrophoretic mobility decreased; a possible result of increased viscosity of the buffer solution with increased pH. The increased EOF makes it a factor to be considered in all separations at high pH.

The mobility of gramicidin s is plotted as a function of pH in Figure 16b. The mobility decreased with increasing pH. As pH increased, the degree of ionization of the peptide decreased slowing the migration of the peptide through the column. In Figure 16c, there is a break in the plot of mobility of bradykinin versus pH. The most probable explanation is that at pH 8.1, deprotonation of the n-terminus is complete (*i.e.* it is neutral) while deprotonation of the two highly basic arginine guanidinium groups is not yet significant.

At pH 9.6, the mobilities of the two peptides were similar resulting in an unresolved peak for the two peptides. As an example of the advantage of MS detection over absorbance detection, MS was used on line with CZE downstream from the absorption detector. The sheath-flow design electrospray probe was used with a sheath liquid of 49.9% methanol, 49.9% water and 0.02% acetic acid at a flow rate of 3 $\mu\text{L}/\text{min}$. The electrospray voltage was 4.2 kV. The CZE electric field strength was 331 V/cm. The TIE is shown in Figure 17a with the later migrating peak evident on the shoulder of the earlier one. The identity of the peaks was determined by extracting the +2 ions of both gramicidin s and bradykinin as shown in Figures 17b and c.

Figure 17. a) Total ion electropherogram (TIE) of gramicidin S (117 μM) and bradykinin (193 μM) in a 10 mM ammonium buffer (pH = 9.6). Injection was performed electrokinetically for 5 s at 331 V/cm and analysis was performed at 331 V/cm. Electrospray voltage was 4.2 kV.
 b) Extract of ion of bradykinin at $m/z = 531$.
 c) Extract of ion of gramicidin S at $m/z = 571$.

3.2.3 Proteins in High pH Buffers

At high pH's many proteins are negatively charged. Interestingly, only a small amount of work has been reported on CZE of protein anions (45,46) and virtually none on CZE-MS of protein anions. CZE analysis of protein anions would seem an easy method to eliminate wall-analyte interaction without any modification to the column or additions to the background electrolyte.

The proteins chosen to study CZE and CZE-MS of anions were ubiquitin, pepsin and myoglobin. The pI's of these proteins are all below 9. The background electrolyte was 0.3% piperidine in water giving a pH of 11.3. The proteins were dissolved in a solution of 0.18% piperidine in water at concentrations of 53 μ M myoglobin, 3.6 μ M ubiquitin, and 31.4 μ M pepsin. The separation of these three proteins with absorbance detection is shown in Figure 18. The drop in the baseline at approximately 3 minutes was a marker for the electroosmotic mobility due to a decrease in the concentration of piperidine in the sample plug. The migration order in this case was the reverse of that for cationic CZE: in the order of decreasing charge/size when the capillary was under a positive electric field gradient. Because the electroosmotic mobility was larger than the mobilities of all the protein ions, their net migration was toward the cathode at the detection end.

Selected ions of the proteins were monitored by MS in the selected ion

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Figure 18. Absorbance detection of CZE separation of A) ubiquitin (3.6 μM), B) pepsin (31 μM) and C) myoglobin (53 μM) prepared in 0.18% piperidine, pH 11.3, in water. Column was a 50 μm ID, PEG coated column filled with 0.30% piperidine in water. Injection was performed electrokinetically for 5 s at 254 V/cm. Analysis was performed at 254 V/cm.

mode. Because these are anions, a negative potential was needed on the electrospray probe. Unfortunately, the sheath-flow design electrospray probe used in the previous experiments did not perform satisfactorily. This was not an uncommon phenomenon in ES-MS (47). It was impossible to get a stable spray before the onset of corona discharge. As a solution to this problem, a second concentric tube was added to supply a sheath gas, air. This is known as pneumatically assisted electrospray. The sheath gas successfully stabilized the electrospray as well as served as an electron scavenger delaying the onset of corona discharge. The sheath liquid was a solution of 2.5% piperidine in methanol at a flow rate of 6 $\mu\text{L}/\text{min}$. The sheath gas was air. The required electrospray voltage was -4.8 kV. Two ions from each protein were chosen to be monitored by MS. The RIE is shown in Figure 19. The counts in the RIE were relatively low which reflected the fact that ES-MS of negative ions is usually more difficult than that of positive ion (47).

By extracting particular ions from the RIE, the identity of the peaks was determined as shown in Figure 20a, b, and c for ubiquitin, myoglobin and pepsin, respectively. Upon extracting the ions, an interesting phenomenon was revealed in the case of pepsin. Pepsin was migrating through the capillary in two distinct bands with the second band co-migrating with the myoglobin anions.

Figure 19. Multiple ion electropherogram of CZE separation shown in Figure 18. Ions monitored include m/z 845, 940, 949, 960, 985, 1068. Electrospray voltage was -4.8 kV and a sheath liquid of 2.5% piperidine in water flowing at $6\mu\text{L}/\text{min}$ was used.

Figure 20. a) Extracted ion from Figure 19 of ubiquitin at $m/z = 1068$.
b) Extracted ion from Figure 19 of myoglobin at $m/z = 845$.
c) Extracted ion from Figure 19 of pepsin at $m/z = 960$.

By analyzing pepsin and myoglobin ions sequentially, it became evident that this phenomenon was present and was revealed in the absorbance traces by analyzing pepsin and myoglobin sequentially (Figures 21 and 22). This was not the first time a double peak for pepsin was observed. Similar results are shown in Figure 23 for pepsin analysis at a low pH under a negative electric field gradient (*i.e.* the anodic end of the capillary was at ground). Pepsin is a very large protein that has an excess of negative charge at all pH's. It is possible that pepsin might have two distinct conformations with different migration times. The conversion between these two conformers might require a time that was longer than that required for a typical CZE separation. Consequently, the two conformations appeared to be 'frozen in time' and their mobilities were so different as to allow them to migrate as two separate bands. Apparently, separation of conformers was possible. This is to be contrasted with the separation of ions of different charge states of the same protein, whose interconversion was rapid and involved little or no conformational change; here, separation was impossible.

Figure 21. Electropherogram of pepsin showing double peak. Sample, 31 μM pepsin in water with 0.18% piperidine, was injected electrokinetically for 5 s at 205 V/cm into a 50 μm ID, PEG coated column containing a buffer of 0.3% piperidine in water. Analysis was performed at 205 V/cm.

Figure 22. Electropherogram of 53 μM myoglobin prepared in water with 0.18% piperidine. Injection was performed electrokinetically for 5 s at 205 V/cm into a 50 μm ID, PEG coated column containing a buffer of 0.30% piperidine in water. Analysis was performed at 205 V/cm.

Figure 23. Analysis of pepsin (187 μM) in 10 mM acetate buffer (pH = 3.7). Injection was performed electrokinetically for 5 s at -313 V/cm into an uncoated column. CZE analysis was performed at -391 V/cm.

3.3 Calibration Curves and Sensitivity

CZE is not only useful in qualitative analysis, as discussed in the previous sections, but also in quantitative analysis. As an example, two calibration curves were prepared - one for cytochrome c and the other for myoglobin. These are shown in Figures 24 and 25, respectively. In both cases, the background electrolyte was 0.4% methylamine in water and the protein samples were prepared in this same electrolyte. Both curves were linear and exhibited good correlation coefficients. The detection limit for both proteins was found to be 0.80 μM . This corresponded to an absolute on-column amount of 841 pg for myoglobin and 669 pg for cytochrome c. The standard deviation in the migration times of the proteins were calculated as 2.9% for myoglobin and 3.4% for cytochrome c. The variations were most likely due to the conditions of sample injection, which was not automated.

There are several common methods to increase the sensitivity of CZE by means of simple modification of the background electrolyte system. Figure 26 illustrates the first of these methods investigated. Sample stacking occurs at the sample-electrolyte interface during electrokinetic injection when the sample is in a buffer solution of lower concentration than in the column. Mobility of sample ions is greater in less concentrated solutions; sample ions rush to the sample/electrolyte interface and enter the column. It is

Figure 24. Calibration curve for cytochrome c using peak height. The buffer was 0.4% methylamine in water and the samples were prepared in buffer. Injections were performed electrokinetically for 5 s at 205 V/cm. Analysis was performed at 205 V/cm. Detection by absorption at 254 nm. Correlation coefficient = 0.99912

Figure 25. Calibration curve for myoglobin using peak height. The buffer was 0.4% methylamine in water and the samples were prepared in buffer. Injections were performed electrokinetically for 5 s at 20 kV. Analysis was performed at 205 V/cm. Detection was by absorption at 254 nm. Correlation coefficient = 0.99842

Figure 26. Electropherograms of 20 μ M of 1) cytochrome c and 2) myoglobin in a) 0.4% methylamine in water and b) 0.16% methylamine in water. The buffer was 0.4 % methylamine in water and each sample was injected for 25 s at 153 V/cm. Analysis was performed at 153 V/cm.

obvious in Figure 26 that the relative buffer concentration between the sample and the running buffer has an important influence on stacking efficiency and peak shape. The two samples injected had the same concentration of 20 μM cytochrome c and myoglobin. When converted to the absolute on-column amount using peak height, 20% more cytochrome c, corresponding to 6.6 ng more sample, was injected under stacking conditions as compared to non-stacking conditions. For myoglobin, 29% more sample was injected onto the column corresponding to an increase of 8.6 ng. The advantage of sample stacking is that a larger amount of analyte can be injected onto the column thus improving detection limits. Depending on the particular analyte or background electrolyte, the increase in detection sensitivity may be as high as 10 to 100 times (1).

The method that gives the most dramatic increase in sensitivity is a relatively new application of an older technique known as on-column CITP preconcentration. The theory behind this effect has been outlined in Section 1.1.4. Briefly, the sample is deposited on column between two zones. The leading electrolyte is of higher mobility than the analyte ions and the tailing electrolyte is of lower mobility. The field strength over the different zones is inversely proportional to the zones' conductivity. The conductivity of the zones is a function of ion mobility and concentration. Highly mobile electrolyte species are conductive thus resulting in decreased field strength and hence decreased electrophoretic velocity. The reverse is true for less mobile electrolyte species

resulting in greater electrophoretic velocity. After a time, an equilibrium is reached in which velocities are normalized. The zones are compressed in the observed on-column preconcentration. The increase in sensitivity as indicated in Figure 27 is approximately 3000 times for cytochrome c and 4500 times for myoglobin. From a previous experiment, it was determined that a sample of $0.75\ \mu\text{M}$ cytochrome c delivered an on-column amount of approximately 627 pg. While from Figure 27, it was calculated that a sample of $0.50\ \mu\text{M}$ delivered 282 ng onto the column under CIP preconcentration. Cytochrome c has an excellent peak shape with minimal broadening. The range of the absorbance detector was set at 4 times larger for this absorbance trace than for all other traces reported previously. From a previous experiment, it was determined that a sample of $0.75\ \mu\text{M}$ myoglobin delivered an on-column amount of approximately 788 pg. While from Figure 27, it was calculated that a sample of $0.10\ \mu\text{M}$ delivered 119 ng onto the column under CIP preconcentration. The peak for myoglobin experienced some zone broadening. This is not surprising as the pH of the background electrolyte was 5 which was only two units below the pI for myoglobin. As the pH of the background electrolyte approaches the pI of a protein, separation efficiency decreases as the protein ions approach neutrality.

Figure 27. Electropherogram of CZE analysis with CITP preconcentration of A) 0.50 μM cytochrome c and B) 0.10 μM myoglobin prepared in water with 0.05 mM formic acid. Injection performed for 20 s at 205 V/cm, followed by preconcentration for 1.5 min at 205 V/cm. Analysis was performed at 205 V/cm. Leading buffer was 40 mM morpholine in water adjusted to pH 5 with formic acid. Tailing buffer was 10 mM formic acid in water.

Chapter 4 - Conclusions

4.1 General Conclusions

In general, proteins have been difficult to analyze because of their large sizes, complex conformations and high charges. PAGE requires difficult staining procedures during detection, and HPLC has difficulty with the ionic character of the proteins. CZE is ideally suited for protein analysis because, by definition, CZE separates charged species in solution by an applied electric field. By adjusting pH the polarity of the ions is influenced as well as the degree of ionization. While there has been a good deal of work on protein analysis at low pH, analysis at high pH has been lacking simply because low pH analysis has been so successful. Two factors hindering analysis at low pH were addressed: electroosmotic flow and protein adsorption. The most effective method to deal with both was the use of a PEG coated column. Fortunately, the same column type was also effective at high pH where most proteins are anions. A large EOF at high pH was necessary to carry the proteins through the column in spite of their mobility toward the anode at the injection end.

The separation of several proteins in their cationic state has been shown. Since each protein is composed of a series of ions each differing by one charge unit and one proton, the separation of these species was not possible. The proposed explanation is that the equilibrium reactions are much too fast with respect to the analysis time of CZE. Protonation/deprotonation reactions are typically very fast. More highly charged ions have higher mobilities relative to those that are less highly charged. Any significant separation of the two groups, and resulting deviation from equilibrium, quickly results in deprotonation of the more highly charged ions to

preserve the equilibrium. One of the proteins studied, pepsin, produced two peaks in its electropherogram. This double peak was evident in both high and low pH buffers. Pepsin is a large protein that is anionic at essentially all pH's. It is likely that these two peaks represent two conformations of the same protein. The time required for interconversion between the two conformers is relatively long with respect to the CZE separation time. The conformations remain unchanged during the CZE separation. Separation of different conformations of the same protein is possible.

With respect to sensitivity, minor changes in the injection procedure and/or buffer constituents can enhance sensitivity. Sample stacking can increase the amount injected by 20 to 30%. On-column CITP preconcentration showed the most dramatic sensitivity enhancement, approximately 3000-fold. While the different modes of CE are quite well understood, CITP preconcentration is an excellent example of the many possibilities still available for advancement in CE research by combining the CE modes in innovative ways and altering buffer constituents from normal CE conditions.

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