BIOANALYTICAL LC-MS: STREAMLINED SAMPLE PREPARATION AND NOVEL AMBIENT IONIZATION TECHNIQUES

by

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ABSTRACT

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Bioanalysis has gained importance in the scientific at an exponential rate. The focus of these works attempt to seek new approaches towards faster and more efficient bioanalysis that require little to no sample preparation. One approach is the use of new restricted access media trap technologies to facilitate an online sample preparation platform. A demonstration of the advantages of RAM involves the trace level detection of bisphenol A, an environmental contaminant. A bulk derivatization strategy used in combination with RAM trap technologies is adopted to boost sensitivity without the use of traditional sample preparation methods. Another strategy is the use of ambient ionization techniques. Novel affinity mesh screen materials are used to facilitate rapid drug discovery. The combination of these novel affinity materials with TM-DESI allows for the eventual creation of a truly high throughput screening process for new antibiotic drug compounds. A new ambient ionization technique, continuous flow – extractive desorption electrospray ionization is introduced in the presented work. Charge state manipulation of protein charge states are performed using CF-EDESI. The CF-EDESI technique is also coupled to high pressure liquid chromatography for a powerful analytical method with great versatility.

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CHAPTER 1

OVERVIEW OF TOPICS

1.1 Introduction

The roles bioanalysis and bioanalytical techniques are indispensable in scientific research. In current times, essentially any research in fields dealing with biological sciences, biomolecular systems, or drug pharmacology/toxicology must heavily rely on bioanalysis in order to obtain meaningful results from their experimental data [1]. Researchers in these fields rely on accurate and sensitive bioanalysis to report their findings with statistical confidence. Because of this critical role, a great amount of research efforts have been devoted towards the development of bioanalytical techniques that are better equipped to handle the challenges that the scientific community is currently facing. At times, scientific progress have been hindered due to limitations from an analytical perspective. This ultimately drives the analytical community to find newer, faster, and more versatile solutions to overcome these problems.

Through the years, development of new more powerful analytical methods has been approached by a number of different strategies. For instance, some developments have focused on improving the analytical work with innovations in chromatography or instrumentation [references]. Data analysis is also an emerging area of development where new data processing software and or mathematical treatments of data are introduced to reduce the amount of time required to process large quantities of compiled data [references]. Another area focuses on enhancing sample preparation to increase efficiency towards a high-throughput analytical workflow [references]. Regardless of the approach, innovations in bioanalysis generally seek to improve on previous methods from the standpoint enhanced accuracy, sensitivity, and efficiency.

The focus of this dissertation centers are exploring new strategies and approaches to streamline sample preparation towards the direction of fully automated sample preparation workflow. At times, sample preparation can represent the majority of consumed resources and required labor causing a significant detriment to method efficiency. In addition, many of the common sample preparation techniques are somewhat lacking in terms of their selectivity and recovery leading to issues with sample loss. Clearly, there must be an effort to seek better, more efficient methods of sample preparation. Two potential solutions to challenges faced with sample preparation are investigated for their potential application in future research. First, the use of restricted access media (RAM) is explored through the form off different liquid chromatography trap technologies. The second approach uses ambient ionization, or atmospheric pressure ionization, techniques are a means another means to circumvent sample preparation entirely. This first section of the dissertation will cover a general overview of the topics to be discussed later in the remainder of the literary work.

1.2 Restricted Access Media

The earliest work featuring the development of restricted access media was first reported many years ago presented by Pinkerton in 1985 [2]. Surprisingly since the mid 1990's, applications demonstrating the advantages of restricted access media had been limited until an explosion of featured applications were demonstrated in the late 2000's. Since then, multiple types of new restricted access media materials have been developed and applied towards rapid analysis in number of viable real world applications [spam ref]. The classifications of the different types of restricted access media are saved for further discussion in chapter 2 of this dissertation. It is important to point out though that the most important aspect of restricted access media its combination of two selectivity mechanisms, size exclusion chromatography (SEC) with another retention mechanism of choice (i.e. reversed phase, weak cation exchange, weak anion exchange). Regardless of the retention mechanisms used, restricted access media

certainly offers unique capabilities that allow the method development to be imported to a streamlined, fully automated platform.

1.2.1 Application of RAM towards Detection of Bisphenol A

Restricted access media (RAM) is used in the development of a technique to detect bisphenol A (BPA). BPA is an environmental contaminant that has gained a great amount of attention since reports revealed the endocrine disruptor capabilities of BPA. The concern was exacerbated by its detection in many consumer plastic products, especially of those in infant care products. Infants, who are undergoing hormone and sexual development, are particularly susceptible to the negative effects of endocrine disruptor compounds. In addition to plastic infant care products, BPA was also being reported in a number of consumer food products as well. The primary aim of this work focuses on developing a novel streamlined method for the rapid analysis of BPA with ultra-high sensitivity.

When considering viable sampling options for the detection of human contamination from ingested food, saliva comes to mind as an attractive route for fluid sampling. Ultra-sensitive detection of environmental contaminants from human saliva has its challenges as well primarily in the form of matrix interferences. Traditional methods have already been demonstrate for the analysis of target compounds from saliva. However, these methods are tedious and expensive in terms of resource costs. To overcome these challenges, a bulk derivatization sample preparation strategy is used in conjunction with RAM trap technologies to achieve accurate and sensitive analysis of BPA from saliva using a fraction of the time and resources that previous traditional methods require. Detection limits as low as part per trillion (w/v) are reported from this work. In addition the development and optimization of this method, a viable real world application is demonstrated where the detection of BPA is reported from canned foodstuffs.

1.3 Ambient Ionization Techniques

Ambient Ionization, or atmospheric pressure ionization, coupled to mass spectrometry techniques have been steadily established themselves as power tools in the analytical toolbox. An important feature of inherent to all ambient ionization is the interfacing of an ion source with a mutually exclusive analyte source. The separation of the sample from the ion source, which can be then be independently optimized, offers a number of great advantages. This leads to another important aspect of ambient ionization techniques, which allows analysis to be performed on a sample in its native, unaltered state with minimal or no sample preparation required. Analytes can be selectively removed from matrix components from the sample for rapid , high through-put analysis through a variety of different extraction or desorption mechanisms.

The hallmark of this area of research is the most mature and well-developed technique, desorption electrospray ionization (DESI) developed by the Cooks group [3]. The number of different applications of DESI is vast ranging from small molecular analysis of drug compounds to imaging applications [4-9]. It is also a crucial part of the current trend towards miniaturization of MS instrumentation given DESI natural robust and versatile characteristics. DESI has also been applied to a great number of separation techniques, including thin layer chromatography[], high pressure liquid chromatography[], capillary electrophoresis[], and solid phase extraction[], to truly create a powerful combinatorial analysis technique [CTMs review]. The fundamental basis of DESI lies in the generation of electrospray droplets separately from a sample source that is positioned on a sampling stage. The electrospray droplets are aimed towards the sample and then bounced off the sample into the inlet of the MS. As the ESI droplets are passed over the sample and bounced into the MS inlet, analytes of interest are desorbed from the surface of the analyte onto the ESI droplet in a “droplet pick-up” mechanism [3]. As a result of this unique desorption mechanism, target compounds can be rapidly analyzed from native or crude samples with little or no sample preparation required, which upholds the principles of

ambient ionization. Another well-established ambient ionization is direct analysis in real time (DART), which can be considered the first demonstration of ambient ionization [10]. DART, like DESI, offers advantages that allow the rapid analysis of compounds directly from native samples despite employing a different ionization mechanism employing a gas stream to generate vapors for detection [10].

Since the presentation of DESI and DART to the analytical community, a multitude of other ambient ionization techniques have been demonstrated each with their own role in the analytical field. Some notable ambient ionization with interesting applications include extractive electrospray ionization (EESI) [11, 12], desorption atmospheric pressure photoionization (DAPPI) [13], transmission mode – desorption electrospray ionization (TM-DESI) [14, 15], continuous flow – extractive desorption electrospray ionization (CF-EDESI) [16], and paper spray ionization [17], among others. Again like DART and DESI, these techniques all offer a separation of analyte sample source from the ionizing source, which leads to direct analysis from samples with little to no sample preparation. This dissertation explores the application of two ambient ionization techniques, TM-DESI and CF-EDESI in order to present new strategies of analysis towards sample preparation. In the case of CF-EDESI, sample preparation is precluded completely by using the unique characteristics of its ionization mechanism and instrumental arrangement.

1.3.1 Transmission Mode – Desorption Electrospray Ionization

A novel approach towards antibiotic drug discovery from natural product extracts is proposed in this work. Currently, drug discovery from natural products in the pharmaceutical industry involves a time and resource consuming screening process, which also has low selectivity for viable potential new chemical entities. The new strategy involves the use of novel affinity mesh screen materials to boost selectivity as well as greatly reduce screening time. TM-DESI is used as an analytical solution to evaluate the use of novel affinity mesh screen

materials. This collaborative work first describes the design and creation of these affinity mesh screen materials. The affinity materials are selective for macrocyclic antibiotic compounds that recognize a specific Kaa tripeptide sequence found in the cell walls of Gram positive bacteria. With this novel affinity material, potential new chemical entities can be extracted from complex mixtures with high selectivity. This circumvents many of the challenges facing drug discovery processes that have shown to be tedious and extremely taxing on labor and resources. TM-DESI-MS is used to rapidly desorb captured analytes from the screen into the mass spectrometer for rapid analysis and identification. An analytical workflow that is specifically tailored towards for this method was designed to facilitate rapid analysis towards drug discovery. Proof-of-concept is demonstrated with antibiotic standards. Both a positive control and negative control experiments were performed to evaluate the proposed drug discovery method. Results were in good agreement with hypothesized outcomes.

1.3.2 Continuous Flow – Extractive Desorption Electrospray Ionization

As mentioned previously, many new ambient ionization techniques are presented to fill roles that were left unoccupied by current analytical technologies. However, there are still certain challenges that the analytical still face without a current solution to address these problems. The first presentation and initial studies of a new ambient ionization technique, continuous flow – extractive desorption electrospray ionization (CF-EDESI) are introduced from work in this dissertation. Instrumental design of the CF-EDESI technique is also described in this inaugural research work. Considerations into its mechanism are also offered. CF-EDESI offers a number of attractive features that overcome limitations of conventional ESI. An application demonstrating the advantages of manipulating protein charge state distributions are featured in these initial studies. The advantage of the separation of sample analyte from electrospray source is clearly seen in this protein charging application.

A follow up research work using CF-EDESI is also presented after its initial studies. Here, a series of protein charging experiments performed on myoglobin indicated the softer ionization characteristic over conventional ESI. The softer ionization allows preservation of protein conformational structures, which implicates the possibility of protein-protein or protein-substrate complexation studies. In addition, a proof-of-principle is demonstrated to show that CF-EDESI can be hyphenated to a high pressure liquid chromatography. The added benefits of a powerful separation technique prior to CF-EDESI allow the creation of method with greater versatility and maximum analytical performance.

CHAPTER 2

RESTRICED ACCESS MEDIA AS A NEW APPROACH TOWARDS ON-LINE SAMPLE PREPARATION: CURRENT ADVANCEMENTS AND APPLICATIONS IN BIOANALYSIS

2.1 Introduction

Sample preparation is an integral part of analytical method development whether the analysis pertains to pharmaceutical, bioanalytical, or environmental settings. In many cases, sample preparation is responsible for the greatest consumption of time and resources during the analytical process. The main purpose that sample preparation serves is to remove or reduce the effects of matrix interferences that are caused by the multiple matrix components of a complex sample. Additionally, sample preparation can be used to facilitate other aspects of analysis, such as preconcentration or derivatization, in order to enhance sensitivity. Nonetheless, the improvement on existing sample preparation strategies is an area of research that is in need of continual development in order to account for the different types of samples and situations encountered during analysis. There are currently a number of methods for sample preparation, the main three categories being solid-phase extraction, liquid-liquid extraction, and protein precipitation. These traditional methods, though effective for completing the task at hand, can often be characterized as having poor selectivity, poor recovery, and low selectivity, as well as being more laborious and cost-intensive for target analyte determination. The problems with traditional sample preparation are compounded with the current movement of streamlined sample preparation towards full automation of analysis for a high throughput process.

In recent years, restricted access media (RAM) has received an increasing amount of attention as a new approach towards solving the problems with traditional sample preparation [18]. Despite being first introduced in 1985 by Pinkerton in the form of the first internal surface

reversed phase column stationary phase [pinkerton ref], restricted access media has only been recently been widely explored as a viable means to expedite sample preparation [2]. Shortly afterwards, other types of RAM materials were introduced featuring different types of stationary phase/surface chemistry. An in-depth coverage of the history of these RAM materials through the decades has already been covered in previous reviews [reviews]. Although studies featuring restricted access media have been presented through the following years, the explosion of interest and research efforts focused on restricted access media in method development occurred within the recent decade. Significant advances towards commercialization of restricted access media trap columns were achieved recently, which allowed greater availability to the research community. Implementation of RAM trap columns in conventional method development has also been facilitated with significant advancements towards instrument hardware and technology. With renewed interest in these types of RAM materials, a wealth of literature featuring the use of RAM in a number of different applications has been presented [1, 19-21]. In general, methods using RAM materials feature the rapid removal of matrix inferences and ion suppression effects from samples prior to analyte determination, which allows more accurate and sensitive quantitation of drug compounds of interest [ref].

Restricted access media (RAM) can be essentially described as a type of stationary phase material that uses a combination of size exclusion chromatography with another selection mechanism (e.g. reversed phase, normal phase, strong cation exchange, strong anion exchange) giving added versatility compared to conventional analytical or trap columns. For instance, with a SEC/RP RAM column, matrix interferences, which are generally in the form of large biomolecules and proteins, are too large to permeate through the pores of a silica gel particle. As large biomolecules are rinsed from the trap, so are unretained salts. However, small molecule analytes of interest can permeate through to the core of the phase. The inner core of this type of RAM material is functionalized with hydrophobic groups, which effectively

retains small molecule analytes through a reversed-phase mechanism. After sufficient washing of the trap to remove unretained matrix interferences, the small molecule analytes of interest can be eluted (or back-eluted, depending on the experimental set-up) out of the RAM trap column into the standard analytical separation platform for rapid and accurate analysis. The inherent nature of to the two orthogonal selection mechanisms implicates greater versatility during analysis. RAM materials can be custom tailored to fit specific situations by varying the retention mechanism of the inner surface/layer of the stationary phase. As a result, sample preparation time is significantly reduced or even eliminated completely. With the correct instrumental setup (e.g. multidimensional chromatography valve-switching system), the entire method from native sample to final analysis can be streamlined in an automated fashion. The unique stationary phase/ solid support chemistry of RAM materials allows it to supplant many types of traditional methods for sample preparation, such as solid phase extraction, liquid-liquid extraction, and protein precipitation. Additionally, the large number of different combinations along with the different types of practical solid support choices leads to a vast number of different types of RAM stationary phase materials that can used in a wide range of different applications.

The objective of this review work aims to elaborate on the major advantages of using RAM trap materials for streamlined sample preparation as well as to offer an overall coverage of the different types of specific RAM trap columns that have been introduced in recent years. More specifically, the research works covered in this review primarily focus on recent applications presented in the timeframe of 2009-2012. The discussion is broken down into three sections based on the major classifications of the different types of currently available RAM traps, which are the internal surface phase (ISP), the semi-permeable surface (SPS), and the molecularly imprinted polymer (MIP) restricted access media. A description of the specific characteristics that define each class is first considered to offer a general overview of the types trap materials that will be discussed. Interesting applications of different RAM materials

representing each classification are then presented to highlight their versatility in the analysis of a wide range of samples. Although, it is clear that there are a number of different subclasses within each major grouping, the discussion is organized in this fashion to provide a more simplified coverage of the many different types of RAM trap materials whether they are commercially available or custom synthesized within a certain research group. Details that differentiate each the specific RAM columns are then solely based on bonding chemistry within the internal surface/layer, which will also be discussed as they are encountered in the various types of applications. The focus of this work is to review viable applications of using RAM development to overcome analytical challenges with efficient, high throughput methods. While the coverage of these different applications will be in-depth, the author apologizes if other viable studies have been overlooked in the organization of this review.

2.2 Internal Surface Reverse Phase RAM

Internal surface reverse phase (ISRP) RAM columns were the first type of RAM to be introduced. The original ISRP by the Pinkerton group used a reverse phase C18 bonded group as the internal surface layer. The principle across all ISRP all still the same. The example mechanism described in the previous section is the exact description on how the ISRP selection/retention mechanism operates. Many applications of ISRP have been demonstrated using a wide range of different bonded internal surfaces ranging from hydrophobic groups, to ion exchange materials, to even aromatic peptide chains.

Many in-depth optimizations of ISRP trap columns have been performed, and an even greater has been applied towards Bioanalysis. In a very elegant work presented by Santos-neto *et. al.*[19], a alkly-diol-silica (ADS) bonded internal phase was used to study a series of benzimidazole drugs directly from two different sample sources, human blood and urine. The RAM was a LiChrospher trap column with capillary dimensions (25 mm; 60 Å pore size) purchased form Merck. The ADS RAM columns have alkyl chains in the internal surface while

diol groups are bonded on the outer surface. The group had also prepared in-house a new albumin-C18 RAM capillary trap column in house as well to compare against the commercially purchased column. The albumin-C18 is a ISRP trap column with a length of mm and a 120 Å pore size. Extensive investigations were performed to explore the effects of backflushing and front flushing to clean the trap after injection of whole blood and urine samples. After optimization of the loading step of the method, a separation was performed on all six drug compounds. An evaluation of the matrix effects was made. Finally the method was used to demonstrate sensitive detection of ten drug compounds including, albendazole sulfoxide, albendazole sulfone, mebendazole,

desipramine, fluoxetine, albendazole, nortriptyline, imipramine, amitriptyline, and clomipramine, with MRM detection on a triple quadrupole mass spectrometer.

2.3 Semi-Permeable Surface RAM

Semi-Permeable Surface RAM trap columns are powerful in their use as well. The MAYI trap column from Shimadzu is a variation on the SPS type and the ISRP. The MAYI uses silica gel particles, but they are coated with a methylcelluclose polymer coating to form a semipermeable surface as well. Liu and authors, demonstrate an application of the MAYI trap where large volumes of urine can be injected for enhancement of sensitivity [20]. The MAYI trap is being treated as a preconcentration device in addition to its role as a sample cleanup trap column. A series of seven estrogen and estrogen-like compounds were analyzed directly from injection of the large volumes of urine. Considerations were first taken into the maximum that the MAYI trap is physically able to handle. A dilution line was added to change the sample solvent composition prior to loading on the MAYI trap column to ensure retention of all analytes. A chromatographic separation was then developed with the RAM-LC-MS to resolve all seven analytes into peaks from an analytical C18 column. Although not every peak was baseline resolved, the selected ion monitoring of the mass spectrometer was used to deconvolute

coeluting peaks. The method was finally subjected to method validation assessing method sample recovery, inter-/intra-day reproducibility, limits of detection, and limits of quantitation.

2.4 Molecularly Imprinted Polymer RAM

Molecularly Imprinted Polymer (MIP) RAM materials are the newest type solid supports for RAM. Essentially, polymers are created with site specific with molecular templates. This leads to a material that is selective through molecular recognition for a particular analyte or class of analytes. In a work presented by Hiroshina and authors [21], a unique MIP RAM material is synthesized to recognize flufenamic acid and the non-steroidal anti-inflammatory drugs (NSAID). In this case, the sample matrix was river water, a sample source with a large amount of matrix interference as well. Flufenamic acid was first used as the test analyte to evaluate the MIP RAM column. Once this NSAID was proven to be recognized by the MIP material, other NSAID compounds were evaluated for molecular recognition as well. Other NSAID compounds include mefenamic acid, indomethacin, etodolac, and ketoprofen. pH effects were first explored in this work to see the variation of retention factor based on changes in pH. Once conditions were optimized, a separation of all NSAID compounds was performed and subjected to method validation. Method validation criteria include intra-/inter-day reproducibility from the calculate RSD values.

These examples are just a few of the many types of different RAM trap columns that are commercially available as well as those that are synthesize in-house. The many applications demonstrate the true power of using RAM sample preparation strategies to create high throughput analytical workflows.

CHAPTER 3

QUANTITATIVE DETERMINATION OF BISPHENOL A FROM HUMAN SALIVA USING BULK DERIVATIZATION AND TRAP-AND-ELUTE LIQUID CHROMATOGRAPHY – ELECTROSPRAY IONIZATION – MASS SPECTROMETRY

3.1 Introduction

Endocrine disruptors are a particular class of compounds that have garnered much attention from the scientific community. The cause for such alarm is due to their ability to cause adverse health effects in the human body such as feminization of the male species [22-24], abnormal sex organ growth [25-27], and cancer [28-30]. Compounding the problem is their nearly ubiquitous presence in all aspects of the environment, the realization of which has become increasingly evident with advancements in analytical instrument sensitivity. Numerous reports have cited the presence of endocrine disruptors in a variety of different sources ranging from natural drinking water to food products to materials used in the manufacture of consumer goods [31-33]. Endocrine disruptors specifically target the hormone balance that is maintained by the endocrine system, which is comprised of an intricate web of hormone receptors and ligands that regulates metabolism and bodily function. The ability of endocrine disruptors to affect this system lies in their structural similarities with endogenous hormones. These similarities confer the ability to bind key interaction sites on target membrane receptors triggering unintended signal transduction through the cell [34-36]. Even more distressing are reports that indicate endocrine disruptors impart epigenetic effects, where negative effects are proliferated from the affected parent through to offspring generations [37].

Given the numerous different types of hormones, the most prevalent and well-studied type of endocrine disruptors are those that mimic the biological function of estrogen. Estrogen is a steroid hormone that plays a critical role in the development and maturation of male and female sexes. Studies have been reported implicating these “estrogen mimics” as the culprit

responsible for causing many disastrous medical anomalies over many decades [38, 39]. Since the early 1970s, a large number of compounds have been identified as having estrogenic properties and as a result, are now tightly regulated. A good example is dichlorodiphenyltrichloroethane (1,1,1-trichloro-2,2-di(4-chlorophenyl)ethane;DT), which was a commonly-used pesticide from the 1930s [38]. Another is diethylstilbestrol (4,4'-(3*E*)-hex-3-ene-3,4-diyldiphenol; DES), which was used as a drug to prevent miscarriages and as a constituent in daily household products such as cosmetics and shampoos [38]. Ultimately, DES was found to be the cause of multiple reproductive abnormalities in both male and female infants and was banned from use [37, 40, 41].

Recently, the use of bisphenol A (BPA) in consumer products has sparked debate and deliberation, especially over the actual repercussions of exposure to BPA and whether the establishment of regulations is justified. BPA is present in the epoxy resins used in the sealants of canned foods [42-45]. It is used as the monomer unit in the synthesis of polycarbonate polymers [46], a material that is used in many manufactured products. In addition, it is also used as a plasticizer in certain polyvinyl chloride and structural polymers [47]. Because of these ubiquitous uses, there is a significant chance of oral exposure to BPA by organisms [31, 48-50]. Despite a wide body of research which reports the deleterious bioactivity of the compound, the actual effect of BPA on the human body is still an area of significant debate. Binding studies show that BPA has a thousand-fold lower binding affinity to the estrogen receptor than endogenous estrogen [51, 52]. Many argue that even though BPA is prevalent in the environment, its effects are negligible in the trace quantities that are generally detected in the environment. On the other hand, biological studies in test animal subjects have already reported adverse side effects due to exposure of BPA, even at very low levels [53]. Another aspect of the debate deals with the effects of long term exposure on the human body. Regardless of the outcome of these debates, a growing need for fast, accurate methods to detect and quantify trace amounts of this endocrine disruptor is apparent. Only then can data

be collected that will allow a fair assessment of BPA exposure and its associated effects for potential establishment of regulations on this compound.

To probe for oral exposure of BPA, a suitable and representative biological fluid from the body must be analyzed. Human saliva fits many of the criteria for this quantitative analysis for a number of reasons. Oral exposure of BPA passes through the mouth before being transferred into the rest of the body; therefore direct sampling of the saliva can indicate oral exposure [54, 55]. In addition, sampling of human saliva is one of the simplest and least invasive routes for biomonitoring, when compared to collection of fluids such as blood and urine, among others. However, saliva still provides similar problems as a matrix for quantitative analysis compared to other biological fluids. Traditional methods of preparation involving biological fluids suffer a wide variety of potential interferences and complexities stemming from these matrices. Consequently, time-consuming sample preparation steps (e.g. liquid-liquid extraction, solid phase extraction, and protein precipitation) are often needed prior to analytical methods to reduce interferences contributed by the sample matrix [55-57]. In addition, these methods can require the use of costly consumables, materials, and chemicals.

Herein, we report a series of methods for quantitative analysis that incorporate a bulk derivatization strategy to greatly reduce sample preparation time, to improve recovery of analytes in biological fluids, and to enhance sensitivity. Emphasis is placed on the reduction of sample preparation time for the quantitative analysis of bisphenol A from biological fluids and the overall increase in efficiency of analysis given by bulk derivatization as compared to previous methods [48, 58-60]. The approach is further aided by the use of a trap-and-elute liquid chromatography – mass spectrometry (LC-MS) instrumental configuration, which features restricted-access separation media [18]. The bulk derivatization strategy involves crude saliva being directly subjected to a derivatization reaction with dansyl chloride as shown in Fig. 1. Dansylated BPA is easily protonated under acidic conditions, which, in addition to the added hydrophobicity conferred to the product, enhances sensitivity in positive ionization mode

electrospray ionization – mass spectrometry (ESI-MS). Three separate analytical methods (standard addition, standard addition with multiple injection loading (MIL), and standard addition with internal standard and MIL) were developed for the quantitative analysis of BPA from human saliva using these proposed strategies. Each was subjected to rigorous method validation. Applications were performed to extend these validated methods to practical case studies in a real-world scenario. In this work, canned food products suspected of BPA contamination were ingested; after which, saliva samples were immediately collected for analysis by the developed methods.

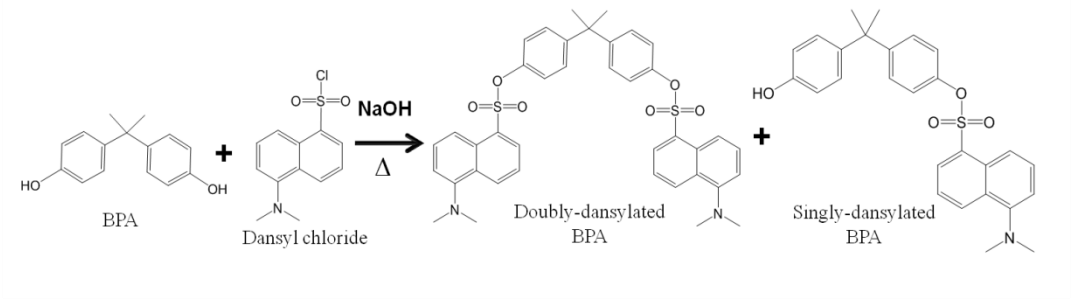

Figure 3.1 - Reaction diagram of derivatization reaction of BPA with dansyl chloride

3.2 Experimental

Chemicals and Materials

Bisphenol A was purchased from Sigma-Aldrich (St. Louis, MO) and the internal standard, d_{16}-bisphenol A, was obtained from Supelco (Bellefonte, PA). Dansyl chloride was obtained from Fluka Analytical (Buchs, Switzerland). Sodium hydroxide was obtained from

EMD Chemicals Inc. (Darmstadt, Germany). Concentrated hydrochloric acid was obtained from Pharmaco – AAPER (Brookfield, CT; Shelbyville, KT) and concentrated formic acid was obtained from J.T. Baker (Phillipsburg, NJ). HPLC grade acetone was purchased from J.T. Baker (Phillipsburg, NJ). LC-MS grade water and acetonitrile were supplied by Burdick and Jackson (Muskeegon, MI, USA); LC-MS grade CHROMASOLV 2-propanol was purchased from Fluka Analytical (St. Louis, MO). Blank saliva and application saliva samples were obtained from the authors of this paper. All saliva samples were collected in glass vials, and contact with plastic products was minimized during every step of the procedure for all samples and chemicals.

Optimized Sample Preparation Method

The optimized method was finalized and is outlined in Fig. 2. The total sample preparation was devised to achieve a final volume of 1 mL after completion of the procedure. Initial volumes of the saliva samples (720 μL) were adjusted to compensate for additional BPA spike and internal standard spike volumes, which were both 50 μL) each, dissolved in acetonitrile. After the standards were added, 40 μL) of NaOH (1 M) was added into the vial, followed by 150 μL of dansyl chloride (8 mg/mL in acetone). Sufficient reaction time was allowed, which can be qualitatively monitored by observing the color changes of the reaction solution (approximately 3 min). After the reaction reached a transparent pale yellow color, the solution was incubated in an oven at 60°C for 15 minutes to insure complete reaction. The sample was then removed from heat, briefly vortexed, treated by addition of 40 μL) of concentrated HCl, and centrifuged for 20 min at 2000 rpm. The supernatant was transferred into a conical bottom autosampler vial for injection onto the HPLC system. Completion of the entire sample preparation protocol is achievable in less than one hour, and multiple samples can be processed in parallel.

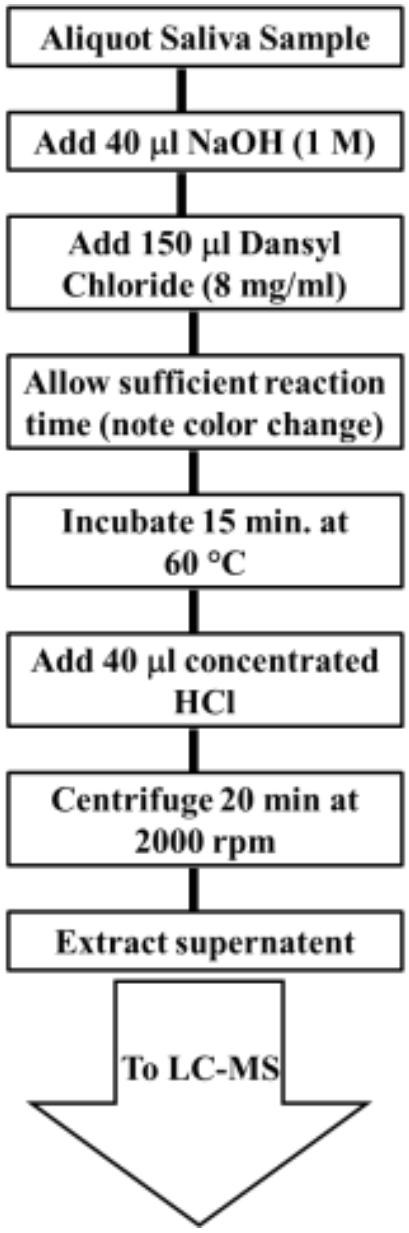

Figure 3.2 – Flow diagram of bulk derivatization sample preparation protocol

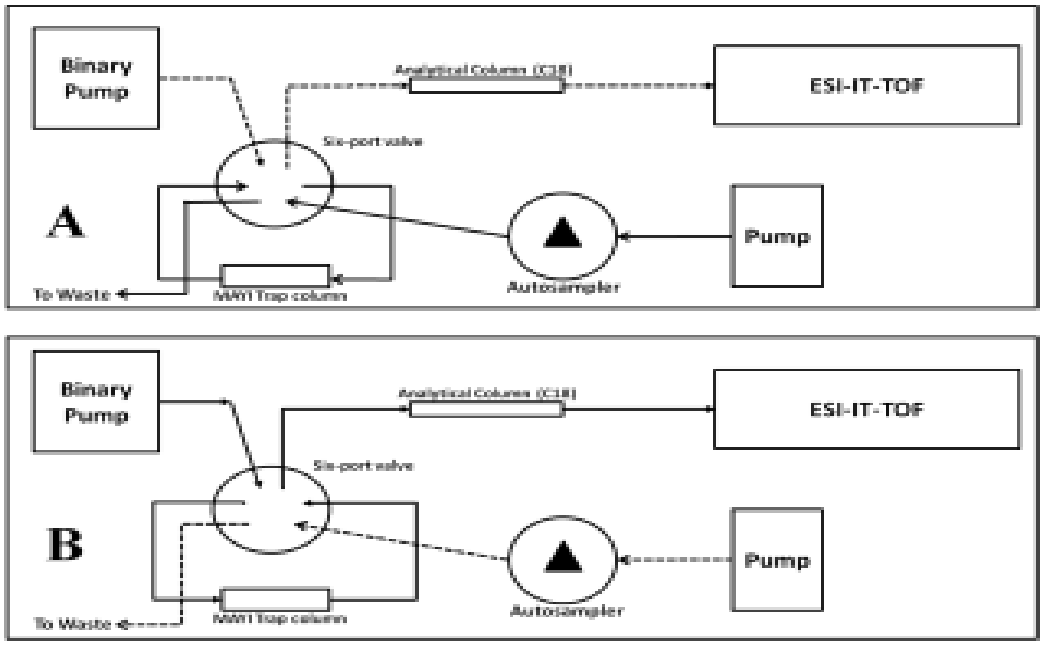

Figure 3.3 - Schematic diagram of trap-and-elute liquid chromatography system in (A) trap column loading phase and (B) analysis phase

Chromatographic method

HPLC analysis was performed using a Shimadzu© Prominence LC system (Shimadzu Scientific Instruments, Inc., Columbia, MD) comprised of four LC-20AD pumps, a SIL-20A HT autosampler with a 40 μL sample loop, and a CTO-20AC column oven with a six-port valve switching system. An instrumental diagram for the LC system is shown in Fig. 3. The trap column used in the analyses was the Shimadzu Shimpack MAYI – ODS (4.6 mm i.d. x 10 mm L, 3 μm d_p); a Varian Inc. Pursuit XRs C18 column (2.0 mm i.d. x 100 mm L, 3 μm d_p) was used as the analytical column. In reference to Fig. 3, the mobile phase compositions used in the binary pump consisted of water/acetonitrile (95:5) + 0.1% formic acid for mobile phase A and acetonitrile/isopropyl alcohol (90:10) + 0.1% formic acid for mobile phase B. At the start of the

chromatographic method, the binary pump mixes the mobile phase at 20% B to equilibrate the column. The loading mobile phase used for injection of the saliva sample through the autosampler was an isocratic composition of water/acetonitrile (80:20). The binary pump was operated at a constant flow rate of 0.1 mL/min. The secondary pump system used for sample loading was operated at 0.6 mL/min to inject the saliva sample into the trap column and wash out unretained biomolecules from the trap. The flow rate was then reduced to 0.05 mL/min after the system configuration was switched from configuration A (trap loading phase) to configuration B (analysis phase). In the multiple injection loading scheme, the samples were successively injected in intervals of one minute each. After five minutes of washing, the six-port valve is switched to configuration B, and the retained analytes were eluted from the trap column, onto the analytical column for separation, and into the ESI-MS instrument for analysis. For each method, to separate components on the analytical column, a gradient program of 20% to 95% organic content in 12 minutes was carried out by the binary pump. The pump was then held at isocratic flow with 95% organic content for 20 minutes. To wash the trap column, the configuration is switched back from the analysis phase (B) to the loading phase (A) to wash the trap column with a mixture of water/isopropyl alcohol (10:90) for 10 minutes before reverting back to the loading mobile phase composition of water/acetonitrile (80:20) for equilibration. Sufficient pre-equilibration of the entire chromatographic system (35 min) is allowed between each analysis to ensure precision.

Mass Spectrometry

A Shimadzu IT-TOF ESI-MS system was used for the mass analysis in this method. Electrospray ionization was performed in the positive ionization mode with a spray capillary voltage of 4.5 kV while the detector voltage was 1.6 kV. Mass analysis was operated in the automatic mode setting with a scan range of 150 - 600 (m/z) and an ion accumulation time of 56 msec. The curved desolvation line (CDL) and heat block were both heated to 200 °C. The

time-of-flight mass analyzer was maintained at 40 °C. Nebulizing gas was introduced at 1.5 L/min, while the drying gas was set to 75 kPa. Data analysis was performed using LCMSolutions (version 3.5) software (Shimadzu). The instrument was regularly calibrated to <5 ppm error in mass accuracy with an external standard of sodium TFA solution (sodium hydrate, 0.1 g/L; trifluoroacetic acid, 0.25 mL/L).

3.3 Results and Discussion

Optimization of Bulk Derivatization Protocol

Initial studies yielded promising results and indicated the viability of the proposed method for biomonitoring applications using a 5 μL injection volume. In these experiments, the free, unmetabolized form of BPA was monitored, as opposed to any possible enzymatically-modified forms. We assume that the time frame between ingestion of BPA and saliva sampling was short enough that the BPA had not been subjected to enzymatic conversion to the glucuronidated form to a significant degree. In addition, the BPA should be in the free form for derivatization by dansyl chloride. The chromatographic peak for dansylated BPA was assigned using the extracted ion chromatogram from its exact mass. One major problem that was encountered was the division of the derivatized BPA between singly-dansylated and doubly-dansylated forms as shown Fig. 4A. Splitting of analyte signal between two forms generally leads to a loss of sensitivity. The cause for the two different forms of BPA could be attributed to an insufficient amount of dansyl chloride available for bulk derivatization. However, adding a large excess of dansyl chloride leads to excessive dilution and loss of sensitivity since a large aliquot of dansyl chloride must be added into the sample mixture. The chosen concentration of 8 mg/mL dissolved in acetone is a concentration that is close to its solubility limit, and thus increasing the concentration of the dansyl chloride was not a viable option. Theoretically, there is a certain threshold of dansyl chloride that, upon reaching, would provide for complete derivatization to convert all BPA to the doubly-derivatized form, even in the presence of other

matrix components. Optimization of the bulk derivatization reaction was performed in order to determine a set of reaction conditions where the derivatization was most efficient. The efficiency of the reaction was evaluated by monitoring the signal response of both derivatized forms of BPA against varying mass quantities of dansyl chloride added. Fig. 5 shows the findings from an investigation of the effects of the dansyl chloride (8 mg/mL in acetone, a concentration close to its solubility limit) mass quantity of dansyl chloride on the BPA signal response (a sum of both singly- and doubly-derivatized forms). 20 μL injections of a spiked sample of 10 μM BPA in blank saliva were used in this experiment, which is a concentration that is well above the expected levels of BPA exposure in saliva. Saliva was collected and combined from multiple sources into a single pool in order to accommodate the variability of different factors (i.e. pH, amylase content) in saliva between each source [61]. From the plot, the optimal volume of dansyl chloride was determined to be 1.2 mg dansyl chloride (150 μL of 8 mg/mL in acetone) for bulk derivatization.

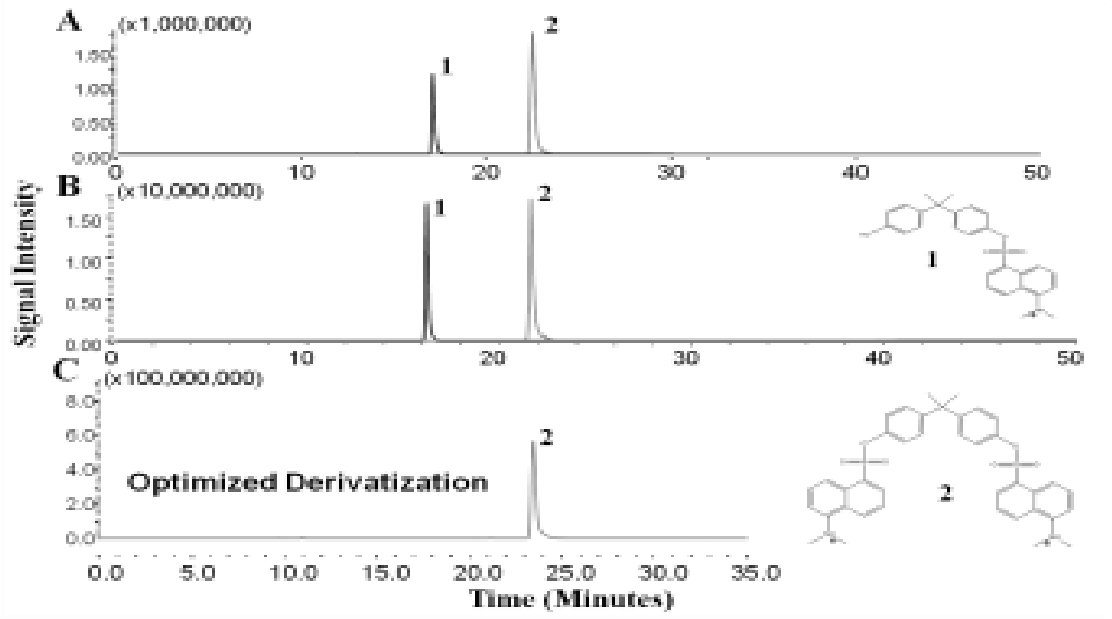

Figure 3.4 - Representative chromatograms demonstrating division of different concentrations of BPA at (A) 1 μM and (B) 10 μM into both (1) singly- and (2) doubly-derivatized forms. (C) Effects of incorporation of optimized derivatization reaction and a 40 μL additive is also shown in comparison to the division of BPA signal.

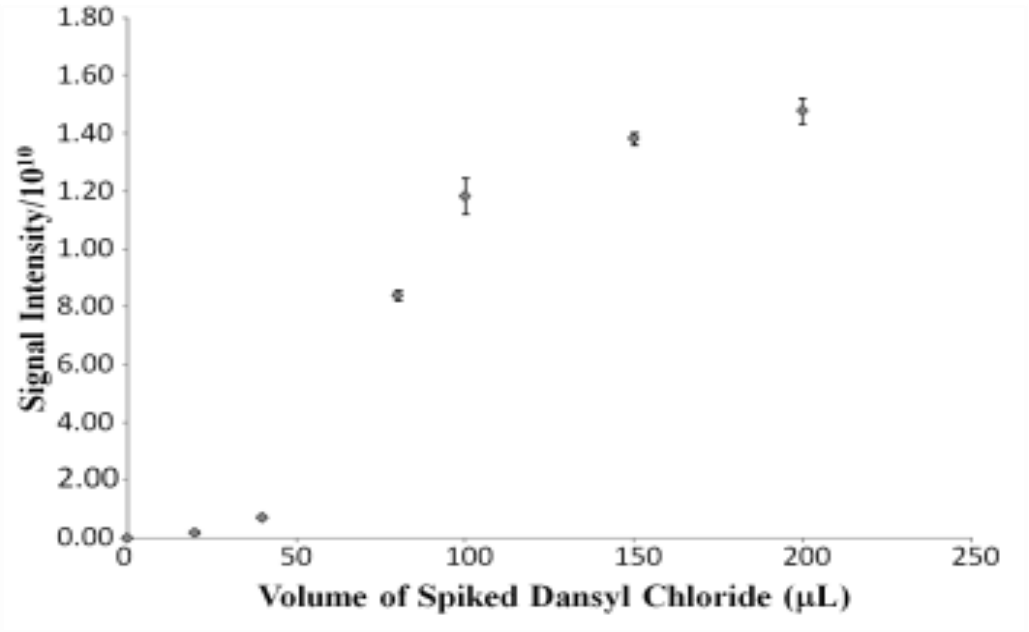

Figure 3.5 - Optimization of aliquot volume for bulk derivatization using an 8 mg mL^{-1} dansyl chloride solution, a concentration that was near to the solubility limit of dansyl chloride in acetone.

To enhance recovery, the effects of post-derivatization additives were also investigated. It was found that the addition of concentrated HCl to the preparation dramatically enhanced the analyte signal. It is speculated that the HCl accentuates signal response by both protonating the dansylated BPA and cleaving the solid salivary protein contained in the biological matrix, thus disrupting hydrophobic-hydrophobic interactions between the dansylated BPA and the solid proteins in the saliva. By releasing the BPA from the solid matter, the BPA is not removed from the supernatant during pellet formation *via* centrifugation. Recovery studies using a sample size of 1 mL showed that 91.9% (± 0.2% RSD) of the BPA was recovered from the sample with this optimized procedure. Fig. 4C shows a representative chromatogram of the final optimized method where the BPA has been completely converted into the doubly-derivatized form and

with the addition of the HCl to the sample preparation. A 10-fold increase in signal intensity was observed from the optimized sample preparation (compare Fig. 4C and Fig. 4B).

The method was subjected to rigorous method validation in accordance to the FDA bioanalytical method validation guidelines [62]. The method limit of detection (LOD) was determined by taking seven replicates of a concentration point near the limit of quantification along with generation of a full calibration curve containing seven points. The LOD was then calculated by the equation, LOD = $t(s)/m$, where t is the t – table value for 7 degrees of freedom (3.1); s is the standard deviation of the seven replicates; and m is the slope of the calibration curve generated from method validation. The LOD is reported as both a concentration (e.g. pg/mL) as well as a mass loaded on column (e.g. pg). The accuracy and precision of the method were determined by sampling three different concentration points (0.01, 0.05 and 0.08 μL) in replicates of five. These particular concentrations were chosen to represent a low, medium, and high region of the calibration curve. Linearity was evaluated based on the correlation coefficient of the best-fit line of the empirical data points. The standards and samples used in method validation were spiked into blank pooled saliva, performed on a single day by a single researcher. Water blanks were injected in between each run; the use of blank-corrected signals for calibration and LOD determination was not necessary, due to the negligible signal for BPA in the blank samples. The results of the method validation are summarized in Table 1. The correlation coefficient (R^2) of the calibration curve was 0.996. The limit of detection was determined to be 3.25 ng/mL (16 pg on column). Accuracy and precision evaluated for quality control standards were judged to be satisfactory.

Table 3.1 - Method validation parameters for single injection method (5 μL)

Limit of Detection (ng/mL)	3.25 (16 pg loaded on column)	
Reported Concentration (μM)	0.500	8.00
Calculated Concentration (μM)	0.60 ± 0.04	7.9 ± 0.1
Accuracy error (%)	20.2	0.24
Coefficient of Variance (%)	6.3	1.7

Multiple Injection Loading Strategy

Extension of the validated method to application studies revealed a significant problem. The signals generated from several application studies were below the reported limit of detection (*data not shown*). New strategies were thus explored to enhance the sensitivity or detectability of BPA. The use of a multiple injection loading (MIL) scheme was explored, where the saliva sample was injected onto the MAYI trap column multiple times, essentially converting the MAYI column into a preconcentration device. The negative aspect to using MIL is the band broadening associated with repeated injections of a large sample slug. An investigation to find the optimal loading scheme was conducted, the results of which are shown in Fig. 6. The figure shows the plots of different injection schemes (10, 20, and 40 μL) along with their respective peak asymmetry factors. The optimal MIL scheme was determined to be three injections of 20 μL. This setting offered a good compromise between increased signal response (sample loading) and reduced peak asymmetry. A second method validation was performed on the multiple injections method, the data for which are summarized in Table 2. The correlation coefficient (R^2) of the calibration curve was 0.996. It is important to note that while the LOD expressed in terms of pg/mL decreased, due to the higher signal intensities seen while using the MIL scheme, the limit of detection of the method when considered in terms of mass loaded on column was not significantly affected by the MIL scheme. The MIL scheme was simply a means by which more saliva sample could be loaded onto the system and preconcentrated by

the trap column. It does not alter the lowest amount of analyte that the analytical system can see. Regardless, acceptable accuracy and precision was again demonstrated from method validation.

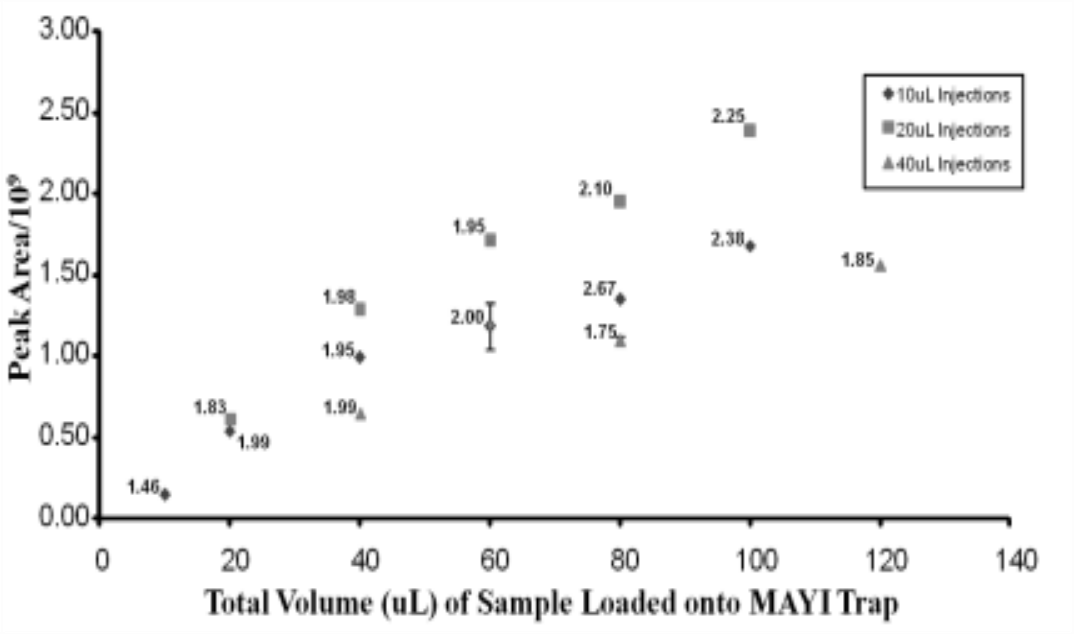

Figure 3.6 - Optimization of multiple loading injection with different injection volumes (10 μL, 20 μL, 40 μL). Area of the doubly dansylated BPA peak was plotted against total injection volume. Peak asymmetry factors are indicated above each injection point.

Table 3.2 - Method validation of multiple loading injection method (3 x 20 μL)

Limit of Detection (pg/mL)	199 (12 pg loaded on column)		
Reported Concentration (μM)	0.05	0.10	0.50
Calculated Concentration (μM)	0.052 ± 0.002	0.096 ± 0.004	0.497± 0.02
Accuracy error (%)	3.4	-3.8	-0.5
Coefficient of Variance (%)	3.6	3.6	3.5

A proof-of-principle application is presented to demonstrate the utility of this validated method in the detection of BPA exposure from human saliva. It is well known that a primary source of widespread BPA exposure is canned food goods [42-44]. A source of BPA in these canned products comes from the epoxy resin lining and sealants used in the preservation and packaging process. A series of different canned goods ranging from different countries, purchased from local supermarkets, were investigated. Each food product was first ingested, and a saliva sample was collected immediately afterwards. To ensure that BPA contamination was solely from the canned product, a blank saliva sample was also collected prior to ingestion of any food substance. From these canned food analysis studies, the product with the highest BPA contamination was determined to be a can of green salsa chili from Mexico (*data not shown*). Concentrations were calculated by generation of standard addition calibration curve and extrapolation of the zero point concentrations from the curve. Despite the larger BPA signal relative to the single 5 μL injection method, the calculated concentration was determined to still be below the reported limit of detection, even though a seemingly reasonable-sized signal was recorded at the retention time of interest.

Incorporation of Internal Standard

A method that incorporates an internal standard, d_{16}-bisphenol A was explored as a solution to the problem. Addition of an internal standard to the analysis allowed normalization of

the analyte signal to the internal standard signal, which significantly enhanced the precision (i.e. lower the standard deviation of each concentration point) of the method. According to the calculations of limit of detection set by the FDA bioanalytical method validation guidelines, precision is directly proportional to the limit of detection, and therefore accentuated precision inherently lowers the limit of detection. The MIL method, now with internal standard, was once again subjected to method validation, the results of which are reported in Table 3. The correlation coefficient (R^2) of the calibration curve was 0.998. With this method, a significantly lower limit of detection of 49.0 pg/mL (2.9 pg on column) was calculated; very good accuracy and precision was also demonstrated.

Table 3.3 - Method validation of multiple loading injection method (3 x 20 μL) with incorporation of an internal standard

Limit of Detection (pg/mL)	49.0 (2.9 pg loaded on column)		
Reported Concentration (μM)	0.01	0.05	0.08
Calculated Concentration (μM)	0.0091 ± 0.0001	0.051 ± 0.001	0.082± 0.001
Accuracy error (%)	-8.6	2.8	3.5
Coefficient of Variance (%)	0.79	1.9	1.6

Another array of different canned food products was tested to screen for BPA contamination in human saliva after ingestion. Interestingly, one canned product, hot and sour soup from the Republic of China (i.e. Taiwan), revealed a high content of BPA contamination. The representative chromatograms are shown in Fig. 7. The final calculations, obtained through standard addition, for the amount of BPA in saliva, presumably from oral contamination by ingestion of the hot and sour soup, was 2.39 ng/mL (or 137 pg on column). The background BPA levels in the pre-exposure samples were negligible. The entire analysis was replicated with a separate can of the same food product purchased from another local supermarket to

ensure repeatability of these results. Instead of another standard addition experiment, a direct calculation of the BPA amount was determined directly by interpolation of the signal intensity of the canned food saliva from the equation obtained from a linear calibration curve of standards in blank saliva. The final BPA concentration was calculated to be 2.58 ng/mL (or 155 pg on column), which was in agreement with the first trial. This provides a proof-of-principle experiment to show that these methods are applicable for detection of BPA exposure sampling of human saliva.

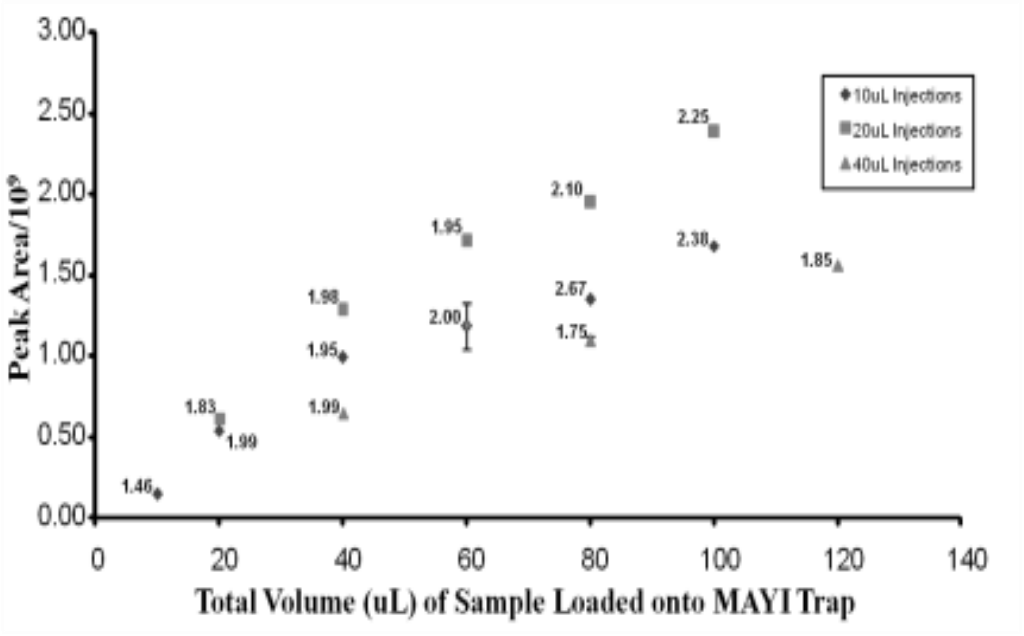

Figure 3.7 - Extracted ion chromatograms of doubly-dansylated BPA (348.1107 m/z) in saliva sample obtained from exposure to Hot and Sour Soup from Taiwan (ROC) using a MIL with incorporation of an internal standard. Figure shows (A) the saliva blank sample before ingestion of food, (B) the saliva zero point of the standard addition curve, (C) the 0.04 µM BPA spike from the standard addition curve, and (D) the representative internal standard spike of 0.05 µM d_{16}-BPA of an exact mass of 355.1557 m/z.

3.4 Conclusions

Widespread BPA contamination from a variety of sources has been an issue of great concern, which increases the need for rapid, high-throughput, and sensitive biomonitoring methods. With such analytical methods, proper steps can be taken to evaluate toxicity and establish appropriate regulations on a compound that is currently receiving a great deal of attention. Three separate, viable methods are presented to serve as tools in the study to detect trace quantities of BPA from a biological fluid such as human saliva. Part-per-trillion levels of oral exposure can be detected directly from sampling of saliva after ingestion of BPA. The extensive work presented in this manuscript has presented a new approach to sample preparation of biological fluids analysis in the form of bulk derivatization coupled to restricted access media as means to expedite the entire preparation protocol when compared to conventional methods. Such methods can be expanded to a wider range of analytes (and metabolites) in addition to a wider range of biological matrices, studies for which are currently being undertaken by our laboratory.

CHAPTER 4

AFFINITY MESH SCREEN MATERIALS FOR SELECTIVE EXTRACTION OF ANTIBIOTICS USING TRANSMISSION MODE DESORPTION ELECTROSPRAY IONIZATION

4.1 Introduction

Current antibiotic drugs are losing their effectiveness in the battle against bacterial infections[63-66]. This trend has caused increasing alarm in medical and scientific communities. As a result, there is a constant need for the development of new drug compounds that circumvent the prevalent bacterial resistances. Natural product extract are a common source for drug discovery[67]; however, the drug discovery process from these natural sources can be slow, tedious, and extremely taxing on limited resources. This provides an impetus to develop new, fast, and efficient methods for the identification, and accurate analysis of potential new chemical entities (NCEs) as drug target leads from natural sources. For this purpose, the use of mass spectrometry has shown promise, due in part to its remarkable versatility and high-throughput capabilities [68-72]. These capabilities have been further enhanced with the introduction of new ambient ionization techniques [3, 5, 73, 74]. The presented work builds on these concepts and introduces a prospective approach for antibiotic drug discovery using a new ambient ionization technique, transmission mode – desorption electrospray ionization [14, 15, 75, 76].

Human health is confronted by mounting bacterial resistance to our current arsenal of antibacterial therapeutics [63-66]. The problem is compounded by the fact that these resistances are hereditary mutations in nature and can be passed down to subsequent microbial offspring. Eventually, the wild type strains will succumb to the antibiotic treatments, but mutant drug-resistant bacteria strains will persist, rapidly multiply, and lead potentially to widespread epidemics if left unchecked. In addition to genetic mutation, bacteria can assimilate

antibacterial resistance through pathways, such as conjugation, transduction, or transformation[77]. In an effort to combat bacterial proliferation, antibiotic drug compounds are sought that can exert their effect through different mechanisms to initiate cell death. This includes binding and inhibition of critical enzymes, proteins, nucleic acids, plasmids, or even the outer cell wall, which protects the structural integrity of the bacterial cell [77, 78]. A clear strategy in antibiotic drug discovery is to identify and isolate new chemical entities that either work through known inhibition mechanisms or reveal new pathways for inducing bacterial cell death.

Drug compounds, such as β-lactams (e.g., penicillin and ampicillin) and macrocyclic glycopeptides (i.e. vancomycin and teicoplanin) are particular classes of antibiotics with well-characterized mechanisms that have greatly aided the medical field in the treatment of infections by Gram positive bacteria [77, 79]. The β-lactams inhibit key enzymes responsible for synthesizing the peptidoglycan, which composes the outer cell wall. On the other hand, macrocyclic glycopeptides prevent cell wall synthesis by binding to key *D*-alanine residues on a growing peptidoglycan chain that are crucial crosslinking points for adjacent peptidoglycan chains [79-83]. Thus, these drugs exert their action by compromising the structural integrity of the protective outer cell wall in Gram positive bacteria.

Vancomycin, in particular, is important because of its current role as a drug of last resort in medical treatment of bacterial infections after all other options have failed. However, the distressing issue is that the efficacy of vancomycin has declined with the appearance of vancomycin-resistant bacteria strains [84-86]. Vancomycin relies on critical hydrogen bonding interactions with the backbone and carboxyl-terminus of alanine residues in a *L*-lysine-*D*-alanine-*D*-alanine (Kaa) tripeptide sequence [87], which is revealed during peptidoglycan synthesis in wild-type Gram positive bacteria [79, 82, 83]. Disruption of these non-covalent interactions results in a loss of the antibiotic activity of vancomycin[79, 82, 83]. Because of its critical importance at the forefront of antibacterial drug discovery, a large volume of research

has explored the details of molecular recognition between vancomycin and Kaa using various analytical methods [88-91]. Importantly, studies that have used depsipeptide mimics (e.g. truncated peptides) of the natural system to probe these non-covalent binding interactions have shown good agreement with *in vivo* experiments and thus confirm the validity of these model systems. Along these lines, our group has recently demonstrated a direct affinity chromatography – mass spectrometry assay, which features use of the Kaa tripeptide to pin-point a chemical compound believed to be responsible for bactericidal activity in an extract of a Caribbean coral [92].

Although it has declined in current times, natural product sources or natural product-derived compounds still comprise roughly half of all new chemical entities (NCE) in the pharmaceutical industry [93-95]. However, Isolation and subsequent identification of natural products from complex extracts can be an arduous task. Traditional methods of drug discovery from natural products suffer from poor selectivity and require extensive resources and labor to identify, to isolate, and to advance a potential hit to the next stage of drug development. Instead, recent trends in drug discovery have shown more effort devoted towards higher efficiency and lower cost strategies that entail combinatorial synthesis approaches with high throughput screening [94-96]. Even so, despite the great benefits these new approaches would provide in theory, the practical results have not generally justified the monetary investment into their development [96]. Thus, there is still a dire need for newer, more efficient methods of identifying potential NCEs. Methods that can enhance targeted identification of NCEs from natural product sources would seem to be particular fruitful given the historical success of such compounds in the drug discovery pipeline.

Electrospray ionization – mass spectrometry (ESI-MS) has enabled sensitive analysis for investigative studies in a wide variety of fields ranging from large biomolecules and polymers to small molecules [16, 97]. Even so, the introduction of ambient ionization (AI) techniques over roughly the past decade has helped to overcome some of the limitations of conventional ESI

and opened the door for even greater versatility in terms of sample types [3, 5, 73, 74]. Desorption electrospray ionization (DESI), first developed by the Cooks lab, paved the way for future ambient ionization techniques [DESI] [3]. A significant amount of literature has demonstrated the capabilities of DESI in a number of different applications, including tissue imaging, trace detection of analytes from complex matrices, and miniaturization for use in hand-held systems [4, 5, 9, 73, 98-100]. A current survey of the literature reveals an ever-growing list of AI techniques available for use. These include: Extractive electrospray ionization (EESI) [11, 12], paper spray ionization [17], leaf spray ionization [101], direct analysis in real time (DART) [10], desorption atmosphereic pressure photoionization (DAPPI) [13], liquid sample desorption electrospray ionization [102], transmission mode – desorption electrospray ionization (TM-DESI) [14], and continuous flow – extractive desorption electrospray ionization (CF-EDESI) [16], among others. Each technique has its specific advantages and limitations, demonstrated in the context of a wide variety of applications.

TM-DESI, developed by the Brodbelt group, offers some attractive features for the purpose of this research work [14, 15, 75, 76]. The technique involves the use of an electrospray source oriented on-axis to the MS inlet. Relative to some other AI techniques, this orientation reduces the number of variables that need to be optimized prior to operation. In TM-DESI, analytes are desorbed from a porous solid support material (i.e. a mesh screen), which is placed in the spray path of the electrospray source. A careful exploration of experimental variables, mesh materials (polymer type, sample size, mesh pore size, etc.), and their effects on analytical performance has been reported [14, 15]. In addition to these studies, an interesting application was demonstrated where sulfhydryl analytes were extracted directly from plasma and urine[75, 76]. Many common drug compounds, such as captopril used in that work, contain free thiol groups and this method allows them to be selectively extracted from biological matrices. In order to do so, a neutravidin coating with a biotinylated thiol capture reagent was attached onto the screen and used to selectively couple with the sulfhydryl compounds by

covalent coupling. The analytes were then photocleaved from the solid mesh support prior to analysis by TM-DESI-MS [75, 76]. The current work builds on this technology to modify mesh materials as affinity capture devices to support rapid drug analysis.

In this work, we report a proof-of-principle for a new approach towards drug discovery through the use of novel affinity mesh screen materials in combination with TM-DESI-MS. The approach is designed on the principle of selective noncovalent recognition for a depsipeptide mimic, immobilized on a mesh screen, by compounds in solution, which are then subjected to mass spectrometric analysis. Poly(propylene) mesh screens were first coated with a polyacrylic acid film using pulsed plasma polymerization [103, 104] to provide the foundation upon which the screen could be functionalized. Synthesized Kaa peptide was then covalently attached through their N-terminus to carboxylic acid groups on the screen through a condensation reaction. Vancomycin was chosen as a model test compound to assess the affinity screen material for binding selectivity while spectinomycin, an antibiotic that inhibits enzyme production in bacteria cells, and does not bind Kaa tripeptide through selective interactions, was chosen as a negative control. A droplet sample preparation scheme was used to apply the antibiotic solutions onto the screen with the aim of eventually facilitating an automated work-flow. Such an application scheme allowed the generation of adsorption and desorption plots in a rapid fashion with which selectivity of the screen for vancomycin could be determined based on mass spectrometric ion response. Besides achieving an analytical platform for TM-DESI analysis, an important goal of this work, to optimize selective binding of model compounds to the functionalized affinity capture screens, was achieved.

4.2 Experimental

Pulsed Plasma Polymerization

The mesh screen substrates were coated with thin plasma-deposited polymeric films using a bell shaped RF plasma reactor. Details of the plasma reactor used for this work are

described elsewhere [104]. An RF power supply (13.56 MHz) was connected capacitively to two external electrodes. The monomer, methyl acrylic acid (MAA), was subjected to three freeze/thaw cycles, to remove dissolved gases, prior to use. The plasma reactor system was evacuated to less than 5 mtorr and then purged three times with monomer vapor. The working pressure was adjusted to 160 mtorr, as controlled by a butterfly valve. The plasma polymerized MAA (PMAA) films produced for this study were all approximately of 100 nm thickness. In prior studies, it had been demonstrated that the surface density of –COOH groups retained during the polymerization of carboxylic acids is readily controlled under pulsed plasma conditions, in which the -COOH surface density increases as the plasma duty cycle is reduced [103]. In the present case, the PMAA films were produced using a gradient layering technique which provides both high surface densities of –COOH groups and excellent adhesion of the plasma generated films to the solid substrates. For this purpose, initial plasma operation was carried out under continuous wave conditions for one minute followed by brief periods of pulsed plasmas of on/off ratios, in milliseconds, of 10/30 and 5/30 to strongly graft the plasma films to the substrates. Subsequently, pulsed plasmas, operated at 3 ms on/30 ms off periods for 15 minutes, were employed to provide an external film having a –COOH surface concentration of approximately 7% of the total carbon content, as documented by XPS analysis. Although the peak power employed during the runs at 3/30 on/off ratio was 150 W, the average power was significantly less in view of the pulsed mode operation. The average power is computed from the plasma duty cycle (ratio of ON time to the sum of the ON plus OFF time) multiplied by the peak power. Thus, the average power input was 13.6 W for the 3/30 pulses.

Fourier Transform – Infrared Spectroscopy and X-ray Photoelectron Spectroscopy

The PMAA film compositions were characterized by FT-IR Spectroscopy and X-Ray Photoelectron Spectroscopy (XPS). FT-IR spectra of the plasma-deposited PMAA films were obtained using a Brucker Model Vector 22, with spectra recorded using 4 cm^{-1} resolution. The

XPS spectra were acquired using a Kratos Axis Ultra DLD instrument equipped with a monochromator operated at a pass energy of 10.0 eV. A neutralizer was used in these measurements since the samples were non-conductive. The neutralizer was operated at a current of 1.7A and charge balance of 3.4V. The high resolution XPS spectra were analyzed (deconvoluted) using Casa XPS software. The binding energy of the carbon atoms not directly bonded to any heteroatoms was centered at 284.6 eV.

Synthesis of L-Lysine-D-alanine-D-alanine and Covalent Coupling to PMAA Coated Screen

The following work was performed by our collaborator Sumit Bhawal. All procedures on synthesis of Kaa tripeptide were developed and performed by him. This is a documentation of his synthetic procedure

Boc-*D*-Ala-*D*-Ala-OBzl: To a solution of pre-activated Boc-*L*-Ala-OH (1.89g , 10 mmol) in DMF (30 mL) was added 3.51 g (10 mmol) of the tosic acid salt of *L*-Ala-OBzl and i-Pr_2NEt (5 Eqv). The pre-activation of the acid and the work-up was performed with the following coupling reaction procedure. Boc-*D*-Ala-OH/Boc-*L*-Lys-OH, EDC (1.5 Eqv) , HOBt (1.1 Eqv, based on EDC) were added and stirred at 0 ^{O}C in DMF or dichloromethane (10-40 mL) depending on the scale of the reaction for 30 min. In a separate vial, the TFA or tosic acid salt of benzyl protected amine and N,N-diisopropyl ethylamine (5 Eqv; unless and otherwise stated) was mixed and stirred at 0 ^{O}C for 30 min. The amine was then added slowly to the pre-activated acid and stirred for 16 hr. When the reaction was complete (monitored by TLC, 95:5 DCM /Methanol), the reaction mixture was further purified by column chromatography.

D-Ala-*D*-Ala-OBzl: To the previous compound, 0.97g (5.63 mmol) of Boc-*D*-Ala-*D*-Ala-OBzl in 10 mL anhydrous DCM, 2.2 mL TFA was added (5 Eqv, based on compound 1a) at 0 ^{O}C. The reaction was allowed to run for 14 hr at room temperature and was monitored by TLC for completeness of the reaction. The excess TFA was removed by following the general workup procedure. Ethyl acetate (25 – 50 ml) was added to the reaction mixture, which was

then washed with saturated NH_4Cl (5 x 25 – 50 mL), followed by saturated NaCl (2 x 25 – 50 mL) and DI water (2 x 25 – 50 ml). The organic layer (ethyl acetate) was concentrated under reduced pressure to obtain the protected di- or tri- peptide. The resulting compound was further dried under high-vacuum for 3 – 4 hr. The dried compounds in some cases were washed with cold diethyl ether to remove any hydrophobic impurities. The TLC was checked for progress of the reaction and purity (95:5 DCM:Methanol). If required, flash column chromatography was performed (95:5 DCM/Methanol) to obtain the purified compound.

N-Boc-N^{e}-CbZ- *L*-Lys-*D*-Ala-*D*-Ala: 20 mL DCM was added to N-Boc-N-CbZ-*L*-Lys-OH, 2.1 g (5.5 mmol) and CDI 1 g (6.19 mmol). After evolution of CO_2 subsided, 0.77 ml (5.52 mmol) Et_3N, followed by 2 g (5.5 mmol) of 1b was added. The reaction was allowed to run for 24 hr.

$N^{\square}$-CbZ-(*L*-Lys-*D*-Ala-*D*-Ala): N-Boc-$N^{\square}$-CbZ- *L*-Lys-*D*-Ala-*D*-Ala 0.5g (0.815 mmol) when treated with excess (160 Eqv) TFA and DCM in 1:1 ratio afforded the Boc-deprotected tripeptide 0.46 g (0.73 mmol) in 90% yield as a white solid after crystallization from ethyl acetate and diethyl ether (TLC Rf = 0.39). Ethyl acetate (25 – 50 ml) was added to the reaction mixture, which was then washed with saturated NH_4Cl (5 x 25 – 50 mL), followed by saturated NaCl (2x 25 – 50 mL) and DI water (2 x 25 – 50 ml). The organic layer (ethyl acetate) was concentrated under reduced pressure to obtain the protected di- or tripeptide. The resulting compound was further dried under high-vacuum for 3 – 4 hr. The dried compounds in some cases were washed with cold diethyl ether to remove any hydrophobic impurities. The TLC was checked for progress of the reaction and purity (95:5 DCM/Methanol). If required, flash column chromatography was performed (95:5 DCM/Methanol) to obtain the purified compound. Characterization and assessment of purity of the synthesized tripeptides were performed using NMR on a JEOL 500 MHz NMR instrument and thin layer chromatography after each synthetic step to ensure completion of each.

Coupling N□-Cbz-(*L*-Lys-*D*-Ala-*D*-Ala-OBzl) to the solid support (PMAA grafted on polypropylene): Excess EDC (0.5 mmol) and 0.05 mmol of NHS was used to activate the carboxylic acid groups on the solid support, which was immersed in 5 mL of DCM. Then 0.05 mmol of Boc-deprotected amine (N-CbZ-*L*-Lys-*D*-Ala-*D*-Ala-OBzl), pre-mixed with 0.25 mmol N,N-diisopropyl ethylamine, was added to the reaction flask under inert conditions. The reaction was allowed to run for 24 hr. The screens were then washed 3 times with 50 mL water and methanol respectively. The BBr_3 deprotection was performed (after the Boc-deprotected tripeptide was tethered on to the carboxylic acid-grafted solid support) to remove the CbZ and BzL protecting group. Next, the screen was placed in 2.5 mL DCM at -10 ^{O}C, and 2.5 mL (50 Eqv. based on amine) of 1 M BBr_3 per screen, was added dropwise. After 1 hr, the ice bath was removed and 4 hr later, the reaction was quenched with ice – cold water, and then with water several times, and air dried. **Figure 1** shows a schematic flow diagram, beginning from PPP and progressing to Kaa coupling steps, to obtain the expected end product.

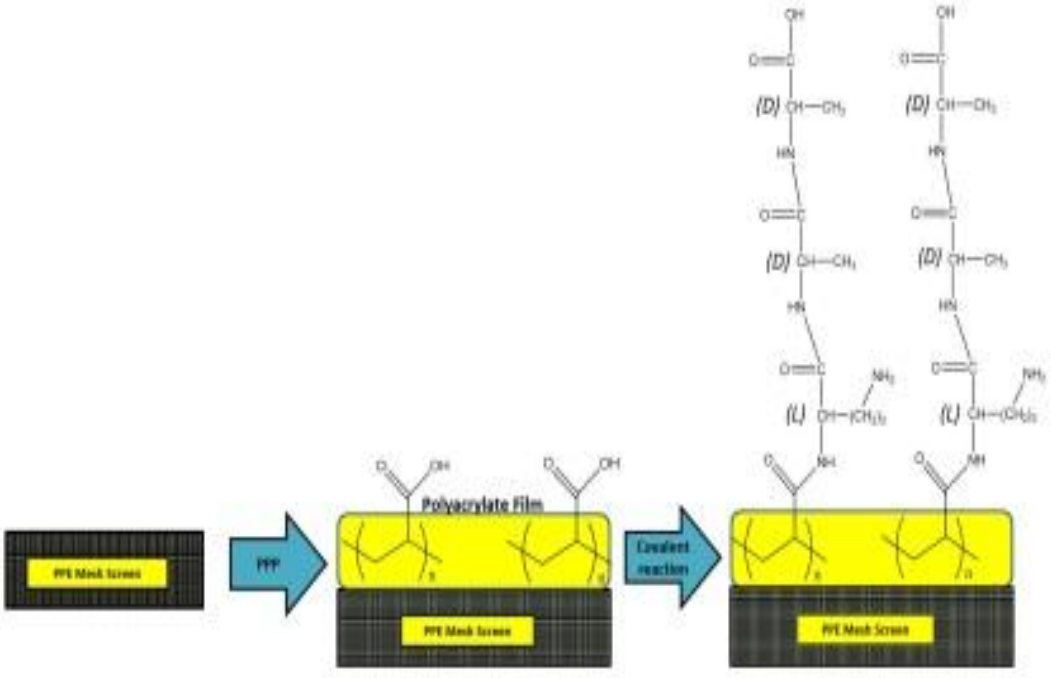

Figure 4.1 – Schematic diagram of the synthesis of the affinity mesh screen materials starting from a generic polypropylene mesh screen, to coating of the polyacrylate film by pulse plasma polymerization (PPP), and finally to covalent attachment of the Kaa tripeptide.

Chemicals and materials

Vancomycin hydrochloride was purchased from Calbiochem (EMD chemicals, Inc, San Diego, CA) and spectinomycin was purchased from Duchefa Biochemie (Haarlem, The Netherlands). LC-MS grade water, acetonitrile, and methanol were supplied by Burdick and Jackson (Muskeegon, MI, USA). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO) and acetic acid was obtained from J.T. Baker (Phillipsburg, NJ). Vancomycin and spectinomycin were both dissolved in 100% water at 1 mM stock concentrations; the samples were then diluted to a working concentration of 100 μM prior to use in analysis. The wash solution was chosen to be 5 mM ammonium acetate dissolved in water. To clean the entire

screen after completion of analysis, the screens were soaked in 100% methanol and dried three times to prevent carry-over from the previous trial.

Sample preparation: Application of Antibiotic Solutions to Affinity Screens

A droplet application sample preparation scheme was devised to investigate the difference in uptake between vancomycin and spectinomycin on the functionalized screens, as well as to provide a means for potential automation of sample preparation in future work. The details of the analytical workflow are shown in **Figure 2**. The area of the mesh screen was divided into eight regions, designated by a coordinate system with two rows (A and B) and four columns, in numerical order. Antibiotic solutions were applied as droplets in aliquots of 25 μL at a consistent rate. Gentle vacuum suction was placed directly underneath the screen in order to facilitate the transfer of the droplet across the screen. For the design of the adsorption experiments, the number of droplets was increased incrementally from one region to the next. The screen was then allowed to dry for 5 min and was mounted onto the TM-DESI apparatus for analysis.

For the solvent desorption experimental design, a different scheme was followed. First, each of the eight spot regions on the screen was exposed to five aliquots (25 μL) of antibiotic solution in succession. After drying, the spots were exposed to 10 μL applications of the 5 mM ammonium acetate wash solution. The number of applied wash aliquots was incrementally increased from one region to the next. The screen was then again allowed to dry for 5 min prior to TM-DESI analysis, where each region was individually sampled.

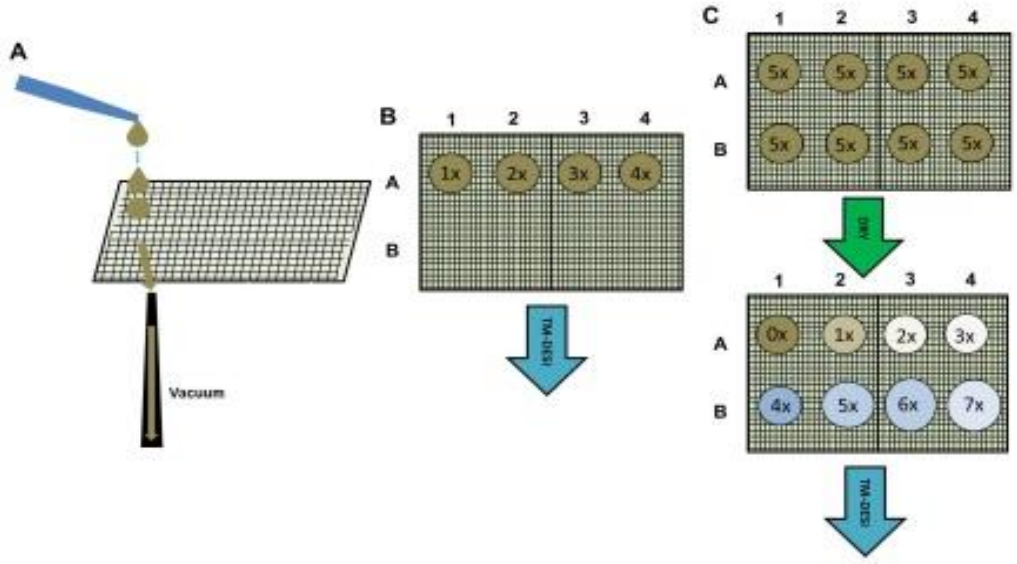

Figure 4.2 – Analytical workflow of antibiotic sample preparation for TM-DESI-MS showing the (A) visual representation of the application process using aliquots of antibiotic solution onto screen. Experimental design of (B) the adsorption measurements with incrementing aliquots of 50 μL of antibiotic solution and (C) the desorption measurements with incrementing aliquots of 10 μL of wash solution are also visually outlined. In the desorption experiments, the antibiotic solutions were first uniformly applied onto the screen and then dried for 5 minutes. Application of the wash solution was performed following the drying step.

TM-DESI Mass Spectrometry

A Thermo Scientific LCQ Deca XP (San Jose, CA) ion trap mass spectrometer with a custom-mounted on-axis electrospray ionization source was used for the TM-DESI analysis. Instrumental settings were as follows. Electrospray ionization was performed in the positive ionization mode with a spray capillary voltage of 5.0 kV and a nitrogen sheath gas flow rate of 80 arbitrary units. The mass spectrometer capillary inlet temperature was set at 200° C. Two minute scan times were taken per measurement with a scan range was set from 200-1800 m/z with 3 μscans and 200 ms scan times. Data analysis was performed using Thermo Xcalibur

Data Analysis software (ver 1.5). The instrumental configuration of the TM-DESI apparatus is shown in **Figure 3.** Distances for each variable indicated on the figure were optimized for maximum ion response using vancomycin deposited on an unmodified mesh screen prior to analysis. Positioning and movement of the desorption sample spot was controlled by a home-built *xyz*-positioning stage. The electrospray flow rate was maintained at 20 μL/min with a solution of water/MeOH (50:50) + 0.1% HOAc.

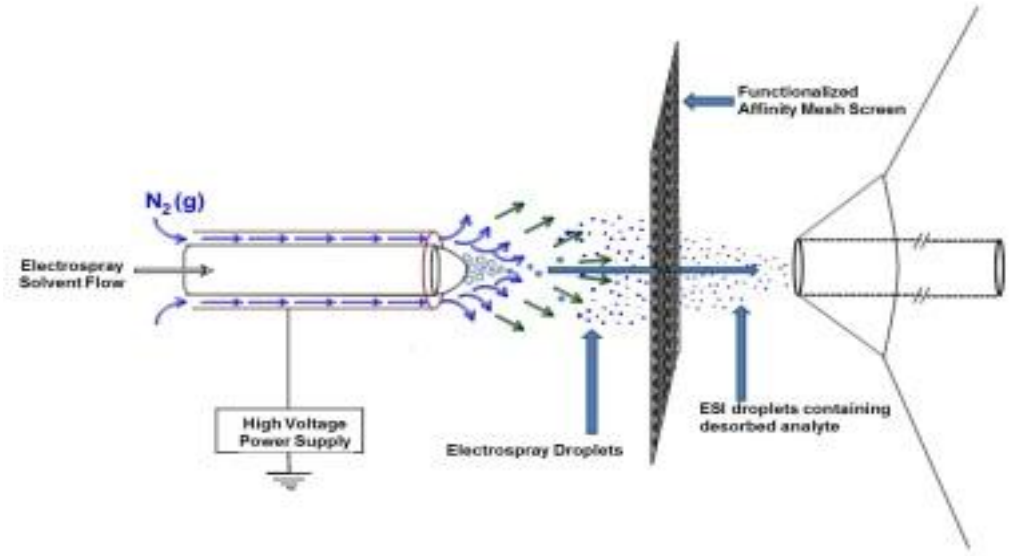

Figure 4.3 – Instrumental diagram of the TM-DESI-MS apparatus where the affinity mesh screen is placed in the path of the electrospray droplet flow towards the MS inlet

4.3 Results and Discussion

The overall goal of this work is to create a new analytical strategy with the potential for NCE discovery from natural extracts. We first designed mesh screen materials that could select for compounds that recognize the Kaa tripeptide. To do so, PPP was used to coat a generic polypropylene mesh screen with a polyacrylate polymer film. The Kaa tripeptide was then covalently attached onto the screen through the carboxylic acid group of the film coating. After application of different sample solutions to this functionalized screen, TM-DESI was used to rapidly desorb analytes off the screen and into the mass spectrometer for analysis.

Confirmation of the PMAA polymer coating by PPP onto the screen was crucial before continuation onto the next synthetic step. Extra precautionary measures were necessary because the carboxylic acid coating is the foundation on which the Kaa tripeptide would be coupled. XPS was performed on the coated screen to determine carbon, nitrogen, and oxygen content. The presence of oxygen from the XPS data points towards successful coating with a polyacrylate film layer. FT-IR was performed on the screen to successfully verify the characteristic absorptions of the carboxylic acid.

Next, a method was needed to assess the attachment of the Kaa tripeptide to the PMAA-functionalized screen. A perfluoroaniline test compound was chosen to assess the coating of the PPP step as shown in **Figure 4a**. The perfluoroaniline compound was attached onto a test sample section of the functionalized screen by covalent reaction through the carboxylic acid groups similar to how the Kaa tripeptide was attached. After reaction and rinsing of the prepared material, XPS analysis was performed to examine for the presence of a fluorine signature. The distinct fluorine signature shown in **Figure 4b** confirmed successful coupling of the perfluoroaniline group onto a PMAA coated screen, and thus provided the confirmation required to proceed onto the next step of the synthesis of selective screen

materials. The screen materials containing Kaa were prepared as described in the Experimental section.

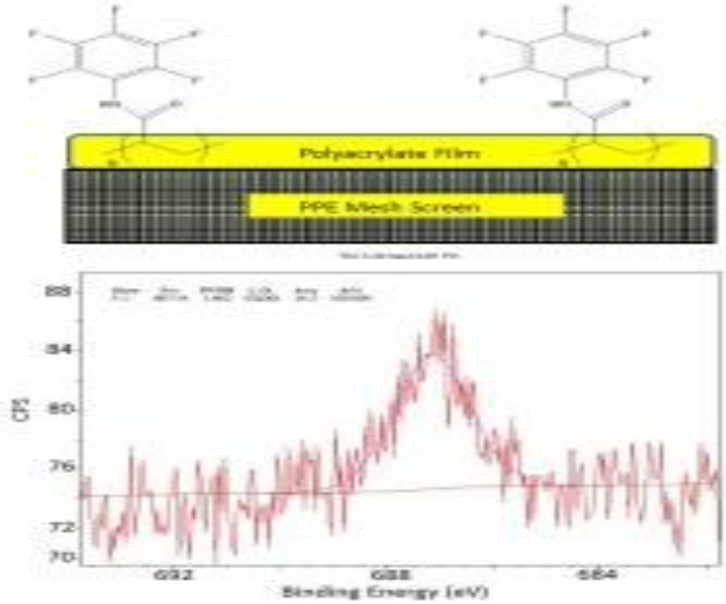

Figure 4.4 – XPS data showing the fluorine signature that was taken of the functionalized screen after covalent attachment of the perfluoroaniline test marker for indirect verification of attachment of Kaa tripeptide. Presence of the fluorine signal in XPS data indicates successful covalent attachment.

The adsorption and desorption experiments were carried out following the workflow outlined in **Figure 2**. Vancomycin was chosen as a positive control model test compound based on its known binding affinity for Kaa. Spectinomycin, an antibiotic known to inhibit ribosomal function of bacteria, not cell-wall synthesis, was chosen as the non-binding negative control. To monitor adsorption, the antibiotic solutions were applied onto the screen in increasing volumes of 25 μL. As the volume of applied antibiotic solutions increases, it was expected that the screen would

preferentially bind vancomycin over spectinomycin. Although it is non-specific for the Kaa tripeptide sequence, spectinomycin was still expected to be retained on the screen to some degree due to non-specific hydrogen bonding, Coulombic, and hydrophobic interactions. The results of the adsorption experiments are shown in **Figure 5a**. Because the droplet sizes, volumes, and removal rates were maintained in a consistent fashion during the sample preparation process, the number of droplets equates to a total volume of antibiotic solution applied onto the screen. This allows the data to be represented by tracking the concentration of antibiotic on the screen as a function of volume. Signal intensity acquired from the instrument was converted to concentration based on individually measured vancomycin and specintomycin response factors, which were determined to be 2.7×10^5 and 2.8×10^6 on average, respectively. Based on the comparison of these two plots, we see that our hypothesis has been supported by the data. Vancomycin concentration retained on the screen is seen to steadily increase with increasing application of solution sample, while spectinomycin shows a lesser accumulation with increasing application volume, indicating that non-specific interactions indeed occur, but are likely saturated at low concentrations.

The results of solvent desorption experiments further support the selective uptake of the targeted analyte. A wash solution of 5 mM ammonium acetate was chosen to desorb analytes from the screen by disrupting non-specific interactions that inevitably occur between the analytes with the screen. However, the pseudo-physiological 5 mM ammonium acetate solution also mimics the solution conditions of the recognition system in nature in order to preserve the specific hydrogen bonding interactions, making it the ideal wash solution for this experiment. The results of the desorption experiments are shown in **Figure 5b**. As seen from the results, vancomycin gradually desorbs from the screen as the applied volume of wash solution increases, indicating the presence of selective interactions of the screen with vancomycin. Conversely, spectinomycin, which did not bind in high concentrations to the screen in initial adsorption experiments, was almost completely removed from the screen within initial

applications of the wash solution. From these results, it is clear that the synthesized screen has selective extraction capabilities for vancomycin over spectinomycin. These findings provide the conclusive evidence to demonstrate proof-of-principle for this novel affinity extraction and analysis strategy.

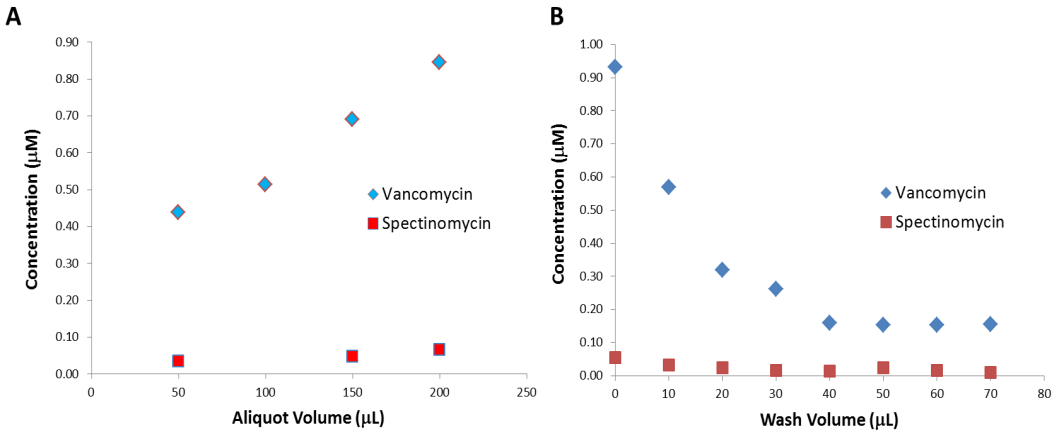

Figure 4.5 – (A) Adsorption and (B) desorption profiles showing preferential selection of vancomycin over spectinomycin by the affinity mesh screen. Vancomycin measurements are indicated with (◆) and spectinomycin measurements are indicated with (■). Concentrations of each antibiotic were obtained from conversion of relative signal intensity with response factors for each antibiotic.

4.4 Conclusions

In this work, we have introduced a new approach which may be applied towards antibiotic drug discovery using novel mesh screen affinity materials for rapid extraction and analysis of potential NCEs from natural product sources. The selectivity of the screen is based

on the recognition motif between the Kaa tripeptide and macrocyclic antibiotic compounds such as vancomycin. Proof-of-principle was demonstrated through adsorption and desorption profiles of model test compounds. Further optimization of this strategy still must be performed, an effort that will be crucial for their eventual use to extract bioactive compounds from natural product extracts. For instance, a more detailed wash protocol will be needed when screen materials are applied to a mock natural extracts solution. The synthesis of the affinity materials must also be optimized to maximize selectivity of target analytes. Other variables must also be fine-tuned in order for the screen to selectively extract multiple analytes that recognize the binding motif as well, which are currently being conducted within our research group.

CHAPTER 5

MANIPUATION OF PROTEIN CHARGE STATES THROUGH CONTINUOUS FLOW – EXTRACTIVE DESORPTION ELECTROSPRAY IONIZATION: A NEW AMBIENT IONIZATION TECHNIQUE

Mass spectrometry is a powerful tool for the analysis of large biomolecules and polymers. Various studies have demonstrated its capability for accurate molecular weight determinations with high specificity [105-107]. Importantly, electrospray ionization - mass spectrometry has enabled analysis of high mass molecules through the generation of multiply-charged ions.[108, 109] Multiple charging of high molecular weight proteins shifts their mass-to-charge (m/z) ratio to ranges that are within the limits of conventional mass analyzers and makes them more amenable to analysis by top-down tandem mass spectrometry techniques [110, 111]. For example, the availability of increased charge states facilitates more efficient electron capture dissociation (ECD) and electron transfer dissociation (ETD) by quadratically increasing the electron capture cross-section of the protein. This phenomenon has been shown to be useful for investigation of post-translational modifications and protein sequence determination [112-114].

Protein charge state distributions give insight into protein structure, as well as their folding/unfolding kinetics.[115-117] Much progress has been made to better understand and utilize charge state manipulation to optimize protein analysis.[118-124] Many of these techniques use a variety of chemical compounds, such as supercharging agents[120-122, 125, 126] or denaturing agents,[126, 127] to control the distribution of charge states observed in mass spectra. Performing such analysis, however, invokes certain challenges that are difficult to overcome by conventional ESI-MS. A major limitation in conventional ESI-MS is that the

additives must be directly incorporated in solution and infused together with the proteins of interest. As a result, analyses of these samples become complicated as many of the additives are not amenable to the electrospray process and can cause signal suppression. In addition, some proteins are highly sensitive to their solution environment and can be drastically affected by these additives. Only a limited number of ESI-friendly solvent systems are suitable for mass spectrometric analysis of proteins.[125] To overcome these obstacles, new strategies must be explored.

Many atmospheric pressure ionization (API) techniques, such as desorption electrospray ionization (DESI),[3] direct analysis in real time (DART),[10] desorption atmospheric pressure photoionization (DAPPI),[13] and extractive electrospray ionization (EESI),[11] have been developed in recent years. Each has been demonstrated for analysis of a wide variety of analyte and sample types. Ambient ionization techniques facilitate the formation of molecular ions by interfacing an ion source with a mutually exclusive analyte source. In other words, ambient ionization techniques generally allow for separation of the sample and source, enabling independent optimization of each for maximum analytical performance.

EESI, developed by the Cooks group,[11, 128, 129] has many admirable qualities. It has shown potential to alleviate problems with obtaining charged proteins due to its inherently soft ionization nature.[130] EESI interfaces two spray sources, a conventional electrospray and a simple aerosol sample spray, placed at defined angles and aimed towards a central focal point. Analytes can be extracted from their original solution by the electrospray and transferred into the inlet of the mass spectrometer, leaving behind unwanted matrix components and possible interferences.[12] Another new development in the field of ambient ionization is transmission mode – desorption electrospray ionization (TM-DESI), which was recently introduced by the Brodbelt group.[14, 15, 75, 76] In TM-DESI, analytes are desorbed from a solid support material (i.e., a polymeric mesh screen), which is placed in the spray path of the

electrospray source. The designation of transmission mode indicates a fixed 180 degree angle between the ESI source and the MS inlet. Such a configuration alleviates a great deal of optimization, which is often required for other ambient ionization approaches.

Drawing from aspects of both EESI and TM-DESI, we sought a novel ionization technique for ambient ionization. Continuous flow - extractive desorption electrospray ionization (CF-EDESI) features an electrospray source set on-axis with the MS inlet and an additional continuous flow needle set orthogonal to the ion-spray path. An instrumental diagram of the CF-EDESI apparatus is shown in **Figure 1**. In this ionization technique, the ESI process can be independently optimized, and the analyte of interest is only subjected to the shrinking droplet portion of the electrospray process. The analyte is introduced to the system by continuous flow through a hypodermic needle. The continuous stream of liquid, which is focused to the tip of a hypodermic needle, is placed directly in the path of the electrosprayed solvent. The hypodermic needle is critical to efficient CF-EDESI. The continuous flow is focused to the tip of the needle where it can be extracted/desorbed by the spray emitting from the Taylor cone of the ESI source.

The exact mechanism of CF-EDESI is still unclear. The technique is akin to combining EESI and TM-DESI ionization sources. In one aspect, the sample analyte can be speculated to be desorbed from the bulk liquid phase of the continuous sample flow by the electrospray droplets, similar to that shown previously for DESI of liquids.[131] Yet, the CF-EDESI source is set in the transmission mode (the ESI source and MS inlet are 180° from each other). Extractive processes can certainly be conceivable as well, given the mixing between the two liquid media. Yet, no nebulization of the sample is performed or required, differentiating this technique from EESI. More work is needed to elucidate the exact mechanism of CF-EDESI. Regardless, analytes may be introduced in a particular medium through the CF needle, and are only subjected to the additives contained in the ESI solvent for a brief period of time following extractive desorption. Here we show that the charge states of a protein, introduced through the

CF needle in a non-denaturing solution, can be manipulated by desorption/extraction with acidified electrospray droplets.

In reference to **Figure 1**, the indicated distances between the electrospray source needle and the continuous flow needle (1.5 mm), and between the electrospray source and the inlet of the mass spectrometer (8 mm), were optimized with in-line injections of a standard solution of 100 μM bradykinin through the CF needle to obtain the most abundant signal response from the system. Needle positioning was controlled by a home-built xyz stage. Samples were prepared and introduced through the CF-needle by direct infusion. The flow rate through the electrospray source was 10 μL/min, and the flow rate through the CF needle was also 10μL/min, for all experiments.

The CF-EDESI apparatus was interfaced to a Thermo LCQ Deca XP quadrupole ion trap mass spectrometer. A source housing was designed and constructed from an aluminum block to orient the Thermo ESI probe on-axis, and directly in-line with the inlet to the mass spectrometer. The spectra were acquired in the positive ionization mode with a spray voltage of 4.0 kV. From five to ten minute direct infusion sample runs, one minute of acquisition time (approximately 50 scans from 150-2000 m/z) were averaged to obtain the data presented. The transfer capillary temperature was set to 250 °C and the sheath gas was set at 60 arbitrary units.

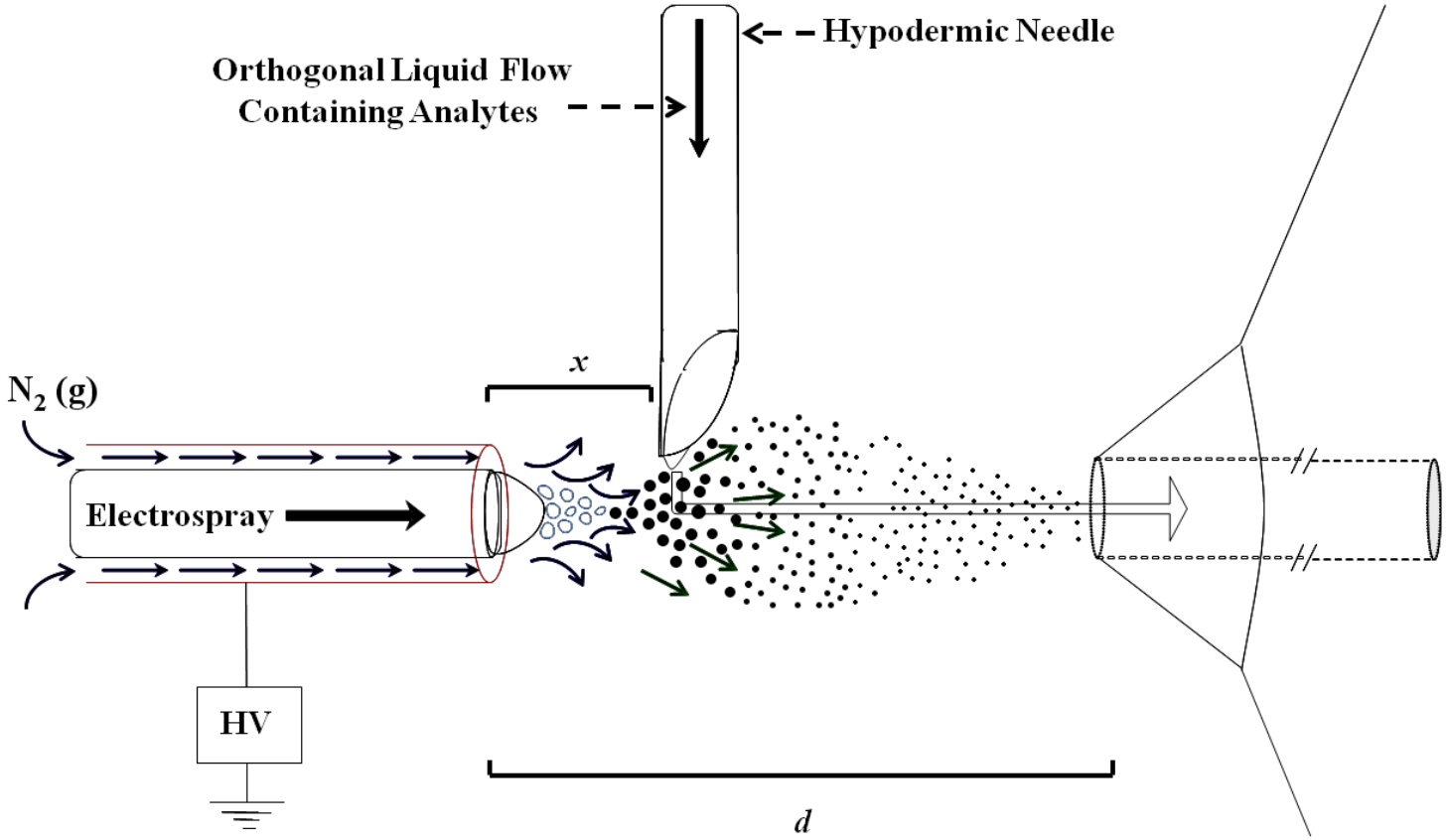

Figure 5.1 - Instrumental diagram of the CF-EDESI apparatus where the distance between the ESI source and the continuous flow needle (*x*) is 1.5 mm; the distance between the ESI source; and the distance between the ESI source and the inlet to the mass spectrometer (*d*) is 8.0 mm

A series of experiments was performed to manipulate the charge state distributions of cytochrome c using the CF-EDESI technique. Cytochrome c is a common protein standard used in ESI-MS experiments.[115, 116, 119, 120] Solutions of 50/50 water/methanol with increasing concentrations of acetic acid (0%, 2%, 6%, and 10%) were prepared and electrosprayed. From the CF needle, direct infusions of 100 μM cytochrome c in 100% water were made. Representative spectra of the CF-EDESI experiments are shown in **Figure 2 (A-D)**. For comparison, a ESI-MS mass spectra of 100 μM cytochrome c, generated in 50/40/10 methanol:water:acetic acid with the on-axis spray source, is shown in **Figure 2E.**

Clear evidence of the ability to alter the charge state distribution of cytochrome c by CF-EDESI was demonstrated. Addition of 2% acetic acid to the ESI source produced a base peak with a charge state of +9 and a dramatic decrease of the lower charge states relative to that seen in the spectra acquired with no acetic acid. This shift is due simply to additional protons available in the system, and is not believed to be due to denaturing of the protein.[115] According to previous studies, denatured cytochrome c exhibits characteristic shifts of the charge state distribution to much higher states centered around the +16 through +18 states.[115, 116] Partially denatured cytochrome c exhibits a bimodal distribution between low-charge-state folded and high-charge-state unfolded states, the extremes of which are heavily dependent on the pH of the solution.[115] As more acid is introduced to the CF-EDESI set-up, higher charge states become populated, and the spectra begins to clearly show the appearance of a bimodal distribution of charge states, giving the indication that the cytochrome is becoming partially denatured. This loss of native cytochrome c structure is not unexpected, as the folding kinetics of cytochrome c is reported to be on the microsecond time scale,[132] which is faster than the millisecond time scale of the electrospray process. We can only infer that the unfolding kinetics, observed here, are also quite rapid. A clear contrast is shown between data collected

by CF-EDESI with 10% acetic acid (Figure 2D) and that collected by direct ESI-MS (Figure 2E). With conventional ESI, the base peak moves to a much higher charge state. Clear evidence of bimodality, due to the majority of cytochrome c ions being denatured, can be observed in the spectrum.

The results from this work can be compared to work contributed by the McLuckey group where the manipulation of charge state distributions was achieved by exposure to gaseous acids.[124] This technique used acidic vapors, leaked into the nitrogen drying gas of the electrospray interface, to manipulate the charge state distributions of several proteins. The resulting data from this work also yielded dramatic shifts in the charge state distributions similar to the results shown with CF-EDESI. Although this is a different approach to manipulation of charge state distributions, both techniques essentially introduce excess protons to alter the charge state of a protein molecule. Even the actual timeframe of the charge state alteration process may be similar. The acidic vapors affect the protein charge states after droplet formation. Proteins on the surface of the ESI droplets would be exposed to the acidic vapors, even as some of the acidic vapors are likely dissolved into the droplets during the spraying process. In CF-EDESI, the acidified electrospray droplets are formed just prior to extracting/desorbing and ionizing the protein from the continuous flow needle. Overall, the effects on cytochrome c charge state distribution incurred by introducing acid vapors are largely consistent with the data obtained from the CF-EDESI experiments.

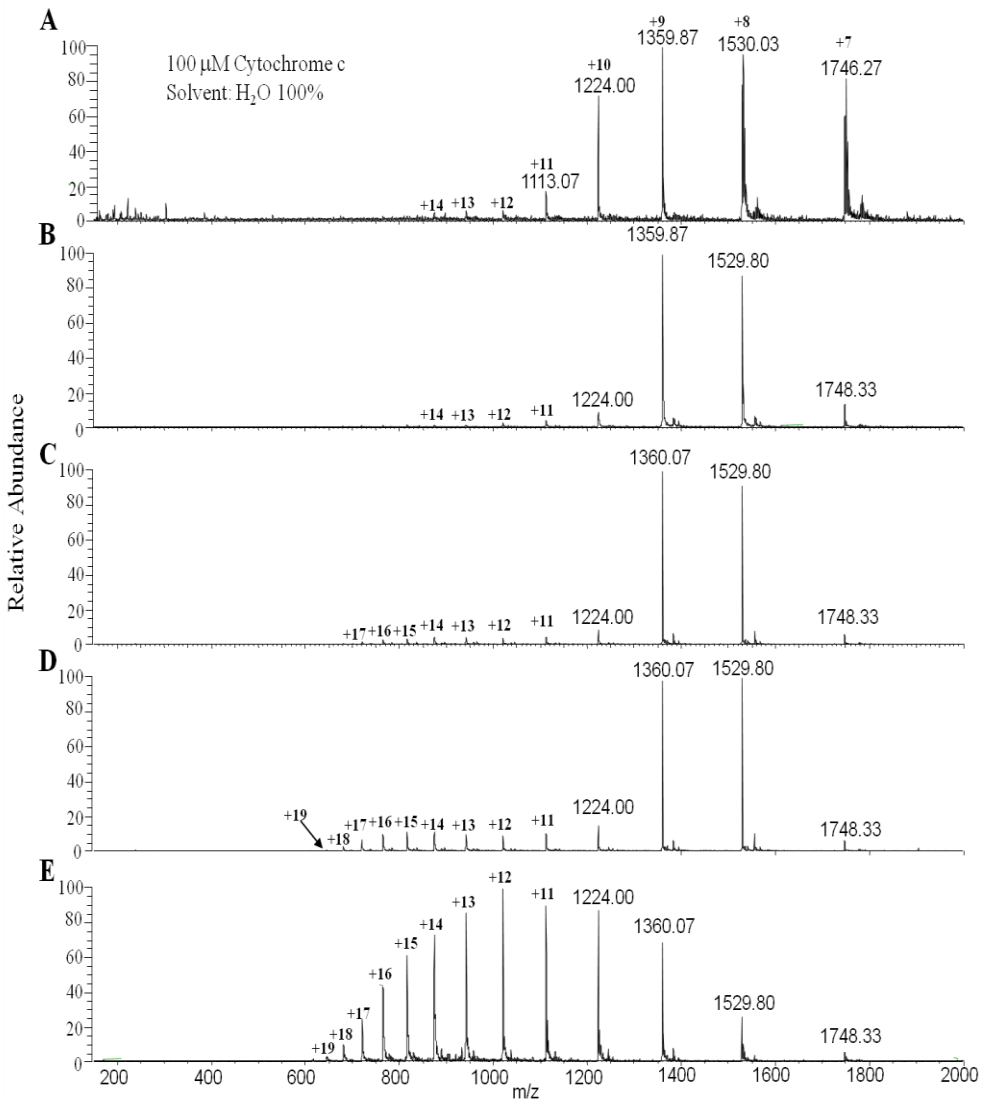

Figure 5.2 - Representative spectra of the series of CF-EDESI experiments with 100 μM cytochrome c introduced orthogonally from the continuous flow needle to (A) 0% acetic acid content, (B) 2% acetic acid content with water/methanol (48:50, v/v), (C) 6% acetic acid content with water/methanol (44:50, v/v), and (D) 10% acetic acid with water/methanol (40:50, v/v) content in the electrospray solvent system. Charge states are assigned accordingly. A comparative spectrum from experiments conducted with direct infusion in conventional electrospray is shown in (E) with 10% acetic acid in water/methanol (40:50, v/v).

Table 1 gives a numerical representation of the data in the form of the calculated average charge state (Z_{avg}), the maximum charge state (Z_{max}), the base charge state (Z_{base}), and the average molecular weight calculated by standard deconvolution practices. Average charge state was calculated as shown in previous work.[119] In CF-EDESI, the base peak of the distributions were consistently observed at the +9 charge state, while the weighted average charge state increased with increasing acetic acid concentration. Thus, manipulation of the charge state distributions was accomplished.[115, 122] In contrast, the distribution of cytochrome c obtained in 10% acetic acid with conventional ESI showed Z_{base} = 12 and Z_{avg} = 12.3, more consistent with gross denaturing of the protein. Another interesting observation is the enhanced precision of the molecular weight calculations given by distributions obtained following the addition of acetic acid into the electrospray. This can be attributed to the narrower peak widths obtained, presumably to a higher degree of protonation, as opposed to cationization by or inclusion of other species, which broaden signals for native-folded proteins analyzed by conventional ESI-MS.[133] The enhanced precision obtained by the CF-EDESI approach accentuates the possibility to more definitively probe or identify post-translationally-modified or point-mutated proteins when spectral charge state deconvolution is used.

Table 5.1 - Charge state and molecular weight calculations determined from CF-EDESI (and conventional ESI-MS) spectra

Mode	% Acetic Acid	Z_{avg}	Z_{max}	Z_{base}	Molecular Weight
CF-EDESI	0	8.79	14	9	12215 ± 78
CF-EDESI	2	8.63	14	9	12224 ± 19
CF-EDESI	6	9.06	16	9	12243 ± 25
CF-EDESI	10	9.95	19	9	12267 ± 17
ESI-MS	10	12.3	19	12	12310 ± 180

In addition to the experiments performed with cytochrome c, another series of data was collected to investigate the manipulation of lysozyme charge states using a common supercharging agent, sulfolane.[125] Lysozyme is another well-studied protein that is reported to exhibit folding kinetics on the millisecond timescale, much slower than cytochrome c.[134] Lysozyme (100 μM in 100% water) was introduced through the CF-needle, and sulfolane was introduced in different concentrations through the electrospray source. **Figure 3** illustrates the effects of sulfolane concentration on individual lysozyme charge states when analyzed by CF-EDESI. Each charge state was plotted to show increase or decrease as the sulfolane content in the electrospray solvent was varied. The signal intensities were normalized against the base peak signal intensity of each charge state distribution. The resulting data indicates that the charge states are indeed affected by sulfolane. It is important to note that the higher charge states, especially the +12, +13, and +14 charge states, steadily increased in intensity with increasing sulfolane concentration while the lower charge state intensities showed variable alteration. Lower charge states appear to sacrifice intensity for slightly higher charge states at

low concentrations of sulfolane. However, at high concentrations of sulfolane, lower charge states largely recover in intensity. The use of a high concentration of sulfolane in this arrangement appears to be beneficial for both increasing charge state magnitude and maximizing charge state intensity. Spectra for this series of experiments are provided in Supplementary Figure 4.

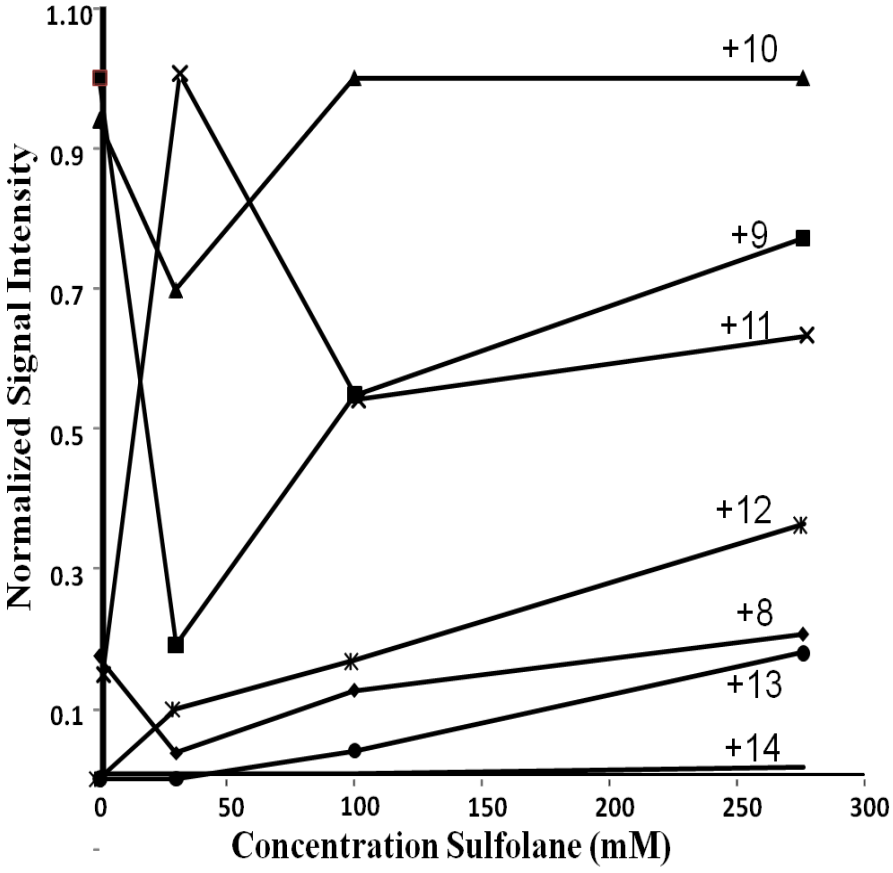

Figure 5.3 - Manipulation of lysozyme charge states with increasing sulfolane concentration using CF-EDESI-MS. Lysozyme (100 μM in 100% H_2O) was introduced through the CF-needle, whereas sulfolane was introduced through the electrospray source (50/50 H_2O/MeOH).

In summary, we have presented and termed a new ambient ionization technique. CF-EDESI incorporates key aspects derived from EESI and TM-DESI, but is different. The ESI source and mass spectrometer inlet are set in an 180° orientation, which minimizes some optimization parameters. However, an important component for which the placement must be optimized is the hypodermic CF-needle set orthogonal to, and terminating directly in the ESI spray plume. No pneumatic nebulization is used to assist the sample delivery through the CF-needle. Manipulation of cytochrome c charge state distributions was demonstrated with these initial experiments using acetic acid. In addition, the effect of sulfolane on the charge state distribution of lysozyme was also investigated. Further experiments to a) extend this study and b) obtain a better understanding of the ionization process are underway. Additionally, this novel ionization technique can be extended to a wide range of applications where the ESI source conditions and the CF solvent conditions can be independently optimized.

CHAPTER 6

REVERSED PHASE LIQUID CHROMATOGRAPHY HYPHENATED TO CONTINUOUS FLOW – EXTRACTIVE DESORPTION ELECTROSPRAY IONIZATION – MASS SPECTROMETRY FOR ANALYSIS AND CHARGE STATE MANIPULATION OF UNDIGESTED PROTEINS

6.1 Introduction

Electrospray ionization mass spectrometry (ESI-MS) is a powerful analytical tool that has facilitated numerous scientific studies [68-71, 135]. The versatility of soft ionization analysis was further enhanced with the development of new ambient ionization, or atmospheric pressure ionization (API), techniques [3, 5, 73, 74]. Two hallmark AI techniques, desorption electrospray ionization (DESI) [3] and direct analysis in real time (DART) [10], demonstrated in a wealth of literature that AI techniques are capable of analysis of a wide variety of analytes directly from different sample types. Since DESI and DART, new AI techniques have been presented, each occupying their own niche in the analytical field [11, 13, 14, 16]. Despite their differences, these ambient ionization techniques generally follow certain principles. An important feature of ambient ionization is the interfacing of an ion source with a mutually exclusive analyte source. This arrangement allows for the separation of the sample from the ion source, which can be then be independently optimized. This inherently leads to another important aspect of ambient ionization techniques, which allows analysis to be performed on a sample in its native, unaltered state with minimal or no sample preparation required.

The coupling of ambient ionization to separation techniques offers even greater advantages. For example, complex mixtures that involve multiple analytes of interest can be isolated prior to ambient ionization for accurate and sensitive analysis without the use of a high resolution mass analyzer capable of high mass accuracy [136]. In addition, ambient ionization techniques allow sensitive analysis from non-polar solvents, which are used in normal phase

liquid chromatography. Nonvolatile additives and buffer salts, such as phosphoric acid/phosphate or citrate, which are commonly used to improve chromatography, can be coupled to MS detection without their associated ion suppression effects. These advantages are particularly useful in overcoming challenges encountered when hyphenating conventional ESI to high performance liquid chromatography (HPLC). However, there is a limited amount of applications featuring the combination of ambient ionization with HPLC [136]. Currently, only DESI and DART, the most developed techniques, have clearly demonstrated viable examples of chromatographic analysis in conjunction with ambient ionization[136, 137]. DESI, in fact, has also been applied to a number of different separation techniques including thin layer chromatography [138-140], capillary electrophoresis [141], and solid phase extraction [142, 143] in addition to its application with HPLC. Thus, it would be greatly beneficial to explore the hyphenation of separation techniques such as HPLC with other ambient ionization approaches for the creation of methods with greater analytical performance.

Recently, our group has introduced a new ambient ionization technique called continuous flow, extractive desorption electrospray ionization (CF-EDESI)[16]. Following principles similar to other ambient ionization techniques, CF-EDESI features a dual sample source, which allows for separate introduction of sample analyte from the electrospray ion source. The electrospray source is aligned on-axis with an MS inlet while a hypodermic needle is positioned orthogonal to the electrospray. Through the hypodermic needle, sample is introduced in a continuous stream of liquid flow into the path of the electrosprayed solvent. The generated electrospray droplets come into contact with the sample at the tip of a hypodermic needle and analytes of interest are extractively desorbed, ionized, and transferred to the MS inlet. In addition to the dual sample source, the fixed 180 degree angle between the ESI source and MS inlet is a particularly attractive feature of CF-EDESI in that it circumvents further optimization, which is often required for other ambient ionization techniques.

Initial work presented an application using CF-EDESI in order to perform manipulation of protein charge states [16]. Increases in the protein charge state distribution was achieved by taking advantage of the dual sample source of CF-EDESI, where separation of the sample protein from the charging additive prior to analysis was possible. Increasing the protein charge states offers a number of advantages. The availability of increased charge states facilitates more efficient electron capture dissociation (ECD) and electron transfer dissociation (ETD) by quadratically increasing the electron capture cross-section of the protein, which greatly benefits investigations of post translation modification [112-114]. Also, increasing charge state distributions lowers high molecular weight proteins down within the limited ranges of certain mass analyzers. As a result, much research effort has been devoted towards finding new ways of manipulating protein charge state distributions using an extensive list of different, viable charging additives [120-122, 124-126, 144]. However, the majority of work in this area has focused on denatured proteins using conventional ESI/nano-ESI. A primary limitation of supercharging in conventional ESI-MS is that the supercharging agents, which generally are also protein denaturants, must be directly incorporated in solution and infused together with the proteins of interest. With the extensive list of viable charging additives, only a limited number of ESI-friendly solvent systems are suitable for mass spectrometric analysis of proteins in their folded, native form [122, 145]. Recently, new efforts are devoted towards achieving the same enhanced protein charge states with the added feature of maintaining the native protein structural conformation [122, 145]. Preserving the protein folded structure has its merits as it allows characterization of noncovalent protein-protein and protein-substrate interactions. With its capability to separate protein analyte from charging additive, CF-EDESI would naturally be able to accommodate protein charge state manipulation of natively folded proteins.

In this work, we present an application of CF-EDESI demonstrating its ability to handle protein charging experiments while preserving native structural conformation. The experiments focus on myoglobin and observation of its charge state distribution as it transitions from holo-

enzyme to apo-enzyme with varying concentrations and types of charging additives. The experiments were performed on a LCQ Deca XP, which admittedly may not be the most ideal instrument to study protein conformational studies. However, the results still indicate softer ionization capabilities of CF-EDESI over conventional ESI. In addition to protein charging and conformational studies, we also report a proof-of-principle of HPLC-CF-EDESI, demonstrating the hyphenation of CF-EDESI to liquid chromatography. A reversed phase liquid chromatography (RPLC) method was developed to resolve seven protein analytes on conventional ESI. The method was then directly imported onto the CF-EDESI platform for a side-by-side comparison of the two techniques. Chromatographic effluent coming from the column was directed through the hypodermic needle into the path of the electrospray solvent. Different concentrations of charging additives were introduced through the electrospray source to show that manipulation of protein charge states can be performed simultaneously with chromatographic separation. Considerations into sensitivity and reproducibility are taken as well when comparing LC-CF-EDESI method to LC-ESI. The results show a clear example of the advantages offered from hyphenation of an ambient ionization technique to HPLC.

6.2 Experimental and Materials

Chemicals and materials

Protein standards, including cytochrome c, lysozyme, transferrin, myoglobin, hemoglobin, lactalbumin, and bovine serum albumin (BSA), were purchased from Sigma-Aldrich (St. Louis, MO). LC-MS grade water, acetonitrile, and methanol were supplied by Burdick and Jackson (Muskeegon, MI, USA). Formic acid and acetic acid were obtained from J.T. Baker (Phillipsburg, NJ), while ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO). Sulfolane and *m*-nitrobenzyl alcohol were purchased from Sigma-Aldrich (St. Louis, MO).

Progesterone and bradykinin standards were also purchased from Sigma-Aldrich (St. Louis, MO).

CF-EDESI Mass Spectrometry

A Thermo Scientific LCQ Deca XP (San Jose, CA) ion trap mass spectrometer with a custom-mounted on-axis electrospray ionization source was used for the CF-EDESI analysis. Instrumental settings were as follows. Electrospray ionization was performed in the positive ionization mode with a spray capillary voltage of 5.0 kV. The nitrogen sheath gas was set at a flow rate of 60 arbitrary units for direct infusion measurements, while it was adjusted to 80 arbitrary units to handle the faster flow rates of the chromatographic separations. The mass spectrometer capillary inlet temperature was set at 200° C. Two minute scan times were taken per measurement with a scan range was set from 800-2600 m/z on the high mass range setting of the instruemtn with 3 μscans and 200 ms scan times. Data analysis was performed using Thermo Xcalibur Data Analysis software (ver 1.5). The instrumental configuration of the CF-EDESI apparatus has been described previously [CF-EDESI]**.** In a similar fashion as in previous works, the distances between the electrospray source needle and the continuous flow needle (1.5 mm), and between the electrospray source and the inlet of the mass spectrometer (8 mm), were optimized with in-line injections of a standard solution of 10 μM progesterone and 100 μM of bradykinin through the CF needle to obtain the most abundant signal response from the system. For direct infusion experiments, electrospray flow rate was maintained at 20 μL/min while the sample analyte through the CF needle was set also set at 20 μL/min using two syringe pumps.

Sample Preparation

All protein stock standards, except hemoglobin, were dissolved in 100% water at 1 mM stock concentrations; the samples were then diluted to a working concentration of 100 μM prior to use in analysis charging analysis. Hemoglobin was dissolved in 5 mM NH_4OAc in 100%

water to prevent precipitation because being diluted to a working concentration of 100 μM. In protein chromatography, mixtures of all seven proteins were prepared from 100 μM stocks. The response factor measurements required three measured concentrations of each protein. The concentration scheme of the three mixtures is listed as follows: Mixture 1 consisted 10 μM lysozyme, 5 μM cytochrome c and transferrin, and 1 μM myoglobin, lactalbuin, hemoglobin, and BSA; Mixture 2 consisted of 15 μM lysozyme, 8 μM cytochrome c and transferrin, and 5 μM myoglobin, lactalbuin, hemoglobin, and BSA; Mixture 3 consisted of 20 μM lysozyme, 12 μM cytochrome c and transferrin, and 10 μM myoglobin, lactalbumin, hemoglobin, and BSA. Electrospray solvents were prepared in water/MeOH 50:50 (v/v) composition with volume adjustments made to accommodate the varying concentrations of charging additive.

Reversed Phase Chromatography

Reversed phase liquid chromatography was performed using a Surveyor LC quartenary pump system from Thermo Scientific (San Jose, CA). A Phenomenex Aeris WIDEPORE XB-C18 (2.1x100 mm; 3.6 mm) column was chosen as the analytical column for this work. Flow was set at 0.350 mL/min; column Temperature was 40°C. A 25 mL injection volume was used. A binary mobile phase of MPA: water + 0.1% formic acid and MPB: ACN + 0.1% formic acid was used for the separation. A gradient program was used to resolve the protein analytes. The program began with a one minute isocratic segment with a mobile phase composition of 15%B. Then a gradient was carried out starting from 15%B to 30%B in 19.5 minutes; a second gradient was then carried out from 30%B to 50%B in 12.5 minutes. The pump was then held in a 10 minute wash segment at 99%B followed by a reequilibration step at the end for 5 minutes. When coupling the chromatographic method to CF-EDESI, extra considerations must be taken into account. A 0.350 mL/min flow rate eluting from the CF needle caused droplet formation at the tip of the CF needle. As mentioned in previous studies [CF-EDESI], a continuous liquid flow must be maintained from the CF needle for optimal CF-EDESI analysis. Droplet formation can

be attributed to the high flow rate of the chromatographic method. To remedy this problem, a 5:2 flow split was introduced post column so that the liquid flow rate from the CF needle was roughly 0.120 mL/min. An electrospray solvent flow rate of 0.120 mL/min was used to match the flow rate from the CF needle.

6.3 Results and Discussion

Charge State Manipulation of Myoglobin using Direct Infusion

Charge state manipulation experiments were conducted with CF-EDESI using myoglobin as the representative protein. The goal of these experiments is to demonstrate the softer ionization capabilities offered by CF-EDESI over conventional ESI while using charging agents to achieve increased protein charge states. Myoglobin is a small molecular weight protein similar to cytochrome c and lysozyme, which were used in previous studies of protein charging with this ambient ionization technique. Myoglobin is unique from the latter two proteins in that it exists in a holo-enzyme form, where the protein molecule has maintained its native folded conformation around a prosthetic heme group, and in a apo-enzyme form, where the molecule is denatured and interactions with the heme group have been lost. The structural integrity of the molecule can be clearly distinguished from its charge state distributions seen from MS analysis. The folded protein complex has less accessible protonation sites and thus occupies lower protein charge states than those observed in a completely denatured protein molecule. Molecular weight calculations from the m/z values of the charge states also indicate loss of the heme group in the higher charge state distributions of the apo-myoglobin. In CF-EDESI, indicated additive concentration is separately electrospray through the ESI source, while in conventional ESI, the additive concentration must be premixed with the protein analyte prior to direct injection through the electrospray source.

Initial measurements of myoglobin using conventional ESI without the presence of denaturing agents revealed that the protein exists in a partially denatured state when electosprayed, where a certain percentage of protein molecule occupy the holo-myoglobin state

with the remaining percentage in the apo-myoglobin state. The same finding was observed with initial CF-EDESI measurements of myoglobin under the same solvent conditions. This indicates that the particular instrument used for these measurements may not be ideal for preserving native protein conformations. However, this does not preclude the use of the instrument to demonstrate the application of CF-EDESI for these studies. A comparison can be drawn between CF-EDESI and conventional ESI when considering the ratio of holo-/apo-myoglobin forms. A lower ratio indicates that the starting equilibrium between holo- and apo-myoglobin has shifted towards a larger population of denatured protein molecules, while a conservation of the ratio value indicates that protein folded structure has been preserved during the charging process. Based on this premise, the two ionization techniques can be compared.

Figure 1 shows the graphical representation of the results obtained from the protein charging experiments. Acetic acid, *m*-nitrobenzyl alcohol (mNBA), and sulfolane were chosen as charging agents to facilitate increased protein charge states. The plot of the holo-/apo-myglobin ratios from CF-EDESI (indicated with ◆) seen in the acetic acid measurements (**Figure 1A**) is held constant with increasing concentrations of acetic acid, while the ratios from conventional ESI (indicated with ◇) decreases dramatically with the exposure of the protein to high concentrations of acid. Sulfolane experiments (**Figure 1B**) also confirm this finding with a conservation of the holo-/apo- ratio observed in CF-EDESI and a decrease in the ratio in ESI with increasing sulfolane concentration. Experiments with mNBA (**Figure 1C)** did not follow this trend seen from the acetic acid and sulfolane experiments. This result was expected and in agreement with previous studies where mNBA was shown to preserve protein complexes and native protein structure when directly infused with protein analytes [Loo]. All three charging agents were able to successfully increase protein charges as seen from the plots of both holo-myoglobin (CF-EDESI (■) and ESI (□)) and apo-myoglobin (CF-EDESI (●) and ESI (○). It is clear from the results that the apo-myoglobin form is more easily increased to higher charge states due to greater number of accessible protonation sites of an unraveled protein chain.

However, increases in holo-myoglobin charge states were also observed especially from CF-EDESI analysis. This provides evidence that CF-EDESI allows for the preservation of native protein structure while manipulating protein charge states with denaturing, charging additives.

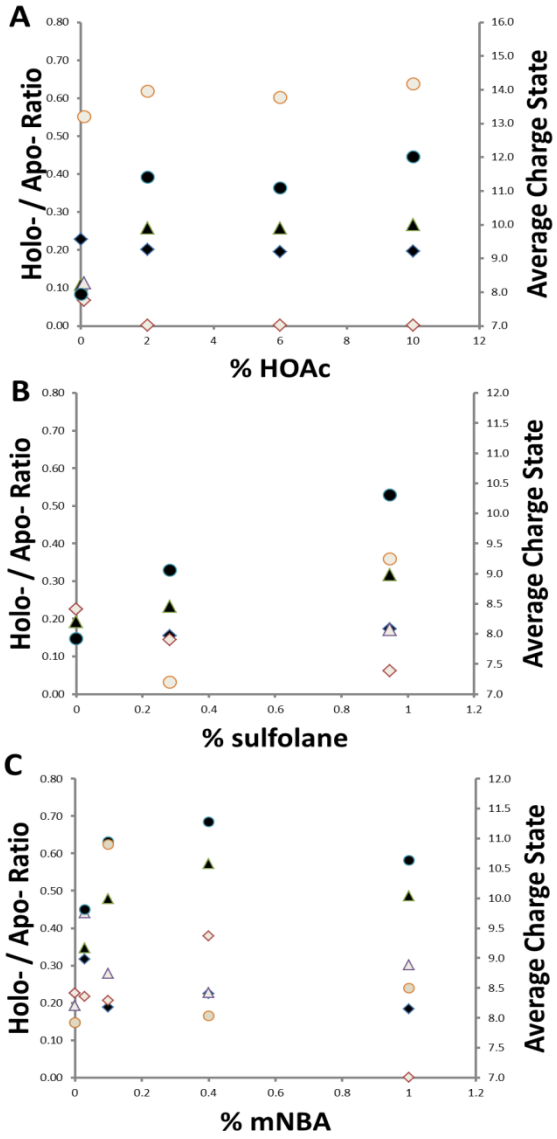

Figure 6.1 – Protein charging experiments conducted on myoglobin with varying concentrations of (A) acetic acid, (B) sulfolane, and (C) *m*-nitrobenzyl alcohol (mNBA). (◆) represent the holo-/apo- ratio obtained in the CF-EDESI mode while (◇) represent the holo-/apo- ratio obtained in the ESI mode. The observed average charge state of holo-myoglobin in (■) CF-EDESI and (□) ESI are also plotted with the axis on the right hand side. Observed average charge states of apo-myoglobin obtained with CF-EDESI (●) and with ESI (○) as also shown for comparison.

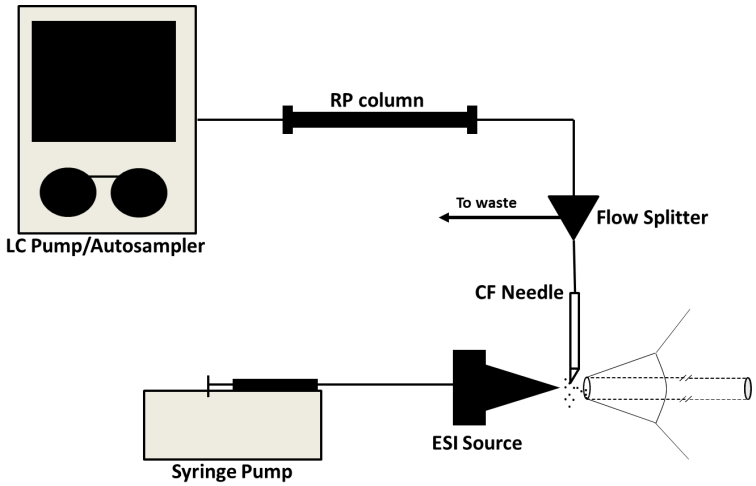

Figure 6.2 – Schematic diagram of the HPLC-CF-EDESI instrumental setup.

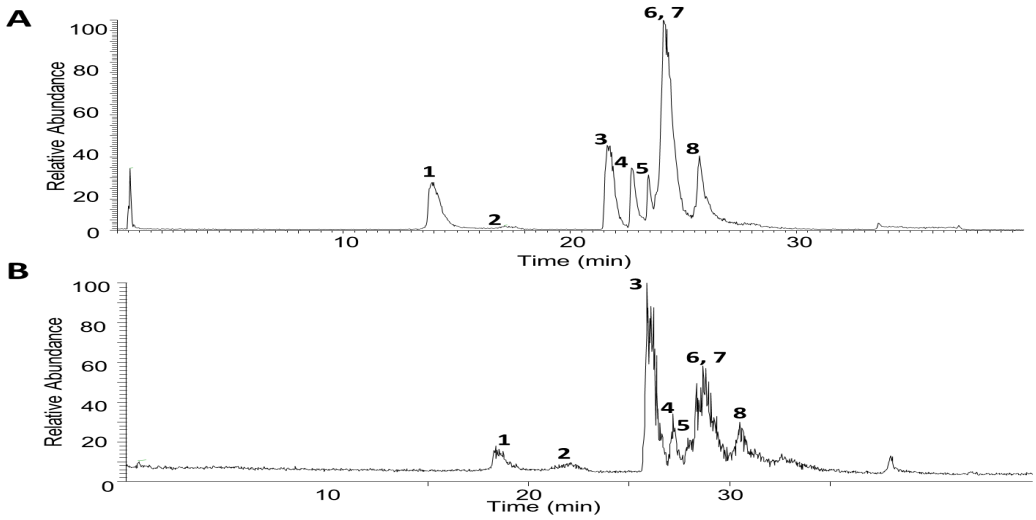

Figure 6.3 - Representative chromatograms of the developed chromatographic method for protein separations performed in (A) conventional ESI and (B) CF-EDESI. Analyte peaks are assigned as listed: (1) cytochrome c; (2) lysozyme; (3) transferring; (4) myoglobin; (5) hemoglobin Form A; (6) lactalbumin; (7) bovine serum albumin (BSA); (8) hemoglobin Form B

RPLC chromatography coupled to CF-EDESI

The previous protein analysis using CF-EDESI was extended to demonstrate proof-of-principle of the hyphenation of reversed phase liquid chromatography to CF-EDESI. First, method development was performed using conventional ESI to establish a chromatographic separation of a mixture of seven protein standards. The representative chromatogram is shown in **Figure 2A.** Eight analyte peaks were monitored during the separation due to fact that hemoglobin fragments into two forms, which will be denoted Form A and Form B for the rest of this discussion. The developed method was then imported into a CF-EDESI platform. The arrangement of the HPLC system with the CF-EDESI source is depicted in **Figure 3**, where the column is connected in-line with the CF needle. Considerations with the split ratio have been discussed in the experimental section where a rough 5:2 v/v split was set in place after the column yielding about 0.120 mL/min flow rate eluting from the CF needle. The electrospray flow rate and sheath gas flow rate were adjusted to accommodate this increased flow rate from the CF needle. A representative chromatogram of RPLC-CF-EDESI is shown in **Figure 2B**.

From a comparison of the two chromatograms, we can see a good agreement between the two ionization techniques. All eight protein analytes were detected in CF-EDESI as they were in conventional ESI with the same elution order. A qualitative assessment of method sensitivity was conducted to compare CF-EDESI to conventional ESI in the form of calculated response factors, which are presented in **Table 1**. The response factors were determined from the summation of all detectable protein charge states. The concentration points indicated in the experimental section were also measured in triplicate to evaluate reproducibility of the method. To have a fair comparison, a third set of response factor measurements were taken where the chromatographic separation was performed in conventional ESI with a post column infusion of matching acid additive concentration as well. Naturally, the addition of an acidic modifier facilitates positive mode ionization and will thus enhance response factor values. The results show that the LC-CF-EDESI method has comparable if not better response factor values than

those obtained in ESI with a post column infusion. The CF-EDESI response factor values taken with 10% acetic acid in the electrospray solvent demonstrate that the method can be easily adjusted to obtain optimal conditions by simply changing the composition of the electrospray solvent.

Table 6.1 – Calculated response factors comparing the four different methods

	R_f: ESI (conv.)	R_f: ESI (0.1% HOAc – Post column)	R_f: CF-EDESI (0.1% HOAc)
Cytochrome C	$1.09 \times 10^6 \pm 2.35 \times 10^5$	$9.20 \times 10^6 \pm 3.51 \times 10^6$	$4.17 \times 10^6 \pm 6.58 \times 10^5$
Lysozyme	$6.71 \times 10^4 \pm 5.91 \times 10^3$	$2.05 \times 10^5 \pm 4.96 \times 10^4$	$2.79 \times 10^6 \pm 8.40 \times 10^5$
Transferrin	$1.49 \times 10^7 \pm 1.85 \times 10^5$	$1.72 \times 10^7 \pm 8.85 \times 10^5$	$1.53 \times 10^9 \pm 2.26 \times 10^8$
Myoglobin	$3.93 \times 10^6 \pm 2.14 \times 10^5$	$7.19 \times 10^6 \pm 3.26 \times 10^5$	$1.33 \times 10^7 \pm 2.51 \times 10^5$
Hemoglobin	$2.03 \times 10^6 \pm 3.72 \times 10^4$	$6.84 \times 10^6 \pm 3.72 \times 10^5$	$1.17 \times 10^7 \pm 4.49 \times 10^6$
Lactalbumin	$2.42 \times 10^6 \pm 1.93 \times 10^5$	$5.55 \times 10^6 \pm 4.75 \times 10^5$	$1.86 \times 10^7 \pm 7.03 \times 10^5$
Bovine Serum A	$6.28 \times 10^7 \pm 1.94 \times 10^6$	$9.74 \times 10^7 \pm 6.38 \times 10^6$	$1.93 \times 10^8 \pm 1.10 \times 10^7$

To further elaborate on the advantages of LC-CF-EDESI, a series of charging experiments were then conducted in using increasing concentrations of acetic acid to manipulate protein charge states simultaneously with the chromatographic separation. Similar previous charging experiments, the protein charge distributions state of cytochrome c were monitored with increasing acetic acid composition in the electrospray solvent. It is important to note that previous discussions of softer ionization do not apply to LC-CF-EDESI, as proteins are effectively denatured when subjected to the denaturing conditions of an HPLC separation. Regardless, charging phenomena are still observed as shown in **Figure 4**, where the cytochrome c charge state distributions are shifted towards higher states with increasing

amount of charging additive. These results provide a clear demonstration of the power and versatility given from HPLC-CF-EDESI analysis. With the presented method, complex mixtures of protein analytes can be rapidly separated with liquid chromatography and then immediately subjected to protein charging for a high throughput screening process.

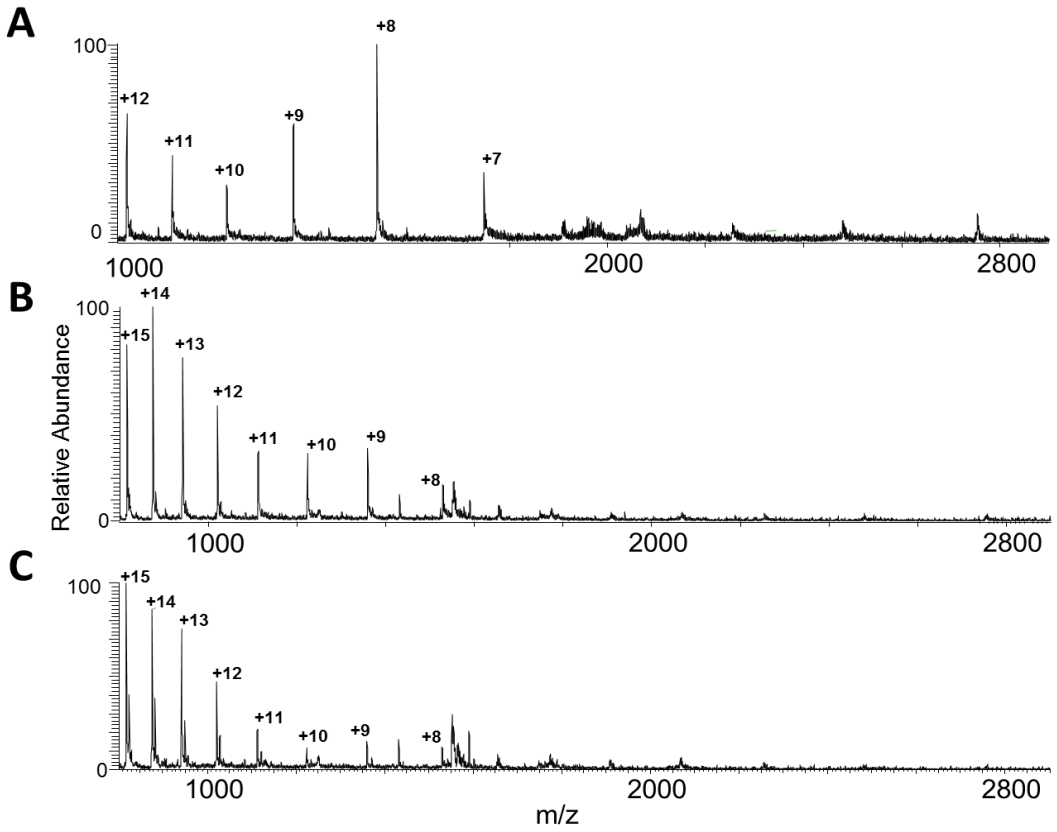

Figure 6.4 – Representative spectra of LC-CF-EDESI experiments monitoring the charge state distribution of cytochrome C with increasing concentrations of (A) 0% acetic acid content, (B) 0.1% acetic acid content, and (C) 10% acetic acid content added to the electrospray solvent.

6.4 Conclusions

Multiple advantages of CF-EDESI are featured in this presented work. In manipulating protein charge state distributions, the ability to perform separate introduction of sample analyte from charging additive imparts the ability to preserve native-folded conformations when using strongly denaturing charging conditions. In addition, a definitive proof-of-principle is demonstrated of hyphenation of CF-EDESI to a separation technique such as HPLC. Examples of the versatility of LC-CF-EDESI were also demonstrated. Further investigations into the uses of LC-CF-EDESI are being conducted to take advantage of the benefits of performing CF-EDESI analysis. Optimization of the LC-CF-EDESI method is still needed to build upon this proof-of-principle and obtain even more accurate and sensitive analysis. Hyphenation to other potential separation techniques is also being currently explored with our research group.

CHAPTER 7

SUMMARY AND FUTURE WORK

Throughout the work of this dissertation, clear examples and applications have been presented on the benefits of using novel approaches in sample preparation. Sample preparation has the potential to be the most time and resource consuming step of the analysis procedure. Two approaches were taken in the presented work. The first used restricted access media and novel trap technologies to circumvent the need to perform sample preparation prior to liquid chromatography. An application studying the trace detection of BPA is presented to demonstrate the viable of these designed RAM methods. The second approach focuses on exploring the use of ambient ionization techniques in order to perform accurate analysis with little to no sample preparation. Ambient ionization techniques including TM-DESI and CF-EDESI were used in the work of this dissertation. Novel affinity mesh screen materials that were designed for the purpose of facilitating more efficient drug discovery was used in conjunction with TM-DESI to create a high throughput drug discovery platform. CF-EDESI was also first introduced with initial studes that demonstrate its use in protein charging experiments. Proof-of-principle was also presented to demonstrate the hyphenation of CF-EDESI to HPLC analysis. Clearly, these presented analytical methods using ambient ionization could use further optimization to obtain even greater analytical performance. More optimization of the screening workflow using affinity capture materials and TM-DESI will also allow better selection of compounds that recognize the Kaa tripeptide sequence. It has also not escaped our notice that other forms of chromatography can be coupled to CF-EDESI to overcome a number of challenges that conventional currently faces.

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BIOGRAPHICAL INFORMATION

Samuel Hsiao-chieh Yang was born August 22, 1984 in the city of Tulsa, Oklahoma. At the age of 5, he moved to Arlington, Texas where he grew up in the Texas public school system. He then attended the University of Texas at Austin where he obtained his Bachelor's of Science in Biochemistry. After graduating in the spring of 2007, he enrolled in the bachelor's to Ph.D program at the University of Texas at Arlington. In the early years of his graduate work, Samuel began work in Biochemistry. As an undergraduate, he had been involved in a number of protein enzyme studies and had wished to continue using the skills learned from the undergraduate research on towards his graduate degree. After a year and a half of graduate work in Biochemistry, Samuel then switched his focus towards Analytical Chemistry under the mentorship of Dr. Kevin A. Schug. It was there he continued to pursue his interests in Analytical Chemistry and obtained his doctoral thesis. During the time spent working in the Schug lab, Samuel has explored a number of different areas of research within the realm of Analytical Chemistry. The primary focuses of his research involved the use of high performance liquid chromatography (HPLC) and mass spectrometry (MS). A great part of his research was devoted towards developing applications of these two powerful techniques in the use a wide variety of real-world problems. He then wishes to continue on in the pharmaceutical field using such developed skills in a professional setting.