FUNDAMENTAL AND APPLIED STUDIES OF AN AMBIENT IONIZATION TECHNIQUE: CONTINUOUS FLOW-EXTRACTIVE DESORPTION ELECTROSPRAY IONIZATION - MASS SPECTROMETRY

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April 9th, 2014

Abstract

FUNDAMENTAL AND APPLICATION STUDIES OF A AMBIENT IONIZATION METHODS: CONTINUOUS FLOW-EXTRACTIVE DESORPTION ELECTROSPRAY IONIZATION

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Continuous flow extractive desorption electrospray ionization (CF-EDESI) is a novel ambient ionization technique that was developed to help analyze samples from non-ESI-friendly solvents, manipulate protein charge states, and couple to a flowing sample solution stream. CF-EDESI is performed by introducing a sample solution through a hypodermic needle set orthogonal to an on-axis electrospray ionization source. Samples dissolved in non-ESI-friendly solvents such as hexanes, ethyl acetate etc. can be successfully detected in CF-EDESI which then makes it an attractive detection technique for normal phase separation. Additionally, introducing samples separated from electrospray has provided freedom to optimize both the sample solvents and spray solvents. Additives such as acetic acid, sulfolane, m-nitrobenzyl alcohol were used in spray solvents to manipulate the protein charge state, which also minimized the post column band broadening for separations when coupled with separation. Besides, ammonium acetate additive in spray solvents can greatly reduce the sodium adducts in lipid analysis from MTBE.

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Chapter 1

Overview of Topics

1.1 Ambient Ionization and Separations

There are various methods to generate ions for mass spectrometry. Classical methods such as electron impact (EI) [1], chemical ionization (CI) [2], field ionization (FI) and field desorption (FD) [3] require the sample to be introduced into a vacuum environment prior to analyte ionization. Often, for large molecule analysis, fast-atom bombardment (FAB) [4] and matrix-assisted laser desorption ionization (MALDI) require the samples to be dispersed or prepared in certain matrix. Even the recent atmospheric pressure ionizations such as electrospray (ESI) [5], atmospheric pressure chemical ionization (APCI) [6], atmospheric pressure photoionization (APPI) [7], and atmospheric pressure-matrix assisted desorption ionization (AP-MALDI) [8] prefer samples to be diluted in certain solvents (or solid) that are compatible with the ionization process before analysis. Due to all the limitations, none of these ionization techniques could provide direct analysis of samples *in situ*.

A term called “ambient ionization” (AI) coined by Graham Cooks describes a method to analyze samples in their native state. The first AI was introduced at the end of 2004 and was named desorption electrospray ionization (DESI) [9] which was an electrospray based (derived from ESI) method. Soon after, direct analysis in real time (DART) [10] was reported in 2005 and was plasma-based (derived from APCI) method. The apparent potential of DESI and DART was demonstrated their fast and effective capabilities in various fields such as analyzing samples from pharmaceutical testing (quality control/assurance or counterfeit identification [11]), chemical weapons [12], explosive residues, latent fingerprints [13], forensics [14], fragrances [15] and food

inspection [16], among others. These applications initiated an exponential growth of ambient ionization techniques derived from either spray-based or plasma-based ambient ionization techniques to adapt to specific analytical needs. Easy sonic spray ionization (EASI) [17], extractive electrospray ionization (EESI) [18], desorption atmospheric pressure chemical ionization (DAPCI) [19], and desorption atmospheric pressure photoionization (DAPPI) [20] were among the top list of new ambient ionization techniques.

Besides the ability to directly analyzes samples in their native state, AI coupling with different modes of separation both on- and off- line has also been highlighted [21]. This is an emerging area of research which has a strong potential for novel applications, especially given the capability for generating sensitive mass spectral signals from complex samples. However, in many cases, whereas the possibility for coupling efficient separations with current ambient ionization mass spectrometry exists, much work still remains to comprehensively demonstrate the effectiveness of such hyphenated techniques, so that they can be used more routinely.

1.2 Introduction to CF-EDESI

Inspired by recent developments in ambient ionization (AI) techniques, we developed a novel ion source in our lab, which can circumvent many of the limitations of the ESI process [22,23]. It involved with introducing sample solutions through a hypodermic needle independent from electrospray. And it provides flexibility in optimizing the spray solution and the sample solution. The design of CF-EDESI maintains efficient coupling of a continuously flowing stream to the source, an aspect which is not possible in many AI techniques reported in the literature. CF-EDESI is not only amenable to coupling with liquid chromatography and flow injection analysis, but more importantly, the

source can also be used to generate ions from solutions composed from non-ESI-friendly solvents and additives.

The interest was first initiated on manipulation of the protein charge state with different additives, such as acetic acid and sulfolane. The manipulation of protein charge state has significant meaning in the study of protein interactions, post-translational modifications and protein sequencing etc and is often hard to achieve with conventional methods of analysis. In CF-EDESI, the charge state manipulation was achieved by introducing sample solution through a hypodermic needle and changing the super charging agent through electrospray. The data obtained from CF-EDESI was compared to conventional electrospray ionization. Good agreement with previously reported studies of cytochrome C folding studies was found. The flexibility in optimizing electrospray solvent in CF-EDESI provided a unique way to manipulate charge state for proteins.

1.3 Reverse Phase Separation of Proteins Coupled with CF-EDESI

To expend the application of continuous flow – extractive desorption electrospray ionization (CF-EDESI), which demonstrated previously for protein charge state analysis (Anal. Chem. 2011, 83, 643-647), was used as the interface for coupling reversed phase protein separations to mass spectrometry. By introducing super charging agents through electrospray independently from sample eluent through hypodermic needle, CF-EDESI has avoid the charging agent interference of separation and any side effect from post column band broadening. Additionally, another two advantages of CF-EDESI were demonstrated in this part of the research. Firstly, CF-EDESI has demonstrated softer ionization capability in monitoring the ratio of holo-myoglobin and apo-myoglobin when subject to a series of different charging additives. Secondly, a proof-of-principle is presented to demonstrate the applicability of hyphenation of liquid chromatography to

CF-EDESI. The LC-CF-EDESI-MS set-up is shown to be as sensitive, and for some proteins more sensitive than LC-ESI-MS.

1.4 Analysis from "non-ESI-friendly" Solvent and Mechanism

Due to their low polarities and dielectric constants, analytes in solvents such as hexane, chloroform, and ethyl acetate exhibit poor ESI efficiency. These are deemed to be "non-ESI-friendly" solvents. CF-EDESI was found to efficiently generate analyte ions from samples contained in non-ESI-friendly solvents. This feature makes it attractive to general analytical community due to its apparent potential for facilitating the coupling of normal phase separations to mass spectrometric detection, or the direct analysis of nonpolar solvent solutions. In this context, interest was subsequently initiated on determining the mechanism for its success with the comparison to extractive electrospray ionization (EESI) in the negative ionization mode through the analysis of a series of fatty acids. A liquid-liquid mixing of phases in CF-EDESI is more likely to be the dominant process over liquid-extraction interactions in EESI as the obvious different results obtained. A partial factorial design experiment was performed to determine the experimental variables most important for efficient operation. The effects of sample and electrospray solvent flow rates, nebulizer gas, spray voltage, and sample solution temperature on signal intensity for progesterone in hexane were examined. While sample flow rate was confirmed to be among the most significant factors to affect sensitivity, in general, CF-EDESI consistently provided higher signal intensity in these experiments compared to conventional electrospray ionization.

1.5 CF-EDESI Coupled with Normal Phase Separations and Lipid Extract Analysis

Normal phase liquid chromatography (NPLC) is a common mode for chiral separations. Many chiral amines are used as drugs or are important intermediates for drug synthesis. Electrospray ionization mass spectrometry (ESI-MS) is well-known for its high sensitivity. However, when using NPLC, ESI-MS detection is hampered by the poor ionization efficiency of analytes from NPLC eluents. Continuous-flow extractive desorption electrospray ionization (CF-EDESI), which introduces the eluents through a hypodermic needle into the electrospray plume is demonstrated for its success to interface NPLC to mass spectrometry detection. Such an approach was shown to be more sensitive than UV detection. Also demonstrated is the direct infusion of cell extracts to monitor phospholipids from three different bacterial cells. Despite their presence in non-ESI-friendly extraction solvents, CF-EDESI enabled sensitive detection of phospholipids and the ability to tune ion forms through incorporation of different spray modifiers.

Chapter 2

Ambient Ionization and Separations

2.1 Introduction

This review highlights the combination, or potential combination, of various separation methods with ambient ionization – mass spectrometry [24]. The benefit of coupling separation devices with mass spectrometry has been recognized as an extremely useful approach for real-world samples analysis, where complex mixtures are the norm. Typical liquid phase separation techniques, including high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC) and capillary electrophoresis (CE), have been successfully combined with various mass spectrometry interfaces, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) to create high performance analytical methods [25]. Ambient ionization (AI) refers to a series of relatively new ionization techniques, which are rapidly advancing towards use in many fields for direct in situ screening of analytes with minimal or no sample preparation. The popularity of these new ionization techniques is indicated by the explosive growth of the new designs and applications in this area. The ability to separate and independently optimize the sample introduction and the ionizing radiation provides significant advantages compared to more traditional ionization techniques. Even so, without removing large background interferences, we should be aware that high resolution and mass accurate mass detectors are required to unambiguously discriminate the target mass from background noise. This is why additional hyphenation of AI with separation devices can promote these new techniques for wider applications. Significant advances can be found with these new hyphenated techniques, compared with traditional coupling

combinations (e.g. HPLC and ESI), however, to a large extent, very few demonstrations and applications of this promising technology currently exist.

From the standpoint of separation science, incompatible factors which can restrict the range of the analysis in current separation - mass spectrometry combinations still exist in various aspects. For example, nonvolatile buffers (e.g., borate and phosphate buffers) adopted for better separation in chromatography can cause ion suppression in ESI [26]. Also, non-polar solvents, which are typically used in normal phase liquid chromatography, are poorly compatible with ESI, due to their low conductivity and/or low dielectric constants [27]. On the contrary, this is another regime where AI sources could broaden the compatibility between separations and mass spectrometry. At least, with respect to matrix tolerance and samples present in non-ESI friendly solvents, AI has been shown to have to provide a higher tolerance to such conditions, compared with tradition atmospheric pressure ionization [28].

In this review, we cover the developments and trends of recent research achievements in AI techniques. Some popular cases will be illustrated with regard to experimental arrangements, mechanisms of ionization, and applications. Following this, some successful combinations between AI and separation techniques will be narrated. Importantly, the potential for this coupling in future work to handle complex sample analysis will be discussed.

2.2 Recent Ddevelopments in Ambient Ionization

Due to the virtue of increased throughput and less stringency in sample form, AI techniques have become an increasingly useful part of the analytical tool box. AI has

been widely used in environmental, food, flavor, fragrance, forensic, homeland security, and pharmaceutical areas for trace and real-time analysis [29]. A large number of set-ups have been proposed in the past few years and this effort has been accompanied by an equally long list of new acronyms to remember. Generally speaking, ambient ionization techniques can be classified into two groups based on their associated ionization process. Nominally, these include spray-based (ESI source-modified) and electric discharge-based (APCI source-modified) set-ups [30] [24] [31]. Some other variations exist, such as those inspired by atmospheric photoionization (APPI) [32], and a multimode ionization technique which integrates benefits of both ESI-based and APCI-based ionization techniques [33].

2.21 Spray-based Ambient Ionization

Spray-based AI techniques often take advantage of conventional ESI to generate charged species, applying it to various situations for gas, liquid, and solid samples analysis, with and without sample pretreatment. Desorption electrospray ionization (DESI) is one of the most prominent and commonly-used examples of spray-based AI techniques. It provides direct solid and liquid sample analysis. The complete design and application was reported by Cooks and coworkers in 2004 [9]. The general configuration is shown in Figure 2-1A. Success of DESI was first demonstrated with small molecules that can be both polar and non-polar on solid surfaces. The variation of spray solvents (from various aqueous to non-aqueous solvents) plays a critical role for target analyte selection based partly on analyte polarity and solubility [34]. While DESI from solid samples is most common, DESI from liquid samples and flowing liquid samples has also been reported [35].

A droplet pickup model is hypothesized to be the predominant sampling process in DESI. The sample surface is first wetted by the spray, forming a thin film of liquid on

top of the sample. Analytes partition into this thin film and subsequent spray droplets "pick up" the analyte into the ESI droplets, which will continue to desolvate, subdivide, and ultimately lead to release of analyte ions into the gas phase. DESI has been used to analyze a multitude of different analyte types, including non-volatile pyrolysis products [36], polymer oligomers [37], inks [38], carbohydrates [39], peptides [40], explosives [41] [42] [43], human breast cancer tissue [44], porcine and rabbit adrenal glands [45], lipids in mouse brain tissue [46] [47], and counterfeit antimalarial drugs [48], among many others [49]. Commercial DESI sources are available that are designed to interface to mass spectrometers produced by a variety of manufacturers.

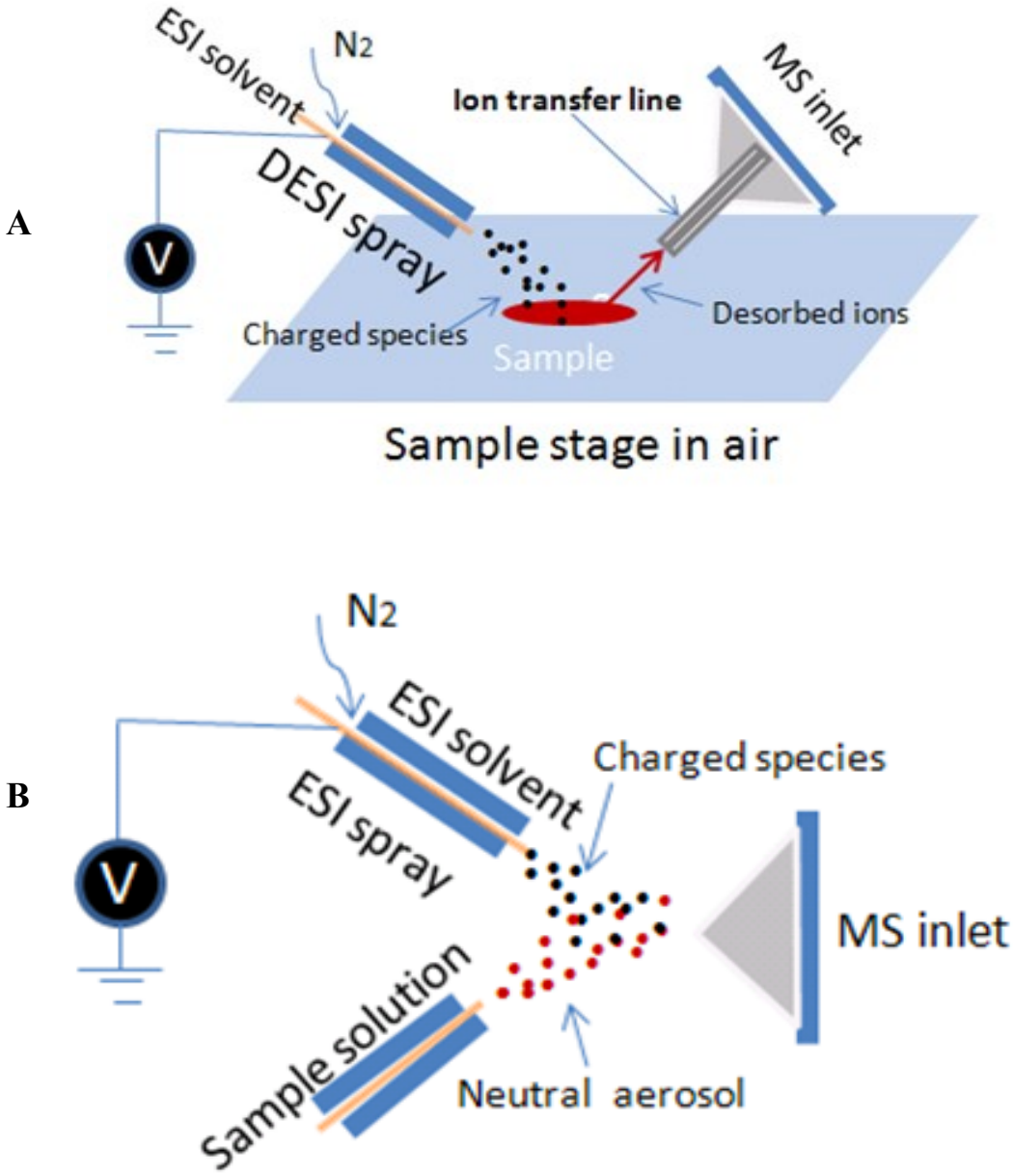


Figure 2-1 (A) desorption electrospray ionization (DESI) (B) extractive electrospray ionization (EESI) (adapted from reference [18]).

Extractive electrospray ionization (EESI) is another popular spray-based AI technique, which was also introduced by the Cooks group [18]. EESI, as shown in Figure

1B, is typically suited for analysis of liquid samples or aerosol samples, which can be nebulized. The sample solution is isolated from the ESI source and its high voltage, by virtue of its introduction through a second nebulizer. Neutral sample droplets intersect with charged aerosols from the ESI source in front of the MS inlet [50].

Applications have included the analysis of undiluted urine[18], milk [51], diet effect on biofluids [52], perfume classification [53] and human breath [54], all with minimal or no sample preparation. Zhu [55] and McCullough [56] described the use of EESI for monitoring chemical reactions. Sampling was designed to ensure that the bulk reaction mixture was sampled rather than just the headspace by using a second nebulizer to create an aerosol of the bulk solution using a Venturi pump. The compatibility with liquid samples or aerosols makes EESI a good partner with separation techniques such as liquid chromatography.

In 2008, a modification of the EESI sampling method that uses a neutral desorption (ND) sampling gas beam to gently acquire analyte from sample surfaces, called NDEESI, was proposed by Chen and his coworkers [57]. This development broadened the EESI application base to trace analysis on biological surfaces, and has been demonstrated for use with cheese [58], skin, tissue, muscle, and vessels [59].

2.22 Chemical Ionization-based Ambient Ionization

A significant number of chemical ionization-based AI techniques have been reported, including direct analysis in real time (DART) [10], desorption atmosphere pressure chemical ionization (DAPCI) [60], flowing atmospheric pressure afterglow (FAPA) [61], low temperature probe (LTP) [62], dielectric barrier discharge ionization (DBDI) [63], and microplasmas [64], among others. They basically involve the generation of an electrical discharge across a pair of electrodes in the presence of a flowing gas, such as in nitrogen, helium or vapor in air. Under the high electric field or radiofrequency

excitation, a series of ionized molecules, radicals, excited state neutrals, and electrons will be produced that, in turn, can be used to ionize analytes of interest.

DART, schematically depicted in Figure 2-2, was one of the first ambient ionization sources brought to the market and it has been demonstrated for numerous applications, including counterfeit pharmaceutical analysis [65], drug discovery [66], organic reaction monitoring [67], serum metabolite fingerprinting [68], hormone detection [69], and malaria control [70]. DART has also been used to directly characterize analytes in conjunction with separations by TLC [71] and HPLC [72].

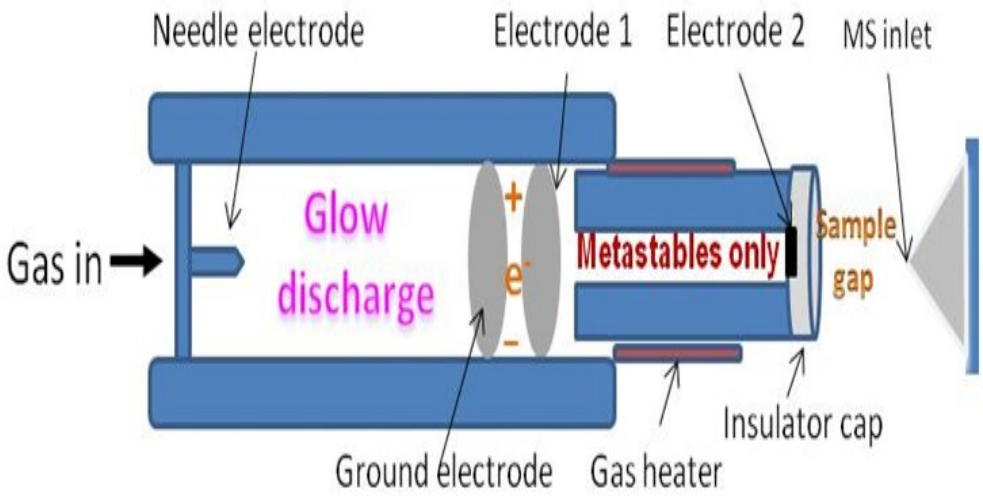


Figure 2-2. Direct analysis in real time (DART) ion source (adapted from reference [10]).

Desorption atmospheric pressure chemical ionization (DAPCI) was first reported in 2005 [60]. It was originally investigated as a complementary method to study the ionization process in DESI. DAPCI uses moisture in air or nitrogen to generate the charged species following their passage across a discharge needle. Subsequently, Chen and coworkers have successfully applied this technique to the analysis of food, skin, and clothing [73].

Additionally, sheath gas is not necessary for good performance in DAPCI, so the analysis of samples in powder form can also be realized [31].

2.3 Hyphenation of ambient ionization with various separation methods

AI (e.g. DESI and DART), which provide versatile interfaces between liquid phase separations (e.g. HPLC) and MS have been highlighted by recent reports [71, 72,74]. Significant advantages were demonstrated in terms of the ability to tolerate harsher buffer salts and high flow rates, increase sensitivity and selectivity, and achieve high throughput. These applications are very promising and should help increase interest in coupling AI with a wider array of separation techniques, in general. In an era where chromatographic separations are being pushed to higher dimensionality, the added specificity and versatility of coupling to MS detection through AI sources will serve to further multiply capabilities. Existing and potential hyphenation of AI with various separation methods are listed in Table 2-1. Further details and highlights of these achievements and their potential are discussed below. The reader should understand that this may not serve as an exhaustive coverage of this technology.

Table 2-1. Overview of work highlighting the coupling of AI with separation techniques

	Acronym	Name	Ionization Format	Year introduced	Coupling
Spray based	DESI	Desorption electrospray ionization	Charged spray; desorption from solid/liquid surface	2004 [9]	TLC [75,76,77,86 ,87 ,88 ,89], SPE [78], SDME [90], HPLC [79,74], CE [91]
	EASI	Easy ambient sonic-spray ionization	Spray; desorption from surface	2006 [80,81]	TLC [17]
	CF IR MALDESI	Continuous flow infrared matrix-assisted laser desorption electrospray ionization	Charged spray, matrix, laser	2008 [82]	Not yet demonstrated
	EESI	Extractive electrospray ionization	Charged spray, Extraction from a neutral sample	2006 [18]	
	Nano-EESI	Nano extractive		2009 [83]	

Table 2-1 continued

		electrospray ionization	spray/aerosol		
	FDESI	Fused droplet electrospray ionization		2002 [84]	
	ND-EESI	Neutral desorption extractive electrospray ionization	Charged spray; Gas jet neutral desorption	2007 [57]	
	CF-EDESI	Continuous flow-extractive desorption electrospray ionization	Charged spray; Extraction from bulk liquid flow	2011 [22]	
Chemical ionization based	DART	Direct analysis in real time	Neutral excited atoms/molecule	2005[10]	HPTLC[71], HPLC [72]
	DAPCI	Desorption Atmospheric Pressure Chemical Ionization	Charged species from air or nitrogen	2005 [60]	Not yet demonstrated
	FAPA	Flowing Atmospheric Pressure Afterglow	Excited discharge gases	2008 [61]	
	LTP	Low Temperature Probe	Low temperature	2008 [62]	

Table 2-1 continured

	DBDI	Dielectric Barrier Discharge Ionization	plasma	2007 [63]	

Table 2-1 continued

2.3.1 Coupling Separations with DESI

The coupling of liquid chromatography with mass spectrometry through a DESI source was recently demonstrated by Chen and coworkers (Figure 3). Characteristics include a wide range of elution flow rates, online derivatization via reactive DESI [48], and further combination with electrochemistry experiments [79]. Three neurotransmitter compounds (3 mg/mL, each), norepinephrine (NE), normetanephrine (NM), and dopamine (DA) were separated in isocratic mode at pH 3.0, and fused silica tubing was used to deliver fractions for DESI analysis. Ionization could be readily generated through the interaction between the charged spray and the HPLC eluent. HPLC flow rates as high as 1.8 mL/min were utilized in this experiment without compromise of sensitivity.

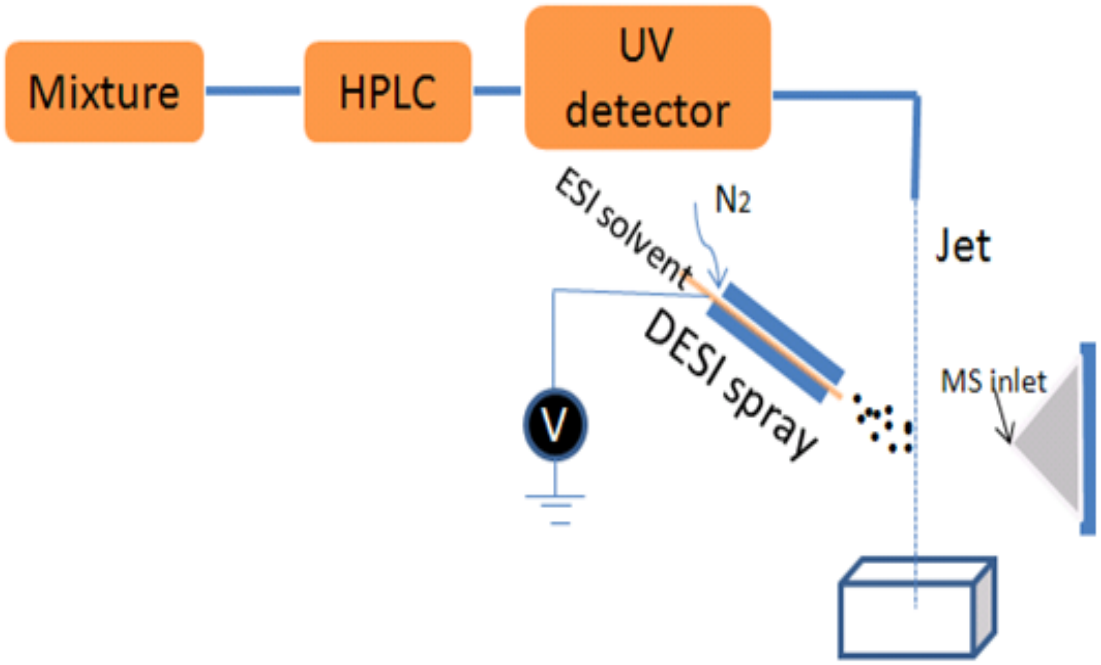


Figure 2-3. Schematic of a prototype HPLC-DESI-MS apparatus (Adapted from reference [79]).

Besides coupling with HPLC, DESI in combination with thin layer chromatography (TLC) is a more natural marriage, due to the surface analysis format. Surfaces such as nanoporous silicon, ultra-thin layer chromatography plates, polymethyl methacrylate (PMMA), and polytetrafluoroethylene (PTFE) have been investigated and compared by Cooks and coworkers [85]. For all four materials, limit of detection (LODs) were obtained in the fmol-pmol (pg-ng) range.

Van Berkel *et al.* have taken advantage of this simple and fast method for the direct analysis of gangliosides from TLC plates. Importantly, they demonstrated the potential for automation of this technique by coupling normal phase TLC with DESI-MS through a computer-controlled scanning device [86]. Other applications for analysis of porcine brain lipids [87] and peptides [88] were also reported. Of course, a significant advantage is that DESI can be used to sample and analyze separation bands without removing or extracting them from the plate. Additionally, the sampling area can be sufficiently small so that the plate can be retained for further analysis by other techniques, if desired.

Similar to a TLC surface, pre-concentration and purification techniques such as solid phase extraction (SPE) and single droplet micro-extraction (SDME), where extracted analytes can be enriched on a bead or a frit, respectively, are also amenable for DESI analysis. Direct coupling of SPE with DESI was shown to improve the overall sensitivity up to 6 orders of magnitude if enough sample was collected [78], Rhodamine 116 was used as the analyte in this study. The lower limit of detection obtained (100 fg/mL-100 pg/mL) was comparable to ESI. For SDME, single aqueous or organic droplets, enriched with trace amounts of methamphetamine (MA) from water, served as a good substrate for DESI analysis [89]. The limit of detection (LOD) for MA in the droplet was determined to be 51 ng/mL.

Another classic separation technique is capillary electrophoresis (CE). CE is used for separation of ionic species in solution, and recently a unique coupling with DESI was reported by Zare and coworkers [90]. Two sets of analyte mixtures, rhodamine dyes and cardiac drugs prepared in high concentration of buffer solutions were separated by CE and then deposited on a rotating stage (Figure 4A). The researchers were able to show that ion suppression and MS inlet contamination were minimal, despite the high salt concentration. Additionally, the rotating stage format is amenable for automated and high throughput operations.

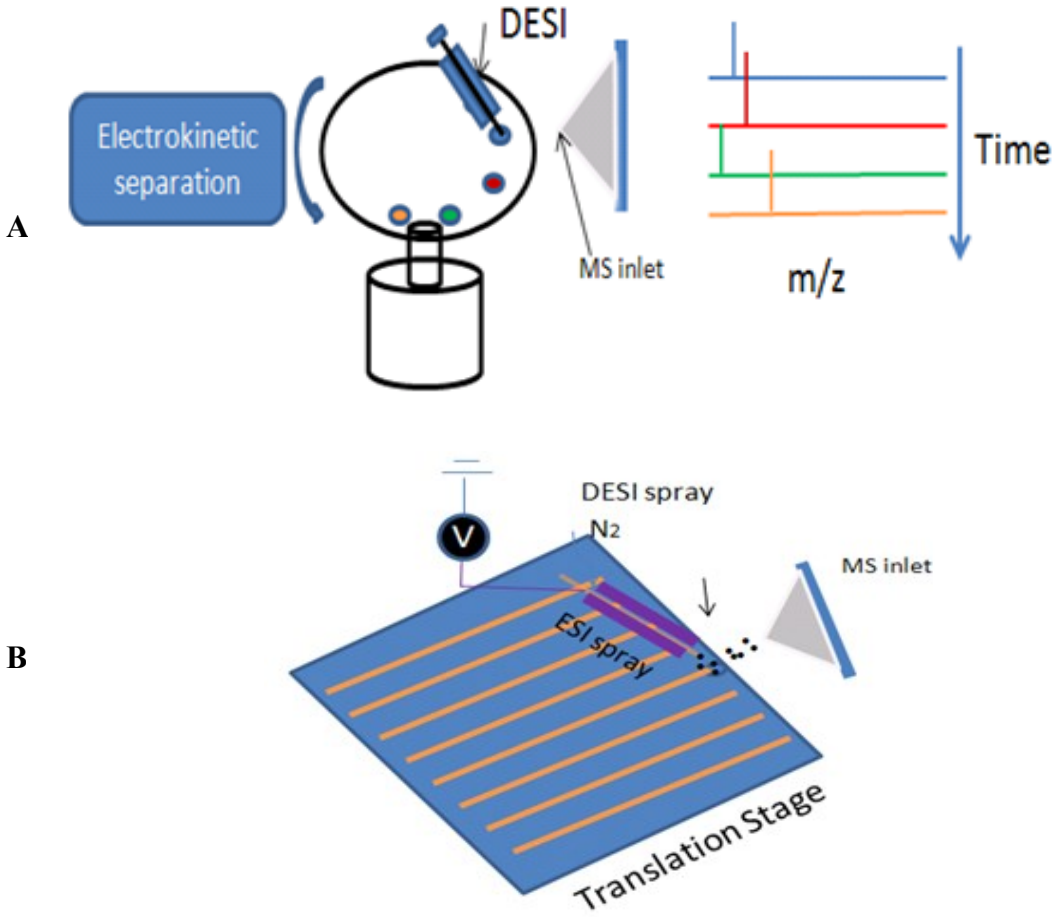


Figure 2-4. A) Schematic of capillary electrophoresis coupled with DESI through a rotational spotting/sampling stage (Adapted from reference [90]). B) Multichannel device that invented to DESI for high throughput analysis (Adapted from reference [91]).

The capability for increased throughput has always recognized as a very important aspect to be considered for a new method to be developed, especially if it is to be amenable for use in industry. To this end, a new high throughput device was developed by Ma *et al.* [91] that features a multichannel device with 16 parallel capillaries which can be interfaced with DESI-MS detection (Figure 4B). Of course, the potential for

HPLC separations in the capillaries is a significant advantage. The authors indicated that if each sample analysis requires 1.6 min, then an overall throughput of 600 samples/h can be achieved with this technology.

From these examples, it is clear that DESI has enjoyed the most development in terms of interfacing separations with MS detection through an ambient ionization source. Clearly, the ability to sample directly from separation media in various automatable and high throughput formats, in some cases alleviating potential band broadening effects associated with elution, makes these techniques extremely attractive for use in both routine and high impact research scenarios.

2.3.2 Coupling Separations with DART

Of all of the AI techniques, DART is second only to DESI in number of studies where it has been coupled to various separation formats. Similar to DESI, the coupling of HPLC with DART exhibited significant tolerance to buffer salts in the chromatographic separation [72]. As high as 120 mM of phosphate buffer were used in this combination without evidence of any source contamination or ion suppression. Limits of detection from 20 - 55 µg/L were achieved for a series of parabens as analytes in the negative ionization mode in this study. The outlet of the HPLC was connected to a stainless steel or fused silica capillary, and the effluent was directed into the ionizing radiation (Figure 5A). As high as 1mL/min of flow was introduced through the needle into the DART source for MS analysis. Fraction collection may realized by the HPLC-DART combination. After MS analysis, further characterization of the HPLC fractions can be carried out.

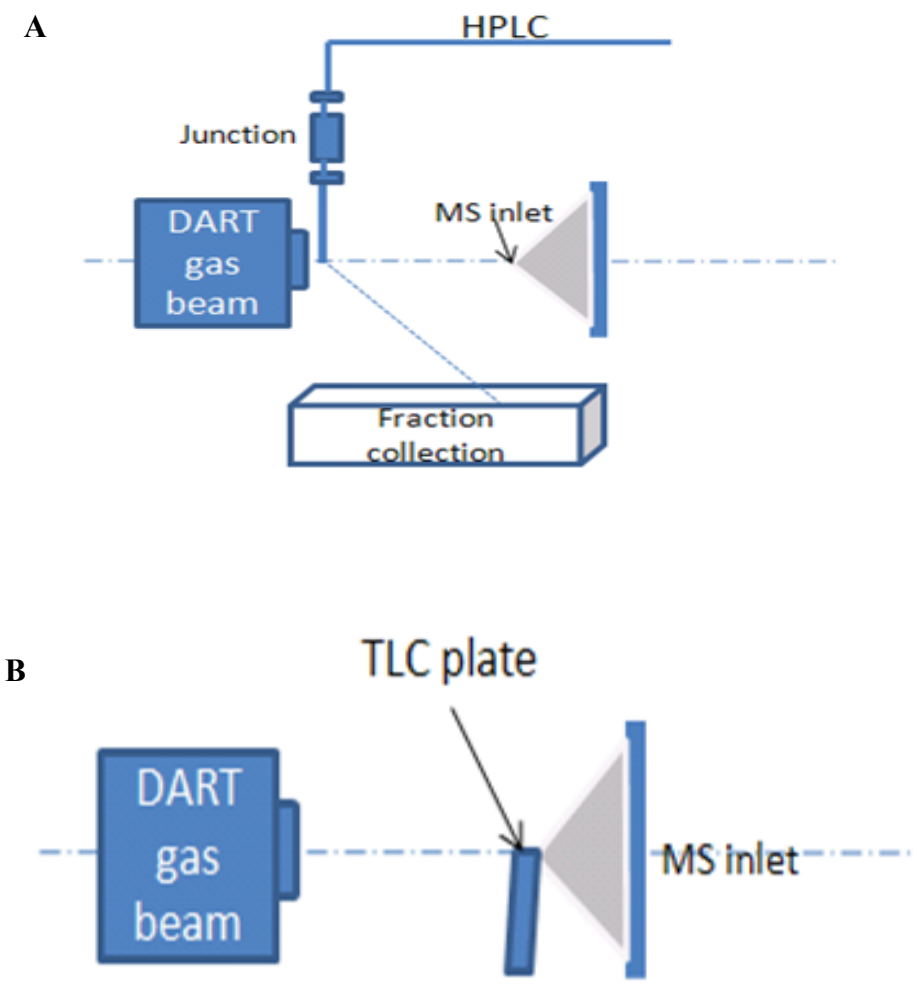


Figure 2-5. Schematics showing the coupling of A) HPLC [72] and B) HPTLC [71] to a DART ion source. In the latter, the TLC plate was positioned manually in the set-up.

High performance TLC is also easily coupled with DART-MS [71], as depicted in Figure 5B. Strips of plates containing the separation bands were cut and the edge of each band was exposed to the metastable gas ion beam. Optimal response was observed at approximately 1 mm distance from the MS inlet at an angle of about 160° vertical to the gas flow. The coupling was applied in qualitative and quantitative analysis

of isopropylthioxanthone. Quantitation was difficult initially, but good linearity (R^2 0.9892) and repeatability (RSD < 5.4%, n = 6) was achieved for caffeine detection by introducing an isotopically-labeled internal standard. Spatial resolution was shown to be better than 3 mm in this coupling system.

2.4 Potential for the Future

EESI [18] and nanoEESI [83], continuous flow infrared matrix-assisted laser desorption electrospray ionization mass spectrometry (CF IR MALDESI) [82], and continuous-flow extractive desorption electrospray ionization (CF-EDESI) mass spectrometry [23] have been demonstrated for on-line analysis of liquid samples in recent literature. A natural next step is to couple these with separation techniques, like HPLC and CE. Some work to this end is undoubtedly already being undertaken. Besides, DAPCI, which can ionize an analyte without need for a charged spray and nebulizer gas, holds significant potential for combinations with GC separations.

2.5 Perspective

The capability to independently tune and optimize sample introduction and ionizing radiation in AI-MS holds great promise for further developments in this area. Additionally, since such a high degree of tolerance to solution conditions which hamper traditional API techniques has been demonstrated, the coupling of separations to MS through these AI sources is immensely attractive. In the earlier years of HPLC, before MS detection was developed into a routine approach, many separations were developed that featured high concentrations of nonvolatile salts. It appears that these high quality separations could now be revisited. As separations advance to higher complexity and dimensionality, the fewer the limitations with detection, the better. With robustness,

versatility, and increased throughput capabilities, it can be expected that the number of studies featuring the combined use of high efficiency separations in virtually all phases and formats with the vast array of AI techniques continually being developed, will amass at a significant rate.

Chapter 3

Introduction to CF-EDESI

3.1 CF-EDESI Set-up

Inspired by developments in DESI, TM-DESI, and EESI, we have developed a new AI source termed continuous flow-extractive desorption electrospray ionization (CF-EDESI) (Figure 3-1). In CF-EDESI, sample solutions are introduced into the ESI source (set on-axis with the MS inlet) by pumping flow through a hypodermic needle set orthogonal to the source. A LCQ Deca XP quadrupole ion trap mass spectrometer equipped with a conventional ESI source (all from Thermo-Fisher Scientific, Inc., San Jose, CA, USA) was used and modified in-house to construct the arrangement. A homemade XYZ stage was constructed and used to carefully position the hypodermic needle (22 gauge; SGE Analytical Science, Victoria, Australia) in the spray path. The position the CF needle was optimized by monitoring the response of a 10 μM bradykinin solution in water to achieve a stable and high intensity signal. The distances between the electrospray source needle and the CF needle (1.5 mm) and between the electrospray source and the inlet of the mass spectrometer (8.0 mm) are used based on optimization.

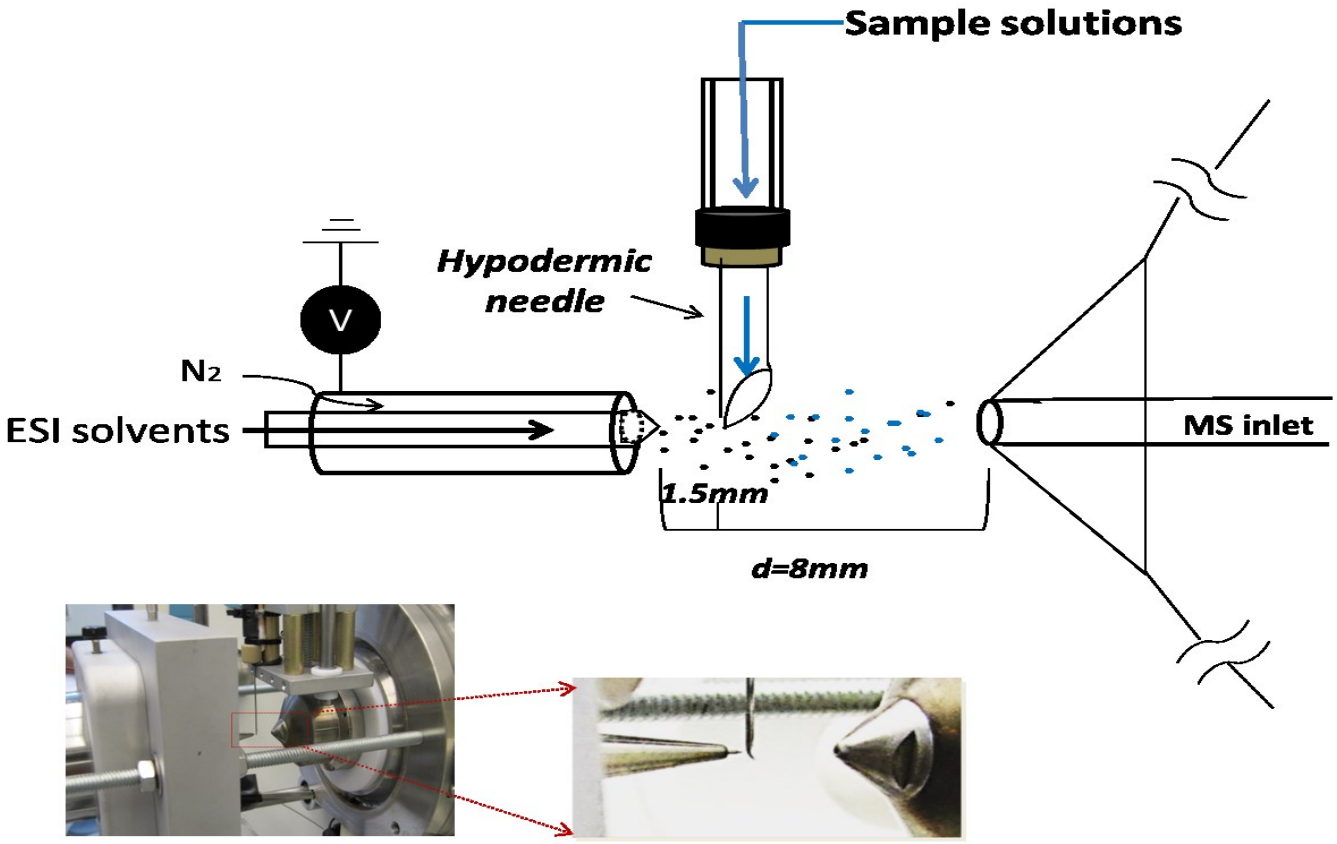


Figure 3-1. Configuration of Continuous-Flow Extractive Desorption Electrospray Ionization (CF-EDESI)

3.2 Initial Study on Protein Charge Manipulation

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. [92, 93, 94] Importantly, electrospray ionization mass spectrometry has enabled analysis of high mass molecules through the generation of multiply charged ions.[95, 96] 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.6,7 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 [97, 98].

CF-EDESI as one of the ambient ionization technique in mass spectrometry which characterized by introducing spray solvents independently from sample solution has showed great potential in manipulation of protein charge state. It can easily change the charging agent concentration in spray solvents without affect the protein introduction through an isolated source (hypodermic needle). 100 μM of Cytochrome C in water was introduced in hypodermic needle, acetic acid additives at the concentration 0%, 1%, 2%, 6% and 10% were tested in spray solvents. The results were compared with 100 μM of Cytochrome C in 10% acetic acid in spray solvent in ESI Representative spectra of the CF-EDESI experiments are shown in Figure 3-2. For comparison, an ESI-MS mass spectra of 100 μM cytochrome c, generated in 50/40/10 (v/v/v) methanol/water/acetic acid with the on-axis spray source, is shown in Figure 3-2E. 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 9þ 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 [11]. According to previous studies, denatured cytochrome c exhibits characteristic shifts of the charge state distribution to much higher states centered around the 16þ through 18þ states.11,12 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.

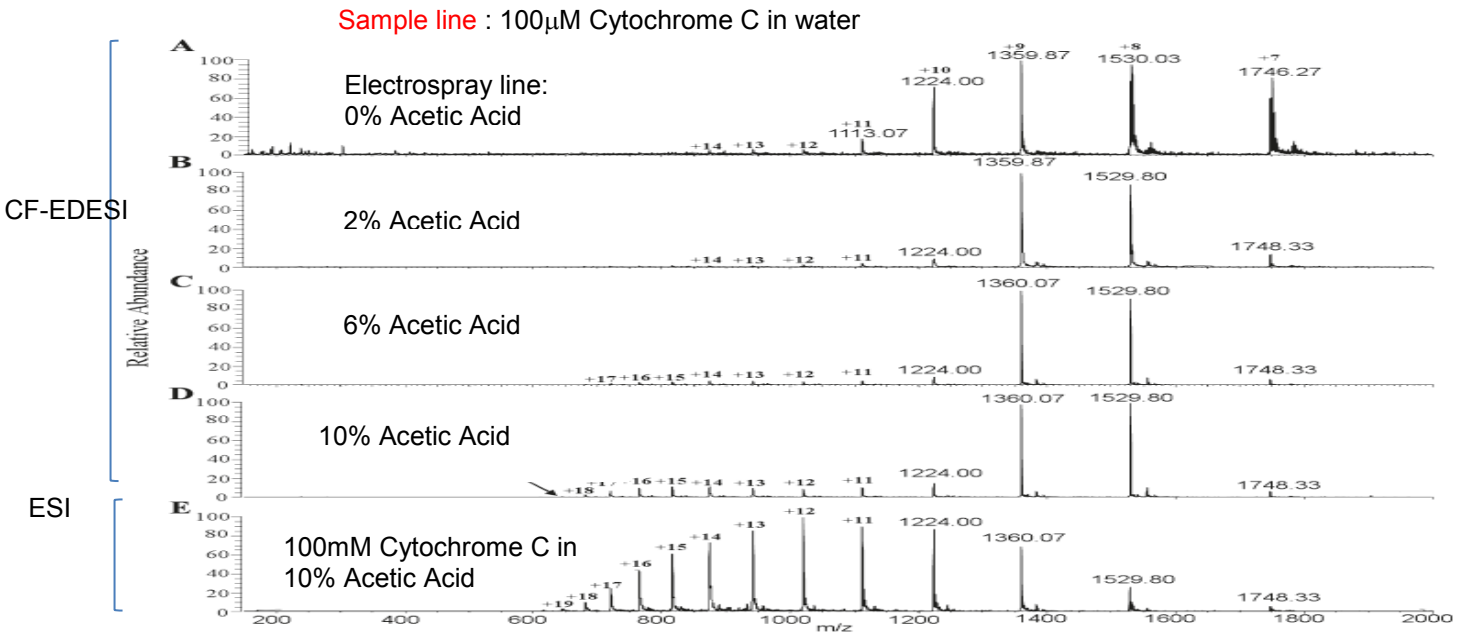


Figure 3-2. Representative mass spectra of the series of CF-EDESI experiments with 100 μMcytochrome c introduced orthogonally from the continuous flow needle to (A) 0% acetic acid content, (B) 2% acetic acid content with water/methanol (48/50, v/v), (C) 6% acetic acid content with water/methanol (44/50, 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 part E with 10% acetic acid in water/methanol(40/50,v/v).

CF-EDESI incorporates key aspects derived from DESI and EESI 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.

In spite of the advantages showed in protein charge manipulation, CF-EDESI also has wide use in other fields such as chiral separation, lipidomics and the capability of coupling with separations. We will demonstrate its use in following chapters.

Chapter 4

Reverse Phase Separation of Proteins Coupled with CF-EDESI

4.1 Introduction

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. According to previous study of protein charge state, CF-EDESI showed clear advantage in preserving proteins in higher charge state (or tend to keep protein in natural state), and produce less complicated spectrum than traditional ESI. A further investigation of CF-EDESI coupled to reverse phase separation of proteins was demonstrated in this chapter. Additional advantage of CF-EDESI in protein analysis has been explored. Also, the supercharging mechanism investigated in CF-EDESI provided insight to a highly debated supercharging process in ESI. The results indicate that the charge state distribution (CSDs) of either folded or unfolded proteins are not affected by the surface tension of the solvent as predicted by the Rayleigh equation.

The coupling of ambient ionization to separation has several advantages. Firstly, it can isolate each analyte from the sample complex to increase the detection sensitivity without the requirement for high accuracy mass detector. Secondly, most of additives and buffers that added in chromatography to improve separation such as phosphoric acid/phosphate or citrate could affect the detection efficiency heavily. The formation of adduct peaks and suppression of signal were quite common issues that caused by those additives and buffers in ESI. The coupling of ambient ionization technique to separations such as thin layer chromatography [20-22], capillary electrophoresis [23], and solid phase extraction [24, 25], in addition to its hyphenation to HPLC has already been highlighted in

recent papers. It would be great beneficial in coupling high performance liquid chromatography (HPLC) to AI techniques in consideration of its limitations in coupling with ESI.

CF-EDESI as one of the ambient ionization featured with introducing samples independent from electrospray was used in our group to demonstrate its coupling with reverse phase protein separations. In CF-EDESI, the electrospray charged ion species come into contact with the sample at the tip of a hypodermic needle where the 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 a significant need for optimization of the source/inlet geometry, which is often required for other ambient ionization techniques.

A reversed phase liquid chromatography (RPLC) method was developed to resolve seven protein analytes using conventional ESI. The method was then adapted onto the CF-EDESI platform to compare the two techniques. Chromatographic effluent 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 between the HPLC-CF-EDESI-MS and HPLC-ESI-MS methods are given. In the meantime, we investigated the effect of solvent surface tension on protein CSDs by means of introducing protein and super charging agent through different combinations in CF-EDESI set up.

4.2 Experimental

4.2.1 Chemical and Materials

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

CF-EDESI set up was described in previous chapter, and we only describe the parameters set for this experiment. The electrospray ionization was performed in the positive mode with the voltage set at 5.0 kV. The nitrogen sheath gas flow was set at nominal value 60 on Thermo LCQ deca for direct infusion from CF needle, while we used 80 for coupling to HPLC (which normally involve higher flow rate). The sampling inlet temperature was set at 200 ° C. Two minute scan times were taken per measurement with a scan range of 500-2600 m/z on the high mass range setting of the instrument with 3 μscans and 200 ms scan times. Data analysis was performed using Thermo 157 Xcalibur Data Analysis software (ver 1.5).

4.2.2 Sample Preparation

Protein standards except hemoglobin, were dissolved in pure LC-MS water to make a concentration at 1mM stock solution; the samples were then diluted to 100 uM working solution. Hemoglobin was dissolved in 5 mM NH_4OAc aqeous solution to prevent precipitation before diluted to 100 μM. The mixtures of all seven proteins were prepared from 100 μM working solutions. The ratio for each protein were varied from 3 different mixture since we need at least three measured concentrations of each protein. The concentration data for each protein in three mixtures is listed as follows: Mixture 1

consisted of 10uM lysozyme, 5μM cytochrome c and transferrin, and 1 μM myoglobin, lactalbumin, hemoglobin, and BSA; mixture 2 consisted of 15 μM lysozyme, 8 μM cytochrome c and transferrin, and 5 μM myoglobin, lactalbumin, 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 spray solutions with 0.1%, 1%, 2%, 10% acetic acid, 0.1% 0.2%, 0.5%, 1% sulfolane, and 0.1%, 0.2%, 0.5%, 1% m-nitrobenzyl alcohol solutions were all made individually in 50/50 (v/v) MeOH/H_2O solutions.

4.2.3 Supercharging mechanism study

A set of experiments were designed to investigate the supercharging mechanism in CF-EDESI and ESI. Cytochrome C was used as the test protein. The protein and supercharging agents were added in all different combinations from electrospray line and CF line in CF-EDESI set-up. Experiment 1: protein in pure water introduced through CF line, supercharging agent from electrospray line. Experiment 2: supercharging agent introduced through CF line, protein introduced through electrospray line. Experiment 3: Protein and supercharging agent were premixed and introduced through electrospray, blank solvent (MeOH: H2O=50:50) through CF line. Experiment 4: Protein and supercharging agents were premixed and introduced through CF line, blank solvent from ESI. The flow rate was all set at 10 μL/min.

4.2.3 Reversed Phase Chromatography

The separation was performed on a Surveyor LC quaternary pump system from Thermo Scientific (San Jose, CA). A Phenomenex Aeris WIDEPORE XB186 C18 (2.1 x 100 mm; 3.6 μm) column was used for the protein separation. And the flow rate was set at 0.35 mL/min, column temperature was set at 40 °C. The mobile phase A consisted of pure LC-MS water with 0.1% formic acid and B consisted of pure acetonitrile with 0.1% formic acid. A gradient program starting with 15% B hold for 1min, gradient from 15% B to 30% B

in following 19.5 minutes, then from 30% B to 50% B in another 12.5 minutes. The pump then was hold in a 10 minute wash at 99% B followed by 5 minutes re-equilibration of the column with 15% B. A flow split ratio at 5:2 was introduced post column so that the eluent from CF needle was finally reached around 0.120 mL/min. A electrospray flow rate of 0.12 mL/min was used to match the flow rate from the CF needle. See the configuration below.

4.3 Results and Discussion

4.3.1 RPLC Coupled to CF-EDESI

Method development on separation of seven proteins was first performed with ESI. Eight analyte peaks were observed on the chromatogram and was shown in Figure 4-2A. Under the separation condition, hemoglobin fell apart into two forms, denoted Form A (earlier eluting) and Form B (later eluting) in this work. Then the developed method was transferred on CF-EDESI. A representative chromatogram for the HPLC-CF-EDESI was shown in Figure 4-2B.

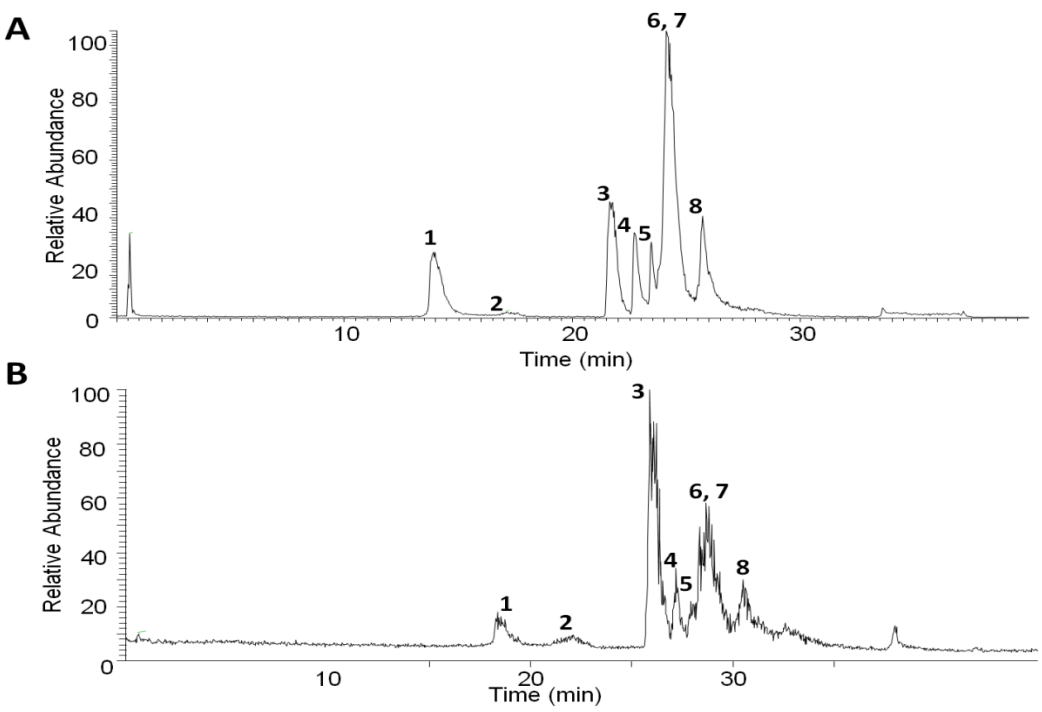


Figure 4-2. Representative chromatograms of the developed chromatographic method for protein separations performed using (A) conventional ESI and (B) CF-EDESI. Analyte peaks are assigned as listed: (1) cytochrome c; (2) lysozyme; (3) transferrin; (4) myoglobin; (5) hemoglobin Form A; (6) lactalbumin;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

Good agreement is seen between the two techniques when comparing those two chromatograms. The difference in retention time could be explained by a little bit slow in flow rate in CF-EDESI. The method sensitivity was compared between CF-EDESI and ESI by measurement of response factors of each analyte. The results presented in Table 4-1. The response factors for each protein were determined from three protein mixtures with various concentration of each protein and calculated from the summation of all detected peaks for each protein. Each concentration points were reproduced for three times. For

fair comparison, the third time measurement was done with the chromatogram separation on ESI. A post column addition acidic modifier could gain better response. The results showed that CF-EDESI showed comparable response factor than ESI with a post column infusion if not better. From CF-EDESI, we could see that response factor can easily be improved by increasing the concentration of acetic acid in electrospray solvent.

Table 4-1. Response factors (R_f in ion counts/μM) determined for proteins analyzed by LC-ESI-MS, LC-ESI-MS with post-column addition of acetic acid, and LC-CF-EDESI-MS with acetic acid introduced through the ESI source.

Protein	R_f: ESI (conv.)	R_f: ESI (0.1% HOAc – Post column)	R_f: CF-EDESI (0.1% HOAc)
Cytochrome C	$(1.1 \pm 0.2) \times 10^6$	$(9.2 \pm 0.4) \times 10^6$	$(4.2 \pm 0.7) \times 10^6$
Lysozyme	$(6.7 \pm 0.6) \times 10^4$	$(2.0 \pm 0.5) \times 10^5$	$(2.7 \pm 0.8) \times 10^6$
Transferrin	$(1.49 \pm 0.02) \times 10^7$	$(1.72 \pm 0.09) \times 10^7$	$(1.5 \pm 0.2) \times 10^9$
Myoglobin	$(3.9 \pm 0.2) \times 10^6$	$(7.2 \pm 0.3) \times 10^6$	$(1.33 \pm 0.03) \times 10^7$
Hemoglobin	$(2.03 \pm 0.04) \times 10^6$	$(6.8 \pm 0.4) \times 10^6$	$(1.2 \pm 0.4) \times 10^7$
Lactalbumin	$(2.4 \pm 0.2) \times 10^6$	$(5.6 \pm 0.5) \times 10^6$	$(1.86 \pm 0.07) \times 10^7$
Bovine Serum A	$(6.3 \pm 0.2) \times 10^7$	$(9.7 \pm 0.6) \times 10^7$	$(1.9 \pm 0.1) \times 10^8$

In LC-CF-EDESI-MS, proteins were subject complete denaturation due to the HPLC conditions before the mass detection. However, when you increase the acetic acid concentration in its electrospray, increasing charging was still observed in Figure 4 (as you can see for cytochrome C).

Additional experiments were also carried out with super charging agent sulfolane and mNBA on the chromatographic method in CF-EDESI. With the same concentration of additive (mNBA and sulfolane) that were used in myoglobin denaturation experiments, however, did not observe significant charge state shift, instead, increased the background noise. This results seem to indicate that the mechanism of CF-EDESI were different from conventional ESI. In CF-EDESI, the super charging agent interaction with the analyte droplets from outside and instead to be trapped inside charged droplet with analyte in ESI.

4.3.2 Supercharging mechanism study

With the supercharging agent (sulfolane and mNBA), people were first proposed the mechanism based on charge residue model (CRD) and Rayleigh equation. They believed that the increased surface tension caused by those high boiling point agents will directly attribute to protein unfolding or conformational change. But this proposal was not widely accepted [99]. Several recent studies also demonstrated the preservation of noncovalent complexes in ESI-MS upon SC [100][101]. However, the most recent paper proposed that a “binding” mechanism that the supercharging agent bonded to certain site of protein causes the supercharging [102].

In this experiment, we provided insight into protein supercharging through CF-EDESI set up. In CF-EDESI, the charging agent is not present in the (clarify) electrospray droplet to augment the surface tension and facilitate increased charging (as it proposed in some paper), as it does in conventional ESI. Four experiments (1, 2, 3, 4) were

designed with introducing protein and supercharging agents at different combinations through CF line and electrospray line. Charging agents such as sulfolane and mNBA did not show obvious impact in increasing charge states in CF-EDESI, characteristic charge state for native state cytochrome C centered at m/z (+8) was observed in most of the results in cytochrome C experiments. However, at high concentration of (1%) sulfolane, adduct peaks caused by sulfolane itself completely suppressed the signal from protein in experiment 1 (Normal CF-EDESI) set up, no protein and charging agent binding peak was observed. This result was different from that produced in ESI. So the surface tension mechanism for super charging protein sounds reasonable based on our results. However, we cannot rule out the possibility of "binding" mechanism completely, since the contact of super charging agent and protein was instant in CF-EDESI.

When using acetic acid as the supercharging agent, identical result was obtained with introducing protein solution through different line showed in experiment 1 and 2, see Figure 4 A and B. When premix the protein and acetic acid in experiment 3 and 4, however, showed different spectrum by introducing the premix through different line. In experiment 3, two cones of supercharging distribution were observed, see figure 4C. The first one centered at m/z 888.97 (+14) indicating the denatured form of cytochrome C. The second cone was calculated as the dimer form of cytochrome C which also centered at m/z (+14). The aggregation should have happened before the protein was supercharged as it premixed with acid. The supercharging capability is more likely to depend on the contact time (for cytochrome C and its dimer are the same), not the surface area or the binding sites availability. Thus, either cytochrome C or its dimer form has the same chances to acquire the same amount of charges. This conclusion was confirmed in experiment 4. When premixed protein was introduced through electrospray, protein as well as any aggregation (the dimer) could obtain sufficient charge as it could.

The spectrum we got was only with one cone distribution, see Figure 4D. From this result, we could propose that contact time of protein mix and electrospray is critical for protein to be sufficiently charged. The binding mechanism which they proposed with mNBA and sulfolane may support by this result.

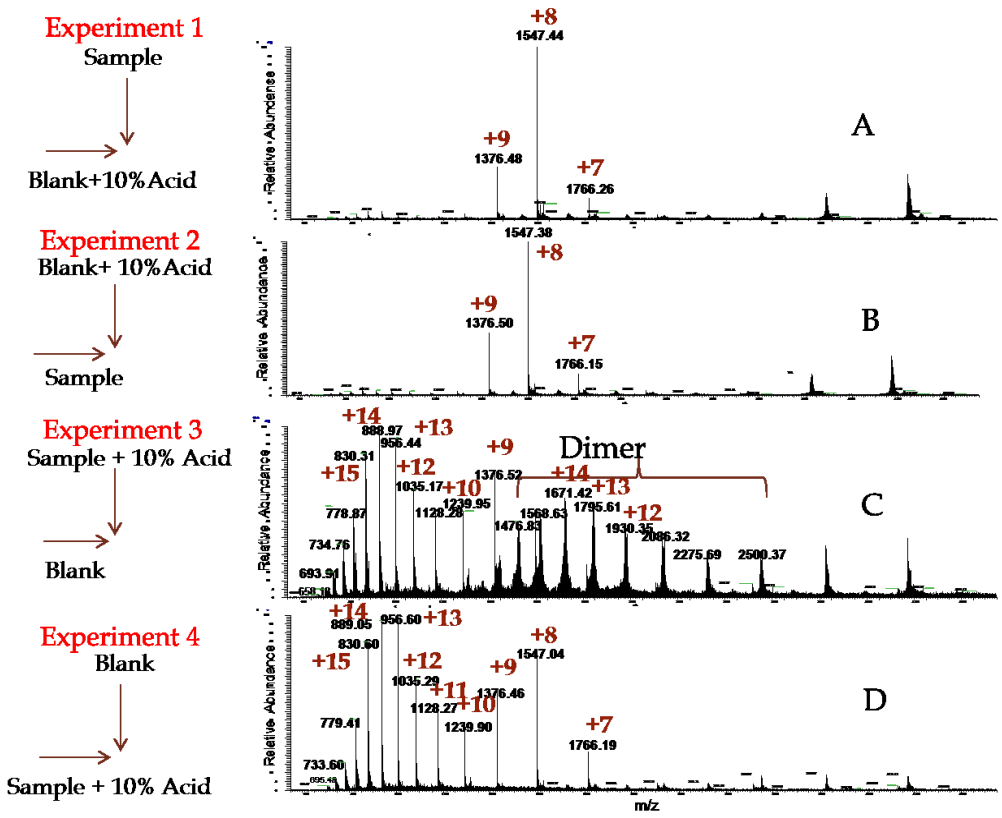


Figure 4-3. The protein and supercharging agents added in all different combinations from electrospray line and CF line in CF-EDESI set-up

From CF-EDESI, some insight was provided for the argument of supercharging mechanism. The “surface tension” mechanism and “binding” mechanism may both contribute to protein supercharging. And the process may complicated by several factors combination.

4.4 Conclusion

This experiment successfully demonstrated the separation of seven proteins and their detection in CF-EDESI. The advantage over ESI has particularly showed in the flexibility in changing (or optimization of post-run additives) in post-run separation. Also,charging agent affect the droplet dynamics and protein charge state is different from conventional ESI enlightened us to figure out the mechanism of CF-EDESI which supposed to be different than conventional ESI. More work has to been done to elucidate the difference.

Chapter 5

Analysis from Non-ESI-friendly Solvents and Mechanism

5.1 Introduction

Electrospray ionization (ESI) has made a significant impact on the development of mass spectrometry (MS) due primarily to its application as a soft ionization source for analysis of small and large biomolecules from aqueous media [96, 103]. Besides, ESI can easily be coupled with high performance liquid chromatography (HPLC) and other liquid separation modes to facilitate MS analysis of complex mixtures. Wider application has been achieved for a multitude of analytes in pharmaceutical [104], environmental [105], forensic [106], and clinical [107] analysis, among many others.

Even considering its many contributions, ESI still has limitations. One of the limitations is the choice of solvent. Typical ESI solutions are aqueous mixtures of polar organic solvents. They represent good compromise for ionization process as too high surface tension (pure water), on the contrary too low surface tension and low dielectric constant (many pure organic solvents). For the solvents exhibiting low conductivity, dielectric constant, and surface tension, the main reasons to cause the low efficiency are that a) it is difficult to achieve a stable spray with solvents possessing low surface tension, b) charge separation is hindered at the spray capillary tip, and c) nonpolar or low polarity solvents insulate the analyte from efficient charging. Further, nonpolar or too low polarity solvents do not dissolve electrolytes that would ensure conductivity of solution necessary for proper spraying. Consequently, nonpolar or low polarity solvents which are widely used in organic reactions (primary solvents in radical reactions), sample extraction, normal phase chromatography eluents, and as well as their great potential in lipidomics, chiral and LC-NMR/MS analyses, are less amenable for use in ESI.

That said, there have been accounts in the literature focusing on the use of non-ESI-friendly solvents for ESI-MS analysis. Such experiments have generally relied on augmenting the solvent through the addition of more ESI-friendly components. Non-polar solvents with low conductivity can be used in HPLC-ESI-MS, but they generally require post-column addition of a polar solvent to attain satisfactory sensitivity [108,109]. Noncovalent complexes and supramolecular assemblies, which are stable in low polarity solvents can be promoted into the gas phase by ESI for interrogation by MS, but success in this regard can be highly system dependent [110,111]. Gas phase ionic metal-ligand complexes can be generated directly from solvents such as chloroform or dichloromethane using ESI, but the vast majority of reports for such systems, some of which may require an anhydrous environment to remain intact, utilize acetonitrile as a dominant component in the spray solvent [112,113]. The use of ionic liquids as an additive to hydrocarbon solvents (such as hexane and toluene) was reported to facilitate ionization of metal complexes by McIndoe and coworkers [114]. Also worthy of note, Van Berkel and coworkers have published a number of papers focused on redox charging of analytes using ESI in combination with halogenated solvents [115,116,117]. So, while analytes in nonpolar and low polarity solvents have been successfully studied using ESI-MS, these solvents can still be considered to be non-ESI-friendly in the context of the vast majority of routine and highly sensitive applications.

Recently, a plethora of ambient ionization (AI) techniques have been introduced as alternate means to generate ions for mass spectral analysis [28]. Independent optimization of the ionization and sample introduction processes are generally achievable. Often, samples can be introduced into the ion source in their original state, with minimal sample preparation.

For liquid samples, especially in the form of a continuous flowing stream, extractive electrospray ionization (EESI) is an AI technique worthy of note. EESI involves the comingling of the electrospray plume with a pneumatically-nebulized sample solution or aerosol in front of the MS inlet to achieve ionization and detection of analytes. EESI has been shown to be viable for analysis of undiluted urine [18], milk [51], perfumes [51], and human breath [54], with minimal or no sample preparation. Non-ESI-friendly solvents such as hexane, cyclohexane, benzene, chloroform were successfully used in a mechanistic investigation of EESI, focused on fatty acid analysis [50].

Compared to CF-EDESI developed in our group, sample solutions are introduced into the ESI source (set on-axis with the MS inlet) by means of a pumped flow through a hypodermic needle set orthogonal to the source. Previously, this AI technique has been demonstrated for the manipulation of protein charge state distributions [22], but little effort was given to characterizing the mechanism associated with its successful performance. In this study, we have sought to demonstrate the advantages of CF-EDESI compared to ESI and to systematically examine the effect of experimental variables on CF-EDESI response in both positive and negative ionization modes. A comparison was also made to EESI in the context of fatty acids analysis [50]. . Meanwhile, in the case of the various analytes targeted in this study, a significantly higher response was obtained by CF-EDESI compared to ESI, indicating the promise of this technique for future applications.

5.2 Experiment

5.2.1 Chemicals/ Reagent

LC-MS grade water (H_2O) and acetonitrile (ACN) were obtained from Burdick & Jackson (Muskeegon, MI, USA). LC-MS grade methanol (MeOH) and glacial acetic acid (HOAc) were obtained from J. T. Baker (Phillipsburg, NJ, USA). Hexanes were purchased from Fisher Scientific (New Jersey, USA). Chloroform was from Pharmco-AAPER (Connecticut, USA). Ethyl acetate, isopropanol, progesterone, hydrocortisone, and menadione (Vitamin K_3) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Myristic acid, palmitic acid, oleic acid, stearic acid, arachidic acid, and linoleic acid (marketed as GC standards) were purchased from Fluka (St. Louis, MO, USA).

5.2.2 Instrumental

A LCQ Deca XP quadrupole ion trap mass spectrometer equipped with a conventional ESI source (all from Thermo-Fisher Scientific, Inc., San Jose, CA, USA) was used and modified in-house to construct the CF-EDESI arrangement depicted in Figure 3-1. A homemade XYZ stage was constructed and used to carefully position the hypodermic needle (22 gauge; SGE Analytical Science, Victoria, Australia) in the spray path. The position the CF needle was optimized by monitoring the response of a 10 μM progesterone solution in hexanes to achieve a stable and high intensity signal. The indicated distances between the electrospray source needle and the CF needle (1.5 mm) and between the electrospray source and the inlet of the mass spectrometer (8.0 mm) are generally consistent with the optimum arrangement. Samples were prepared and introduced through the CF needle by direct infusion with a 500 μL SGE syringe and syringe pump (KD scientific, Model KDS-108, Holliston, MA, USA). Typical ESI solvents were introduced in spray line with a 500 μL SGE syringe and the syringe pump housed

on the LCQ Deca instrument. A 200 μl stainless steel injection loop immersed in a hot water bath was integrated into the CF sample solution line when temperature studies were performed.

A commercial factorial design software Design-Ease version 8.0 was used to predict the impact and the interactions of five main variables deemed important for the experimental arrangement. The five factors (independent variables) (and respective chosen maximum and minimum settings) included sample and electrospray solvent flow rates (5, 50 μl/min), nebulizer gas flow rate (40, 80 arbitrary units), electrospray voltage (3, 5 kV) and sample solution temperature (20, 50 ˚C). The monitored output was the mass spectral signal intensity, obtained for analysis of solutions containing 10 μM progesterone in hexane and in chloroform. Representative maximum and minimum values of each parameter were provided to the software to generate an experimental design matrix. A half-factorial experimental design (25/2) was performed in triplicate (randomized) for a total of 48 analytical determinations under the defined conditions (Half numbers of runs was picked from full factorial design by the DOE software which leaves the subject interest of runs). Additional experiments under a more extended range of settings for variables identified by the study to be the most important were also investigated.

5.3 Results and discussion

Several analytes including progesterone, hydrocortisone and vitamin K_3 in different ESI-non-friendly solvents were evaluated for analysis using CF-EDESI. As a bioactive small molecule of significant interest [118,119,120], we chose progesterone as our primary model compound for initial experiments. While a plethora of methodologies have been published for the analysis of progesterone and related chemical compounds

[121,122,123,124], its hydrophobic nature generally dictates the use of hydrophobic media for its isolation from various sample types. Thus, strategies for analysis of progesterone, and other compounds of similar chemical nature, directly from such media might be considered beneficial for reducing sample preparation such as evaporate off less polar extractions and reconstitute in standard ESI solvents.

Based on solubility, some other combinations of analytes and non-ESI-friendly solvents were also investigated. Overall, excellent signal intensity for the combinations tested was obtained by CF-EDESI, as shown in Table 5-1. A solution of 49/49/2 H_2O:MeOH:HOAc was used as an optimal electrospray solvent after some preliminary tests. For comparison, the same sample solutions were directly infused for analysis using conventional ESI-MS (in the standard orthogonal spray geometry on the LCQ instrument). No discernable signal response was observed in conventional ESI for the analytes tested in hexanes and chloroform. Low signal intensity for progesterone was observed in ethyl acetate, but the analysis signal was dwarfed by an interference signal from the solvent (m/z 177 was the observed base peak when EA was used as sample solvent).

Table 5-1. Comparison the capability of CF-EDESI and ESI in generating ions from non-ESI-friendly solvents. 49MeOH/49H_2O with 2% acetic acid was used to generate electrospray.

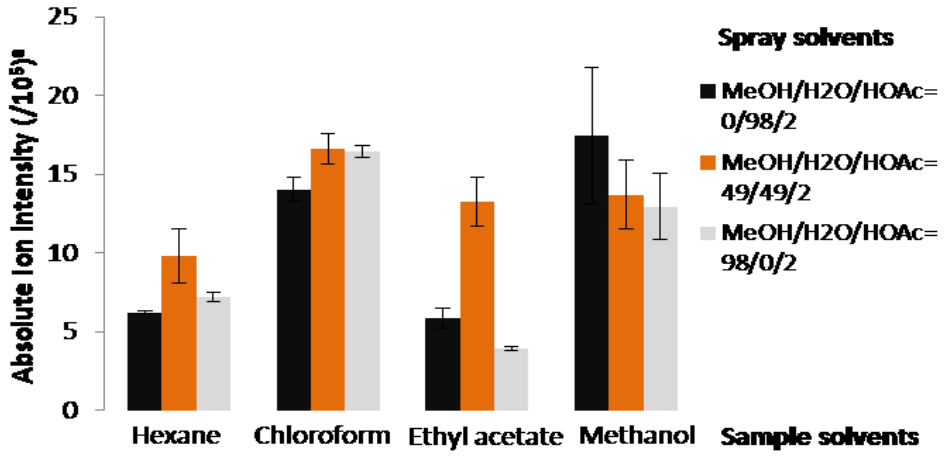
Entry	Analyte	Solvent	Concentration	ESI Response	CF-EDESI Response (/10^5)[a]
1	Progesterone	Hexane	10 μM	ND	6.5 ± 0.5
2	Progesterone	Chloroform	10 μM	ND	16.9 ± 0.9
3	Progesterone	Ethyl Acetate	10 μM	1.7±0.3	14 ± 1
4	Vitamin K_3	Hexanes	1 μM	ND	1.5 ± 0.3
5	Hydrocortisone	Chloroform	10 μM	ND	21 ± 2

[a] Results presented as average ± standard deviation (n = 3). 'ND' denotes not detected.

An optimal ESI spray solvent (49/49/2 H_2O:MeOH:HOAc) was found to maximize signal response in the CF-EDESI mode. Such modified aqueous organic solvents give a good compromise between conductivity, volatility, and charge separation to facilitate ion formation through the ESI mechanism in the positive ionization mode. However, the notion of mixing between the spray and sample solvents, or the possibility of contributions from microextraction processes prompted us to consider the effect of miscibility of the sample and spray phases on signal quality. Ethyl acetate (EA) and chloroform ($CHCl_3$) are partially miscible with a 49/49/2 H_2O: MeOH: HOAc spray solvent, whereas hexanes is largely immiscible with it. These assertions were made based simply on mixing the solvents together in a vial, however, they do not account for how charged spray droplets might possibly effect the interaction between the spray and sample solvents. In general, better results were obtained from ethyl acetate and chloroform (Table 5-1), but a more thorough investigation of phase miscibility was also performed.

To investigate the effects of relative miscibilities of sample and spray solutions on analyte response, the analyte progesterone was introduced through continuous flow in hexanes, chloroform, ethyl acetate and methanol, while the composition of the electrospray solvents was varied (0%, 49% and 98% MeOH in aqueous solution with 2% HOAc). According to the data displayed in Figure 2, when the sample was dissolved in hexanes and ethyl acetate, the use of 49/49/2 MeOH:H_2O:HOAc clearly provided the highest signal response. For other sample solvents, there was no significant difference among performance of spray solvents, within experimental error. A slight preference for an ionization spray with high water content when the sample was present in 100% methanol is notable. Taken together, these ionizing sprays are either miscible (and facilitate mixing of the phases; e.g. high methanol) or immiscible (and facilitate extraction of the sample solvent by the ionization solvent; e.g. high water) with the sample solvent.

Regardless, optimal response appears to be achieved with the use of a spray solvent, which facilitates ionization according to the general ESI mechanism. In other words, the use of a 49/49/2 H_2O:MeOH:HOAc spray solvent provided the optimal response in these experiments.



[a]Responses are represented as average ± standard deviation for n = 3 runs

Figure 5-1 : Effect of methanol composition in the ionizing spray on the signal intensity for progesterone in CF-EDESI. 10 µM progesterone in 100% hexane, chloroform, ethyl acetate or methanol were introduced through the sample line.

The CF-EDESI moniker was chosen to describe an AI technique that was believed to have similarities with other established techniques, namely DESI (particularly TM-DESI, given the on-axis arrangement of the source and MS inlet) and EESI. A greater similarity is apparent for EESI (Figure 3), which allows the coupling of the source to a continuous flow and has been shown to be a powerful tool for analysis of aerosols, complex solutions, and suspensions without additional sample pretreatment.

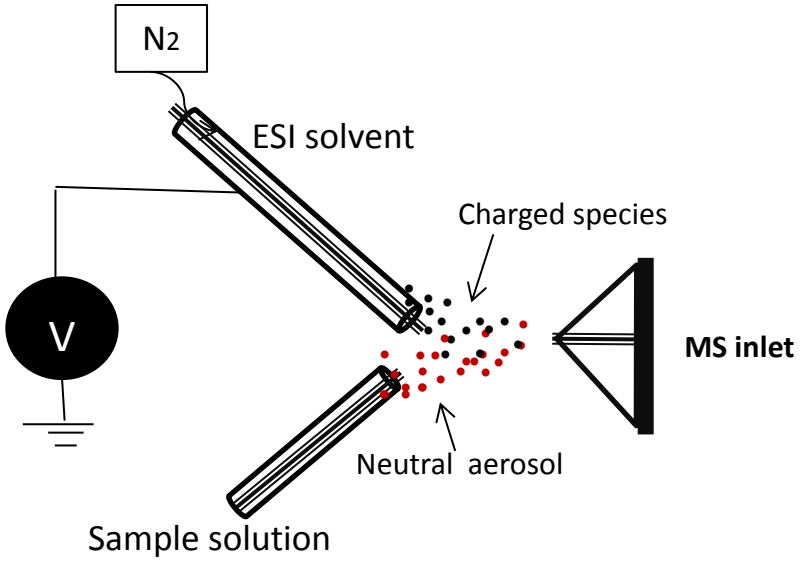


Figure 5-2. Schematic of an extractive electrospray ionization (EESI).

Recent experiments designed to understand the mechanism of EESI have revealed that extraction occurs between the nebulized sample aerosol and charged spray droplets in the intersection region between the two phases, just in front of the MS inlet []. In other words, the key factor governing EESI response is that the analyte should be soluble in the charged spray solvent to facilitate extraction from the sample aerosol and subsequent ion formation. This finding was based on the analysis of a series of fatty acids present in a range of sample solvents. During this process, matrix interferences can be reduced if they are extracted to a lesser degree in the spray phase compared to the analytes of interest. Thus, EESI is well suited to analysis of particular analytes from complex samples.

In order to establish the similarities or differences between EESI and CF-EDESI, an experiment complementary to that reported by Zenobi and coworkers [50] was carried out in our lab. Solutions of four fatty acids (1 μg/ml) including myristic acid (14:0), palmitic acid (16:0), stearic acid (18:0), linoleic acid (18:2) in hexane, cyclohexane, chloroform,

acetonitrile, and methanol were examined using CF-EDESI in the negative ionization mode. A reduced analyte set in our work compared to the previous work was necessary due to limits in solubility for some higher chain fatty acids. The spray solvent (80/19.5/0.5 MeOH:H_2O:NH_4OH) was chosen to be identical to that used for EESI. Figure 4 provides a visual comparison between the results of the two experiments.

Our experiments demonstrated a clear difference between EESI and CF-EDESI for the analysis of fatty acids in the negative ionization mode. Consistent with its moniker, EESI is believed to be largely an extraction process. The solubility of fatty acids has been reported to be higher in methanol compared to acetonitrile [125]. Additionally, it has been shown that the ESI response of fatty acids in protic solvents is much higher than aprotic solvents based on previous reports [126, 127]. In EESI, acetonitrile provided the highest signal. Because of the higher solubility of the fatty acids in methanol, the analytes are efficiently extracted into the spray phase from their original acetonitrile sample solution. In CF-EDESI, fatty acids displayed the highest response in methanol, a protic solvent; this result is consistent with that expected for conventional ESI, and it is consistent with other experiments presented in this work. Another obvious difference of CF-EDESI and EESI is the droplet-bulk solution interaction (charged solvent spray interacted with continuous flow of sample solution) in CF-EDESI compared to droplet-droplet (solvent aerosol and sample aerosol) interaction in EESI. This difference clearly leads to changes in ionization efficiency under similar operation conditions, and asserts that CF-EDESI operates under an independent mechanism.

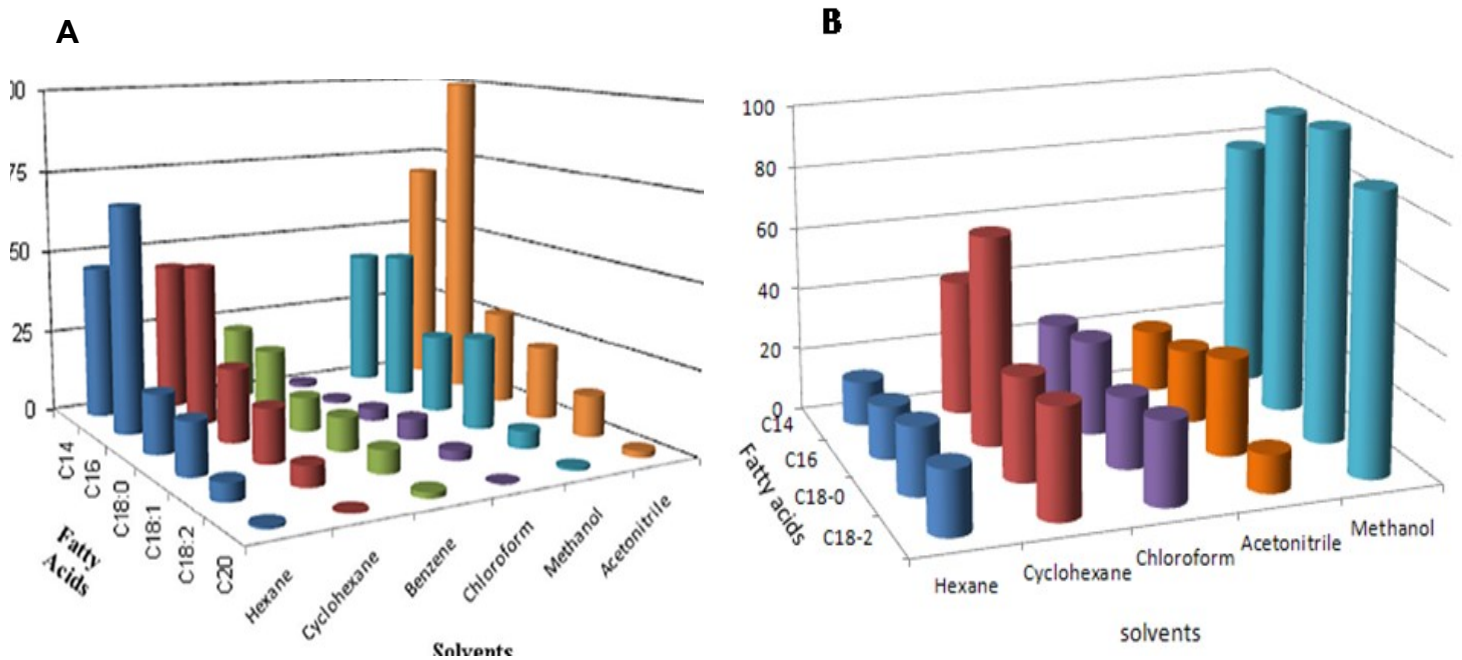


Figure 5-3. A: Effects of different organic solvents on the signal intensity of fatty acids in EESI [10] B: Effects of different organic solvents on the signal intensity of fatty acids in CF-EDESI.

In an effort to more comprehensively characterize the importance of experimental variables, a partial-factorial experimental design was performed to predict the effect of individual and multiple factors on the CF-EDESI signal intensity. Commercial software was used to process the results obtained from experiments to evaluate a matrix of five prominent variables in a series of main 48 analytical runs out of 96 full runs (Half factorial design is more economic and maintains the optimization capability as full factorial design), which included triplicate analysis of each condition specified.

The results of the experiment are given in the form of a half-normal probability plot, shown in Figure 5-4. The points that deviate from the linear region, toward the right of the plot, are considered significant positive and negative effects (depending on the variable specified), whereas the effects close to the “zero region” (on the line) are categorized as insignificant effects relative to experimental error. The positive effects represent a direct relationship of the design factors with the system outcomes (i.e., these variables aid response), whereas the negative effects represent an inverse relationship (i.e., these variables hinder response). Additional quality control parameters provided by the software indicated that the data set used to generate these results was of high quality.

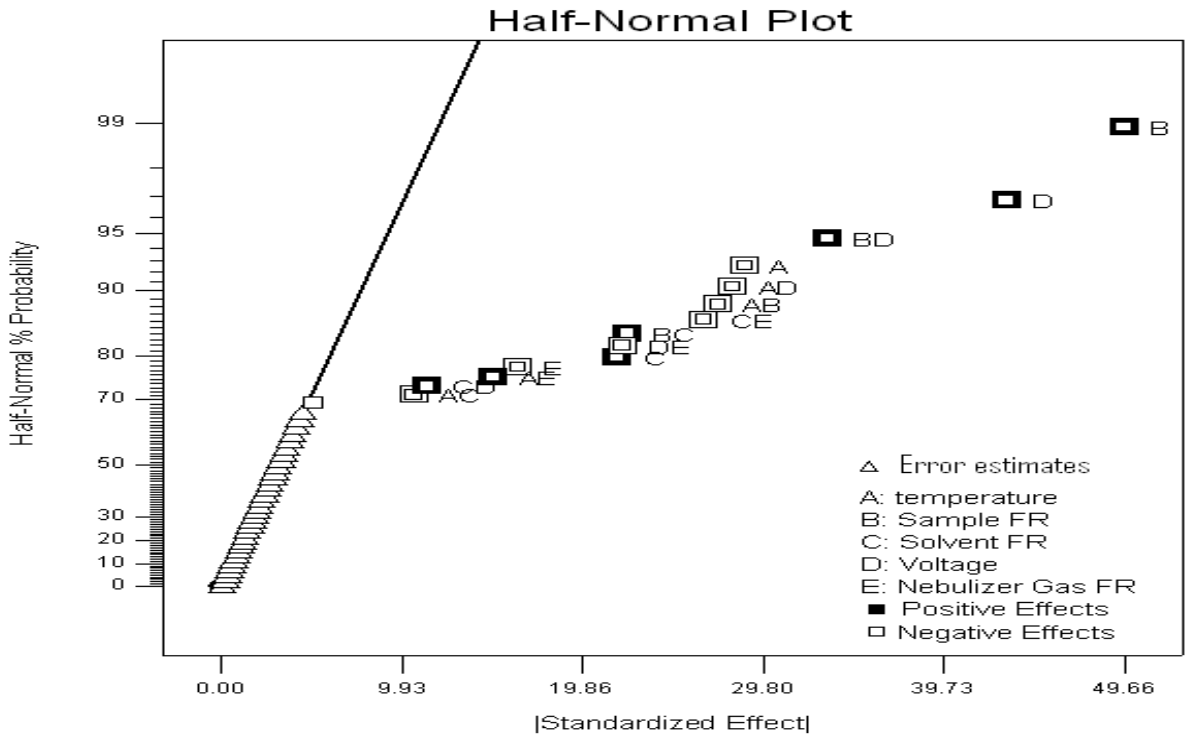


Figure 5-4. Half-normal plot. The absolute value of an effect on the x-axis and estimates of errors are represented as squares and triangles, respectively. A-E are five factors that we investigated which indicated on the plot.

According to this analysis, a higher setting for sample flow rate (B) and spray voltage (D) displayed the most significant positive effect (an order of magnitude increase) on signal intensity. A combined increase of these two variables (BD) provided the next most significant positive effect. An increase in temperature (A) returned the most significant negative effect on signal intensity. Any combinations of variables with temperature (e.g. AB, AC, AD, and AE) produced negative effects. Other factors had lesser effects. An increase in spray solvent flow rate (C) had a positive effect, whereas an increase in nebulizer gas flow rate (E) gave a negative effect. Interestingly, the combination of a high sample flow rate (alone, a strong positive effect) with a high nebulizer gas (alone, a weak but significant negative effect) showed no effect on intensity; this point fell on the zero line of the plot.

Additional confirmation experiments (see Figure 3-3) were done in case there was non-linear relationship of the response and factors. Nebulizer gas flow shows a maximum at around 60bar, and temperature shows some random variation from low to high both were not proportional to the response. Samples were prepared and introduced through the CF needle by direct infusion with a 500 μL SGE syringe and syringe pump (KD scientific, Model KDS-108, Holliston, MA, USA). Typical ESI solvents were introduced in spray line with a 500 μL SGE syringe and the syringe pump housed on the LCQ Deca instrument. A 200 μL stainless steel injection loop immersed in a hot water bath was integrated into the CF sample solution line when temperature studies were performed.

The results showed disagreement with the prediction by the software. But both sample and solvent flow rate were showed good linearity with intensity, so the prediction was reliable.

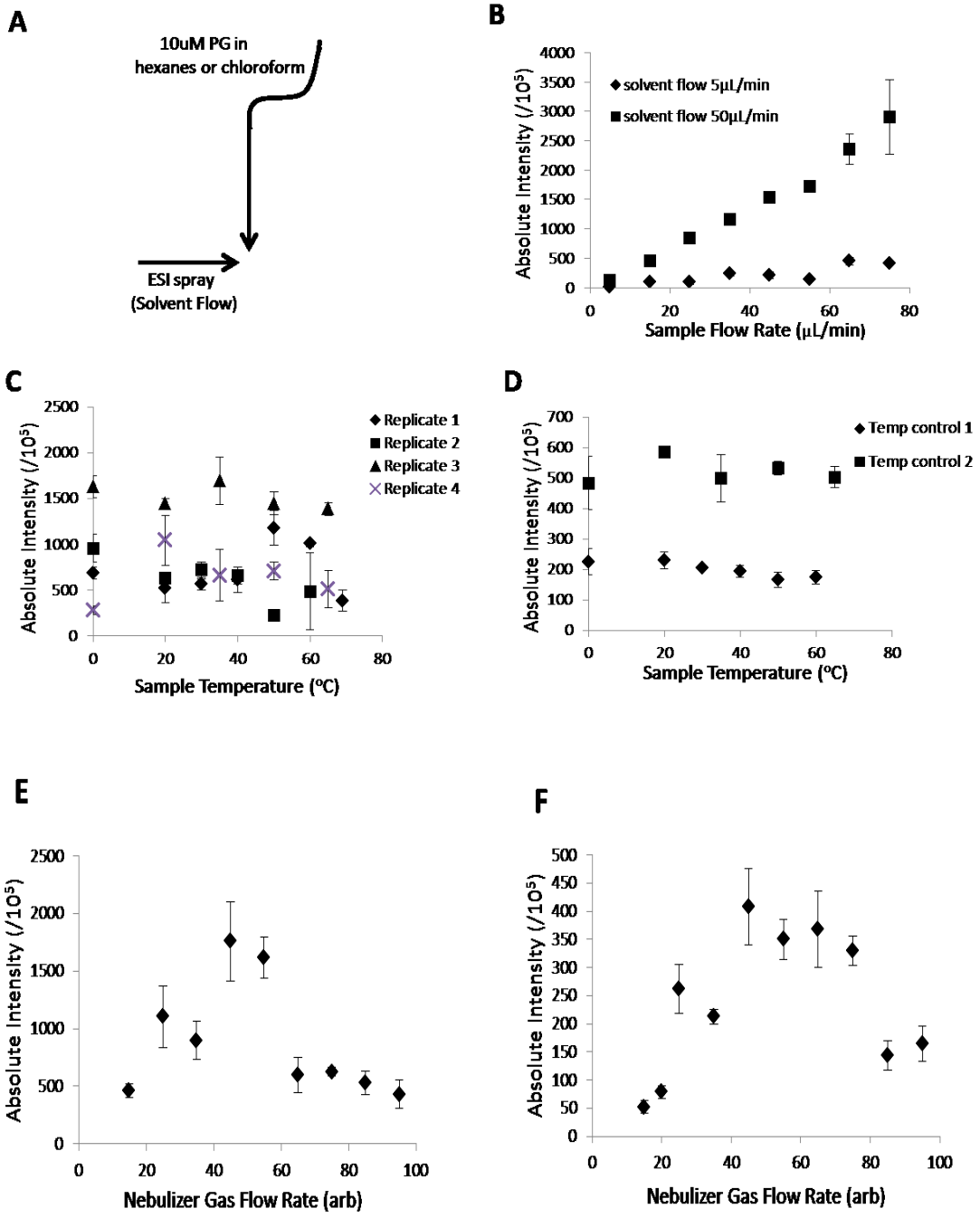


Figure 5-5. Effect of sample flow rate, sample temperature and nebulizer gas on

responses in CF-EDESI. A: Schematic diagram of CF-EDESI; B: Sample flow rate vs solvent flow rate with 10mM PG in hexane; C: Sample temperature tested from 0- 69˚C (Hexane b.p.: 69 ˚C) with 10mM PG in hexane (realized by sample solution went through a 200 ml metal injection loop which submerged in water bath); D: Sample temperature tested from 0- 60˚C (Chloroform b.p.: 61 ˚C) with 10mM PG in chloroform (set up same with C); E: Nebulizer gas flow tested from 15-95bar with 10mM PG in hexane; F: Nebulizer gas flow tested from 15-95bar with 10mM PG in chloroform. (If not mentioned, the other parameters were at sample flow rate 10mL/min, solvent flow rate 10mL/min, voltage 5KV, nebulizer gas flow 40 bar, sample temperature 25 ˚C).

The experiments presented have demonstrated a significant advantage in the use of CF-EDESI for the generation of ions from non-ESI-friendly solvents. The potential for independent optimization of the ionizing spray flow and the sample flow is well apparent in this technique. The best sensitivity is obtained when the electrospray solvent is chosen on the basis of ESI theory for efficient ion generation (e.g., acidified aqueous methanol). Further, where conventional ESI was not able to generate appreciable signal for analytes in nonpolar or low polarity solvents, CF-EDESI could. In cases where partial miscibility of spray and sample solvents was apparent, the signal intensity was higher. This prompts the proposition that some degree of mixing between the phases is optimal for efficient ion generation. Even so, a component of extraction is also possible, given that ions could still be observed using phases that are generally immiscible. Further work, using an expanded set of analytes will be pursued in the future to clarify the relative contribution, limits, and advantages for systems that better align with a total mixing vs. phase extraction mechanism for ion formation.

5.4 Conclusion

CF-EDESI, as a novel ambient ionization technique was applied to the analysis of analytes from non-ESI-friendly solvents, such as hexanes, chloroform and ethyl acetate. A careful systematic investigation of important variables, along with comparisons to results reported on the mechanism of EESI, have helped solidify the unique nature of this new AI source. Further applications of this technology are underway, and should broaden the scope of analyte systems which can be investigated using a commercial mass spectrometry system with a modified ion source. Specifically, we are exploring applications, such as tracking organic synthesis product reactions (from the reaction flask or the NMR tube; including reaction kinetics) and normal phase liquid chromatography separations (e.g., lipidomics and chiral separations).

Chapter 6

Coupled to Normal Phase Separation

6.1 Introduction

Ambient ionization (AI) mass spectrometry is a technique that has grown exponentially since 2004 due to its virtue of providing rapid results, gentle ionization, and little need for sample pretreatment [31]. Often, samples can be introduced in their original state without further preparation and independent of the ionization source. Therefore, more options exist for optimization of ionization source and sample conditions. AI techniques are revolutionizing the field of mass spectrometry and making possible many applications that were previously thought intractable.

Two common techniques used in ambient ionization are known as desorption electrospray ionization (DESI) [9] and direct analysis in real time (DART) [10]. These provide a reasonable delineation between two categories of AI, those that are spray-based and those that are plasma/chemical ionization-based, respectively. Both DESI and DART have been commercialized.

In DESI, a spray of charged microdroplets (usually ESI spray solvents) bombards a sample surface and forms a film to dissolve or extract analyte. Subsequent sprayed mircrodroplets impinge on the liquid film and pick up the analyte as they proceed to the MS inlet. This ionization process was believed to be “softer” than conventional ESI due to the secondary charge transfer from the spray species to analyte. Another popular spray-based AI technique called extractive electrospray ionization was designed and mostly useful for liquid or aerosol sample analysis. It equipped with additional nebulizer. The sample solution is isolated from the ESI source and its high voltage, by virtue of its introduction through a second nebulizer. Neutral sample droplets intersect with

charged aerosols from the ESI source in front of the MS inlet [50] to generate analyte ions.

The application of spray-based AI has been widely explored. DESI has been used to analyze a multitude of different analyte types including carbohydrates [39], peptides [40, 42], explosives [41][43], polymer oligomers [37], inks [38], non-volatile pyrolysis products [36], human breast cancer tissue [44], porcine and rabbit adrenal glands [45], lipids imaging or profiling in tissue and bacteria [43] [47][44][128],counterfeit antimalarial drugs [38], human bladder carcinomas[129], among many others [49]. Applications for EESI have included the analysis of undiluted urine [18], milk [51], diet effect on biofluids [52], perfume classification [51], human breath [54], and chemical reaction monitoring [55][56].The compatibility with liquid samples or aerosols makes EESI a good partner with separation techniques such as liquid chromatography. A modification of the EESI sampling method that uses a neutral desorption (ND) sampling gas beam to gently acquire analyte from sample surfaces, called NDEESI, was further demonstrated by Chen and his coworkers [57]. This development broadened the EESI application base to trace analysis on biological surfaces, and has been demonstrated for use with cheese [58], skin, tissue, muscle, and vessels [59].

Inspired by the initial discovery of ambient ionization techniques (DESI, DART, EESI etc.), numerous other spay-based ambient ionization methods are emerged during the last decade, including fused-droplet ESI [84], continuous flow infrared matrix-assisted laser desorption electrospray ionization (CF IR MALDESI) [82], electrospray laser desorption ionization (ELDI) [130], laser ablation electrospray ionization (LA-ESI) [131] and laser-induced acoustic desorption/electrospray ionization (LIAD-ESI) [132], to name a few.In our laboratory, continuous flow-extractive desorption electrospray ionization (CF-EDESI) was devised. It features introduction of sample solution through a

hypodermic needle (CF needle) set orthogonal to the electrospray source. The electrospray source is aligned on-axis with the MS inlet. CF-EDESI was first reported for supercharging native state proteins [22]. It was also demonstrated for its ability to analyze samples from non-ESI-friendly solvents [23].

Recently, AI techniques have been reported hyphenated with separations such as high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), capillary electrophoresis (CE), and solid phase extraction (SPE) [74, 78,79]. However, the combination of AI with normal phase separation has not been widely explored and is among the most attractive application field, since conventional ESI-MS provides limited compatibility with normal phase eluents. DART was among one of the first AI techniques to be demonstrated in combination with normal phase separations [133], but no spray-based AI has yet been reported.

One aim of this research was the application of CF-EDESI for coupling with normal phase liquid chromatography separations. We demonstrated coupling to normal phase chiral separation; five pairs of enantiomers were separated on 3,5-dimethylphenyl-functionalized cyclofructan 7 stationary phase (LARIHC CF7-DMP chiral column), according to conditions published in the literature [134]. A high percentage of heptane was used to ensure full separation of chiral aromatic amines. The liquid chromatography eluents were subjected to a post column flow splitting then introduced through the hypodermic needle into the ESI source. The configuration for this analysis is shown in Figure 6-1.

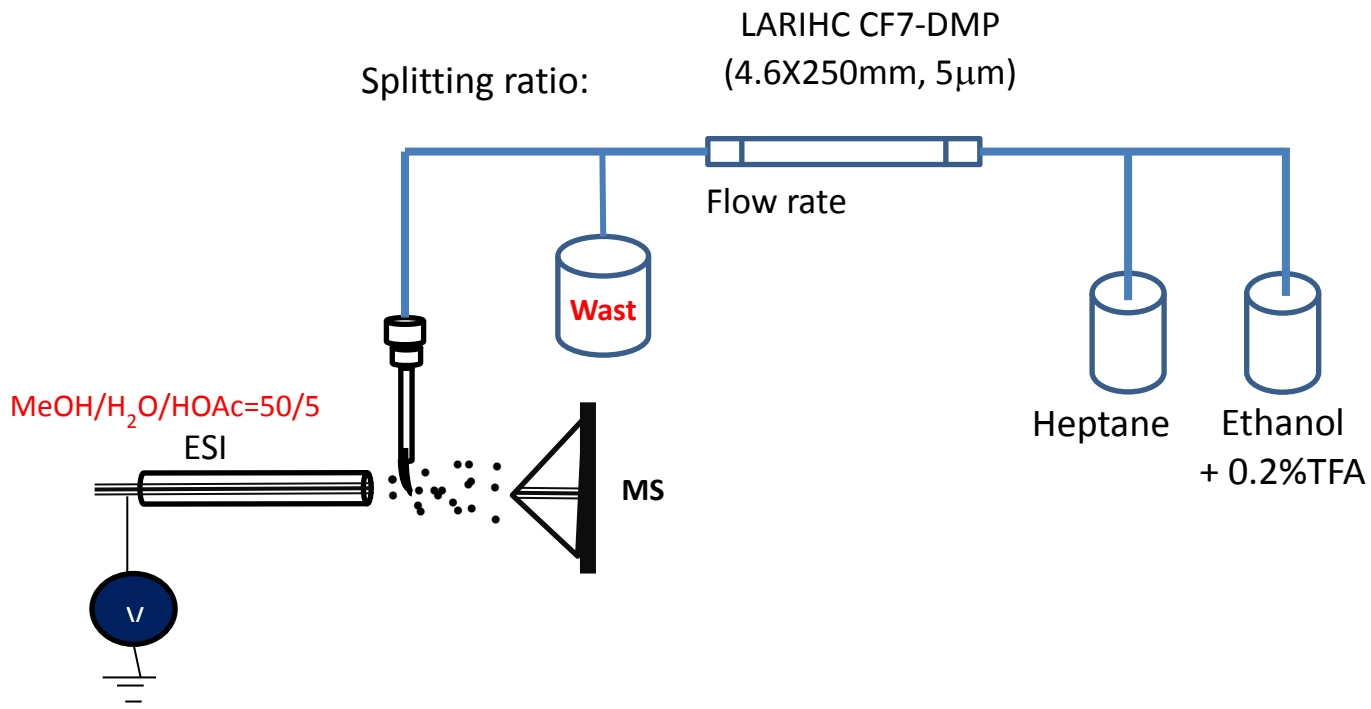


Figure 6-1. Normal phase separation coupled to continuous flow-extractive desorption electrospray ionization

A secondary aim was the direct analysis of cellular lipid extracts by CF-EDESI-MS. Less polar solvents such as hexane, ethyl acetate, diethyl ether, and methyl tert-butyl ether (MTBE) are common solvents for cell or tissue analysis. Extraction, evaporation, and reconstitution are regarded as the standard steps to prepare such extracts. Yet, due to the non-polar property of MTBE, it has poor ionization efficiency in ESI. Most of the MTBE extract has to be evaporated and reconstitution in a proper polar solvent for further mass spectrometry analysis. However, time, recovery, and stability of extracts can limit this approach. Methodology for direct infusion of the extraction solution for MS analysis would be useful for streamlining lipid analysis work-flows. Direct infusing cell extract in MTBE through CF-EDESI hypodermic needle to further distinguish the lipids difference was demonstrated as a viable alternative approach.

6.2 Experimental

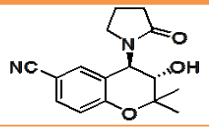
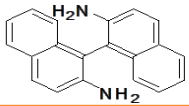
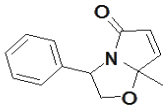
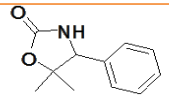
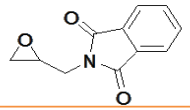
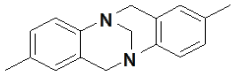
The instrumental configuration of the CF-EDESI has been described previously [22]. A LCQ Deca XP quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source (Thermo-Fisher Scientific, Inc., San Jose, CA, USA) was modified to align the ESI source with the MS inlet. A home-built and electronically controlled XYZ stage was used to manipulate the continuous-flow hypodermic needle position for sample introduction into the ESI plume. The distance between the electrospray source needle and the continuous flow needle was set at 1.5 mm. The distance between the electrospray source and the inlet of the mass spectrometer was set at 8 mm. The hypodermic needle position was optimized during direct infusion of a standard solution of 10 μM progesterone in methanol by achieving highest signal intensity. Electrospray was recorded in the positive ionization mode with the spray capillary voltage at 5.0 kV.

Nitrogen sheath gas flow was set at 60 arbitrary units. Sample flow rate was subject to a splitting ratio of 5:1. Final flow rate of approximately 0.2 mL/min was directed through the continuous flow needle. An electrospray solvent of 50/50 MeOH/H_2O with 2% acetic acid at a flow rate of 10 μL/min was used to generate the electrospray plume. The mass spectrometer capillary inlet temperature was set to 250° C. Data analysis was performed using Thermo Xcalibur Data Analysis software (ver 1.5).

6.2.1 NPLC Separation

The LC-CF-EDESI-MS system included a Surveyor autosampler, a Surveyor MS binary pump, and an LCQ Deca XP ion trap mass spectrometer (Thermo-Fisher Scientific, San Jose, CA, USA) equipped with a modified ESI source as describe above. The normal phase LC eluent was introduced through the hypodermic needle into the electrospray plume. Separation of chiral amines was performed on a LARIHC CF7-DMP (4.6 x 250mm, 5μm d_p) (AZYP, LLC, Arlington TX, USA) chiral column in isocratic mode, with mobile phase A (heptane) and mobile phase B (ethanol with 0.1% trifluoroacetic acid). The compounds and conditions were adapted from reference [134] and are listed in Table 6-1. Injection volume and on-column flow rate were set at 10 μL and 1 mL/min, respectively. Separations were carried out at room temperature.

Table 6-1. Compounds list and separation conditions for NPLC.

#	Compounds name	Structure	Concentration mg/mL	Mobile phase[a]
1	Cromakalim		0.05	90H10E
2	2,2'-Diamino-1,1'-binaphthalene		0.1	80H20E
3	2,3-Dihydro-7a-methyl-3-phenylpyrrolo[2,1-b]oxazol-5(7aH)-one		0.05 0.005	99H1E
4	5,5-dimethyl-4-phenyl-2-oxazolidinone		0.05	95H5E
5	N-(2,3-Epoxypropyl)-phthalimide		0.05	98H2E
6	Tröger's base		0.01	90H10E

a) 90H10E indicates 90% heptane and 10% ethanol with 0.2%TFA

During the course of evaluating the coupling of NPLC to CF-EDESI, we also compared the sensitivity of CF-EDESI-MS and photodiode array detection. Each analyte sample was prepared in a series of diluted solution at 250, 5, 0.5, and 0.1 μg/mL in the appropriate normal phase eluent solvent. Direct infusion of different concentrations of solutions into the CF-EDESI-MS and PDA detectors at the flow rate of 0.2 mL/min was used to generate a truncated calibration curve for both. Limits of detection (LOD) were calculated using the standard deviation of the signal at a low concentration and the slope of calibration curve (3 s/m). A Shimadzu PDA M20A detector at the wavelength of 254 nm was used for this analysis.

6.2.2 Cell Extraction and Direct Infusion for Lipid Analysis

Methanol (1.5 mL) was added to bacteria cells in a 15 mL centrifuge tube and vortexed. Then, 5 mL of MTBE was added, the mixture was sonicated for 5 min, then incubated for 1 h at room temperature. Phase separation was induced by adding 1.25 mL of water [136]. Upon 10 min of incubation at room temperature, the sample was centrifuged at 13,000 rpm for 10 min. The upper organic phase was collected. In separate CF-EDESI experiments, the electrospray solvent was varied between 50/50 MeOH/H_2O, with and without the addition of 5 mM NH_4OAc. Fragmentation was carried out for some of the most intense peaks to identify high abundance lipids in the range of *m/z* 600 - 800 by data dependent acquisition. The electrospray flow rate was maintained at 10 μL/min with the integrated LCQ syringe pump, while the sample solution was introduced through the CF needle at 20 μL/min using a separate syringe pump. Other source conditions were maintained as described above.

6.3 Results and Discussion

CF-EDESI was introduced previously to efficiently ionize analytes introduced orthogonal to the electrospray probe in a continuous flow format from the tip of a hypodermic needle [22][23]. The relative velocities of the intermixed aerosol and liquid can be varied by changing flow rates and needle dimensions. A factorial experimental design was previously carried out to delineate and statistically validate important and interacting variables, en route to their optimization and understanding important mechanistic aspects of the new source. Sample flow rate was confirmed to have the highest impact on signal intensity. It was concluded that a liquid-liquid mixing of phases in CF-EDESI is more likely to be the dominant process over, in contrast, liquid-extraction interactions in EESI [23], as results obtained comparing these two AI sources were markedly different. In the course of that work, CF-EDESI was demonstrated to be very effective for analyzing samples composed from “non-ESI-Friendly” solvents. Here, we extend the use for coupling normal phase separations to mass spectrometry and direct cell extract analysis.

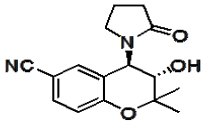
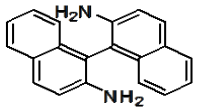
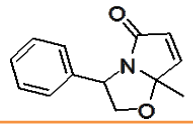
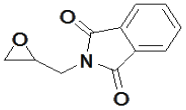
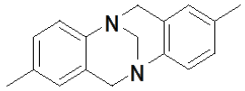
6.3.1 NPLC Chiral Separation

Amine-containing chiral compounds are ubiquitous in nature; they are often biologically active and are commonly encountered in pharmaceutical science. The chiral separation of primary, secondary, and tertiary amines was previously demonstrated on a cyclofructan-based stationary phase column by Armstrong and co-workers [134]. A high percentage of the non-polar solvent heptane (most above 90%) as mobile phase was used for the separation of those compounds in conjunction with UV detection. However, not all the targeted compounds had strong UV absorbance and alternative techniques would be needed for optimal detection, especially if trace determination were necessary.

ESI is a versatile detection technique that generally provides higher

sensitivity than UV detection. It provides the additional benefit of identifying a compound by the mass, which adds significant specificity that cannot be matched by UV detection. Yet, it cannot be used as an efficient interface for normal phase chromatography. A high heptane content is particularly poor for facilitating ionization efficiency. Five enantiomer compounds were selected from a prior study [134]. Separations, detected in that study by UV detection, were reproduced and detected with CF-EDESI. The separation showed good agreement with literature, but better sensitivity. Data acquired from CF-EDESI is shown in Table 6-2.

Table 6-2. Separation of five chiral compounds.

#	Structure	Concentration mg/mL	k_1	α	Rs
1		0.05	22.97	1.1	1.47
2		0.1	2.56	1.84	5.16
3		0.05 0.005	6.29	1.05	0.73
5		0.05	12.6	1.05	0.92
6		0.01	1.95	1.46	5.38

The LOD of each compound detected by CF-EDESI and PDA are compared in Table 6-3. We chose target compounds bearing aromatic rings with good UV absorbance, but also amine bearing compounds which might be favorably ionized by CF-EDESI. This provided us a good way to compare the two detection methods. From Table 6-3, CF-EDESI-MS provided significantly lower (approximately an order of magnitude) LOD than did the PDA. Of course, mass spectrometry will generally be favored for compounds that can assimilate charge through association, dissociation, or adduct ion formation, especially, if a lack of chromophore exists.

Table 6-3. The limit of detection in CF-EDESI and PDA detector for each enantiomer in their corresponding separation solvent.

#	Compounds name	CF-EDESI (LOD) mg/mL	PDA (LOD) mg/mL
1	Cromakalim	1.01E-4	1.04E-4
2	2,2'-Diamino-1,1'-binaphthalene	5.32E-5	1.48E-4
3	2,3-Dihydro-7a-methyl-3-phenylpyrrolo[2,1-b]oxazol-5(7aH)-one	2.17E-4	6.50E-4
5	N-(2,3-Epoxypropyl)-phthalimide	1.95E-5	9.56E-4
6	Tröger's base	1.82E-5	3.98E-4

6.3.2 Direct Infusion of Cell Extracts

Phospholipids are major constituents of membranes in plants, animals and microorganisms. In addition to their structural role, some phospholipids also participate in biological processes, such as cell signaling [135]. Several different families of neutral lipids are present in all cells, including diacylglycerols (DAGs), triacylglycerols (TAGs) and cholesteryl esters (CEs). In addition to the diacylglycerol phospholipids, a large number of plasmanyl and plasmenyl phospholipids also commonly exist. MTBE-based extraction can provide accurate profiling of lipidomes which allows faster and cleaner lipid recovery [136]. One of the important facts about MTBE extraction is that, it has low density than normally used solvents (e.g., chloroform). Lipid-containing organic forms on upper layer during phase separation, which simplifies its collection and minimizes losses. The unwanted phase can be easily removed by centrifuge. Rigorous testing demonstrated that the MTBE extraction gave similar or better recoveries of most major lipid classes compared to classic methods such as those by Folch and by Bligh and Dyer [137, 138].

However, analytes in MTBE will not be sufficiently ionized by direct ESI analysis, due to its nonpolar nature and low dielectric constant. Moreover, it is risky to run highly flammable and explosive solvent such as MTBE through a high voltage capillary. While in CF-EDESI, MTBE extraction infused through hypodermic needle with no high voltage attached, charged species from electrospray solvents ionized the analytes following mixing of the phases.

In Figure 2, we compared the analysis of three bacteria cell extracts in MTBE by CF-EDESI with spray solvent MeOH/H_2O (Column A) and 5 mM ammonium acetate in MeOH/H_2O (Column B). The mass range from *m/z* 600-800 were extracted and

displayed. With addition of ammonium acetate in electrospray solution, the ion intensity of the three cell extracts in CF-EDESI increased approximately 5-10 times in comparison to use of MeOH/H_2O alone. The observation of sodiated adducts peaks were also greatly reduced. For example, in the *E. coli* cell extracts, signals at *m/z* 686.77, 714.77, 726.73, 740.70 (sodiated forms, in A) were shifted to *m/z* 663.79, 692.80, 704.78, and 718.75 (protonated forms, in A'), respectively. For *Pseudomonas putida, m/z* 712.67, 726.69, 740.74, and 752.61 (B) shifted to 690.80, 704.73, 718.75, and 730.71 (B'), upon addition of ammonium acetate to the spray solvent. The same occurred for *Pseudomonas aeruginosa* (C to C'). Adduction of lipid ions greatly complicates spectral interpretation. By adding ammonium acetate in ESI spray solution, we can directly read out the difference between the three bacterial cell extracts from A'- C'.

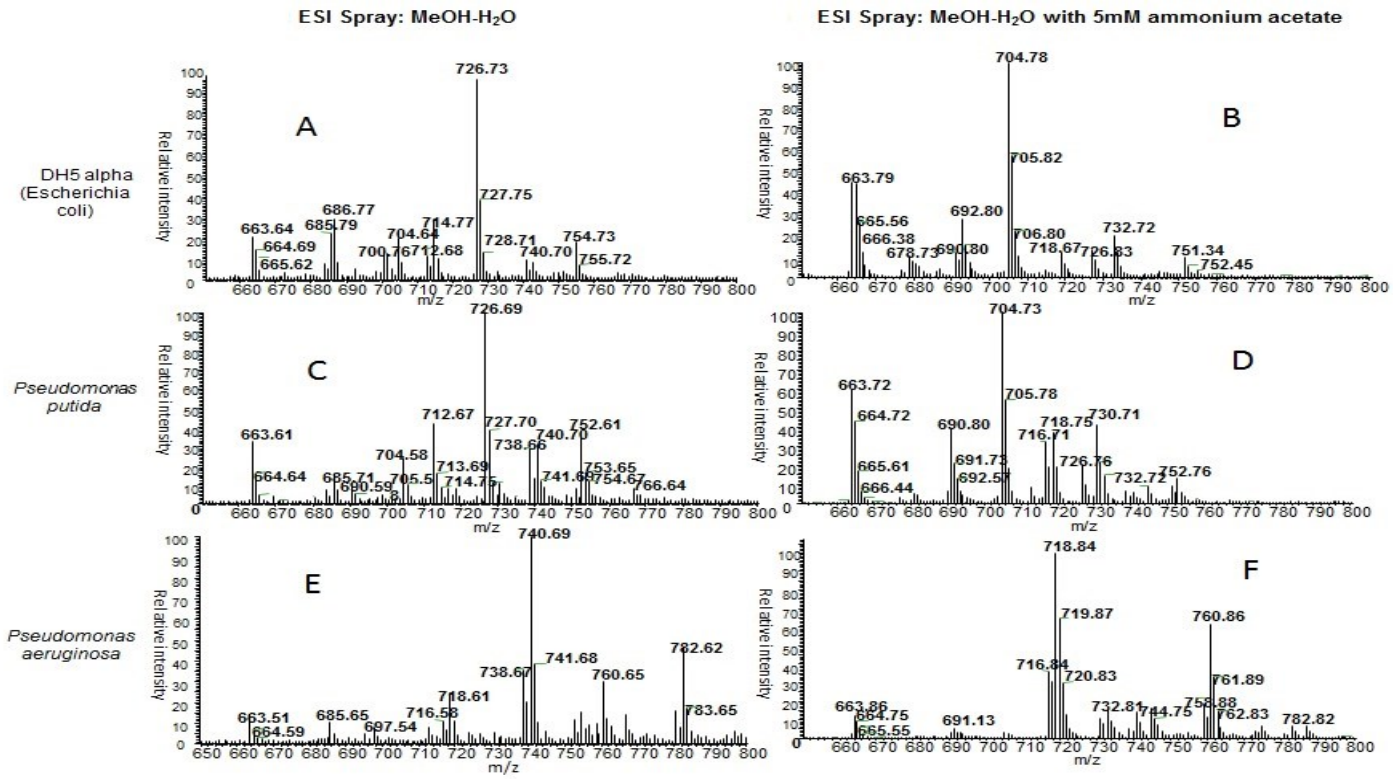


Figure 6-2. Analysis of three bacteria cell extracts in MTBE by CF-EDESI with spray solvent MeOH/H_2O and 5mM ammonium acetate in MeOH/H_2O

For the purpose of identification, a fragmentation experiment of some lipid peaks has also been carried out. Positive ion ESI-MS/MS spectra of phospholipids from ion trap instruments are likely to yield lyso-phosphocholine (PC) fragmentation products [139], which reveal the fatty acid composition of the lipid. Fragmentation of phosphatidylethanolamines (PEs) exclusively yielded one peak, an $[M+H-141]^+$ ion from the neutral loss of the PE head group. By referring to a glycerolipids library [140] and the fragmentation information, the lipids information of three types of bacteria cell extracts in the mass range *m/z* 600-800 was clearly identified.

Extracts from *E. Coli* and *P. putida* produced similar mass spectra. In both cases, a base peak at *m/z* 704.7, with a fragment at *m/z* 563.3 (-141.4), was observed. According to information from lipids library, this species could be 30:1 PC, 34:0 plasmenyl PE or 34:1 plasmanyl PE. But from the fragment information, it is more likely to be PE than PC, so we can confidently exclude the possibility of 30:1 PC with the added data-dependent fragmentation strategy. In P. aeruginosa, *m/z* 718.34 was the dominate peak, and it exhibited a fragment peak at *m/z* 577.35 (-141.35). Then according to the reference, this peak could be 34:1 PE. This strategy could be continued to be applied throughout the spectral analysis, and if the CF-EDESI were set up on an instrument capable of accurate mass and high resolution, it is not difficult to imagine such a set-up as a high throughput process for lipid profiling. Here, we simply have provided proof of principle that CF-EDESI can provide an effective way for directly measuring phospholipid cell extracts. In this case, analysis of extracts in MTBE, a non-ESI-friendly solvent was demonstrated, but one can imagine use of other extraction solvents, if desired. Additionally, the ability to alter the quality of the spectrum my inducing or reducing adduct formation, as desired, can be accomplished by varying the spray solvent.

6.4 Concluding Remarks

The presented CF-EDESI method provided a good example in bridging normal phase separation and mass spectrometry. CF-EDESI also poses the characteristic as one of the ambient ionization method in direct analysis with minimum sample preparation. The feature of CF-EDESI makes it attractive to general analytical community due to its apparent potential in lipidomics, chiral, and LC-NMR/MS analyses. Given that prior work has also demonstrated its use for protein analysis, CF-EDESI appears to be a versatile, simple, and economical AI technique with a wide application for flow through sample analysis.

Chapter 7

Summary and Future Work

In this dissertation, a novel ambient ionization technique, CF-EDESI, was developed and applied to solve various problems associated with protein, lipid, nonpolar small molecule, and chiral compound analysis. Its advantages were exhibited in the context of protein charge state manipulation,intact protein separations, analysis from "non-ESI-friendly" solvents, and coupling with normal phase liquid chromatography separations. The comparison was made between CF-EDESI and conventional ESI on each experiment.

In the initial study of protein charge manipulation, CF-EDESI showed a softer ionization capability than conventional ESI. With the same amount of charging agent, proteins were detected in less charged state and tended to be conserved in their native state. However, in ESI, the charge state progressed to higher values as the concentration of charging agent increased. The proteins were detected in completely denatured state in ESI. And then we confirmed this result by measuring the holo-/apo-myoglobin (native/denatured) ratio in conjunction with the addition of charging agents in CF-EDESI-MS and ESI-MS analysis.

Another virtue of CF-EDESI was its ability to analyze samples from "non-ESI-friendly" solvents such as hexane, ethyl acetate, chloroform, and MTBE. High sensitivity spectra were obtained with minimum interference from the solvents. Due to the poor ionization efficiency of those solvents in electrospray. poor analyte signals were observed for model analytes when performing conventional ESI in these solvents. CF-EDESI was applied for normal phase chiral separation of amine- and aromatic-bearing enantiomer mixtures. Better sensitivity was shown using CF-EDESI detection compared

with PDA-based UV detection. We also demonstrated the analysis of lipid extracts in MTBE directly. The different lipid components in three bacteria cells were successfully compared. The optimization of spray solvent by adding 5 mM ammonium acetate has provided cleaner spectrum with much reduced sodium adducts peaks. Additional fragmentation information helped to immediately identify the lipid components from different cells.

So far, CF-EDESI has demonstrated its use in proteomics, chiral compounds separation, lipidomic. However, more and more applications are waiting to explore for this easily achieved device, such as reaction monitoring, food analysis, pharmaceutical or environmental analysis. It can directly analyze the sample as long as in liquid form. The improvement of CF-EDESI device for high accuracy and reproducibility is another goal of us. An auto XYZ stage was in design to accurately pinpoint the hypodermic position to achieve best performance.

Appendix A Publication Information of Chapter 2-5

1. Chapter 2: A manuscript published in Current Trends in Mass Spectrometry, Li, L.; Schug, K. A. 2011, 2–8. Copyright @2011 reproduced with permission from Advanstar Publications
2. Chapter 3. A manuscript published in Analytical Chemistry, Yang, S. H.; Wijeratne, A. B.; Li, L.; Edwards, B. L.; Schug, K. A. 2011, 83, 643–647. Copyright © 2011, American Chemical Society
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Biographical Information

Li Li was born July 2nd ,1984 in Jingxian, Anhui, China. She grew up and stayed in Jingxian until finished high school at the age of 19. She then attended the Shenyang Pharmaceutical University in Shenyang, Liaoning province where she obtained her Bachelor's of Science in Pharmacy Engineering. After graduation in 2006 spring, she joined a collaboration program from Shenyang Pharmaceutical University and Shanghai Institute of Organic Chemistry and obtained her Master's of Science in Medicinal Chemistry. And then she enrolled the Ph.D program in analytical chemistry at University of Texas at Arlington in 2009 fall. She actively participated in research since she was an undergraduate student. She has been involved in a drug discovery project with partial synthesis of Fidarestat Aldos, an inhibitor of Aldose Reductase. And then, as a Master student, she focused on the synthesis of isoindolinone by using a new coupling method CuI-catalyzed cascade reaction as well as the synthesis of a novel small molecule regulator for guanine nucleotide exchange activity, which was under the supervising of Dr. Dawei Ma & Xiaojing Zhang. When she got accepted by graduate school in University of Texas at Arlington, she switched her focus to analytical chemistry under mentorship of Dr. Kevin A. Schug. During the time spent working in the Schug lab, Li has explored a number of different areas of research within the realm of Analytical Chemistry. The primary work was done on two projects. The first one was a fundamental study of a new ambient ionization source CF-EDESI along with its applications. The other one was identify and characterize unknown fluorescent molecules with various analytical tools (such as LC-MS, MALDI, NMR, X-ray etc.) for analytical and bioengineering use. Besides, she also has gained a lot of separation experience by participating in

quantification of estrogen in various biofluid samples with LC-MS-MS. Except research, she also working in pursuit of ON-TRAC Practitioner status (Organizational Network for Teaching as Research Advancement and Collaboration at UT Arlington, a member of the Center for the Integration of Research, Teaching and Learning (CIRTL) Network.