CAPILLARY ELECTROPHORESIS FOR HIGH SPEED SMALL MOLECULE ANALYSIS

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by
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Abstract

by

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Small molecule analysis has been critical in developing an understanding of biological processes. Small molecule metabolites are the products of enzyme activity within an organism and study of these molecules, collectively called the metabolome, paints a clearer picture of the phenotype of an organism than genomics, transcriptomics or proteomics. In addition, study of drug metabolism is critical in diagnosing efficacy and safety of potential drug candidates. Recent advances in detection technology have facilitated highly sensitive analysis of the metabolome from samples as small as single cells. This work has revealed a complexity far beyond the simple metabolism charts depicted in biochemistry textbooks. Because of the complexity of the metabolome, a separation technique is often utilized to simplify the sample before analysis. Throughput is of particular interest to metabolomics research, as biological variation between the metabolome of individual organisms is high, requiring studies with large sample sizes and significant instrument run-time.
In this work, I present capillary electrophoresis as a complementary separation technique to high-performance liquid chromatography and gas chromatography for the separation of metabolite samples. In particular, I focus on demonstrating the usefulness of capillary electrophoresis for performing rapid separations lasting less than ten minutes. Capillary electrophoresis is especially well suited for the analysis of small, polar metabolites because of its high resolution and aqueous running conditions. In addition, I demonstrate and investigate new technologies for coupling capillary electrophoresis to mass spectrometry and ionizing molecules for mass spectrometric analysis and detection. As a final demonstration of the capability of capillary electrophoresis to rapidly analyze the metabolome, I present a study of the metabolism of early *Xenopus laevis* embryos.
To Grandma. I got my smarts from you.
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CHAPTER 1:
INTRODUCTION

1.1 The Metabolome

The metabolome is the collection of small molecule reactant and products acted upon by enzymes in a biological system. [1-2] This collection includes molecules involved in endogenous metabolic processes, such as glycolysis and the tricarboxylic acid cycle, and molecules exogenous in origin, such as food and drug metabolites. [3] Because of the wide variety of origin for metabolites, the metabolome is incredibly complex. Limiting the scope of the study of the metabolome to endogenous compounds still yields a very complex mixture of molecules. The Human Metabolome Database has over 30,000 entries for endogenous metabolites alone. [4] Metabolites have been quantified in a range of concentrations from 1 pM to greater than 150 mM, and it is likely that many metabolites exist outside of that range. The wide variety of chemical properties of metabolites such as hydrophobicity, acidity and polarity make analysis of the metabolome particularly difficult. Further complicating metabolome analysis is the wide sample-to-sample variation and ubiquity of contaminating species. [5] Metabolism varies widely from individual to individual, even with controls placed on common physiological differences like weight, sex and age. In addition, small organic acids common in the metabolome are also common
contaminants and additives in many analytical techniques. One analytical technique is unlikely to provide information on the complete metabolome, so large scale metabolomics labs often utilize a number of complementary techniques to generate as wide a picture of the metabolome as possible. [6-7]

Study of the metabolome provides additional information on top of genomic, transcriptomic and proteomic analyses. [3] As the product of the proteome, the metabolome is a closer representation of the phenotype of an organism. Typical proteomic analyses measure only the concentration of a protein and not its activation state, which can be modified by very delicate post-translational modifications such as phosphorolysis, SUMOylation and glycosylation. Similar problems exist upstream, where genes can be present but not translated, or translated but not transcribed into protein due to a number of complex, competing regulatory factors. Study of the metabolome, when taken in context with other upstream analysis techniques, helps to complete the picture of biochemical activity in an organism. [8] Figure 1.1 depicts the relationship between the metabolome and other –omics fields.

1.1.1 Metabolomics Experimental Design

Design of most metabolomics experiments fall into one of two categories, targeted or untargeted. [9] Untargeted experiments are large-scale hypothesis-generating experiments, where targeted experiments are narrow focused hypothesis-testing experiments. Breadth and depth of metabolome coverage are crucial factors for untargeted experiments. Typical
concerns for targeted experiments are selectivity and sensitivity for the desired metabolites. Analytical separation of the complex metabolome is usually required in both cases. HPLC, GC, and CE are the most commonly used techniques to perform this separation. [3] NMR spectroscopy and MS are the two most commonly used detection techniques. [10]

Untargeted metabolomics experiments seek to identify key components of the metabolome that change in expression as a function of gene or disease state within an organism. [11] This information can be used to generate diagnostic tests, or to identify key enzymes or gene mutations that result in disease. Untargeted metabolomics typically utilizes a separation technique such as HPLC, GC or CE coupled to MS to analyze the metabolome. [12] Two groups of samples are analyzed, healthy (control) and disease/knockdown (experimental). Statistical analysis tools are used to generate a list of candidate “features” that are dysregulated between the two sample groups. Tandem MS data are used to generate putative identifications of the features. Feature identification can then be confirmed using a targeted metabolomics experimental design, where samples are spiked with standards and checked for comigration or coelution. Sample number is a major factor in the quality of data generated by untargeted experiments. The metabolome varies widely from individual to individual, and even from cell to cell. In addition, because many metabolites are involved in multiple pathways of the metabolism, a defect in one pathway results in a relatively small change in observed concentration of key metabolites, thus observed fold-changes are often much smaller than those observed in proteomics and transcriptomics. Both of these confounding factors can be mitigated by the use of large numbers of samples from many
biological replicates. Because of the large number of samples required to confidently identify dysregulated features, analysis time is an important consideration when developing methods for metabolomics analyses. Identification of dysregulated features is also difficult, as accurate intact mass data do not resolve isomers, and tandem mass spectra databases are sparse.

Targeted metabolomics experiments are used to more finely probe the metabolism for small changes in specific compounds or classes of compounds. They require purchase or synthesis of standards for the compounds of interest, and previous knowledge of what compounds to monitor. Mass spectrometry remains useful for these types of experiments, especially when configured in a multiple reaction monitoring (MRM) mode. [13-14] This mode takes advantage of the sensitivity gained by selectively monitoring for specific parent and product ion pairs, but requires advanced knowledge of the fragmentation pattern of analytes of interest. NMR spectroscopy can be used to structurally characterize unknown metabolites, but sensitivity is usually poor, and the spectra can be very difficult to interpret when taken in the presence of other, contaminating metabolite species. [15] However, these targeted experiments are the only reliable way to confidently identify metabolite species, and produce much more accurate quantitative data than typical untargeted experiments.

1.1.2 Metabolomics by Mass Spectrometry and Data Analysis

Mass spectrometry is commonly used in metabolomics analyses because of its high sensitivity and ability to generate some structural information about a metabolite that can lead to identification. Still, the sensitivity of MS is limited when compared to techniques
such as fluorescence and Raman spectroscopy, so these techniques see occasional use in the metabolomics field. [16-17] Another major limitation of MS is the speed at which tandem spectra can be acquired. Because of the importance of analysis speed in typical metabolomics workflows, the acquisition rate of tandem spectra often dictates the speed at which the analytical separation techniques can be performed. Tandem spectra contain structural information that is crucial to generating identification information for isomeric metabolites. This need drives the demand for high speed mass spectrometers with good mass accuracy to perform metabolomics experiments.

Through the use of high resolution, high speed mass spectrometers coupled to high resolution separation techniques, the data sets generated in metabolomics are quite large. [11] Identification of signals corresponding to metabolites, called “features”, is a daunting task to perform by hand. Adduct formation and in-source fragmentation of metabolites generate many false features that need to be excluded from analysis. Many computer programs exist to perform feature identification and statistical analysis. XCMS is used predominantly in this thesis to perform feature identification and analysis. [18-20] XCMS performs its analysis in three general steps:

- Feature Identification – A continuous wavelet transform algorithm (centWave) is used to identify regions of interest (peaks) in the data. These regions of interest are termed “features.”
• Feature Grouping – Features are grouped according to retention or migration time. This process attempts to group together features corresponding to the same metabolite due to adduct formation or in-source fragmentation.

• Statistical Analysis – Feature groups are analyzed using a pairwise t-test algorithm and ranked by significance. This procedure identifies feature groups that significantly change between populations, termed “significant features.”

Raw feature count is often reported as a figure of merit when discussing metabolomics datasets, but it is relatively meaningless. The centWave algorithm detects many false positives even when operating correctly [18], and feature count takes no account of duplications due to adduction or in-source fragmentation. More meaningful is a count of feature groups with confirmed molecular identities or a count of features for which significant change between populations has been detected. Identification of a feature is achieved through a combination of matching a tandem mass spectrum to one from a database or standard, and by verifying coelution or comigration with a standard. Thus, standard libraries are crucial to analysis of the metabolome by MS. Typically, only features that are differentially expressed between samples are subjected to identification verification. Quantitation of the identified metabolites can be improved by performing experiments with standard additions, or the use of isotopically labelled standards.
1.2 Mass Spectrometry

Mass spectrometry is a highly sensitive analytical detection tool that is used to identify and quantify ions in a sample stream. It generates a diagram called a mass spectrum, which is a representation of the mass-to-charge ratio and abundance of ions in a sample ion stream. A mass spectrometer consists of three fundamental parts: the ion source, mass analyzer and ion detector. Classical physics provides basic equations by which charged particles can be manipulated by electromagnetic fields, and all mass spectrometer designs utilize these principles. [21] Crucial to the ability to detect and analyze real world samples is the ability to generate ionized species from the sample. A number of different ion sources have been implemented in mass spectrometers, but two ionization techniques have become crucial to the analysis of biomolecules, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI). [22-23] Early mass spectrometers used large, heavy, expensive magnets to perform mass analysis, but these instruments have been mostly replaced by TOF, quadrupole and ion-trap mass analyzers. The most common ion detection apparatuses are electron multiplier tubes and multichannel plate detectors.

1.2.1 Electrospray Ionization

Prior to the development of ESI, techniques such as electron impact ionization (EI) and chemical ionization (CI) were used to provide charge and get analyte molecules into the gas phase. These two ionization techniques shared a disadvantage in that they both tended to cause larger and more fragile biomolecules to fragment in the ion source, before mass analysis
and detection. This fragmentation resulted in complicated mass spectra where one molecular species may be represented by many ion peaks in the mass spectrum. Interest in analysis of complex mixtures of biological compounds such as proteins and lipids drove the search for ionization techniques that would not fragment molecular species before mass analysis. Nevertheless, EI and CI remain important for gas-chromatographic metabolomic analysis.

The principles of electrospray ionization were laid out in the late 19th and early 20th centuries by Lord Rayleigh, John Zeleny, Charles Wilson, and Sir Geoffrey Ingram Taylor. [24-26] Lord Rayleigh’s experiments on charged droplets generated an estimate for the maximum amount of charge a droplet may hold before producing jets of liquid, known as the Rayleigh limit (not to be confused with the Rayleigh limit when discussing optical resolving power of lenses). Sir Taylor was first to describe the physics that produce the cone of liquid protruding from the tip of an electrospray emitter, known as the Taylor cone. [27] Malcom Dole first coupled an electrospray ionization source to a mass spectrometer [28], but it was John Fenn who received a share of the 2002 Nobel Prize for the use of electrospray ionization to produce ions of large biomolecules. [22]

In electrospray ionization, a solution containing the analyte of interest is pumped through a tapered needle with small orifice called the emitter. The solution is held at a fixed electric potential higher or lower than the mass spectrometer, depending on whether positive or negative ions are desired. Electric potential is usually applied through a conductive electrospray emitter, or directly to the analyte solution, if it is sufficiently conductive. Electric potential of the mass spectrometer counter-electrode may also be adjusted to
produce electrosprays. Addition of conductive species to the electrospray solution makes charge carrier species available to provide and distribute charge to analyte molecules. Volatile organic species such as methanol and acetonitrile are frequently added to the electrospray solution for two reasons. First, they aid in the evaporation of solvent from the droplets produced by the electrospray, and second, their effect in reducing surface tension of the droplets decreases the droplet charge Rayleigh limit and applied electric potential necessary to produce an electrospray. [29]

Analyte solution at the orifice of an emitter produces a meniscus that is deformed as electric potential at the emitter is increased. The meniscus becomes more conical in shape until it reaches a limit just before electrospray occurs. This shape, known as a Taylor cone, has a unique angle to define the cone shape called the Taylor angle. The Taylor angle for DC electrosprays is ideally 98.6°. At the electric potential where the Taylor cone first forms, the force acting on the meniscus due to surface tension and electric field are equal. Increasing the electric potential beyond this point produces a cone-jet formation characteristic of electrospray. The potential at which the cone-jet electrospray forms is called the electrospray onset potential. The jet emitted from a Taylor cone quickly disperses into a plume of charged droplets. These droplets are exposed to atmospheric gas within the ion source, and solvent evaporates from the droplets, concentrating the analytes and charges within the droplets. [30] When the charge within the droplet reaches the Rayleigh limit, an event termed the Coulombic explosion occurs, separating the droplet into many smaller droplets containing some, or none, of the original analytes and charges. [31] Current theory suggests
that ions eventually reach the gas phase through two competing processes. The ion evaporation model (IEM) suggests that as the droplet becomes small enough, charge present throughout the droplet becomes concentrated enough to produce a potential field gradient that assists in the desolvation of the analyte ion. [32-33] The charge residue model (CRM) suggests that analyte ions remain solvated in the droplets through repeated Coulombic explosions, until all the droplets on average contain only one or no analyte ions. [28] The remaining solvent simply evaporates from the single ion containing droplets to produce gas phase ions. Current theory suggests that a combination of the two processes actually occurs, with small molecules reaching the gas phase through a predominantly IEM mechanism, and large molecules through a CRM mechanism. [34-35]

Current is self-regulated in a cone-jet mode electrospray. [36] The net movement of charge from the ion source electrode, to the counter electrode of the mass spectrometer is the only source of current draw in a stable cone-jet electrospray. However, when the applied electric potential is too great, other discharge modes become predominant, and change the nature of the electrospray and ions produced. Corona discharge becomes an issue when the applied electric potential is too great. This discharge is a form of electrochemical discharge where gases in the atmosphere are ionized and react with other atmospheric species. In electrospray sources, the ionized species may also react with the analyte molecule ions, producing unwanted in-source fragmentation and adduction. [37] When very high potentials are applied, arcing occurs, which can cause damage to the mass spectrometer, ion source or both.
Before entering the mass analyzer portion of the mass spectrometer, the ion stream passes through a charged, heated inlet capillary to aid in desolvation, and enters a chamber containing ion optics held at rough vacuum to shape the ion beam and reject uncharged particles. A crucial step to the mass analysis process, regardless of the mass analyzer technology, is to ensure the ion beam is narrowly focused and does not contain neutral species with which ions may collide and alter trajectory. Electrosprays are usually very sensitive to the presence of salts and surfactants in the analyte solution. These species readily associate with charged species in the electrospray, stealing charge from the actual analyte molecules or creating adduct ions, which increase the complexity of the mass spectra and reduce the sensitivity of the instrument for a particular molecular ion. Salts and surfactants also cause corrosion within the mass spectrometer. Surfactants also change the surface tension of droplets, impacting electrospray performance. For these reasons, sample preparation and clean-up are extremely important when analyzing biological samples by mass spectrometry.

A more recent advancement in ESI technology was the development of low-flow ESI. In 1993, Gale and Smith utilized an ESI interface with flow rates as low as 200 nL/min and observed a significant increase in sensitivity, and in 1996, William and Mann coined the term “nano-ESI” for ESI performed at flow rates around 25 nL/min. [38-39] They utilized glass capillaries pulled to an emitter orifice opening of a few micrometers. Reduced flow rates in electrospray emitters reduced dilution of sample effluent from HPLC and CE capillaries and increased sensitivity for many analyses.
1.2.2 Mass Analysis

Three mass analyzer configurations have become popular for use in metabolomics and proteomics laboratories. They are TOF, quadrupole and ion trap. TOF mass analyzers work on the principle of applying kinetic energy to an ion using an electric field and measuring the time the ion takes to traverse the flight tube. The potential energy of a charged particle in an electric field is described by:

\[ E_p = zU \]  (1.1)

where \( E_p \) is the potential energy, \( z \) is the charge of the particle, and \( U \) is the voltage. The kinetic energy of any particle can be described by:

\[ E_k = \frac{1}{2}mv^2 \]  (1.2)

where \( E_k \) is the kinetic energy, \( m \) is the mass, and \( v \) is the velocity of the particle. Ideally, all the potential energy is converted into kinetic energy, so \( E_p = E_k \). By definition, \( v = \frac{d}{t} \), so combining this with Equation 1.1 and Equation 1.2 and solving for \( t \) yields:

\[ t = \frac{d}{\sqrt{2U}} \sqrt{\frac{m}{z}} \]  (1.3)

The first term of Equation 1.3 is constant for a particular flight tube and acceleration voltage pulse, so the flight time of an ion is proportional to the square root of the mass-to-charge ratio (\( m/z \)). [40] An ion detector detects the time and intensity of ions to generate a mass spectrum. Resolution of a TOF instrument can be increased by employing a reflectron unit, which uses a constant electric field to deflect the ion beam towards the ion detector. [41]
The reflectron minimizes the distribution of kinetic energy of ions with identical $m/z$ because ions with differing kinetic energy take slightly different deflection paths.

TOF mass analyzers are excellent at gathering high resolution parent mass spectra, and can operate at very high scan rates because typical flight tube transversal times are on the order of microseconds. However TOF-TOF tandem mass spectrometry is difficult, requiring two flight tubes, or a complex ion beam path geometry to allow for selection of parent ion, fragmentation and mass analysis of the product ions. [42] Often, a TOF mass analyzer is preceded by quadrupole mass analyzers that filter parent ions and perform fragmentation when tandem mass spectra are desired. Also frequently used are MALDI-TOF instruments, which has many applications in simple, robust analysis of proteins and peptides, and has become the predominant imaging mass spectrometry platform.

A quadrupole mass analyzer consists of four parallel rods that separates ions based on their stability in a RF oscillating electric field with a DC offset applied to the rods. The RF field and DC offset may be chosen such that the quadrupole acts as a high-pass, low-pass or band-pass ion filter. [43] The DC offset may also be disabled entirely so that the quadrupole does not perform mass filtering. This operation mode is used when the quadrupole acts as a collision cell for tandem mass spectrometry. A quadrupole acting in band-pass mode may also be scanned, to selectively pass ions of particular $m/z$ as a function of time and generate a mass spectrum. The resolution of a quadrupole is limited by the scan rate and the band-pass filter width.
One of the most popular configurations of mass spectrometer is the triple quadrupole. [44] Three quadrupoles are put in series and mass filtering or selection may be performed by any of them. In the most common configuration, the first quadrupole performs parent mass selection, the second acts as a collision cell and the third selects a particular product ion, or scans through product ions. When a triple quadrupole is fixed on a particular parent-product ion pair, it is said to be operating in single reaction monitoring (SRM) mode. The quadrupole filters can be rapidly iterated through a list of parent-product ion pairs, and in this case, the instrument is operating in multiple reaction monitoring mode (MRM). MRM is a very valuable technology for the highly selective and highly sensitive analysis in many experiments, including proteomics and metabolomics. However, MRM-MS requires knowledge of which parent-product ion pairs to monitor, and is not typically used as a discovery or hypothesis-generating tool. Triple quadrupole instruments operating in MRM mode are among the most sensitive mass spectrometers, and because of their relatively simple construction, are often the least expensive. [45]

The linear quadrupole ion trap is an evolution of the quadrupole mass filter pioneered by Wolfgang Paul, who shared the Nobel Prize in 1989 for his invention. [46] Similar to the quadrupole mass filter, the ion trap uses superimposed AC and DC electric fields to manipulate ions. However, in the linear ion trap, ring electrodes with DC potentials are used at the entrance and exit of the quadrupole to confine the ions axially within the trap, where they follow complex contours of the electric field generated by the quadrupoles. [47] The ions trapped in a linear ion trap oscillate along complex pathways within the trap.
geometry. The stability of this oscillation is dictated by the applied electric field and the mass-to-charge ratio of the ions within the trap. The electric fields can be modulated to act as a mass filter, or to selectively eject trapped ions of a particular mass-to-charge ratio. [48] A further advancement in trap design traps ions with RF electric fields in all three dimensions. [49] These traps are called 3D traps. These trap designs improve the ability of the ion trap to trap and eject specific masses, increasing the resolution of the instrument. 3D traps can also incorporate a Fourier transform detection scheme, where oscillating ions in the trap induce an image current in a central electrode. [50] The frequencies generated by the oscillating ions depends on the m/z of the ions. Because of the very precise machining requirements and incredibly complex ion paths in 3D ion trap instruments, these mass analyzers are typically the most expensive of the mass analyzers discussed in this document, but provide incredible mass resolution and selectivity, and are easily coupled to other mass analyzers for tandem mass spectrometry work. [51]

1.2.3 Ion Detectors

Common ion detectors are the multichannel plate electrode, the electron multiplier tube, and image current generation. [52-53] The first two ion detection schemes are closely related and rely upon a phenomenon called secondary emission. When a charged particle hits the surface of a metal plate in a vacuum, the impact can knock electrons free from the metal surface. The electrons emitted from the metal plate are called secondary electrons. In an electron multiplier tube, the metal plates, called dynodes, are arranged in a geometry such
that secondary electrons emitted from one dynode crash into another dynode to release more electrons. This process continues a number of times to amplify the signal from a single ion impact event. Voltages are applied to the dynodes to constantly accelerate and guide electrons from each dynode to cause a cascade of electrons to crash down the dynode path. Detection is accomplished when the avalanche of electrons crash into an anode plate and the pulse current flow is detected by an electrometer.

Multichannel plate electrodes also take advantage of an electron cascade configuration. A multichannel plate electrode is fabricated from a plate of glass into which an array of microchannels typically 5-10 micrometers in diameter is constructed. The insides of the microchannels are coated with a semiconductor that will undergo secondary emission of electrons when impacted. The channels are placed at a slant to the incoming ion beam to guarantee impact by incoming ions. The secondary electrons emitted then crash into the other side of the microchannel, and make their way through the microchannel, crashing their way down in an electron avalanche. An anode placed at the end of the microchannel measures the current pulse and performs detection. The multichannel plate array maintains spatial resolution of the ion beam, so it frequently sees use in magnetic sector and TOF instrumentation. Spatial resolution is usually not needed in triple quadrupole or ion trap instruments, so electron multiplier tubes are used in those instruments.

A third type of detection scheme exists for 3D trap instruments. [50] In some 3D traps, ions are confined around a small central electrode by the outer electrodes. The orbiting ions induce an image current in the central electrode that oscillates as a function of the m/z
of the ions within the trap. This current is measured and a Fourier transformation of the
current yields a plot that depicts the frequencies of oscillations within the trap and the

 corresponding \( m/z \) values of all ions within the trap. The resolution of a 3D trap operating in
this configuration is limited primarily by the length of time the trap can keep the oscillating
ions stable. Long spectrum acquisition times yield very high resolution mass spectra, but the
acquisition time can be intentionally shortened to speed acquisition rate at a cost to
resolution. Fourier transform mass spectrometers have high mass accuracy and high
sensitivity, but are typically very expensive.

1.2.4 Tandem Mass Spectrometry

Tandem mass spectrometry aids in the identification of ions detected by the mass
spectrometer by pairing fragment ion \( m/z \) information with intact ion \( m/z \) information.

Fragment ions are produced within a mass spectrometer collision cell by colliding isolated
parent ions with high energy gas molecules or occasionally, electrons. [54-56] The fragment
ions produced during this process are characteristic of particular structural motifs within
ions, and so can be used to identify ions injected into the mass spectrometer. Identification is
done by either database matching or \textit{de novo} structural characterization. Database matching
is the easiest method to determine molecular identity from mass spectra, but requires that the
compound be entered into the database. [57] Fragmentation spectra can vary based on the
instrument being used to perform the analysis, collision chemistry and collision energy. All
of these parameters must be tuned appropriately to generate tandem spectra that will match a
database. *De novo* structural characterization is a much more daunting task, and usually must be performed by hand. Some tools exist to predict structure from tandem spectra by using a database of common fragments, but these tools will be confounded by any contaminants that make their way into the fragment spectra. However, much work has been completed in the area of *de novo* peptide sequencing from fragment spectra, and peptide fragmentation is reasonably predictable. [59] These comforts do not exist for metabolomics researchers, however, and the complexity and variety of the metabolome make identification of metabolites from tandem spectra the biggest challenge in metabolomics today.

1.2.5 Mass Spectrometers in This Work

Three mass spectrometers are used predominantly in this thesis: the Thermo LTQ XL linear ion trap mass spectrometer, the Thermo LTQ Orbitrap Velos Fourier transform ion trap mass spectrometer, and the Thermo Q-Exactive HF Fourier transform ion trap mass spectrometer. The LTQ XL mass spectrometer is a relatively inexpensive instrument with good resolution and sensitivity. It is primarily a test-bed for new separation methods and ionization techniques. The Orbitrap Velos mass spectrometer is a high resolution instrument suitable for deep proteomic and metabolomic analyses. It contains both a linear and 3D ion trap that are used in tandem to generate parent and fragment mass spectra. The Q-Exactive HF mass spectrometer drops the linear ion trap found in the Orbitrap series but retains the 3D ion trap. It can acquire parent and tandem spectra faster than the other two mass spectrometers and is used when speed and sensitivity are a priority.
1.3 Capillary Electrophoresis

Electrophoresis is an electrokinetic phenomenon where charged particles move in a fluid as a response to a constant electric field. A particle’s velocity in the fluid is dependent on charge, electric field strength and frictional forces experienced by the particle as it moves through the fluid. Particles may be separated by electrophoresis based on their size and charge. This phenomenon was first described in 1807 by Ferdinand Frederic Reuss. [60] Arne Tiselius developed the first free solution electrophoresis apparatus and received a Nobel Prize for his work in 1948. [61] Zone electrophoresis methods were quickly developed that implement a gel or solid matrix to stabilize electrophoretic bands, minimize diffusion and provide the ability to perform high resolution separation of analytes in solution. [62] During his work describing electrophoresis, Reuss noticed another important electrokinetic phenomenon called electroosmotic flow (EOF). [60] EOF is the bulk movement of an ion-containing solution in response to the application of an electric field. EOF occurs because of the electric double layer preset at boundaries between a charged surface and an ion-containing solution. EOF drives solution through borosilicate glass tubes and fused silica capillaries. Jorgenson described the first use of fused silica capillaries with micrometer internal diameters to perform capillary zone electrophoresis. [63] Since its introduction, capillary electrophoresis has seen rapid development for the separation of many biomolecules, including peptides, proteins, lipids, carbohydrates and other metabolites. [64]
1.3.1 Capillary Zone Electrophoresis

In capillary zone electrophoresis (CZE), analytes are separated based on their Stokes radius and net charge in solution. [65-67] Analytes with identical size-to-charge ratio migrate together in tight bands, or zones, where zone width is dictated by diffusion and injection volume. Electrophoresis is generally performed in fused silica capillaries with uncoated walls. In this configuration, the bare walls of the fused silica are negatively charged when exposed to pH greater than approximately 1.5. A double layer of positively charged ions from the background electrolyte (BGE) associate with the inner wall of the capillary. When an electric field is applied, the loosely associated positive ions in the double layer are forced away from the positive potential. This movement drags the bulk solution along in the same direction to balance the net movement of charge. This phenomenon is called electroosmotic flow. In addition to experiencing EOF, analytes move through the bulk solution according to their electrophoretic mobility. The net result of these two forces results in the movement of analytes toward the detector, where the system is usually fixed at ground electric potential. Small, positively charged analytes migrate quickest, neutral species are not separated and travel in a plug with EOF, and small, negatively charged analytes migrate slowest, or may not be detected at all if their electrophoretic velocity is greater than the EOF. Larger, less charged analytes have less electrophoretic mobility than smaller, more charged analytes.

Electrophoretic mobility of an analyte depends on the net charge of the analyte in solution and its Stokes radius. These parameters are difficult to predict, so electrophoretic mobility is generally calculated from experimental measurements. The apparent mobility
(\(\mu_{\text{app}}\)) of an analyte through a capillary filled with a BGE is the sum of two mobilities: electrophoretic mobility (\(\mu_p\)) and electroosmotic mobility (\(\mu_o\)):

\[
\mu_{\text{app}} = \mu_p + \mu_o
\]  
(1.4)

The apparent migration velocity, \(U_{\text{app}}\), of an analyte is a function of its apparent mobility, \(\mu_{\text{app}}\), the applied voltage, \(V\), and the capillary length, \(L\):

\[
U_{\text{app}} = \mu_{\text{app}} \cdot V / L
\]  
(1.5)

The apparent migration velocity can be easily calculated from experimental results:

\[
U_{\text{app}} = L / t
\]  
(1.6)

where \(L\) is the length the analyte traveled from injection to detector, and \(t\) is the time taken for the analyte to reach the detector. Combining Equation 1.5 and Equation 1.6 and solving for apparent mobility give:

\[
\mu_{\text{app}} = \frac{L \cdot L_t \cdot t}{t \cdot V}
\]  
(1.7)

where \(L_t\) is the total length of the capillary and \(V\) is the applied separation voltage. Neutral species by definition have no electrophoretic component to their apparent mobility, \(\mu_{\text{app}} = \mu_o\). Thus, to calculate electroosmotic mobility, a neutral species must be added to the analyte mixture. From the migration time of the neutral species, \(t_o\), the electroosmotic mobility of all analytes in the separation can be calculated:

\[
\mu_o = \frac{L \cdot L_t}{t_o \cdot V}
\]  
(1.8)

From Equation 1.8, Equation 1.7 and Equation 1.4, the electrophoretic mobility of any analyte in the separation may be calculated.
Capillary zone electrophoresis is a very high resolution separation technique due to the limited sources of band broadening present. Consider the van Deemter Equation:

\[ H = A + \frac{B}{U} + CU \]  

(1.9)

The eddy diffusion parameter \((A)\) and mass transfer term \((CU)\) are both zero in CZE, leaving diffusion \((B/U)\) as the only on-column source of band-broadening when determining theoretical plate height \((H)\). Eddy diffusion and mass transfer are not considered in CZE because the electrophoresis is performed in free flowing solution, with no stationary phase media for analytes to interact with. In addition, the flow profile of electrokinetically pumped CZE is different from hydrodynamically pumped separation techniques such as HPLC. EOF flows with a flat, plug shaped flow profile, while solutions within a HPLC column flow with the laminar flow profile typical of hydrodynamic flows. Theoretical plate counts for CE separations are usually between 50,000 and 500,000 but can exceed 1,000,000. Theoretical plate counts are determined experimentally by the following equation:

\[ N = 5.54 \left( \frac{t_r}{W_h} \right)^2 \]  

(1.10)

where \(t_r\) is the retention time of a peak, and \(W_h\) is the full width of the peak at half the maximum height (FWHM).

Band broadening is limited in capillary electrophoresis by careful optimization of the separation parameters. Voltage and capillary dimensions play a major role in this optimization. Joule heating of the BGE and capillary is a result of the high voltage dissipated down the length of the capillary. Joule heating leads to band broadening by heating the BGE
and increasing the rate of diffusion of analyte bands. Joule heating is a function of current passed through the capillary and BGE, and the system’s resistivity. Higher applied voltages lead to more Joule heating. The impact of Joule heating can be mitigated by reducing electric field strength or by reducing the capillary diameter. A reduced capillary diameter increases the surface to volume ratio of the capillary system and improves heat dissipation. Some CE systems also incorporate thermal controls such as refrigeration to minimize the impact of Joule heating. Too low of an applied electric field strength also leads to band broadening, however. Under low field strength conditions, analytes migrate very slowly and bands have a long time to diffuse. Construction of an Ohm’s plot aids in the determination of ideal separation voltage. Because the resistivity of a capillary and BGE system \((R)\) is constant at a fixed temperature, Ohm’s law \((V = IR)\) dictates that as voltage \((V)\) is increased, the measured current flow in the system \((I)\) must increase linearly with it. By plotting current as a function of applied voltage, the applied voltage at which Joule heating becomes an issue will be evident as a deviation from linearity in the Ohm’s plot. As the BGE in the capillary is heated, the conductivity of the system increases, increasing the measured current draw. For best separation results, CZE separations should be performed at the highest voltage that does not cause Joule heating issues. [68-69] BGE for CZE should have a higher ionic strength than the analyte solution to minimize the effect of electric field strength differences in analyte zones. The rule of thumb is to ensure the BGE is an order of magnitude greater in ionic strength than the analyte.
Sample is introduced into the capillary one of two ways. [70] An electrokinetic injection may be performed, where voltage is applied to a reservoir containing the analyte and capillary entrance. The distal end of the capillary is held at ground potential. Analyte solution migrates into the capillary by EOF and electrophoresis. Very small injections can be performed in this way, however electrokinetic injections bias sample injection based on the analyte’s electrophoretic mobility. Ions with greater forward mobility will be present at artificially high concentration in the injection while ions with low or backward mobility will be present at lower concentrations. Hydrodynamic injections utilize pressure in an injection chamber to force analyte solution into the capillary. Hydrodynamic injections are simple, robust and repeatable, and do not bias the injection like electrokinetic injections. However, very small injections are more difficult to perform, and the laminar flow profile of hydrodynamic injection can introduce a small amount of band broadening. Injection conditions should be optimized to ensure that no more than 1% of the capillary volume is replaced by analyte upon injection. The equation for calculating injection volume of hydrodynamic injections is:

\[
V = \frac{\Delta P \pi d^4 t}{128 \eta L_t}
\]  

(1.11)

where \(\Delta P\) is the applied pressure, \(d\) is the capillary inner diameter, \(t\) is the time in seconds, \(\eta\) is the sample viscosity, and \(L_t\) is the total length of the capillary. For applied pressure in psi, capillary inner diameter in microns, time in seconds, capillary length in centimeters,
injection volume in nanoliters, and assuming sample viscosity identical to water at 25°C, Equation 1.11 simplifies to:

\[ V = 1.901 \times 10^{-5} \left( \frac{\Delta P d^4 t}{L_t} \right) \]  

(1.12)

Optimizing the injection volume and sample concentration are critical to performing high resolution capillary electrophoresis.

1.3.2 Micellar Electrokinetic Chromatography

Micellar Electrokinetic Chromatography (often abbreviated MEKC or MECC) is a separation mode for capillary electrophoresis that utilizes both electrophoresis and phase partitioning of analytes to provide resolution of neutral analytes. [71] A surfactant is added to the BGE at a concentration greater than the critical micellar concentration. The surfactant forms spheres with the highly hydrophobic tail portion of the surfactant molecules pointing toward the center of the sphere. This creates a highly hydrophobic environment into which analytes can partition. The micelles have their own inherent electrophoretic mobility, and when the analyte molecules are inside the micelles, their own electrophoretic mobility is altered. Analytes, including neutral species, migrate in a tight band as a time-averaged result of partitioning between the two phases. For some lipid species, adding cyclodextrins to the BGE presents a third phase for partitioning and can aid in lipid separations. Lipid species interact with the inner portion of the cyclodextrin molecules and their electrophoretic mobilities are altered. MECC allows CE to resolve neutral species, but the addition of surfactant makes most MECC separations incompatible with mass spectrometry.
1.3.3 Capillary Isoelectric Focusing

Capillary isoelectric focusing (cIEF) is a mode of separation for separating analytes based on their isoelectric point (pI). [72] The capillary is filled with a mixture of electrolytes called an ampholyte solution. These electrolytes are selected such that the ampholyte solution contains a wide variety of ions with various pI. When an electric field is applied to the ampholyte solution, ions move in response to the electric field until they are titrated by neighboring ions and lose charge. This process generates a pH gradient within the capillary. Analytes are then injected onto the capillary and voltage is reapplied. Analytes focus into tight bands in the same way as the pH gradient is generated. Diffusion is not a major factor in cIEF because analyte bands are focused by the pH gradient. Diffusion out of the band causes an analyte molecule to gain charge, and move in response to the electric field back into the analyte band. Finally, the entire contents of the capillary are mobilized through either destroying the pH gradient or pumping the contents out to a detector. Since analytes are focused in cIEF, it can handle large injection volumes, making cIEF appropriate for the analysis of dilute samples. However, the presence of ampholytes in the BGE contributes significant background noise in mass spectrometry and makes cIEF difficult to couple to MS.

1.3.4 Capillary Electrophoresis with Laser Induced Fluorescence

Laser induced fluorescence (LIF) provides a highly sensitive and selective detection technique for use with CE. Sheath-flow LIF setups perform detection off-column, which reduces the impact of diffraction from the capillary walls and provides very high sensitivity.
The first on-column LIF detectors reported by Gassman and Zare produce detection limits around 200 attomoles, but our group has used sheath-flow designs to detect zeptomoles of fluorescently-labelled amino acids and single molecules of b-phycoerythrin. LIF detection requires analytes be fluorescent or fluorescently labelled, which can become complex when dealing with real-world samples.

In our group’s sheath flow cuvette design, the separation capillary is threaded into a rectangular quartz cuvette. The cuvette is filled with liquid, and attached to a reservoir of liquid that flows through the cuvette, around the capillary and to a waste beaker, driven by siphon flow. This creates a sheathing flow around the exit of the capillary that acts to contain the sample as it migrates off the capillary and confine it such that it passes through a laser beam in a narrow stream. Fluorescence is collected at a 90 degree angle to the incoming laser light by a high numerical aperture lens, transmitted along fiber optics and detected by an avalanche photodiode. A band-pass filter is placed in the system to filter stray laser light from reaching the detector. This setup creates exquisite sensitivity because the laser light, and emitted fluorescence are not significantly scattered by the capillary or cuvette because of their high angle of incidence to the flat cuvette surfaces. In addition, the entire sample stream is confined to pass through the laser volume. This setup can produce sensitivity for a single fluorophore molecule, and can be designed to have sufficient dynamic range to also detect micromolar concentrations of fluorophore with the same setup.
1.3.5 Capillary Electrophoresis with Mass Spectrometry

The first CE-MS interface was developed by the Smith group in 1987. [78] It directly coupled the separation capillary to a stainless steel emitter held at a fixed potential for ESI. With the aid of a counterflow desolvation gas, this setup produced stable electrospray for the separation and analysis of quaternary ammonium salts. Since the development of this interface, rapid advancement of coupling technology has occurred. [79-83] The designs can be separated in to two general categories, sheathed and sheathless. [84] Like Smith’s original design, sheathless designs rely on the BGE to support stable ESI, and the EOF must drive fluid toward the mass spectrometer at sufficient rate to drive ESI. These requirements limit the usefulness of sheathless interfaces. Sheath flow interfaces were developed to address this issue. [85] In traditional sheath flow CE-ESI interfaces, the capillary is placed in a stainless steel ESI emitter filled with a solution compatible with ESI. The effluent from the CE separation is passed into the emitter tip, mixes with the ESI solution and is sprayed into the mass spectrometer. The ESI solution is pumped into the emitter to drive the ESI. However, this technique can greatly dilute the sample concentration prior to MS analysis and detection.

Both emitter designs are in widespread use today. Moini has commercialized a sheathless design where the distal end of the separation capillary is etched to a thin, porous wall, where electrical contact is made. [86] This design provides a very stable and robust electrical connection for ESI and CE, but electrolyte choice is still limited. The Dovichi group has pioneered a novel, electrokinetically pumped sheath flow interface. [87-88] In our
interface, the emitter is made of borosilicate glass. The electric connection for CE and ESI is made by an electrode in an ESI solution reservoir vial. This voltage drives both the electrospray, and EOF in the borosilicate emitter. By pumping fluid through the emitter using EOF, rather than mechanical pumps, the analyte dilution rate at the interface is drastically reduced. This interface has seen widespread use for the highly sensitive analysis of the proteome. In 2015, the interface was modified. [89] The distal end of the capillary was etched to reduce the outer diameter, so the capillary fits farther in the emitter, reducing the distance from the capillary to the tip. In addition, the tip opening size was increased, greatly improving the reliability and durability of the interface.

1.4 Introduction to This Work

This thesis presents work in three major areas: CE-LIF for metabolic analysis, CE-MS for metabolic analysis, and the study of ionization techniques for MS. The CE-LIF work presented in Chapter 2, highlights the usefulness of CE for the separation of the products of lipid metabolism, and demonstrates the need for a label-free approach to analyzing the metabolism to avoid modifying endogenous metabolism by the inclusion of a fluorescent label. Chapter 3 demonstrates high speed CZE coupled to MS as a tool for analyzing small molecule metabolites. Since small, acidic compounds play a role in many key metabolic pathways, a negative mode ESI interface for CZE-MS is identified as a critical area of research. The development of this interface, and a demonstration of its usefulness for generating metabolomic data sets are discussed in Chapters 4 and 5. Chapter 6 presents an
alternative method of generating ions for MS analysis called AC-ESI. AC-ESI generates mass spectra with reduced background ion intensity, and improved signal-to-background ratios for many analytes, which may prove useful in the analysis of the metabolome.
Figure 1.1: Diagram of the relationship of the metabolome with other upstream –omics fields. The metabolome more closely represents the phenotype of an organism because changes in regulatory factors at upstream stages will be represented by changes in metabolite concentrations.
1.5 References


CHAPTER 2:
PREPARATION AND SEPARATION OF BODIPY-FL-LABELED GLYCOSPHINGOLIPIDS

2.1 Introduction

Glycolipids constitute over 80% of the conjugated saccharides in the mammalian brain, making them one of the most abundant molecules on neuronal cell surfaces [1]. Glycosphingolipids consist of a polar sugar head group and a ceramide hydrophobic lipid tail made up of a sphingosine and a fatty acid. Sialic-acid containing glycosphingolipids are termed gangliosides and play a crucial role in cellular signaling [2-5], cell differentiation [6-8], oncogenesis [9], auto-immune disorders [10], infectious disease [11-15], and lysosomal storage disorders such as Tay-Sachs and Sandhoff Diseases [16]. The structure of the ganglioside GM1 is shown in Figure 2.1A.

Thin layer chromatography [17,18] and high performance liquid chromatography [19,20] are the traditional approaches for the separation of glycosphingolipids. Glycosphingolipids are amphiphillic, which complicates their separation.

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TLC is rapid, simple, and requires little sample preparation [21], but glycosphingolipids with the same sugar moiety can migrate to different positions on the TLC plate due to differences in carbon chain length within the ceramide tail [22]. HPLC offers several advantages over TLC including improved resolution, reproducibility, and the ability to easily interface with mass spectrometry [23]. Mass spectrometry is frequently used as the detection method to produce detailed structural information about these complex glycolipids. However, gangliosides can be difficult to analyze by mass spectrometry due to the fragile nature of metastable sialic acid-containing ions [24]. In addition, it is difficult to obtain proper separation conditions that are compatible with mass spectrometry [23,25]. Limits of detection for these hyphenated mass spectrometry techniques often range between 0.1 and 1 pmol [24,26-29], which is approximately 8-10 orders of magnitude poorer than what is required for monitoring metabolism in single cells.

Our lab coined the term “metabolic cytometry” to describe the process of probing metabolism within single cells by capillary electrophoresis with laser induced fluorescence detection [30,31]. In a typical experiment, the fatty acid of a glycosphingolipid is replaced with a fluorescent label to create a fluorescent substrate [32]. Cells are incubated with this fluorescent substrate, and any metabolic products due to the addition (anabolism) or removal (catabolism) of sugars will also be detected (Figure 2.1B). After incubation, cells are injected onto the capillary, lysed, and the identities of the metabolic products are determined by co-migration with standards [32,33]. We have used this approach to investigate metabolism in
mammalian cellular homogenates [34] and single cells [35], and lipid uptake by the malaria-causing parasite Plasmodium falciparum [36].

The separation of glycosphingolipids by capillary electrophoresis is complicated because it requires the separation of anionic gangliosides and several neutral metabolic products including lactosyl ceramide (LacCer), glucosyl ceramide (GlcCer), and ceramide (Cer). Most of the previous work focused on capillary zone electrophoresis based on a borate, phosphate, and α-cyclodextrin buffer [37-40]. Because glycosphingolipids are amphiphilic, they are known to form micelles in solution [41]. α-cyclodextrin adds improved separation through an equilibrium partitioning process [25,38]. This composition is excellent at separating ganglioside isomers, but neutral glycosphingolipids co-migrate [38].

We have previously used a micellar electrokinetic capillary chromatography (MEKC) approach to separate charged and neutral glycosphingolipids based on a borate/deoxycholate/methyl-β-cyclodextrin buffer [42,43]. This buffer allowed for rapid separation (<5 min) of tetramethylrhodamine labeled mono-sialylated and neutral glycosphingolipids with theoretical plate counts between 300,000 and 550,000 [32,34,35]. However, here we show that this buffer composition is insufficient to separate higher ordered gangliosides (i.e. GD and GT series). Here we report on the preparation and separation of several Bodipy-Fl (boron dipyrromethene difluoride, $\lambda_{ex} = 505$ nm) labeled glycosphingolipids (Figure 2.1C). Bodipy-Fl is a green-emitting dye ($\lambda_{ex} = 505$ nm) that has been used to study glycosphingolipid transport within cells [44,45]. Here we show that MEKC based on a TRIS/CHES/dodecylsulfate/α-cyclodextrin buffer separates
polysialylated, monosialylated, and neutral glycosphingolipids in a rapid, highly efficient, and ultrasensitive manner.

2.2 Experimental

2.2.1 Preparation of Bodipy-Fl-Labeled Glycosphingolipids

BODIPY®-FL-C5 succinimidyl ester (Bodipy-NHS ester) was purchased from Invitrogen (San Diego, California). D-Sphingosine and C18-lyso-lactosylceramide (LacCer) were purchased from Avanti Polar Lipids (Alabaster, Alabama). C18-lyso-GM1 were prepared as previously reported[32]. N,N-Diisopropylethylamine (DIEA) and β-galactosidasess (from E. coli and from bovine testes) were purchased from Sigma (St. Louis, MO). All small molecule salts and solvents were also purchased from Sigma-Aldrich (St. Louis, MO). Sialyltransferase (MalE fusion protein from Campylobacter jejuni) were expressed and purified as previously reported[46,47]. Cytidine 5′-monophospho-N-acetyl-β-D-neuraminic acid (CMP-Neu5Ac) was purchased from IEP GmbH (Wiesbaden, Germany). Alkaline phosphatase was purchased from Roche (Basel, Switzerland). C18 Sep-Pak cartridge was purchased from Waters Corporation (Milford, MA) and activated by washing with MeOH (20 mL) followed by water (20 mL) before use. MALDI-TOF MS spectra were recorded on Bruker (Billerica, MA) Microflex instruments using a α-Cyano-4-hydroxycinnamic acid solution[20 mg dissolved in 1 mL of 50/50 (v/v) acetonitrile/water with 0.1 % TFA] as matrix.
Cer-Bodipy: To a mixture of D-sphingosine (1.5 mg, 5.0 μmol) and Bodipy-NHS ester (1.5 mg, 3.6 μmol) in DMF (1.0 mL) were added DIEA (2.0 μL, 12 μmol). After stirring at room temperature for 24 h, the reaction mixture was evaporated with toluene and then purified by column chromatography on silica gel (chloroform/MeOH = 50/1) to give the product (2.0 mg, 92%).

LacCer-Bodipy: To a mixture of C18-lyso-LacCer (2.0 mg, 3.2 μmol) and Bodipy-NHS ester (2.4 mg, 5.8 μmol) in DMF (1.0 mL) was added DIEA (5.6 μL, 32 μmol). After stirring at room temperature for 2 days, the reaction mixture was evaporated with toluene and then purified by column chromatography on silica gel (chloroform/MeOH/water = 8/1/0.1 to 4/1/0.1). The purified product was loaded on double connected C18 Sep-Pak cartridges with 40% aq. MeOH (20 mL) and the cartridges were flushed with 40% aq. MeOH (20 mL), 60% aq. MeOH (60 mL), 80% aq. MeOH (80 mL) and MeOH (50 mL). The product was eluted with 80% aq. MeOH giving 2.3 mg (77%).

GM1-Bodipy: To a mixture of C18-lyso-GM1 (2.3 mg, 1.8 μmol) and Bodipy-NHS ester (1.8 mg, 4.3 μmol) in DMF (1.0 mL) was added DIEA (3.1 μL, 18 μmol). After stirring at room temperature for 2 days, the reaction mixture was evaporated with toluene and then purified by column chromatography on silica gel (chloroform/MeOH/water = 2/1/0.1 to 1/1/0.1). The purified product was loaded on double connected C18 Sep-Pak cartridges with 40% aq. MeOH (20 mL) and the cartridges were flushed with 40% aq. MeOH (20 mL), 60% aq. MeOH (60 mL) and 80% aq. MeOH (80 mL). The product was eluted from 60% to 80% aq. MeOH giving 2.4 mg (84%).
After each enzymatic reaction, the resulting reaction mixture was loaded on a C18 Sep-Pak cartridge, previously activated with MeOH (10 mL). The cartridges were washed with sample loading solution (100 mL) and the Bodipy-compounds were eluted with MeOH (10 mL). Finally, the eluent was evaporated to yield the desired product.

GlcCer-Bodipy: The reaction was performed at room temperature in a mixture (30 μL, pH 6.8) containing LacCer-Bodipy (0.010 mg, 11 nmol), β-galactosidase from E. coli (1 U), sodium phosphate (100 mM), NaCl (50 mM) and MgCl₂ (6.6 mM). After 18 h of incubation, the resulting mixture was loaded onto Sep-Pak column with 40% aq. MeOH (1 mL) and purified.

GM3-Bodipy and GD3-Bodipy: The reaction was started at room temperature in a mixture (200 μL, pH 7.5) containing LacCer-Bodipy (0.045 mg, 50 nmol), sialyltransferase (0.27 mg), CMP-Neu5Ac (500 nmol), alkaline phosphatase (125 U), MOPS (50 mM), MgCl₂ (60 mM). After 15 and 24 h incubation, enzymes and donor solution[27 μL; sialyltransferase (0.18 mg), alkaline phosphatase (125 U) and CMP-Neu5Ac (250 nmol)] was added to the reaction mixture. After totally 2 days of incubation, the resulting mixture was loaded onto Sep-Pak column with 30% aq. MeOH (1 mL) and purified.

GM2-Bodipy: The reaction was performed at 37 °C in a mixture (20 μL, pH 4.5) containing GM1-Bodipy (0.01 mg, 11 nmol), β-galactosidase from bovine testes (26 mU), sodium acetate (100 mM). After 18 h of incubation, the resulting mixture was loaded onto Sep-Pak column with 30% aq. MeOH (1 mL) and purified.
GD1a-Bodipy and GT1a-Bodipy: The reaction was started at room temperature in a mixture (200 μL, pH 7.5) containing GM1-Bodipy (0.040 mg, 25 nmol), sialyltransferase (0.27 mg), CMP-Neu5Ac (300 nmol), alkaline phosphatase (125 U), MOPS (50 mM), MgCl2 (60 mM). After 15 and 24 h incubation, enzymes and donor solution[25 μL; sialyltransferase (0.18 mg), alkaline phosphatase (125 U) and CMP-Neu5Ac (200 nmol)] was added to the reaction mixture. After totally 2 days of incubation, the resulting mixture was loaded onto Sep-Pak column with 30% aq. MeOH (1 mL) and purified.

2.2.2 Capillary Electrophoresis With Laser-Induced Fluorescence Detection

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All solutions were prepared in 18.2 MΩ distilled deionized water (Barnstead Nanopure System, Thermo Scientific, Waltham, MA) and filtered twice at 0.2 μm. MEKC was used to perform the electrophoretic separations. One buffer consisted of 10 mM sodium tetraborate, 35 mM sodium deoxycholate, and 5 mM methyl-β-cyclodextrin with an operating voltage of 18 kV[36,48]. Another buffer consisted of 100 mM tris(hydroxymethyl)aminomethane (TRIS), 100 mM 2-(cyclohexylamino)ethanesulfonic acid (CHES), 20 mM sodium dodecyl sulfate (SDS), and 5 mM α-cyclodextrin with an operating voltage of 24 kV.

A Spellman (Hauppauge, NY) CZE-1000R power supply was used to supply voltage for the separation on a 34 cm, 30 μm ID/150 μm OD fused silica capillary (Polymicro Technologies, Phoenix, AZ). Sample introduction was performed by electrokinetic injection (1 kV for 1 s), housed in a locally constructed injection block [33]. Unless otherwise stated,
all optical components were purchased from Thorlabs (Newton, NJ) and CVI Melles Griot (Albuquerque, NM). A post-capillary sheath flow cuvette was used for fluorescence detection, as described elsewhere [49,50]. A 473 nm diode pumped solid-state laser (Lasermate Group, Inc., Pomona, CA) at 10 mW was used for excitation of the Bodipy-Fl-labeled glycosphingolipids. The emission was collected with a 0.7 NA microscope objective lens, bandpass filtered (505 ± 10 nm), imaged onto a fiber optic, and detected using an avalanche photodiode (SPCM-AQR-13-FC, PerkinElmer, Vaudreuil, Quebec). All data was collected using locally constructed hardware and software based on the LabVIEW (National Instruments, Austin, TX) programming environment. Data was digitized using a PCI-6035E card (National Instruments) and the fluorescence emission was monitored at 50 Hz.

Data analysis was performed in locally written software using the LabVIEW and MATLAB (Mathworks, Natick, MA) programming environments. All electropherograms were filtered with a 5-point median filter to remove spikes and smoothed with a 0.1 s full width at half height Gaussian function. Peak widths were estimated using a least-squares fit of each peak to a Gaussian function. Separation efficiencies (N) were calculated according to Equation 2.1

\[ N = \left( \frac{t_m}{\sigma_t} \right)^2 \]  

(2.1)

where \( t_m \) is the migration time of the analyte and \( \sigma_t \) is the standard deviation of the Gaussian peak [51]. Apparent mobilities (\( \mu_a \)) were calculated as
\[
\mu_a = \frac{L_{\text{eff}} L}{t_m V_{\text{run}}}
\]  

(2.2)

where \(L_{\text{eff}}\) is the effective capillary length (to the detector), \(L\) is length of the capillary, and \(V_{\text{run}}\) is the running voltage. Concentrations of the Bodipy-Fl-labeled lipids were estimated by comparing the fluorescence intensity to that of a known quantity of free Bodipy-Fl dye.

2.3 Results and Discussion

2.3.1 Preparation of Bodipy-Fl-Labeled Glycosphingolipid Standards

Mass spectrometry was used to characterize the Bodipy-Fl-labeled glycosphingolipid preparations, Table 2.1. The MALDI mass spectra are shown in Figure 2.2. The mass spectrum of the GT1a/GD1a mix showed a peak corresponding to GM1, but an MEKC separation using 10/35/5 (vide infra) showed only two peaks corresponding to a 1.0:1.4 GT1a to GD1a ratio. This result is consistent with the fragile nature of ganglioside metastable ions and highlights the disadvantage of mass spectrometric detection of glycosphingolipids. Comparing peak heights using MEKC, enzymatic conversion of GM1-Bodipy-Fl to GM2-Bodipy-Fl gave a 3:1 ratio of GM1 to GM2, a 4:1 GM3 to GD3 ratio, and a 2:1 ratio of LacCer-Bodipy-Fl to GlcCer-Bodipy-Fl.

2.3.2 Glycosphingolipid Separation in Borate/Deoxycholate/Methyl-\(\beta\)-Cyclodextrin

A mixture containing 1-3 nM Bodipy-Fl labeled GT1a, GD1a, GD3, GM1, GM2, GM3, LacCer, GlcCer, and Cer was separated in a 10 mM sodium tetraborate, 35 mM
sodium deoxycholate, and 5 mM methyl-β-cyclodextrin (10/35/5) buffer at 18 kV. The electropherogram is presented in Figures 3A and 3B, showing poor resolution of the polysialic acid-containing glycosphingolipids.

GM1 migrated as a shoulder on the GD1a peak. In addition, GD3 and GM3 comigrated, even though GD3 contains two sialic acids that are deprotonated in this buffer (pH 9.2). The gangliosides migrated before the neutral glycosphingolipids, but did not follow any predictable order based on size, charge, or hydrophobicity. Gangliosides are known to form complexes with borate in aqueous solution[25] so the observed separation pattern is likely due to a balance between the interaction of the analyte with the outer ionic surface of deoxycholate micelles and the inner structure of neutral methyl-β-cyclodextrin molecules.

For the peaks that were resolved, separation efficiencies ranged between 440,000 to 550,000 theoretical plates except for GlcCer, which had over 1.4 million plates. The measured current across the capillary was 18 µA for the 530 V/cm separation and apparent electrophoretic mobilities of the glycosphingolipids varied between 3.0 and 3.3 x 10⁻⁸ m²V⁻¹s⁻¹. The limit of detection (3-σ, measured with free Bodipy-Fl dye) was 5.8 ± 1.0 pM or 315 molecules injected onto the capillary.

2.3.3 Glycosphingolipid Separation in TRIS/CHES/SDS/α-Cyclodextrin

The composition of the 10/35/5 buffer had previously been optimized for the separation of amphiphillic lipid species [42]. For enhanced separation of polysialic acid
glycosphingolipids, a previously successful TRIS/CHES/SDS buffering system [52] was modified. α-Cyclodextrin was chosen instead of methyl-β-cyclodextrin as an additive because α-cyclodextrin is the only cyclodextrin that has been shown to separate di- and trisialogangliosides [25,38].

The concentration of TRIS and CHES were held constant at 100 mM while the concentration of SDS and α-cyclodextrin varied. It has been shown that ganglioside separations can be enhanced in a buffer with high ionic strength [37]. The composition of the buffer that achieved the best separation was 100 mM TRIS, 100 mM CHES, 20 mM SDS, and 5 mM α-cyclodextrin (henceforth referred to as TCS-α). Lower SDS and α-cyclodextrin concentrations resulted in poor resolution. Increasing the α-cyclodextrin concentration above 15 mM caused severe excessive peak tailing, consistent with literature [38]. The voltage was increased from 18 kV to 24 kV, improving peak efficiencies without excessive Joule heating.

A separation of the same nine Bodipy-Fl-labeled glycosphingolipids in TCS-α at 24 kV is shown in Figures 3C and 3D. Although baseline separation of GM2 and GD3 was not achieved, all species were resolved. Separation efficiencies ranged from 640,000 to 740,000 theoretical plates. Compared to the 10/35/5, the apparent electrophoretic mobilities of the glycosphingolipids were lower in TCS-α (1.8 to 2.0 x10^-4 m^2V^-1s^-1), slightly increasing the time of the separation.

Besides improving the separation of glycosphingolipids, TCS-α also offered three other advantages over the 10/35/5 buffer. First, the current across the capillary was lower in
TCS-α compared to 10/35/5, 12 µA for the 700 V/cm separation. Second, the migration order of the analytes was predictable, following a decreasing number of sugars attached to the lipid tail (Figure 2.1B). GM2 and GD3 both have four sugars, which could explain why these two analytes had similar migration times. Finally, comparing Figures 2.3A and 2.3C show that TCS-α offers an improved limit of detection. The TCS-α buffer generated lower fluorescence background, improving the limit of detection by half to $3.0 \pm 0.6$ pM or 150 molecules injected onto the capillary.

2.4 Conclusions

Here we have demonstrated the synthesis, electrophoretic separation, and ultrasensitive detection of several Bodipy-Fl-labeled glycosphingolipids. A MEKC separation based on a TRIS/CHES/SDS/α-cyclodextrin buffer provided superior resolution and a lower limit of detection compared to a borate/deoxycholate/methyl-β-cyclodextrin buffer. Since the separation of glycosphingolipids in TCS-α depended on the number of sugars the analyte contained, it is possible that the TCS-α buffer may not separate structural isomers such as the GD1a/GD1b or GT1a/GT1b couple. Fluorescent b-series gangliosides are challenging to synthesize, but when prepared, their separation will be evaluated in TCS-α. These results, taken together with previous work using tetramethylrhodamine[32], should allow for the construction of an ultrasensitive two color laser-induced fluorescence assay for probing multiple complex glycosphingolipid metabolic pathways simultaneously in single cells.
2.5 References


Figure 2.1: Glycosphingolipid structure and metabolism. (A) Structure of the ganglioside GM1. (B) Metabolic pathway of glycosphingolipids (C) Bodipy-Fl-labeled GM1.
Figure 2.2: MALDI mass spectra for the prepared Bodipy-Fl-labeled glycosphingolipid standards.
Figure 2.3: Glycosphingolipid structure and metabolism. (A) Structure of the ganglioside GM1. (B) Metabolic pathway of glycosphingolipids (C) Bodipy-Fl-labeled GM1.
### TABLE 2.1:

MALDI ION INFORMATION FOR BODIPY-FL-LABELLED GLYCOSPHINGOLIPIDS

<table>
<thead>
<tr>
<th>Bodipy-Fl Analyte</th>
<th>Theoretical Ion</th>
<th>Theoretical m/z</th>
<th>Actual m/z</th>
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<tbody>
<tr>
<td>Cer</td>
<td>C_{34}\text{H}_{54}\text{BF}_2\text{N}_3\text{NaO}_3^+</td>
<td>624.41</td>
<td>624.48</td>
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<tr>
<td>GlcCer</td>
<td>C_{46}\text{H}_{64}\text{BF}_2\text{N}_3\text{NaO}_8^+</td>
<td>786.46</td>
<td>786.57</td>
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<tr>
<td>LacCer</td>
<td>C_{46}\text{H}_{74}\text{BF}_2\text{N}<em>3\text{NaO}</em>{13}^+</td>
<td>948.52</td>
<td>948.67</td>
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<tr>
<td>GM3</td>
<td>C_{57}\text{H}_{90}\text{BF}_2\text{N}<em>4\text{O}</em>{21}^-</td>
<td>1215.62</td>
<td>1215.1</td>
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<tr>
<td>GD3</td>
<td>C_{68}\text{H}_{107}\text{BF}_2\text{N}<em>5\text{O}</em>{29}^-</td>
<td>1506.71</td>
<td>1506.4</td>
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<tr>
<td>GM2</td>
<td>C_{65}\text{H}_{103}\text{BF}_2\text{N}<em>5\text{O}</em>{26}^-</td>
<td>1418.7</td>
<td>1418.03</td>
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<tr>
<td>GM1</td>
<td>C_{71}\text{H}_{113}\text{BF}_2\text{N}<em>5\text{O}</em>{31}^-</td>
<td>1580.75</td>
<td>1580.3</td>
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<tr>
<td>GD1a</td>
<td>C_{82}\text{H}_{130}\text{BF}_2\text{N}<em>6\text{O}</em>{39}^-</td>
<td>1871.84</td>
<td>1871.06</td>
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<tr>
<td>GT1a</td>
<td>C_{93}\text{H}_{147}\text{BF}_2\text{N}<em>7\text{O}</em>{47}^-</td>
<td>2162.94</td>
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CHAPTER 3:
HIGH SPEED CZE-MS FOR RAPID ANALYSIS OF AMINO ACIDS AND A PROTEIN DIGEST

3.1 Introduction

High-speed capillary zone electrophoresis (CZE) separations were first described by Monnig and Jorgenson in 1991 [1]. Most high-speed CZE separations have employed laser-induced fluorescence detection [2-5]. Increasing speed while maintaining high separation efficiency requires maximizing the electric field strength and minimizing Joule heating; inefficient heat removal leads to band broadening and separation degradation. Capillaries with a narrower inner diameter increase the surface-to-volume ratio and efficiently remove excess heat. Therefore, a higher voltage can be applied to increase separation speed without drastically increasing the current output [1]. However, the use of narrow capillaries limits the injection volume, which degrades concentration detection limits.

Mass spectrometers are attractive detectors for fast CZE separations. Smith’s group pioneered the interface of CZE with electrospray ionization (ESI) for mass spectrometry

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detection [6], and since then interfaces have undergone many redesigns [7]. A common challenge has been creating a stable electrical contact that serves as both the terminal electrode for CE and the emitter electrode for the ESI [8-9].

The mass spectrometer must provide fast scan speed and high sensitivity to follow the rapid separation and detect the low injection amounts employed in high-speed CZE. Time-of-flight mass spectrometers have often been used in high speed CZE due to their high sampling rates. Banks Jr. and Dresch presented the first high speed CE-MS study of proteins and peptides utilizing a pumped sheath-flow interface coupled to TOF-MS [8]. Matysik and Pelzing have worked extensively with a coaxial sheath-flow interface design coupled to TOF-MS with exquisite results [10-13]. The pumped-sheath flow design allowed for detection in positive and negative ionization modes for many classes of compounds.

Ion traps and Orbitrap mass analyzers are interesting alternatives for detection of high-speed CZE separations. Moini and Martinez used an ion-trap mass spectrometer in an ultrafast CE-MS system that employed a short (< 20 cm) and narrow (≤ 5 μm inner diameter) capillary and ≥1000 V/cm electric field [14]. The analysis of standard peptides and protein digests were completed in about 1 min. Wojcik et al. employed an Orbitrap Velos mass spectrometer for the fast separation of tryptic peptides, achieving separation of ten peptides in 300 s [15].

This report employs an electrokinetically pumped, nanospray sheath-flow CE-ESI-MS interface that is quite simple and robust [16]. Briefly, the separation capillary is threaded through a tee union into a glass ESI emitter. Under applied voltage, sheath flow in
the emitter tip is driven by electroosmosis flow. A stable electrical contact for both the
terminal CE electrode and ESI electrode is created at the sheath liquid reservoir. Emitter
lifetime is extended by applying the electrospray voltage at the sheath liquid reservoir, instead
of to the emitter directly, which prevents undesirable electrolysis reactions. Previous work by
Wojcik et al. investigated fast CE-MS separations of amino acids and urine metabolites
coupled with a high resolution, fast acquisition rate time-of-flight instrument. [17]. While
the electrokinetically-pumped interface has been used in many CZE proteomic analyses [18-
23], it has not applied for metabolite analysis in combination with ion trap MS
instrumentation.

3.2 Materials and Methods

3.2.1 Materials and Chemicals

Amino acids, bovine pancreas TPCK-treated trypsin, bovine serum albumin (BSA),
urea, ammonium bicarbonate (NH₄HCO₃), dithiothreitol (DTT), iodoacetamide (IAA) and
acetonitrile (ACN) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid
(FA) and hydrofluoric acid were purchased from Fisher Scientific (Pittsburgh, PA, USA).
LC-MS grade water and methanol were purchased from Honeywell Burdick & Jackson
(Wicklow, Ireland). Fused silica capillaries (10 µm i.d./150µm o.d., 20 µm i.d./150µm o.d.)
were purchased from Polymicro Technologies (Phoenix, AZ, USA).
3.2.2 Sample Preparation

Concentrated stock solutions of each amino acid were prepared in water and kept at -80°C until needed. Two sample mixtures were prepared for CE-ESI-MS analysis. For the first sample, ten amino acid standards were diluted into 1 mL of 200 mM formic acid. The final concentrations of each amino acid in solution are listed in Table 3.1. The second sample consisted of 20 amino acid standards diluted to ~20 µM each in 0.04% (v/v) formic acid containing 30% (v/v) acetonitrile (ACN).

Bovine serum albumin (BSA, 0.5 mg/mL) in 100 mM NH4HCO3 (pH 8.0) containing 8 M urea was denatured at 37 °C for 30 min, followed by standard reduction and alkylation with DTT and IAA. After dilution with 100 mM NH4HCO3 (pH 8.0) to reduce the urea concentration below 2 M, protein digestion was performed for 12 h at 37 °C with trypsin at a trypsin/protein ratio of 1/30 (w/w). After acidification, the protein digest was desalted with C18-SepPak column (Waters, Milford, MA), followed by lyophilization with a vacuum concentrator (Thermo Fisher Scientific, Marietta, OH). The dried sample was resuspended in 0.05% (v/v) formic acid to a final concentration of 1.6 mg/mL and stored at -20 °C prior to CZE-ESI-MS and MS/MS analysis.

3.2.3 High Speed CE-MS Analysis of Amino Acids

Uncoated, fused silica capillaries were used for all experiments (150 µm o.d., 20 µm i.d.). The capillaries were conditioned by sequential washes with methanol for 2 minutes, 100 mM HCl for 5 minutes, and 1 M NaOH for 10-15 minutes, with intermediate water
washes. Finally, the capillary was equilibrated with background electrolyte (BGE) for 5-10 minutes.

The first- and third-generations of the electrokinetically-pumped sheath-flow interface were employed to couple CZE to mass spectrometry [16, 24]. Briefly, high voltage is supplied by two Spellman CZE 1000R high-voltage power supplies via platinum electrodes to a custom-built injection block and electrospray emitter. A Sutter puller (P-1000) pulls emitter tips from borosilicate glass capillaries (1 mm o.d., 0.75 mm i.d.) to an opening size of 7-9 µm or 35 µm. Once the separation capillary is inserted into the interface, the ESI emitter and sheath flow tubing are flushed with sheath liquid prior to each run.

The high-speed CZE separations were coupled to an LTQ XL mass spectrometer (Thermo Fisher Scientific), operated in positive ionization mode. Instrument parameters were optimized for rapid data collection, resulting in an acquisition rate of approximately 4-6 Hz. Scan speed was increased by setting the scan range to 50-200 m/z, in addition to activating the "low" mass range setting. The maximum injection time was 30 ms, and microscans was set to 1.

A mixture of amino acids (Table 3.1) was used to optimize both the CE and MS conditions for subsequent experiments. Ion optics were tuned to asparagine (m/z 133) to maximize signal intensity in the m/z region of the analytes of interest.

For the sample containing ten amino acid standards, the first-generation electrokinetically pumped sheath-flow interface [16] was employed. Sample plugs were electrokinetically introduced onto a 30 cm separation capillary at 5 kV for 1 sec. The BGE
was 200 mM formic acid and the sheath liquid was 10 mM formic acid, containing 50% methanol. The spray emitter inner diameter was 7-9 µm. The applied separation voltage and electrospray voltage were 28.2 kV and 1.2 kV, respectively.

For the sample containing twenty amino acid standards, the recently improved third-generation electro-kinetically pumped sheath-flow CE-MS interface [24] was employed to couple CZE to mass spectrometry. A 45 cm separation capillary (150 µm o.d., 20 µm i.d.) with an etched end (~ 40 µm o.d.) was used. The spray emitter opening size was 35 µm. The distance between the etched tip of the separation capillary and the electrospray emitter orifice was ~50 µm. The BGE was 0.5% (v/v) formic acid and the sheath liquid was 0.5% (v/v) formic acid, containing 10% (v/v) methanol. The sample was dissolved in 0.04% (v/v) formic acid containing 30% (v/v) acetonitrile. Sample was injected with 10 psi for 2 seconds. The separation voltage and electrospray voltage were 16 kV and 2 kV, respectively.

The data were imported into MATLAB for analysis.

3.2.4 High Speed CE-MS Analysis of BSA Digest

The second-generation electrokinetically pumped sheath flow interface [21] was used to couple CZE to a Q-Exactive mass spectrometer (Thermo Fisher Scientific) for a high-speed separation of BSA digest. Approximately 5 mm of the separation capillary (28 cm, uncoated, 10 µm i.d./150 µm o.d.) was etched with hydrofluoric acid to an o.d. of ~ 60 µm. The applied separation voltage and electrospray voltage were 29.5 kV and 1.5 kV, respectively. Sample was hydrodynamically introduced to the capillary via 8 psi for 2 sec.
The BGE was 0.5% (v/v) formic acid (0.13 M) in water. The sheath liquid was 10% (v/v) methanol containing 0.1% (v/v) formic acid. The spray emitter opening size was 7-9 µm.

For CZE-ESI-MS analysis, the Q-Exactive mass spectrometer parameters were as follows. The resolution was 35,000 (at m/z 200), AGC target was 1E6, maximum injection time was 60 ms, microscan was 1, and the scan range was m/z 380-1800. For CZE-ESI-MS/MS, a top 5 data dependent acquisition method was used. For the full MS1 scans, the parameters were the same as those mentioned above except the resolution was set to 17,500 (at m/z 200). For tandem spectra acquisition, the five most intense peaks from the MS1 spectrum were sequentially isolated in the quadrupole (isolation window as 2.0 m/z) and further fragmented in the higher energy collisional dissociation (HCD) cell (normalized collision energy of 28%), followed by Orbitrap analysis. The resolution was 17,500 (at m/z 200), AGC target was 1E6, maximum injection time was 60 ms and microscans was 1. The parent ions with charge states higher than +1 and intensity higher than 5.0E+04 were chosen for fragmentation. Dynamic exclusion was set to 1 sec. Peptide match and exclude isotopes were turned on.

Raw MS files were analyzed by MaxQuant [25] version 1.3.0.5. MS/MS spectra were searched by the Andromeda search engine [26] against ipi.BOVIN.v3.68.fasta database containing forward and reverse sequences. MaxQuant analysis included an initial search with a precursor mass tolerance of 20 ppm, main search precursor mass tolerance of 10 ppm and fragment mass tolerance of 20 ppm. The search included the enzyme as trypsin, variable modifications of methionine oxidation, N-terminal acetylation, lysine acetylation and
deamidation (NQ), and fixed modification of carbamidomethyl cysteine. The minimum peptide length was set to seven amino acids and the maximum number of missed cleavages was set to two. The false discovery rate (FDR) was set to 0.01 for both peptide and protein identifications. The proteins identified by identical sets of peptides were grouped, and reported as one protein group.

3.3 Results and Discussion

3.3.1 High Speed CE-MS for Amino Acid Analysis

Figure 3.1 presents a high-speed separation of ten amino acids. The amino acids migrate within a ~50 second separation window, framed by lysine and proline, and a total analysis time of less than 100 seconds. Plate counts and detection limits are presented in Table 3.2. Plate counts range from 50,000 to 85,000 and appear to be dominated by injection volume; an Ohms plot of current vs. applied potential was linear (Figure 3.5), which indicates that the resistance of the solution-filled capillary was constant across the range of 5-29 kV. Joule heating was removed as the source of degrading peak resolution in this experiment.

Concentration detection limits were in the low micromolar range and mass detection limits were in the high attomole to low femtomole range, Table 3.1. The superior mass detection limits reflect the very small injection volumes used in this experiment, which were a few hundred picoliters. This small injection volume is a result of the use of a very narrow inner diameter capillary (20 µm) and the absence of any stacking used in the injection.
Improved concentration detection limits are anticipated from the use of stacking or a pH junction in future experiments, which allow use of larger injection volumes without a significant increase in peak width [27, 28].

A twenty-amino-acid-standards mixture was next analyzed by the CZE-MS system. In order to improve the separation efficiency of the CZE-MS system, we made several major changes. First, the analysis employed the third-generation electro-kinetically pumped sheath-flow interface. This interface includes a large spray emitter opening size and very short distance between separation capillary etched tip and spray emitter orifice [24], which reduces the sample diffusion in the spray emitter. Second, a longer separation capillary (45 cm) and lower separation voltage (14 kV across the separation capillary) were used. Third, the sample solution was 0.04% (v/v) formic acid containing 30% (v/v) acetonitrile, and its conductivity is much lower than the BGE (0.5% (v/v) formic acid), which produces significant sample staking during separation, improving the separation efficiency.

Figure 3.2 shows the extracted ion electropherogram of 20 amino acids analyzed by the improved CZE-MS system. The separation can be completed in around 7 min, which is longer than that in Figure 3.1, but the improved CZE-MS system produced much longer separation window (~3 min vs. ~1 min) and much higher number of theoretical plates (on average >200, 000 vs. ~60, 000), Table 3.3. It is important to note that almost-baseline resolution of structural isomers, leucine and isoleucine, was obtained with the improved system, Figure 3.3, demonstrating the high separation efficiency.
In terms of the stability of the CZE-MS system, the main concern is the electrospray emitter. The first and second-generation of electro-kinetically pumped sheath-flow interfaces [16, 21] employ spray emitters with \(~8\,\mu\text{m}\) opening size, which are susceptible to plugging. The lifetime of the \(8\,\mu\text{m}\) spray emitter is typically one day. The third-generation interface [24] employs much larger spray emitter (up to \(~35\,\mu\text{m}\) opening size), which produces a much longer lifetime. In previous work, the third-generation interface-based automated CZE-MS system generated over 5,000 min continuous analysis of BSA digest with good reproducibility, suggesting the good stability and reproducibility of the system [24].

3.3.2 High Speed CE-MS for BSA Digests

The second-generation electrokinetically pumped sheath flow interface [21] based CZE-MS system was evaluated for an ultrafast separation of a BSA digest. 1000 V/cm was applied to a short separation capillary (28 cm) with a small inner diameter (10 µm) and was coupled to a Q-Exactive mass spectrometer with maximum acquisition speed of 12 Hz (at 17,500 resolution).

Figure 3.4 shows the base peak electropherogram of a BSA digest analyzed by the CZE-ESI-MS system in triplicate, demonstrating system stability and reproducibility for multiple runs. It needs to be noted that the peptide intensity in the third run is significantly lower than the first and second runs, which is most likely due to the tandem spectra acquisition performed in the third run. The separation requires less than two minutes and is also reasonably efficient; extracted ion peaks of five peptides produced efficiencies between
50,000 and 100,000 plates, Figure 3.6. Peak widths, estimated as the standard deviation of a
Gaussian function fit to the peak, averaged roughly 700 ms, which corresponds to a peak
capacity of roughly 40 across the one-minute separation window.

CZE-MS/MS analysis of the BSA digest generated 31 BSA peptide identifications
Corresponding to 52% sequence coverage from 2 fmole of BSA digest. The result suggests
that the ultrafast separation and detection system will be valuable for high-throughput
Protein digests analysis. Around 300 tandem spectra were acquired from a single two-minute
run of BSA digest, and 85 tandem spectra were matched to peptides. The number of
Matched tandem spectra and peptide identifications can be improved after further
Optimizations of the mass spectrometer parameters.

It is interesting to compare the present results with the recent work from Moini and
Martinez [14]. The present work employs an electrokinetically pumped sheath flow interface
[16, 21, 24] to couple the CZE separation to the mass spectrometer. This interface is
Compatible with a wide range of BGEs because the sheath liquid, rather than the BGE,
supports electrospray, which gives great flexibility. In contrast, Moini and Martinez
Employed a sheathless CE-MS interface [14] where the BGE must also support electrospray,
which limits the choices of the BGE.

Additionally, the distal end of the capillary in the present interface was etched to a
20-μm thickness, allowing the capillary to remain quite robust. In contrast, the porous
electrospray emitter used by Moini and Martinez was etched to a wall thickness of 2 μm or
less [14], which makes it very fragile.
Finally, the present high-speed separation took twice as long (2 min vs. -1 min), due to the use of a longer capillary, and employed a larger i.d. capillary (10 µm vs. ≤ 5 µm), which allows a four-fold larger injection volume and proportional improvement in the concentration detection limit. The 10 µm i.d. capillary improves the system robustness over a ≤5 µm i.d. capillary because it is much less likely to become clogged over time.

3.4 Conclusions

The results demonstrate that CZE with an electrokinetically-pumped nanospray interface is capable of generating very rapid separations. However, mass spectrometer speed and sensitivity are crucial for detection. The Q-Exactive was running twice as fast as the LTQ XL (12 Hz vs. 4-6 Hz), which allowed for faster separations upstream. In addition, the sensitivity on the Q-Exactive permitted smaller injections onto a narrower separation capillary (10 µm i.d. for BSA digests, 20 µm i.d. for amino acids), which reduced peak tailing and overlap. The narrower capillary i.d. also allowed use of higher separation voltages, and consequently increased separation speed, without excessive Joule heating.
3.5 References


Figure 3.1: Separation of ten amino acids. Peaks labeled by a single letter amino acid symbol. Selected ion electropherograms extracted with a resolution of 500. Data were treated with a first order Lowess filter with a span of 10 and a Gaussian kernel.
Figure 3.2: Extracted ion electropherogram of 20 amino acids analyzed by CZE-MS. Migration order (in single letter amino acid code): C, K, R, H, G, A, V, S, N, T, L, W, M, I, Q, E, F, Y, P, D. The electropherograms were treated with a first order LOWESS filter with a span of 10 and a Gaussian kernel.
Figure 3.3: Zoomed extracted ion electropherogram of leucine (L) and isoleucine (I). The electropherograms were treated with a first order Lowess filter with a span of 10 and a Gaussian kernel.
Figure 3.4: Triplicate base peak electropherograms of BSA digest analyzed by high-speed CZE-ESI-MS. BSA digest loading amount was 2 femtomoles for each run.
Figure 3.5: Ohm’s Law Plot. Capillary dimensions were 30 cm long, 150 µm outer diameter, 50 µm inner diameter.
Figure 3.6: Extracted ion chromatograms of 5 peptides from the BSA digest. Peptides A-E m/z values are 495.78268, 732.29779, 409.71646, 820.47290 and 862.92096, respectively. Plate counts range from 50,000 to 100,000. Peak widths average 700 ms, with a peak capacity of 40 across the 1 minute separation window.
TABLE 3.1:

FINAL CONCENTRATIONS OF AMINO ACIDS IN SAMPLE SOLUTION

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycine</td>
<td>150</td>
</tr>
<tr>
<td>Alanine</td>
<td>200</td>
</tr>
<tr>
<td>Serine</td>
<td>135</td>
</tr>
<tr>
<td>Proline</td>
<td>130</td>
</tr>
<tr>
<td>Threonine</td>
<td>150</td>
</tr>
<tr>
<td>Asparagine</td>
<td>125</td>
</tr>
<tr>
<td>Lysine</td>
<td>110</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>100</td>
</tr>
<tr>
<td>Histidine</td>
<td>115</td>
</tr>
<tr>
<td>Arginine</td>
<td>95</td>
</tr>
</tbody>
</table>
### TABLE 3.2:

CHARACTERISTICS OF HIGH SPEED AMINO ACID PEAKS

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Peak width (s)</th>
<th>S/N</th>
<th>Plate count</th>
<th>LOD (µM)</th>
<th>LOD (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>0.3</td>
<td>70</td>
<td>60000</td>
<td>6.1 ± 1.5</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Ala</td>
<td>0.3</td>
<td>110</td>
<td>84000</td>
<td>5.6 ± 0.5</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Ser</td>
<td>0.3</td>
<td>30</td>
<td>59000</td>
<td>15 ± 3</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>Pro</td>
<td>0.4</td>
<td>110</td>
<td>85000</td>
<td>3.5 ± 0.6</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Thr</td>
<td>0.4</td>
<td>100</td>
<td>51000</td>
<td>4.3 ± 0.2</td>
<td>0.92 ± 0.05</td>
</tr>
<tr>
<td>Asn</td>
<td>0.5</td>
<td>40</td>
<td>59000</td>
<td>9 ± 1</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Lys</td>
<td>0.2</td>
<td>180</td>
<td>61000</td>
<td>1.9 ± 0.2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Glu</td>
<td>0.4</td>
<td>90</td>
<td>51000</td>
<td>3.5 ± 0.7</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>His</td>
<td>0.2</td>
<td>440</td>
<td>62000</td>
<td>0.9 ± 0.3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>Arg</td>
<td>0.2</td>
<td>290</td>
<td>58000</td>
<td>1.0 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

Average peak widths were extracted from the selection ion electropherograms of Figure 3.1. Data were fit with the Gaussian function $\text{Signal}(t) = A \exp(-0.5 \times (t- t0)/\sigma^2)$, where $t$ is time, $t0$ is the peak’s migration time, $A$ is peak amplitude, and $\sigma$ is the peak width expressed as the standard deviation of the Gaussian function. Limits of detection are presented as the average ± one standard deviation determined from three successive runs. Peak width is expressed as $\sigma$; to convert to full width at half height, this value should be multiplied by 2.35. Detection limits correspond to that amount of analyte that generates a signal three times above the standard deviation of the baseline.
TABLE 3.3:
CHARACTERISTICS OF AMINO ACID PEAKS WITH IMPROVED CE-MS SYSTEM

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Plate count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cysteine</td>
<td>140000</td>
</tr>
<tr>
<td>Lysine</td>
<td>300000</td>
</tr>
<tr>
<td>Arginine</td>
<td>310000</td>
</tr>
<tr>
<td>Histidine</td>
<td>260000</td>
</tr>
<tr>
<td>Glycine</td>
<td>260000</td>
</tr>
<tr>
<td>Alanine</td>
<td>170000</td>
</tr>
<tr>
<td>Valine</td>
<td>220000</td>
</tr>
<tr>
<td>Serine</td>
<td>240000</td>
</tr>
<tr>
<td>Asparagine</td>
<td>50000</td>
</tr>
<tr>
<td>Threonine</td>
<td>180000</td>
</tr>
<tr>
<td>Leucine</td>
<td>170000</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>280000</td>
</tr>
<tr>
<td>Methionine</td>
<td>360000</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>150000</td>
</tr>
<tr>
<td>Glutamine</td>
<td>150000</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>210000</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>240000</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>230000</td>
</tr>
<tr>
<td>Proline</td>
<td>230000</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>200000</td>
</tr>
</tbody>
</table>
CHAPTER 4:
CAPILLARY ELECTROPHORESIS COUPLED TO NEGATIVE MODE ESI-MS BY
AN ELECTROKINETICALLY PUMPED SHEATH FLOW INTERFACE

4.1 Introduction

Electrospray ionization (ESI) has been crucial to the analysis of biomolecules since its introduction as an ionization technique for mass spectrometry (MS). [1,2] Coupling high resolution separation techniques such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CZE) to mass spectrometry through an ESI interface allows for the analysis of highly complex mixtures frequently encountered in proteomics and metabolomics. Recently, CZE coupled by electrospray to tandem mass spectrometry has received attention as an alternative to nano-LCMS for the analysis of biological samples. [3-6] The electrospray interface is key to the performance of the system; it must control the potential at the distal end of the separation capillary while simultaneously driving electrospray.

There are at least three classes of capillary electrophoresis-electrospray interfaces. A commercial interface from Agilent resembles a conventional HPLC electrospray interface. [7] It uses a mechanically pumped sheath liquid and nebulizing gas. The sheath liquid provides electrical connection to the separation capillary while supporting electrospray. This interface
should be very robust, but can suffer from high dilution due to use of relatively high sheath flow rates.

A second interface was reported by Moini and commercialized by Beckman. [8] This interface eliminates a sheath liquid by making electrical contact to the interior of the separation capillary through a small portion of the capillary wall itself. The distal tip of the capillary is etched to create a very thin wall with sufficient conductivity to drive electrophoresis. The outer surface of the etched capillary wall is in contact with an electrolyte that is held at the electrospray voltage. Acidic electrolytes containing organic solvents, such as methanol or acetonitrile, are often used and occasionally, a low pressure is applied to the proximal end of the capillary to pump electrolyte through the capillary during electrophoresis. This approach eliminates dilution due to the use of a sheath fluid, however manufacture of the capillaries appears to be challenging, and frequent replacement of the capillaries can be expensive.

We reported a sheath flow interface for CZE-MS that operates in the nanoflow regime. [9-10] The interface uses a glass emitter that is filled with an electrolyte. The silanol groups on the interior of the emitter carry a charge and under an applied electric field, electroosmosis acts as a very stable pump in the nL/min regime. The isoelectric point of silanol groups, pI, is less than 2, and under most conditions, the silanol groups are deprotonated, generating anionic groups on the glass surface and a cloud of cations in solution near the glass wall. Application of an electric field propels the cations from the positive to the negative electrode at low nL/min rates. In this flow regime, analyte dilution is
negligible as it exits the distal end of the capillary. Addition of organics, such as methanol or acetonitrile, to the sheath liquid is a convenient means of stabilizing the electrospray without the need to modify the separation electrolyte.

Modern proteomic workflows generally perform electrospray in the positive ionization mode, and our interface has seen rapid optimization for use in positive ionization mode. [11-12] However, many analyte classes ionize better and have more publicized fragmentation data in negative ionization mode. For example, Jayo demonstrates a CEMS experiment using negative mode ESI that allows for the intact characterization of glycans without the need to derivatize or desialylate before analysis. [13] Capillary electrophoresis is also ideal for separating the small, polar molecules that are frequent components of the metabolome. [14] Adapting our CEMS interface to allow stable operation in negative mode extends the usefulness of the technology and allows for the sensitive analysis of a wider range of compounds.

We have previously reported operation of the described interface in negative ionization mode under select circumstances. [10] Applying negative voltages to an electrospray emitter produces corona discharge at lower magnitudes than in positive mode. To compensate for this, organics are added at higher concentration than positive mode to the sprayed solution which reduces surface tension and increases the corona discharge onset potential. In our interface, the emitter solution is replenished by electroosmotic flow; operating the interface in negative ionization mode drives spray solution away from the emitter opening. Capillary action acts to replenish sheath liquid, but capillary action results
in weak, unstable flow. Stable negative mode electrospray was demonstrated only when the separation capillary was coated to reduce forward electroosmotic flow and reduce dilution of the spray liquid. Operating the interface with uncoated capillary rapidly results in discharge onset and destruction of the electrospray emitter due to dilution of the sheath liquid, regardless of the sheath liquid composition. Methanol can be added to the background electrolyte (BGE) to prevent discharge, but this results in unstable electrospray, as shown in Figure 4.1. In addition, methanol as an electrophoresis additive drastically changes the separation characteristics and conductivity of the BGE.

We present an alternative to the previously mentioned workarounds. By modifying the chemistry of the emitter, we can manipulate the electroosmotic flow in the emitter and generate stable negative mode electrospray without modifying the BGE or separation capillary. Sensitive analysis in negative ionization mode utilizing already published BGEs and relatively inexpensive uncoated capillaries is possible when using the modified emitters.

4.2 Materials and Methods

4.2.1 Reagents and Materials

Unless otherwise stated, reagents and standards were obtained from Sigma-Aldrich (St. Louis, USA). All solvents used in the analysis were HPLC and MS grade. Borosilicate glass capillary (1000 OD/ 750 ID) was from Sutter Instrument Company (Novato USA). Separation capillaries were obtained from Polymicro (Phoenix USA). PEEK sleeves and fittings were purchased from IDEX Corporation (Lake Forest, IL, USA).
4.2.2 Preparation of Coated Emitters

The coating process consisted of three steps. First, the borosilicate emitter capillary was pretreated by flushing in series with 0.1 M NaOH for 30 minutes, water until the outflow reached pH 7.0, 0.1 M HCl for 60 minutes, water again until the pH reached 7.0, and finally methanol. The emitter capillary was dried under a nitrogen stream at room temperature prior to coating. The second step of the coating process was to then fill the emitter capillary with a 50% (v/v) solution of 3-aminopropyltrimethoxysilane. Both ends of the emitter capillary were sealed, and the capillary was placed in a water bath at 45°C for 12 hours. The final step was to thoroughly rinse the emitter capillary with methanol then dry under a room temperature nitrogen stream.

The emitter capillary was then pulled into two tapered-tip emitters by a Sutter P-1000 micropipette puller using the following parameters: heat setting is 475, pull setting is 0, velocity setting is 20, delay is 250, pressure is 550, delay mode is yes, safe heat is yes, and ramp is 490. The ramp parameter was tuned before each pulling session using the ramp test feature. These settings pulled tips with an exit orifice diameter between 15 and 20 µm. The size of the emitter opening was measured with an optical microscope. Note that the tip puller heated the distal end of the emitter capillary, destroying that portion of the coating. However, the majority of the coating on the emitter is not damaged by this heating, and supports sufficient EOF to generate stable electrospray in negative ion mode.

An emitter tip was inserted into an appropriately sized PEEK sleeve with a nut and ferrule, which was screwed into a 4-way PEEK union. Opposite the emitter, the separation
capillary (150 µm OD, 20 µm ID, 35 cm length) was inserted into another PEEK sleeve with a nut and ferrule and screwed into the union. The separation capillary can then be threaded through the union and into the emitter. The other two openings of the union are used to attach a syringe attachment for flushing and to connect a tube leading to the sheath reservoir that is connected to a high-voltage power supply. The emitter apparatus is depicted in Figure 4.2.

4.2.3 Amino Acid Calibration Curve

Five amino acids were selected to generate a calibration curve to demonstrate the stability and sensitivity of the interface. The five amino acids were selected based on properties such as isoelectric point and hydrophobicity to be representative of all the amino acids. Stocks were made of each amino acid in MS grade water, then diluted to running concentration in BGE. Concentrations of the initial mixture of standards are listed in Table 4.1. The BGE was 1 M acetic acid and the electrospray solution was 10 mM ammonium acetate in 75% methanol. pH of the spray solution was 6.2. Serial dilutions of the standard mixture were made at 5x, 25x and 250x to generate a calibration curve. Samples were run in triplicate on a Q-Exactive HF mass spectrometer (Thermo Scientific, Waltham, MA USA). Resolution was set to 30,000, AGC target was 3E6, and the m/z scan window was set to be 90 – 500. Sample was introduced onto the capillary by pressure injection at 10 psi for 1 second, resulting in an injection volume of 1 nL. Separation was performed at 26.5 kV (750 V/cm) and electrospray was performed at -1.5 kV. Thermo RAW files were converted to
mzXML by MSconvert. [14] The data were analyzed in MATLAB (Mathworks Inc., Natick, MA, USA). Electropherograms were extracted with 5 ppm tolerance of the target mass. They were then subjected to Lowess filtering, Gaussian convolution and baseline adjustment. The maximum intensity of the peak corresponding to each standard was used to construct the calibration curve.

4.3 Results and Discussion

Spray solution composition and electrospray emitter opening size are important parameters when performing negative mode electrospray ionization. The discharge onset potential in negative ionization mode is much lower than that in positive ionization mode for the same emitter orifice size. Without appropriate modifications, corona discharge rapidly destroys borosilicate emitters by fusing the tip. Increasing methanol content aids in the production of a stable electrospray by reducing the surface tension of the sheath liquid and reducing the electrospray onset potential. Corona discharge was observed before a stable electrospray could be achieved at any voltage when the sheath liquid contained less than 50 percent by volume methanol. Electrospray emitters with larger than a 25 micron opening also discharged easily, with the stable electrospray onset potential being very close to the corona discharge onset potential.

The pH of the sheath liquid determines the EOF in the emitter. A sheath liquid consisting of 10 mM ammonium acetate in a 3:1 methanol:water mixture in a coated borosilicate emitter with a 15 – 20 micron opening produces stable negative mode
electrospray at applied potentials between -1.0 kV and -1.75 kV, and discharge above -1.75 kV. Ammonium acetate solutions usually have pH around 6.2 when freshly made, and ensuring the pH of the spray solution is around 6 is important to maximize charge availability for negative mode ionization while not damaging the emitter coating by exposure to basic pH. When the emitter is uncoated, BGEs without methanol will dilute the methanol content in the electrospray interface and cause discharge in negative mode. Addition of methanol is not necessary when the coating is applied to the electrospray emitter. As a result, 1 M acetic acid was chosen as the BGE for all experiments. Because the coating generates EOF toward the opening of the emitter, spray liquid is not diluted to the same extent as in uncoated emitters and stable electrospray can be maintained without modifying the BGE.

In the production of the coated emitter, the tube was first treated with aminopropyltrimethoxysilane. This pre-coated tube was then pulled to a ~20-µm inner diameter orifice. Figure 4.3 presents a schematic of the emitter. The conical, pulled portion of the emitter is roughly 2-mm long. This portion of the emitter is heated during pulling, which destroys the aminopropyltrimethoxysilane coating. This portion of the emitter will generate electroosmosis that is directed away from the tip. However, >90% of the emitter’s interior surface is not heated during the pulling process, so the unheated aminopropyltrimethoxysilane coating will survive the pulling process, and will generate sufficient electroosmosis towards the emitter orifice. Production of stable electrospray even when the BGE does not contain methanol is evidence that sufficient EOF is generated to replenish spray solution in the emitter tip.
The calibration curves for the five amino acid standards are presented in Figure 4.4. Sensitivity for the amino acids varied based on isoelectric point, with the exception of serine. Aspartic acid, the most acidic amino acid in the standard mixture produced the highest intensity for an equivalent amount injected, while arginine produced the least, besides serine. Intensities for leucine and arginine were linear across three orders of magnitude, while nonlinearity was observed in the 250x dilution for the other three amino acids, and those points were dropped from the calibration. The calibration for arginine and serine remained linear down to 330 and 590 attomoles injected, respectively. The other calibrations remained linear down to 3 to 7 femtomoles injected. Table 4.2 lists the limits of detection for each amino acid. LODs were between 150 and 900 attomoles injected onto the capillary. Nemkov separated and detected amino acids using HPLC and a Thermo Q-Exactive instrument, producing limits of detection for the same amino acids between 1 femtomole and 2.5 picomoles injected in a three-minute separation. [16] Our CE-MS based method improves upon this UPLC-based method with mass LODs one to three orders of magnitude better in a similar separation time. Our CE-MS method has the added benefit of using relatively inexpensive uncoated capillary.

An extracted ion electropherogram for each of the five standards is presented in Figure 4.5. Separation of all the standards is complete in five minutes. Each of the electropherograms demonstrates good peak shape with little or no tailing. Peak widths (FWHM) are between two and five seconds with plate counts between 15,000 and 20,000. Our electrospray interface operating in positive mode produced similar results. [15] The
good peak shape and reproducibility of the analysis demonstrates the stability of the interface when a coated emitter is used.

4.4 Conclusions

Coating ESI emitters with aminopropyltrimethoxysilane reverses the direction of EOF in the emitter and allows for the construction of an electrokinetically driven sheath flow negative polarity ESI interface for CZE-MS. This interface has been successfully applied for the analysis of amino acid standards. The stability and sensitivity of the electrokinetically driven interface for negative mode ESI was demonstrated across three orders of magnitude, and limits of detection were between 150 and 900 attomoles injected for a five-minute separation, a significant improvement over comparable HPLC methods. Forward EOF is maintained in the electrospray emitters allowing the use of BGEs that do not contain organic additives. The electrokinetically driven sheath flow interface for CZE coupled to negative mode ESI allows for independent optimization of BGEs and electrospray solutions without the decrease in sensitivity caused by pumping electrospray solution at high flow rates in traditional mechanically pumped sheath flow interfaces.
4.5 References

Figure 4.1: Base peak electropherogram of negative mode electrospray from an uncoated borosilicate glass emitter filled with an acidic spray liquid to minimize reverse EOF. The BGE contained methanol to prevent immediate discharge. Electrospray is very unstable even with minimized EOF, with rapid oscillation in spray intensity evident. The electrospray does not remain stable enough in this configuration to perform sample analysis.
Figure 4.2: Diagram of CE-MS interface for negative mode ESI. (A) Separation capillary is filled with BGE and threaded through a sleeve into a 4-way PEEK union (B). (C) An amino-coated borosilicate glass emitter is fitted into a sleeve and attached to the 4-way union, and the separation capillary is threaded into the emitter tip. (D) Electrospray voltage is applied to spray solution in a vial. The electrospray voltage also drives EOF in the emitter toward the opening of the emitter and inlet of the mass spectrometer. (E) A syringe containing spray solution is attached to the final opening of the 4-way union to replenish spray solution in the vial and remove air bubbles.
Figure 4.3: Schematic of the borosilicate glass emitter. The cylindrical portion of the emitter remains long relative to the tapered tip to minimize exposure of the coating to heat during the pulling process. By maintaining these dimensions, detrimental effects on EOF in the emitter can be minimized. The ideal size of the emitter opening is 15 to 20 µm.
Figure 4.4: Calibration of five amino acid standards. Calibration is linear for two of the standards across nearly three orders of magnitude. The lowest injection amounts were between 300 and 500 attomoles injected.
Figure 4.5: Extracted ion electropherograms of the five amino acid standards from the 5x dilution. Peak widths (FWHM) were between two and five seconds. Theoretical plate counts were between 15,000 and 20,000.
TABLE 4.1:

CONCENTRATIONS OF INITIAL AMINO ACID MIXTURE

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Initial Concentration (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>190</td>
</tr>
<tr>
<td>Arginine</td>
<td>86.1</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>37.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>152</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>73.5</td>
</tr>
</tbody>
</table>

Concentrations of the initial standard mixture from which all serial dilutions were made. The mixture was run at 1x, 5x, 25x and 250x dilutions to construct the calibration curve.
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>LOD (attomoles injected)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>820 ± 140</td>
</tr>
<tr>
<td>Arginine</td>
<td>190 ± 60</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>870 ± 110</td>
</tr>
<tr>
<td>Leucine</td>
<td>370 ± 60</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>190 ± 50</td>
</tr>
</tbody>
</table>

Values are reported as attomoles injected onto the capillary. LODs were calculated by measuring signal to noise ratio for each injection performed to construct the calibration curve. LODs were averaged across all injections and concentrations. Error values represent the 95% confidence intervals calculated through the standard deviation.
CHAPTER 5:

ANALYSIS OF THE *XENOPUS LAEVIS* EMBRYONIC METABOLOME BY NEGATIVE MODE CE-ESI-MS

5.1 Introduction

*Xenopus laevis* is an essential model organism for the study of early vertebrate development, and has been the main species of study in a number of landmark experiments, including the first nuclear transfer to induce totipotency, the first gene isolation, first total gene nucleotide sequence reported and the first purification of a eukaryotic gene transcription factor. [1-4] *Xenopus* is a particularly useful model system because the embryos develop outside of the mother, and develop synchronously when fertilized *in vitro*. The eggs, and embryos are relatively large, allowing for easy manipulation, and large sample amounts for analysis. [5] Because of its long history as a model organism, rich information already exists about the development of *Xenopus* at the genome and transcriptome level. Recently, work has been published describing changes in the proteome of developing *Xenopus* embryos. [6] L. Sun *et al.* note a dramatic change in the expression of a number of DNA replication factors which occurs during the mid-blastula transition, when the *Xenopus* embryo begins translating its own mRNA into protein, rather than its mother’s. However, the authors also note no significant heterogeneity in the proteome of individual embryos at the same stage of
development from the same mother. [5] However, Vastag et al. note that different female frogs lay eggs with different initial concentrations of metabolites [7], and Onjiko et al. report that differentiation of cells based on metabolite content can be observed at the 16-cell stage [8]. These results suggest that much information can be learned from the study of the metabolome of early *Xenopus* embryos.

Developing embryos consist of rapidly dividing cells that consume a considerable amount of building materials and energy to construct the new cells. Prior to the midblastula transition at stage 13, these building materials come primarily from the mother of the embryo, and Vastag hypothesizes that it is the depletion of dNTPs in the embryo that triggers the midblastula transition. [7] Vastag also finds that alanine is a major source of energy for the early embryo and is consumed during early stage development. Tracking the flux of small molecules during the initial stages of development will provide new insight to the process by which embryo state is communicated to the cell cycle machinery to trigger the midblastula transition.

Vastag detects and quantifies 48 metabolites by LC-MS using negative mode ESI, while Onjiko detects 80 metabolites and quantifies 40 of them in single blastomere cells using CE-MS with positive mode ESI. [7-8] This chapter of my thesis reports the first analysis of early stage *Xenopus* embryo metabolites by CE-MS coupled to an electrokinetically pumped sheath-flow electrospray interface operating in the negative mode. Because small, polar, acidic compounds ionize more readily in negative mode ESI, joining CE-MS by a negative mode ESI will broaden the data set available for metabolomic analysis
when compared to positive mode ESI alone. In addition, to adequately describe heterogeneity in single cells of *Xenopus* embryos, many separation runs will need to be performed, placing an emphasis on separation run time.

5.2 Methods

5.2.1 Materials and Reagents

Acetic acid, 3-aminopropyltrimethoxysilane and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO). Formic acid (FA) and acetonitrile (ACN) were purchased from Fisher Scientific (Pittsburgh, PA). Methanol was purchased from Honeywell Burdick & Jackson (Wicklow, Ireland). A Nano Pure system from Thermo Scientific (Waltham, MA) was used to generate deionized water. Uncoated fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ).

5.2.2 Embryo Collection and Metabolite Extraction

*Xenopus* embryos were fertilized and collected according to a previously reported protocol. [5,9] Embryos were collected at stage 1 and stage 8. The embryos were placed in an Eppendorf tube with 55 µL of 2:2:1 acetonitrile:water:methanol and vortexed to liberate small molecule metabolites. The tubes were centrifuged and the supernatant was removed, clarified and flash frozen in liquid nitrogen. Extracts were stored at -80 °C until directly analyzed by CZE-MS.
5.2.3 CE-ESI-MS Interface

The CZE-MS system consists of two high-voltage power supplies coupled to a mass spectrometer through an electrokinetically pumped sheath-flow interface. To operate the electrospray interface in negative ionization mode, the inside of a borosilicate glass capillary was coated with 3-aminopropyltrimethoxysilane, which reverses the charge on the inner wall, and reverses the direction of EOF, creating stable electrospray in the nanoflow regime. The background electrolyte (BGE) was 1 M acetic acid in water, and the electrospray solution was 10 mM ammonium acetate in 75% methanol. The pH of the spray solution was 6.2.

Samples were run in triplicate on a Q-Exactive HF mass spectrometer (Thermo Scientific, Waltham, MA USA). Resolution was set to 30,000 (m/z = 200), AGC target was 3E6, and the m/z scan window was set to be 90 – 500. Sample was introduced onto the capillary by pressure injection at 10 psi for 1 second, resulting in an injection volume of 1 nL. Separation was performed at 26.5 kV (750 V/cm) and electrospray was performed at -1.5 kV.

5.2.4 Data analysis

Thermo RAW files were converted to mzXML by MSconvert. [10] The data were subjected to pairwise statistical analysis (Student’s t-test) using the XCMS software package. [11] A p-value of less than 0.05 indicated a statistically significant change in metabolite concentration. Putative identifications were generated by matching intact accurate mass to the METLIN database. [12] Adducts of formic acid, sodium, potassium and chloride, and dehydration decomposition products were considered for database matching.
5.3 Results and Discussion

XCMS identified 288 features in common between stage 1 and stage 8 embryos. Of these, 34 had quantifiable differential expression and putative IDs based on MS1 mass have been generated for 14 of them. Figure 5.1 displays box and whisker plots for the four features with the lowest p-value, indicating the highest confidence of a statistically significant difference. In addition, all 20 of the common amino acids were detected in each of the samples. Figure 5.2 is an extracted ion chromatogram for all 18 of the 20 common, proteogenic amino acids. Valine and glycine were smaller than the mass detection window used, and were not detected. In under 10 minutes, CZE resolved leucine and isoleucine to near baseline, and theoretical plate counts were between 40,000 and 80,000.

Glucosamine depletion is observed as the cell consumes it as a source of energy for cell division. A number of changes are observed for metabolites in the amino acid biosynthesis pathway. 3-methylglutaric acid and homocysteine are consumed to generate energy for the early embryo and synthesize the necessary amino acids to contribute to protein synthesis. A number of other small molecule precursors and products of amino acid synthesis are present in higher concentration in stage 8 embryos compared to stage 1 embryos. Table 5.1 lists significantly changing features from stage 1 to stage 8 embryos along with their putative ID and metabolic pathway. It is important to note that the putative ID is based on intact mass alone, which produces IDs with relatively low confidence. ID information can be drastically improved by generating a database of known metabolites that contains migration time information and tandem mass spectra.
5.4 Conclusions

Generating identifications from metabolome data remains the largest challenge to metabolome analysis. Of the 34 features identified by XCMS as differentially expressed, putative IDs were generated for only 14 of them. Tandem spectra database information for these metabolites, and many others, is sparse. CZE-MS operating in both positive mode ESI and negative mode ESI provides a rapid separation technology for generating database information for metabolites and performing subsequent analysis on *Xenopus* embryos. This work has further described the flux of amino acids and their metabolites in early embryo development. Amino acids and their metabolites play a crucial role in providing both energy and building blocks to the early developing embryo.
5.5 References


Figure 5.1: Box and whisker plots for the four most statistically significant features. (A) diaminoheptanedioate, (B) glucosamine, (C) methylgluatric acid, (D) dihydroxyandrostenone sulfate. P-values for each of the four features were less than 0.005.
Figure 5.2: Extracted ion electropherogram for 18 of the 20 amino acids. Glycine and valine are smaller than the mass window used and were not detected. Intensities are normalized to the maximum intensity for each extracted electropherogram. The electropherograms were treated with a first order Lowess filter with a span of 10 and a Gaussian kernel.
<table>
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<th>Identity</th>
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CHAPTER 6:

A COMPARISON OF ALTERNATING CURRENT AND DIRECT CURRENT ELECTROSPRAY IONIZATION FOR MASS SPECTROMETRY

6.1 Introduction

Since its development, conventional direct current electrospray ionization (DC ESI) has been coupled to mass spectrometry as a mainstay technique for the analysis of a wide variety of biomolecules. Its relative softness compared to fast atom bombardment (FAB) and electron impact (EI) ionization, as well as its ability to ionize very large biomolecules, have made it the ionization method of choice for modern proteomic studies alongside matrix-assisted laser desorption ionization (MALDI)[1,2]. A new method of electrospray ionization based on alternating current (AC ESI) has recently been described [3,4], and initial results showed that it produced up to an order of magnitude increase in signal intensity over conventional DC ESI for intact protein analytes under certain spray conditions. However, a detailed study and analysis of the operational voltage range of AC ESI as compared to DC ESI has not yet been conducted, nor a realistic assessment of the operating conditions when
AC ESI outperforms or does not outperform DC ESI. In this work, we explore a wide voltage operating space and the role of the nebulizing gas to determine the relative merits of AC ESI.

In AC ESI, a sinusoidal potential at a frequency of approximately 80 - 400 kHz is applied directly to a stainless steel electrospray emitter through which the analyte solution is pumped, while the mass spectrometer inlet is grounded. Cone formation for AC ESI is significantly different from that for DC ESI, as confirmed by both experiment and theory [5,6], due to a mechanism termed “preferential entrainment.” This process can best be understood by considering the two half cycles separately during the AC ESI of an acidic protein solution. During the half cycle in which the spray emitter is at negative positive potential, more electrophoretically-mobile protons and less-mobile acidified proteins are attracted toward the tip of the spray cone. When the polarity is reversed, the more mobile protons can be driven back into the bulk solution by the electric field. If the cycle duration is relatively short (i.e. at high frequencies), there is insufficient time for the more massive proteins to relax away from the high electric field at the spray tip before the polarity is again reversed. Both sets of species are forced toward the cone tip by the bulk solution flow. In this way, bulky analyte molecules are entrained near the spray cone meniscus where the electric field is high and the proteins become ionized. After many cycles, repulsion forces between accumulated protein ions expel them in a narrow spray axial to the cone. The result is that the AC ESI cone is much narrower than the DC ESI cone with a half angle of approximately 12° for AC ESI [5,6] compared to 49° for DC ESI [7,8]. The preferential entrainment of
analyte ions near the cone meniscus and narrower spray cone shape are thought to improve sensitivity during AC versus DC ESI. Though the spray droplets emitted from an AC ESI cone are larger and carry less charge than those from DC ESI, the combination of preferential entrainment and the axial emission of droplets is presumed to be the main cause of increased sensitivity [3].

Previous work had focused on comparing optimized spray conditions for AC ESI to equivalent conditions in DC ESI [3]. Among a number of other parameters, this entailed application of a DC potential that was equal to the root-mean-square (RMS) of the applied alternating potential ($V_{DC} = V_{AC,RMS}$). It was under these equivalent conditions that the reported order-of-magnitude absolute signal intensity improvement was observed. However, the potential range accessible during AC ESI is limited because gas discharges occur at lower potentials under AC conditions than DC [9], and previous studies reported a range from approximately 300 - 1500 VRMS in which signal decreased as the frequency increased [4]. Therefore, prior comparisons employed a DC ESI potential of $V_{DC} \sim 1 - 1.5$ kV, which is significantly lower than typical DC ESI potentials (2 - 3 kV) for emitters at a fixed distance from the mass spectrometer inlet. Thus, while AC ESI showed greater absolute signal intensity than DC ESI at comparable conditions, the limitations of operating AC ESI at higher voltages prevented a comparison of its overall value compared to DC ESI. Recently, contactless methods of applying electrospray potential, including square-wave and AC potentials, have been described. [10-11] These contactless methods reduce gas discharge limitations and allow the application of potentials in excess of 6 kV, and as such, produce
improved sensitivity over DC ESI. In this study, however, an insulating nebulizing gas is used to extend voltage range instead, as this change is easily incorporated into already existing emitter designs.

In this work, we report a comparison of AC ESI and DC ESI under applied voltage and nebulizing gas conditions individually optimized for each technique. In general, AC ESI only produced higher signal intensities at relatively low applied voltages, and they were significantly lower than those observed under DC ESI conditions when both were independently optimized. However, it was found that they produce comparable signal-to-background noise ratios under most conditions examined.

6.2 Experimental

Mass spectra were collected using a linear ion trap mass spectrometer (LTQ-XL, Thermo Fisher Scientific, Waltham, MA) operating in normal mass range mode. Electrospray was conducted by direct infusion at a rate of 500 nL/min via the instrument’s syringe pump through a stainless steel emitter with a 50 μm internal diameter (New Objective, Woburn, MA) mounted axial to the inlet at a distance of 5 mm and electrically isolated from the instrument. Nitrogen (N₂) or sulfur hexafluoride (SF₆) gas was supplied at varying pressures as specified in the results to act as a nebulizing gas in both AC and DC sprays. The mount, emitter, and nebulizing gas setup were identical for both AC and DC spray conditions.
To generate AC sprays, AC potential was applied directly to the steel emitter using a function generator (model 33220A, Agilent, Santa Clara, CA) connected to a radio frequency (RF) amplifier (500A, Industrial Test Equipment Co., Port Washington, NY) and a custom-made transformer (Industrial Test Equipment Co., Port Washington, NY). The applied potential was monitored using a high-voltage probe (P6105, Tektronix Inc., Beaverton, OR) and oscilloscope (PM3375, Koninklijke Philips Electronics N.V., Amsterdam, Netherlands). To generate DC sprays, DC potential was applied directly to the emitter using an external power supply (model ES-5R1.2, Matsusada Precision, Bohemia, NY). In both cases, the mass spectrometer end plate was kept at ground potential (0 V).

In these comparative studies of AC and DC ESI, I used instrument tune mix recommended by Thermo-Fisher consisting of caffeine (20 µg/mL, 100 µM), the tetrapeptide methionine-arginine-phenylalanine-alanine (MRFA, 15 µM, 7.9 µg/mL), and a mixture of fluorinated phosphazines, Ultramark 1621 (0.01%), in an aqueous solution of acetonitrile (50%), methanol (25%), and acetic acid (1%). This solution was used to tune ion optics parameters and to calibrate mass and resolution parameters prior to optimizing spray conditions. To carry out limit-of-detection (LOD) analyses, serial dilution series of caffeine (12.5 – 100 µg/mL) and MRFA (1.25 – 10 µg/mL) were prepared. To each dilution, a fixed amount of the amino acid asparagine (62.7 µM) was added to act as an internal standard. A blank sample was created to which only the internal standard asparagine was added. The dilutions were analyzed in random order and in triplicate.
Ion optic tuning parameters were constant across both ionization methods, and forty scans of the infused calibration mix were collected and averaged for each experiment. The automatic gain control target was set to 30,000 with a maximum inject time of 10 ms. Three microscans were averaged per spectrum. All studies were conducted in positive ion mode.

6.3 Results and Discussion

While prior studies compared AC and DC ESI performances at equivalent conditions that were only optimized for AC ESI [3], in this work the first aim was to individually optimize each spray technique, thus ensuring a more realistic comparison of likely performance. To do so, we compared both the operating range and the nebulizing gas conditions for both spray methods.

Using N₂ at a pressure of 4.1 bar as the nebulizing gas, the frequency of AC ESI was first optimized by varying it over a range of $f = 80 - 200$ kHz and assessing the applied voltage range over which the peak corresponding to the protonated peptide MRFA ($m/z = 524.2$) was observable before a gas discharge occurred. Consistent with prior studies [4], the optimal frequency was found to be $f = 120$ kHz. Stable mass spectra were produced over the range of VAC = 200 - 1000 V, where VAC is defined as the amplitude of the applied AC potential. Figure 6.1 shows a comparison of intensity across the AC voltage range to DC ESI with all other parameters (flow rate, N2 nebulizing gas pressure, and mass spectrometer settings) identical. In this comparison, we specify that the applied potential is equivalent when the DC potential (VDC) is the same as the amplitude of the AC potential (VAC)
whereas earlier studies specified equivalence as matching the RMS of the AC potential. We revise this comparison here because AC ESI can be effectively analyzed based on a quasi-static half cycle [5], suggesting that the ionization is strongly tied to the peak electric field that corresponds to the voltage amplitude in the half-cycle. The absolute signal intensity of MRFA (m/z = 524.2) for both AC and DC ESI increased with increasing voltage, and AC ESI produced a similar spectral intensity to DC ESI at equivalent applied potentials in the range of VAC = 200 - 1150 V. Beyond this voltage, the signal intensity for the base peak decreased as the AC voltage was continuously increased until reaching discharge at 1350 V. However, DC ESI did not reach its discharge limit until VDC = 2700 kV was applied to the emitter. Because of the lower discharge limit of AC ESI, DC ESI produced an absolute signal intensity of approximately 50% the intensity of AC ESI at voltages not accessible by AC ESI. For example, the maximum signal intensity at the maximum voltage of VAC = 1150 V was only 2% of that achieved by DC ESI at its maximum operating voltage, VDC = 2700 V. While only results for MRFA (m/z = 524) are discussed here, similar trends are observed for all analytes in the tune mix and further detail is presented in Supplementary Materials (Section 6.6).

A brief study of the application of a DC bias on the applied AC potential was performed utilizing N₂ as a nebulizing gas. DC bias was applied by setting the internal power supply of the mass spectrometer to a positive or negative potential to apply a bias. When a relatively small DC bias was applied, the AC discharge limit was reached at a lower potential equivalent to the applied DC bias, and spectral quality degraded. When a larger DC bias was
applied, very little AC potential could be applied on top without inducing discharge, and the spectra greatly resembled DC ESI spectra with no superimposed AC signal. Stable spray without discharge was difficult to maintain across all tested voltage conditions.

It is well known that electronegative gases can delay the initiation of a gas discharge [9], and for this reason they have been used in negative-ion mode DC ESI to extend analysis capability [1012]. In order to extend the operational voltage range of AC ESI, we used SF₆ as the nebulizing gas. In this way, we were able to extend the applied potential before signal reduction to 1850 V at 120 kHz, an increase in accessible voltage greater than 50% that achievable using N₂ as nebulizing gas. However, as shown in Figure 6.2a, while the operating range was increased, the overall signal intensities for AC ESI were still significantly lower than for DC ESI. For example, at VAC = 1150 V in N₂, the AC ESI peak intensity for the peptide MRFA was $2.2 \times 10^4$, whereas at VAC = 1850 V in SF₆, the AC ESI peak intensity was $3.3 \times 10^4$. Further, both of these were lower than the DC ESI peak intensities at their maximum voltage, which were $1.0 \times 10^6$ at 2600 V in N₂, and $2.1 \times 10^5$ at 3200 V in SF₆.

Figure 6.2b shows the calculated signal-to-background (S/B) ratios for the peak corresponding to MRFA as a function of the operating voltage for AC and DC ESI using both N₂ and SF₆ as nebulizing gasses. Here, we refer to, “signal-to-background” to distinguish the fact that the noise is reduced across the m/z dimension, as opposed to, “signal-to-noise,” which typically describes peak quality in a chromatograph. For the operating range of both methods, the S/B is approximately the same, even though the DC ESI absolute signal intensity is much stronger for the same conditions as shown in Figure
6.1. Importantly, the S/B for AC ESI surpassed that of DC ESI, being 54% greater at its peak value using SF₆ as nebulizing gas (VAC = 1250 V) than that of DC ESI at its peak value in SF₆ (VDC = 3100 V). Another interesting feature of the S/B behavior comparison was a difference in how the maximum values were achieved under the two ionization methods. As the applied potential in DC ESI approached its discharge limit, the S/B appeared to have settled at a maximum value in spite of the fact that the absolute signal intensity was still rapidly increasing. This suggests that applying potentials greater than approximately 1200 V in DC ESI produces more signal, but also produces more background chemical noise, resulting in no net increase in S/B. However, the S/B did not appear to approach a plateau under AC ESI conditions as voltage was increased. Rather, the S/B increased with voltage, then sharply decreased, which may be evidence for the discharge limit. However, this evidence for the discharge regime appears at even lower voltage in the S/B plot than in the absolute intensity plot in Figure 6.1 and Figure 6.2. For example, in the absolute intensity plot, the discharge limit for AC ESI in SF₆ appears to initiate at VAC = 1900 V. In the S/B plot, the ratio starts to rapidly decrease at about VAC = 1300 V. This indicates that AC ESI continues to produce more detectable ions up to 1900 V, but weak discharge or other effects begin to create interfering background ions at earlier voltages.

Despite AC ESI operating over a smaller voltage range and producing less absolute signal intensity, the general quality of AC ESI mass spectra appeared to be superior to those acquired under DC ESI conditions. Figure 6.3a shows intensity-normalized spectra for both AC and DC ESI under optimized voltages utilizing SF₆ as nebulizer. The relative intensity of
the background signal was much smaller using AC ESI, indicating that it produces less noise than its DC counterpart by suppressing background peaks. In order to characterize this effect statistically, cumulative distribution functions for the relative signal intensities under both AC and DC ESI conditions were calculated (Figure 6.3b). For AC ESI, 90% of the relative intensity values in the spectrum were less than 0.14% of the base peak intensity. DC ESI resulted in 90% of the spectral intensity values being less than 0.18% of the base peak intensity. These observations suggest that, with respect to S/B ratios, AC ESI produces spectra similar or superior to those acquired using DC ESI, in spite of the fact that AC ESI produces much lower overall signal intensities. To quantify S/B, signal was separated from background by utilizing an outlier rejection algorithm. In this way, m/z intensity values that fell greater than four standard deviations away from the background mean were rejected. Since intensity values corresponding to background dominate mass spectrometry data, the rejected intensity values are signal and not related to background ion effects, experimental variability, or other uncontrollable signal sources. The standard deviation of the remaining intensity values represents an objective measurement of background in the mass spectrum (see Supplementary Material for details on this calculation).

The similar S/B ratio for AC ESI and DC ESI suggests that, even if the absolute signal intensity of AC ESI is less than that of DC ESI, they should have similar quantitative behavior in terms of the concentration range over which a target signal can be distinguished from background noise. To evaluate this hypothesis, calibration curves were generated for both caffeine and MRFA in AC ESI using both N₂ and SF₆ as nebulizing gasses, where
chromatographic signal-to-noise ratio is plotted as a function of analyte concentration. From these calibration curves, limits of detection were estimated for MRFA and caffeine. As conjectured, AC ESI and DC ESI perform very similarly for MRFA when utilizing N₂ (Table 6.1). Switching to SF₆ significantly improves detection limits for MRFA in both techniques. However, this effect is more prominent in AC ESI resulting in a detection limit half that of DC ESI utilizing SF₆, and nearly an order of magnitude less than that for DC ESI utilizing N₂. For caffeine, however, DC resulted in better limits of detection than AC ESI for both nebulizing gases. Switching to SF₆ did not produce significant improvement in limits of detection for caffeine in either AC ESI or DC ESI. This observation suggests that the performance of each method depends, to some extent, on the properties of the analytes and their individual potentials to ionize.

6.4 Conclusion

In this work, a comparison of AC to DC ESI showed that because of the larger operating voltage range, DC ESI produces spectra with significantly more intense signal than does AC ESI. However, at these voltages, the relative intensity of the background noise is also higher, such that both AC ESI and DC ESI produce spectra with similar signal-to-background ratios. For this reason, although AC ESI does not produce signal intensity with magnitudes as great as those observed during DC ESI, they have similar quantitative capability. For one tested analyte utilizing SF₆ as a nebulizing gas, AC ESI outperforms DC ESI, producing spectra with a limit of detection for the peptide MRFA that is one half that
of DC ESI utilizing SF₆. However, DC ESI outperforms AC ESI for the analysis of caffeine. Therefore, the physical characteristics of the analyte dictate the best ionization method for qualitative and quantitative applications. Further study is needed to determine whether or not there are general classes of compounds for which one technique outperforms the other.
6.5 References


6.6 Supplementary Materials

6.6.1 Signal to Background Calculation Details

One of the traditional figures of merit in mass spectrometry data is a measure of signal to noise in acquired spectra. In such a measurement, a relatively quiet m/z region of the spectrum is selected to be representative of noise in the spectrum. The standard deviation of the intensities in this region ($\sigma_N$) is calculated and used along with the peak intensity ($I_{\text{max}}$) and the mean of the noise region ($\bar{x}_N$) in Equation 6.1 to calculate the signal to noise ratio (SNR).

$$\text{SNR} = \frac{I_{\text{max}} - \bar{x}_N}{\sigma_N} \quad (6.1)$$

This method proves problematic when assessing different ionization methods such as AC and DC ESI because AC ESI and DC ESI appear to produce different noise profiles. A relatively quiet m/z region in AC ESI may be relatively noisy in DC ESI when compared to other regions of the spectrum. These differences make it difficult to establish objectively a single m/z region that is relatively quiet in both techniques and not biased toward one technique over the other. One solution is to pick two different m/z regions, one for each technique, but this too can lead to bias in the calculations for one ionization technique over the other.

To develop a more objective figure of merit to quantify the intensity of analyte peaks compared to that of the background, a more rigorous statistical analysis is utilized. The goal of the method is to generate a quantitative figure of merit that objectively describes mass
spectrometry peaks without user input to select quiet m/z regions of a mass spectrum. This figure of merit is called signal to background ratio (SBR) to distinguish it from the traditional signal to noise ratio (SNR). Background rather than noise also better describes the baseline observed in mass spectrometry, as much of this signal arises from contaminate, solvent clusters and other chemical interferences, not true stochastic noise. To perform this analysis, a simple outlier rejection algorithm is retooled to separate background data from peak data. Since background in a mass spectrum arises because of a large collection of independent random factors including chemical noise, ion shot noise and electronic noise, the central limit theorem suggests that background in a mass spectrum can be roughly approximated as normally distributed. Thus, an intensity threshold can be determined based on the sample standard deviation such that data that fall outside the intensity threshold are likely not to belong to the population of background intensities.

Signal is separated from background by an iterative process utilizing this concept. First the sample mean ($\bar{x}$) and sample standard deviation ($\sigma$) of the intensities in the entire spectrum are calculated. A threshold intensity is calculated using these values according to Equation 6.2.

$$I_{\text{thresh}} = \bar{x} + 4\sigma$$  \hspace{1cm} (6.2)

A 4\sigma threshold rather than the typical 3\sigma threshold is chosen because of the large number (>20,000) of data points in a typical mass spectrum. If background data in a mass spectrum were normally distributed, only one datum in 15,787 would have greater intensity than a 4\sigma threshold due to random variance alone. Data that fall above the 4\sigma threshold can
be reasonably excluded from the background population, as it is statistically unlikely to belong to the background population. After exclusion, a new sample mean and standard deviation are calculated. From these updated values, a new threshold is calculated according to Equation 6.2. Data that fall above the new threshold can be excluded and this process is repeated until no more data can be excluded. After the entire process is performed, the remaining data should reasonably describe the background of the mass spectrum. The sample mean ($\bar{x}_B$) and standard deviation ($\sigma_B$) of the final remaining data are used in place of $\bar{x}_N$ and $\sigma_N$ in Equation 6.1 to calculate SBR (Equation 6.3).

$$SBR = \frac{I_{\text{max}} - \bar{x}_B}{\sigma_B}$$  \hspace{1cm} (6.3)

Figure 6.4 depicts a region of an AC ESI spectrum to which this algorithm has been applied. The data plotted in grey are data excluded from the background population by the described algorithm. These data fall above the $4\sigma$ threshold and correspond to peaks. The data plotted in black do not fall above the final $4\sigma$ threshold and belong to the background population. SBR calculations performed with this algorithm correlate with traditional SNR calculations for AC ESI and DC ESI, but SBR requires no additional user input to select regions of baseline. AC ESI performs better than DC ESI in both SNR and SBR calculations by approximately the same amount.
6.6.2 Signal to Background Comparison of All Analytes

Signal to background plots were created for all analytes in the tune mix, including caffeine and MRFA. A similar trend as reported in the main document is observed for most of the tune mix components. In general, AC ESI at its optimum produces much less signal than DC ESI at its optimum but AC ESI produces spectra with a significantly higher SBR than DC ESI when SF$_6$ is used as a sheath flow nebulizing gas. Table 6.2 summarizes SBR calculations for each analyte in AC ESI and DC ESI under optimized conditions for each technique utilizing both nebulizing gasses.

Using sulfur hexafluoride as a sheath flow gas, AC ESI produces peaks with greater SBR than DC ESI for all components of the tune mix except the smallest component of UltraMark 1621 (m/z=1022). There is a strong trend of increasing performance of AC ESI with increasing mass of UltraMark 1621 components. This may be a result of the preferential entrainment mechanism unique to AC ESI. Larger molecules spend more time in the high electric field at the tip of the spray cone and have better chance of being ionized. Caffeine (m/z = 195) and MRFA (m/z = 524) break this trend, but differences in hydrophobicity or hydrophilicity between these species and the UltraMark series may explain this difference. Switching to sulfur hexafluoride from nitrogen as a nebulizing gas improves AC ESI performance for all analytes except caffeine. Caffeine exhibits a peculiar behavior in AC ESI in the discharge voltage regime, and strong signal corresponding to caffeine can still be observed in AC ESI past the discharge limit observed for all other compounds in the tune mix. This peculiar behavior suggests that the ionization of caffeine may be somehow
improved in the discharge regime of AC ESI. The trend observed in these data suggest that sulfur hexafluoride benefits AC ESI more when larger or less charged ions are analyzed.
Figure 6.1: Intensity and signal to background ratio comparison of AC ESI and DC ESI. (a) Absolute signal intensity of MRFA (m/z=524) as a function of the applied voltage for AC ESI and DC ESI. (b) Signal to background ratio as a function of the applied voltage for AC ESI and DC ESI. For all these experiments, N2 was used as the nebulizing gas at 4.1 bar and the AC ESI frequency was 120 kHz.
Figure 6.2: Intensity and signal to background ratio comparison of AC ESI and DC ESI with N₂ and SF₆. (a) Absolute signal intensity of MRFA (m/z=524) as a function of the applied voltage for AC ESI and DC ESI using both N₂ and SF₆ nebulizing gasses at 4.1 bar. The inset magnifies the region below 2000 V. (b) Signal-to-background ratio of MRFA as a function of the applied voltage for AC ESI and DC ESI. For all AC ESI experiments the frequency was 120 kHz.
Figure 6.3: Sample mass spectrum with AC ESI and DC ESI plus cumulative distribution function. (a) Cumulative distribution function of AC ESI and DC ESI intensity data under optimum conditions for both techniques utilizing SF6 as nebulizing gas. Spectral data were normalized to the intensity of caffeine before analysis. (b) Mass spectra of caffeine (m/z=195) and MRFA (m/z=524) in DC ESI (top) and AC ESI (bottom) at each techniques’ respective optimum potential illustrating the reduced intensity of background evident in AC ESI.
Figure 6.4: Sample output of SBR rejection algorithm. Data plotted in grey are excluded from the background population. Data plotted in black are likely members of the background population and are included in subsequent calculations of signal to background ratio (SBR).
TABLE 6.1: LIMIT OF DETECTION COMPARISON

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<th>Technique</th>
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<th>MRFA LOD (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC ESI</td>
<td>N₂</td>
<td>2.0</td>
<td>0.33±0.04</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td></td>
<td>N₂</td>
<td>2.4</td>
<td>0.16±0.01</td>
<td>0.36±0.02</td>
</tr>
<tr>
<td></td>
<td>SF₆</td>
<td>2.4</td>
<td>0.24±0.03</td>
<td>0.09±0.02</td>
</tr>
<tr>
<td></td>
<td>SF₆</td>
<td>3.0</td>
<td>0.168±0.007</td>
<td>0.11±0.01</td>
</tr>
<tr>
<td>AC ESI</td>
<td>N₂</td>
<td>1.2</td>
<td>0.47±0.08</td>
<td>0.30±0.03</td>
</tr>
<tr>
<td></td>
<td>SF₆</td>
<td>1.2</td>
<td>0.62±0.09</td>
<td>0.052±0.002</td>
</tr>
<tr>
<td></td>
<td>SF₆</td>
<td>1.6</td>
<td>0.43±0.06</td>
<td>0.073±0.002</td>
</tr>
</tbody>
</table>

Limits of detection calculated for caffeine (m/z=192) and MRFA (m/z=524) in AC ESI and DC ESI at a variety of voltages and utilizing both N2 and SF6 as a nebulizing gas.
TABLE 6.2:

INTENSITIES AND SIGNAL TO BACKGROUND RATIOS OF ALL ANALYTES

<table>
<thead>
<tr>
<th>m/z</th>
<th>Intensity</th>
<th>SBR</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N₂ AC</td>
<td>DC AC</td>
<td>DC</td>
</tr>
<tr>
<td>195</td>
<td>5720</td>
<td>73600</td>
<td>1600</td>
</tr>
<tr>
<td>524</td>
<td>10,600</td>
<td>292,000</td>
<td>11,000</td>
</tr>
<tr>
<td>1022</td>
<td>1610</td>
<td>74,800</td>
<td>1920</td>
</tr>
<tr>
<td>1122</td>
<td>3970</td>
<td>181,000</td>
<td>5660</td>
</tr>
<tr>
<td>1222</td>
<td>5990</td>
<td>309,000</td>
<td>10,000</td>
</tr>
<tr>
<td>1322</td>
<td>7500</td>
<td>399,000</td>
<td>14,500</td>
</tr>
<tr>
<td>1422</td>
<td>7190</td>
<td>448,000</td>
<td>16,700</td>
</tr>
<tr>
<td>1522</td>
<td>6680</td>
<td>413,000</td>
<td>16,700</td>
</tr>
<tr>
<td>1622</td>
<td>5070</td>
<td>301,000</td>
<td>14,100</td>
</tr>
<tr>
<td>1722</td>
<td>3610</td>
<td>212,000</td>
<td>10,900</td>
</tr>
<tr>
<td>1822</td>
<td>1980</td>
<td>131,000</td>
<td>7510</td>
</tr>
<tr>
<td>1922</td>
<td>974</td>
<td>59,900</td>
<td>4250</td>
</tr>
</tbody>
</table>

Intensities and signal to background ratios of tune mix peaks in spectra gathered by AC ESI and DC ESI in both nitrogen and sulfur hexafluoride nebulizing gas. Voltage used was the optimum for each technique.
CHAPTER 7:
CONCLUSIONS AND FUTURE WORK

7.1 Conclusion

CZE-MS is a powerful tool for the rapid analysis of the metabolome. High resolution separations of common small molecule metabolites can be performed in under 10 minutes for both standard mixtures and biological samples. Mass spectrometry provides a highly sensitive platform without the need to label analytes, and modern mass spectrometry instrumentation can generate quantitative data from untargeted experiments. The largest remaining challenge in using CZE-MS for the routine analysis of the metabolome is the generation of a comprehensive database containing migration time and tandem mass spectra information. IDs of metabolites based on intact mass alone are of low confidence, and the sparse availability of tandem spectra information make MS2 identification difficult for many common metabolites.

Expanding the capabilities of the electrokinetically pumped sheath flow interface for CZE-MS to include operation in negative ionization mode expands the range of molecules that can be ionized and analyzed with high sensitivity. Coating the electrospray emitter provides a means to alter the charge of the emitter surface, and modify the direction of electrokinetic flow in the emitter tip. A stable, negative mode electrospray is generated,
which provides exquisite sensitivity for many molecules, especially the small, acidic molecules that make up a significant portion of many critical metabolic pathways.

7.2 Future Work

7.2.1 Optimization and Comparison of Negative Mode Interface

Only one coating compound was investigated to generate stable negative mode electrospray ionization in the electrokinetically pumped interface. Many other compounds exist that modify the charge of capillary walls, and optimization of the coating materials procedure may produce emitters with improved lifetime and sensitivity. Each coating compound will also have an optimal spray solution pH, which will also impact sensitivity. Coatings may also improve ionization in the positive mode through more control over emitter flow rates, and reduced dilution. A comparison study between uncoated emitters in positive ion mode and coated emitters in both modes will reveal optimal conditions for specific analytes and guide the design of future experiments.

7.2.2 Construction of a Tandem Mass Spectrometry Database

Identification of metabolites based only on their intact mass is unreliable, due to the high numbers of structural isomers involved in most metabolic pathways. Current tandem mass spectrometry databases such as METLIN and LIPIDMAPS are sparsely populated and contain tandem spectra usually acquired on HPLC-qTOF instrumentation. Matching
spectra to data gathered on another instrument is difficult, as fragmentation patterns depend heavily on collision chemistry and collision energy, which are not standardized across the different mass spectrometer types and brands. To generate reliable identifications and quantitative data, a compound database must be generated for the CZE-MS instrumentation. Representative compounds from major metabolic pathways can be purchased and analyzed by CZE-MS in positive and negative mode to collect migration time and fragmentation spectra information to assist in the identification of compounds in the biological analytes. In addition, metabolites of high interest can be purchased containing heavy isotope atoms, so that they may be used as internal standards to generate very accurate quantitative data.

7.2.3 Analysis of Single Xenopus Embryos

The CZE-MS technology can be used to analyze single Xenopus embryos in under 10 minutes in both positive ion mode and negative ion mode. This data will form a comprehensive database of the metabolism of the early Xenopus embryo. By utilizing a large number of individual embryos coupled with a short analysis time, small but important changes in the concentration of metabolites can be observed. Changes in metabolite concentration can be used to direct the study of the proteomic, transcriptomic, and genomic datasets already generated, identifying products of key enzymes which may be switched on or off as the embryo develops.
Combining the positive and negative ion mode datasets will increase metabolome coverage over positive ion mode alone, and should provide much improved sensitivity for small, acidic metabolites. Leveraging the advantages of CZE for rapidly separating small, polar molecules generates data for large sample numbers rapidly, which is critical for quantifying the smaller fold-changes common in metabolomics.
APPENDIX A:

ABBREVIATIONS

A.1 Common Abbreviations Used in this Text

Abbreviations used throughout the text are listed in Table A.1. Often these abbreviations are used hyphenated together, as in “CE-MS”. This hyphenation indicates a technique used in tandem, coupled to one another.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Alternating current</td>
</tr>
<tr>
<td>BGE</td>
<td>Background electrolyte</td>
</tr>
<tr>
<td>CE</td>
<td>Capillary electrophoresis</td>
</tr>
<tr>
<td>cIEF</td>
<td>Capillary isoelectric focusing</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary zone electrophoresis</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current</td>
</tr>
<tr>
<td>EOF</td>
<td>Electroosmotic flow</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HILIC</td>
<td>Hydrophobic interaction chromatography</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LIF</td>
<td>Laser induced fluorescence</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix-assisted laser desorption ionization</td>
</tr>
<tr>
<td>MEKC</td>
<td>Micellar electrokinetic chromatography</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MS/MS</td>
<td>Tandem mass spectrometry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>RF</td>
<td>Radio frequency</td>
</tr>
<tr>
<td>SCX</td>
<td>Strong cation exchange chromatography</td>
</tr>
<tr>
<td>SRM</td>
<td>Single reaction monitoring</td>
</tr>
<tr>
<td>TOF</td>
<td>Time-of-flight</td>
</tr>
</tbody>
</table>