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Improving Protein Analysis by Desorption Electrospray Ionization (DESI-MS)

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IMPROVING PROTEIN ANALYSIS BY DESORPTION ELECTROSPRAY IONIZATION (DESI-MS)

by

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Elahe Honarvar

IMPROVING PROTEIN ANALYSIS BY DESORPTION ELECTROSPRAY IONIZATION MASS SPECTROMETRY (DESI-MS)

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Western Michigan University, 2019

Electrospray ionization mass spectrometry (ESI-MS) is one of the most well-known and versatile techniques for analyzing a broad range of molecules and it has become one of the leading techniques to study biomolecules, such as proteins. ESI-MS can accurately determine the molecular weight of proteins and provide information about their peptide sequence, post-translational modifications as well as their interaction with other molecules.

During ESI-MS analysis, by spraying a sample of proteins, prepared in form of a solution, charged droplets are produced using an electric field. As the solvent molecules gradually evaporate from these droplets, freely hovering bare protein ions remain. The ions are then sampled into the mass spectrometer where they are separated and detected based on their mass to charge (m/z) ratios.

In the recent years, a new extension of ESI-MS has been developed that allows analysis of molecules from their immediate surroundings. The technique is called desorption electrospray ionization (DESI-MS). In DESI-MS the sample preparation steps take place in close proximity to ionization step. Such features also provide the advantage of surface analysis and imaging to study spatial distribution of molecules.

While DESI shares the ionization mechanism of ESI-MS, it lacks its ability to analyze large biopolymers, and struggles to analyze proteins larger than 25kDa.

Previously our research group suggested that the loss in protein signal intensity was not due to problems with physical desorption or ionization, but rather due to incomplete protein dissolution during the desorption step.

The studies conducted in this dissertation address this shortcoming by improving protein dissolution during DESI-MS. Effect of addition of volatile ammonium salts during DESI is studied, among which ammonium bicarbonate shows significant improvement in signal to noise (S/N) ratio of proteins, specifically those with higher isoelectric points (pI). The improved S/N ratio seems to be caused by extensive removal of potassium from the protein ions. While these additives lead to improvements in the performance of DESI, their addition does not cause the same effect in ESI. The different effects of these additives in DESI and ESI are studied in terms of proteins signal intensity, S/N ratio as well as charge state distribution.

The effect of addition of the amino acid of serine to the electrospray solvent of DESI is investigated. For proteins with different molecular weights and pI values, serine shows promising improvements in the signal intensity.

Application of vaporized organic reagents in the nebulizing gas flow of the electrospray solvent of DESI is described. To add these vapors, DESI sprayer is enclosed and the vapor is delivered to the inner environment of the enclosure. By adding the vapor of ethyl acetate during DESI analysis of proteins, the attained signal intensity is increased.

Such improvements can potentially be combined during a single analysis to further better the outcome of protein analysis by DESI-MS.

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CHAPTER I

INTRODUCTION

1.1 Foreword

Desorption electrospray ionization mass spectrometry (DESI-MS) is a quite recent mass spectrometry technique that can analyze molecules from their immediate surroundings, without requiring complex processes of sample preparation and in their native form. DESI-MS produces spectra that are similar to the ones produced by ESI-MS. The technique can be used to obtain the spatial distribution of analytes on a surface. Nevertheless, the advantages of using DESI are bounded to smaller molecules. DESI faces a severe struggle for the analysis of larger biomolecules, such as proteins. My Ph.D. research was mostly dedicated to improving DESI for detection of proteins. The data presented in this dissertation were all collected during my time as a PhD student in Dr. Venter's research laboratory and under his guidance. The major part of the experimental chapters has already been published in well-known peer reviewed journals such as *Journal for the American Society for Mass Spectrometry* (JASMS) and the American Chemical Society journal, *Analytical Chemistry*.

1.2 A brief history of protein analysis by ESI-MS and DESI-MS

Mass spectrometry has become a powerful analytical technique, finding applications in both quantitative and qualitative analysis. Sample molecules are vaporized and ionized to form gas-phase ions. The ions are separated based on their mass to charge ratios inside the mass spectrometer.

The first mass spectrometer was built during the early years of the 20^{th} century when they could only be utilized for analyzing small gaseous organic molecules. Over the following decades,

through development of soft ionization techniques such as electrospray ionization and MALDI the analysis of large, non-volatile molecules by mass spectrometry became achievable[1]. In the late 1980s, in a groundbreaking work, John Fenn and coworkers employed ESI to analyze intact multiply charged proteins by mass spectrometry for the first time[2]. Mass spectrometry of proteins made possible the new era of proteomics and played a phenomenal role in the rapid evolution of the field.

Electrospray ionization mass spectrometry (ESI-MS) can give rapid and accurate protein analysis. The analysis results in a multiply charged protein envelope consisting of different charge states of the same protein which is a favorable fact about this technique when working with mass analyzers with limited mass range. Typically, for ESI-MS analysis of proteins, the sample undergoes extraction, purification and chromatographic separation to yield a mass spectrometry-ready analyte. Then the sample, which now is in solution form, is introduced to the mass spectrometer by being electrosprayed toward the mass spectrometer inlet. A new extension of ESI-MS, allows the sample preparation to be done during the process of analysis[3]. An intact sample surface on a 3-D translational stage, with sample either native to the surface or deposited, is electrosprayed with a pneumatically assisted solvent. The charge bearing droplets are directed at the surface where the sample is readily accessible under ambient condition. Upon the incidence of the droplets, the analyte molecules on the sample surface are solubilized, desorbed and ionized. Finally the gas phase ions of the analyte are formed and transferred into the high vacuum region of the mass spectrometer where they are separated by their mass to charge ratios. This technique is called desorption electrospray ionization mass spectrometry (DESI-MS) which some believe to be the first ambient ionization technique when introduced by Cooks et al. in 2004[4].

DESI-MS soon found widespread applications in biology[5-7], forensics[8] and pharmaceuticals[9,10]. In these cases, DESI provides good sensitivity for detecting analytes of interest which are usually molecules with relatively low molecular weight. However, for detection of large biomolecules such as proteins, DESI suffers from a mass dependent loss of sensitivity[8,11]. Previously, during a study by Douglass and Venter, which broke down DESI into desorption and ionization steps, it was shown that this shortcoming of DESI for analysis of proteins is caused by insufficient dissolution of sample during the millisecond timescale of the analysis[12]. The incomplete dissolution of proteins retains the non-specific interactions of protein-protein and protein-adducts which unfavorably causes distribution of signal over different forms of protein leading to lower sensitivity. This limitation becomes more severe for protein with molecular masses above 25kDa[11]. Considering that the average molecular weight of proteins across the kingdom of life is calculated to be 30-40kDa and that these proteins have an average pI of 6-7[13] this severely limits the scope of DESI, despite its potential valuable ability to analyze proteins under ambient conditions from surfaces. Improving DESI for proteins analysis brings the possibility of using this technique in different fields for accurate qualitative and quantitative analysis closer to reality. One remarkable possibility through DESI can be performing imaging of proteins on surfaces where spatial distribution of proteins throughout the sample can be studied. This was recently demonstrated[14] but alas, only small proteins could be detected, the largest being hemoglobin at 16kDa.

The main objective of my PhD research was to make state-of-the-art DESI practical for larger proteins and get it closer to the point where it can be utilized for imaging of proteins. In the following chapters, different approaches employed for improving DESI for protein analysis were

studied. These approaches may also be combined in future works to reach higher levels of improvement.

1.3 Preview of dissertation chapters

The following chapter, Chapter 2, contains detailed and thorough background information about DESI-MS and its mechanism. Chapter 3 shows optimization of depositing and spray solvents for protein analysis by DESI. Chapter 4 looks into the effect of ammonium bicarbonate as the depositing solvent as well as spray solvent additive to improve DESI-MS of proteins and peptides. The effect of ammonium bicarbonate was studied in comparison to the commonly used additive, formic acid. To better understand the mechanism for improved analysis of high pI proteins with the addition of ammonium bicarbonate in Chapter 5 I investigated different ammonium salts and their related weak acids, and compared their behavior in DESI to their behavior in ESI. Proteins with different physical properties showed dissimilar behavior in the two modalities. The investigation of useful additives for DESI of proteins goes beyond using salts and acid in Chapter 6 where the amino acid of serine is employed as a spray solvent additive to study its effectiveness on DESI of proteins. Serine was successful in improving DESI analysis for proteins with different molecular weights and pI values. Chapter 7 introduces a new method for using organic vapors in the nebulizing gas flow to possibly remove adducts and increase sensitivity of DESI. The chapter shows promising preliminary result for some of the vapors. This chapter is the latest and last experimental work done during my research. Finally, the observations made in Chapters 3 to 7 are concluded in Chapter 8 along with recommendation for the future work.

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CHAPTER II

DESORPTION ELECTROSPRAY IONIZATION: APPLICATIONS, MECHANISM AND LIMITATIONS

2.1. Mass spectrometry for intact protein analysis

Mass spectrometry (MS) is an invaluable tool for precise detection and quantification of compounds. Molecules in a sample are turned into ions before detection by a mass spectrometer, where they are separated by their mass to charge (m/z) ratios. In the past four decades, through the use of electrospray ionization (ESI)[1] and matrix-assisted laser desorption ionization (MALDI)[2], it has become possible to analyze large biomolecules such as proteins by mass spectrometry[3]

In ESI, ions are formed by nebulizing solution exposed to high electric fields[4]. Since polar molecules can undergo such ionization mechanism, ESI becomes available for analyzing a broad range of compounds. With the invention of ESI, it was made easier to detect macromolecules such as proteins by mass spectrometry. This ability of mass spectrometry, developed by John Fenn[1,5-8], has revolutionized the field of proteomics and brought its inventor the Noble prize of chemistry in 2002. In MALDI, exposure of the matrix-embedded sample analyte to the laser pulse produces the gas phase ions[9]. Both ESI and MALDI have a soft ionization nature meaning that the molecule and its noncovalent interactions are not prone to fragmentation during analysis. Macromolecules such as proteins with numerous basic and acidic sites can be multiply charged by ESI-MS[6]. The multiply charged species allows detection at lower mass to charge ratios. This is useful because it makes ESI more compatible with mass analyzers that have limited m/z ranges[10] as compared to MALDI which typically yields singly charged ions[4] and needs mass

analyzers that can scan much higher m/z values. The multiply charged proteins can provide higher sequence coverage and the feasibility of top-down proteomics[11]. Also at higher charge states of protein, unfavorable adductions are usually lessened[12].

2.1.1. Multiple charging of proteins by ESI-MS

The mass spectrum of proteins when analyzed by ESI usually consists of an "envelope" of different charge states of the same protein (Figure 2.1). If a protein is native or folded, its spectrum shows lower charge states than an unfolded protein, and consequently appear at higher mass to charge ratios values on the mass spectrum. It is believed that unfolded proteins bear more charges compared to the globular native proteins due to higher accessibility of their basic and acidic sites[13,14]. Each of the charge states, within the same envelope, indicates a different degree of protein adduction. In positive mode, protonation $(M+nH)^{n+}$ is the most common and simplest form of adduction. The multiple charge states of protein can be used to confidently calculate the molecular mass of the protein by using a deconvolution algorithm [7]. The molecular mass of a multiply charged protein can be calculated by knowing the m/z value of two adjacent charge states in the mass spectrum.

$$P_1\,(m/z)=(M+z_1)/z_1$$

$$P_2\,(m/z)=(M+(z_1+1))/(z_1+1)$$

Where P_1 is the protein peak that carries z_1 charges and P_2 is the adjacent protein peak toward lower m/z values with +1 more charge than P_1. M is the molecular mass of the parent protein. This equation is applicable to proton-adducted proteins. Other types of positively and negatively

charged species are also common during ESI-MS analysis of proteins such as different alkali metal ions or ammonium in positive ion mode [15] and halogen ions in negative ion mode[16]. For these types of charged species added to protein, similar equations to the one above can be derived where the molecular mass of proton (1 amu) is replaced with those of other charged species.

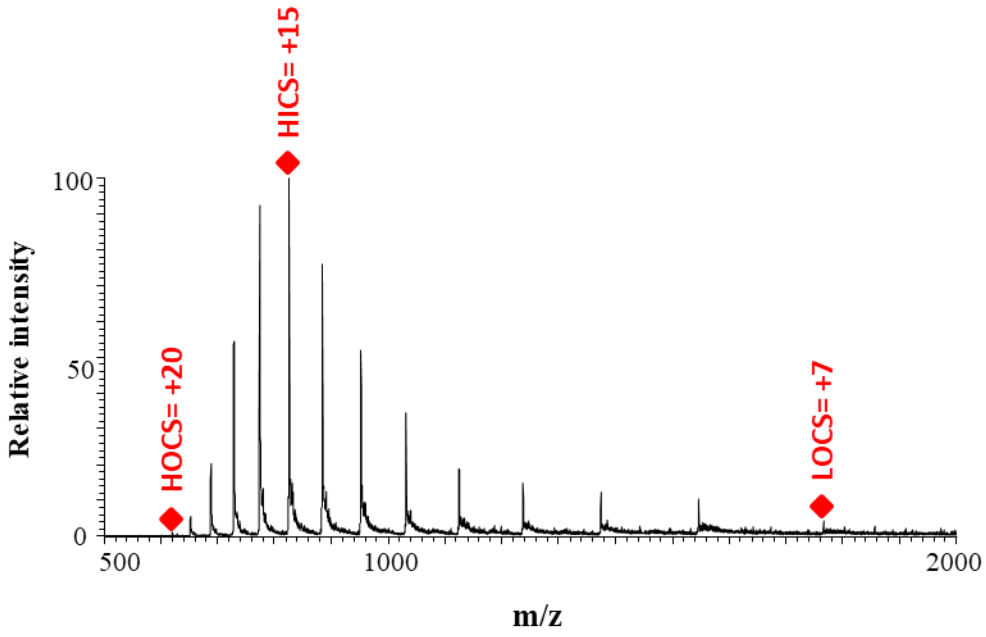

Figure 2.1 Example of protein multiple charging in mass spectrum of α-cytochrome c analyzed by ESI-MS. The highest observed charge state (HOCS), highest intensity charge state (HICS) and lowest intensity charge state (LOCS) are indicated on the protein envelope.

In a multiply charged protein spectrum like the one in Figure 2.1, as we move from peaks on the right to the left of the envelope, the charge state of the protein increases. The charge state distribution (CSD) is dependent on the protein conformation[13]. Each of the charge states shows the relative abundance of a certain adducted form of the protein. The charge state to the far right of the envelope is known as the Lowest Observed Charge State or "LOCS" which indicates the lowest number of charges on the protein molecule. The charge state to the far left of the envelope

is known as the Highest Observed Charge State or "HOCS" which indicates the maximum number of charges that the protein molecule bears[17,18]. The peak of the envelope where a charge state shows the maximum height in signal intensity is known as the Highest Intensity Charge State or "HICS"[19]. The CSD of a protein and the state of its LOCS, HICS and HOCS can provide information about its conformation[17].

2.1.2 Ionization mechanisms during ESI-MS

Different types of analytes are said to ionize through different processes. It is generally accepted that ionization of small molecules follow the ion evaporation model (IEM)[20] and ionization of larger molecules such as globular proteins follow the charged residue model (CRM)[21].

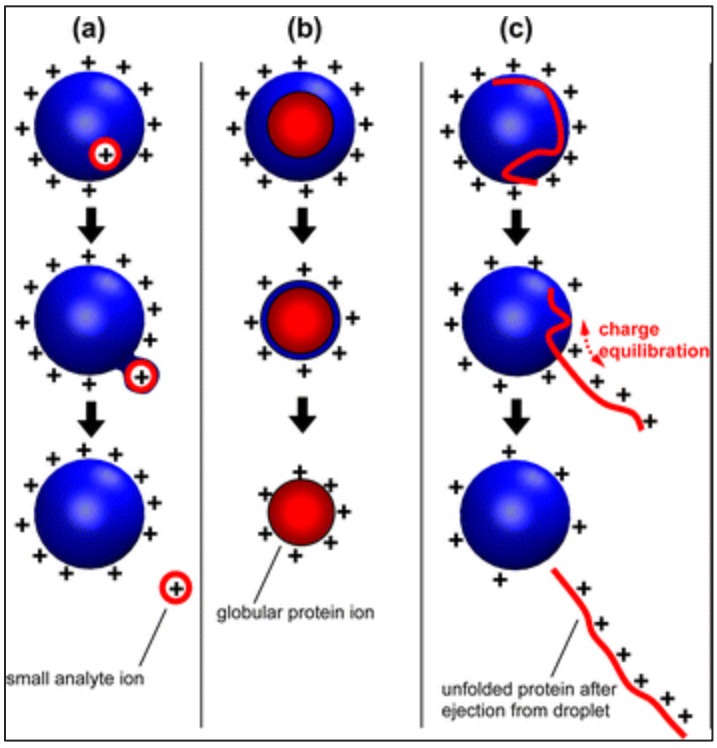

Figure 2.2. Different ionization mechanisms (a) IEM: Small ion ejection from a charged nanodroplet. (b) CRM: Release of a globular protein into the gas phase. (c) CEM: Ejection of an unfolded protein. Charge equilibration in panel c is indicated by red arrows. (Reprinted from Konermann et al. Anal. Chem. 84, 6798-6804, Copyright (2012), American Chemical Society)[25].

More recent work suggested a different model where unfolded protein molecules are ionized by the so called chain ejection mechanism (CEM) [22] (Figure 2.2). In IEM, it is hypothesized that the electric fields emanating from the electrosprayed droplet are strong enough to eject the solvated analyte ion from the surface of the droplet[20]. In CRM, the electrosprayed droplet which carries only one single large analyte ion completely dries out of the solvent, leaving the charges from the final droplet on the analyte ion[23,24]. CEM shares some common features with IEM. In CEM, due to the increase in hydrophobicity of the protein upon unfolding, it becomes unfavorable for the protein to remain within the droplet hence migration to the surface occurs which is followed by sequential ejection of the whole molecule[22].

2.2 Introduction to ambient Ionization techniques

Over the past 15 years, a new field of mass spectrometry techniques has emerged that strives to minimize the sample preparation steps typically required by conventional mass spectrometric methods such as ESI-MS or MALDI-MS. These newer techniques altogether are known as "ambient ionization" techniques where samples are analyzed in their immediate surrounding and open atmosphere[26]. Ambient techniques represent novel application of sample processing which take place in real time and proximal to ionization, while ionization mostly takes place through well-known processes[27]. Traditionally, it was said that ambient techniques require "no sample preparation" [28-30]. However, the novelty of ambient ionization arises from coupling the sample preparation step with sample ionization in time and proximity[27,31]. By definition, sample preparation is not eliminated but has been shifted from *before* ionization to simultaneously *during* ionization.

Some of the popular ambient ionization techniques that have been developed up to now are shown in Figure 2.3. These techniques are grouped into different forms of sample processing (outer skin, green) and physical processes (center brown). The font color used for each technique also indicates its ionization mechanism.

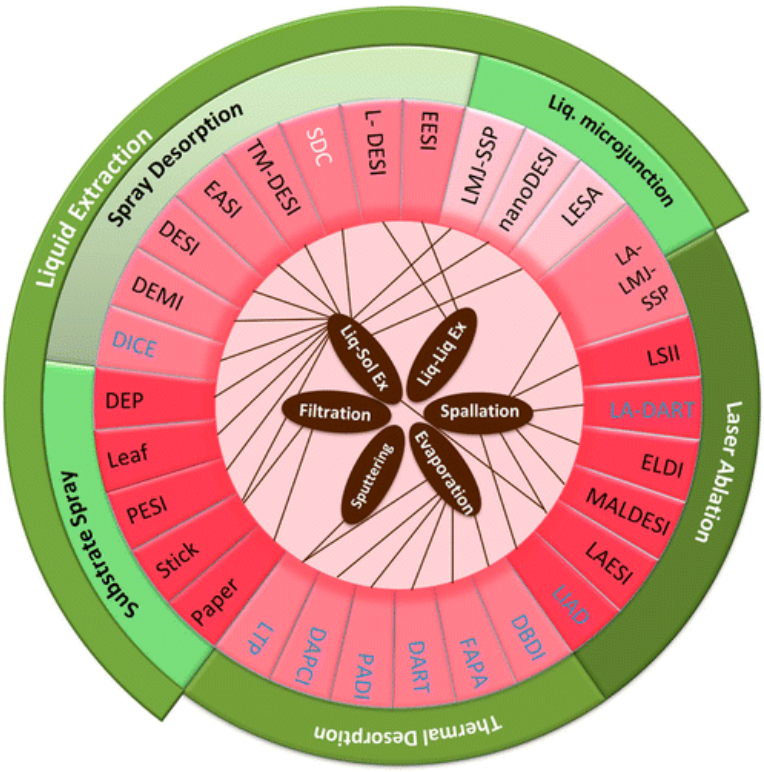

Figure 2.3. Summary of the major classes of ambient ionization methods based on traditional groupings (green), examples of methods (shades of pink), and the various sample processing methods (black). The mechanism of ionization is indicated by the font color for each listed example. Black indicates ESI, blue APCI, and white ambient sample processing without ionization. (Reprinted with permission from Venter, Douglass et al. Anal. Chem., 86, 233–249. Copyright (2014) American Chemical Society)[27].

Formation of ions in ambient ionization techniques commonly follows one of these two methods: atmospheric pressure chemical ionization (APCI), represented with blue font and electrospray ionization (ESI), represented with black font. The white font indicates special cases where

sample processing is not followed by an ionization process but the unique sample processing of an ambient method is used only for sample collection.

2.3. Desorption electrospray ionization mass spectrometry (DESI-MS)

Among the ambient ionization methods developed to date, desorption electrospray ionization has been one of the most widely-used methods. This method was first introduced by Professor Graham Cooks et al in 2004[28] and later commercialized by Prosolia, Inc. Since the introduction of DESI it has become a popular ambient method of analysis that provides high sensitivity and instant spectrum[32]. The success of DESI is in part due to the fact that building a DESI source can be easily done in the lab with relatively low cost and also because of the ability that during DESI, the solvent can be optimized for analyzing different compounds both by choice of the solvent or by adding reagents that can enhance the ionization of the analyte[33-35]. The development of such reagents also forms a large part of this dissertation, as discussed later.

2.3.1. Sample processing during desorption electrospray ionization

The sample processing in DESI starts with spraying an electrically charged solvent at the sample on the surface which is then followed by sample removal from surface and formation of ions and eventual introduction into the mass spectrometer[32].

Usually sample processing during DESI is broken down into 5 steps as described below;

1) Generating a pneumatically-assisted electrospray plume directed at the sample (primary droplets)[36,37]
2) Creation of a thin micro-localized liquid film on the surface[38-41]
3) Dissolution/extraction of the analyte into the liquid film[36,42,43]

4) Desorption of analyte containing droplets (secondary droplets) by momentum transfer between the pneumatically accelerated primary droplets and the liquid film[26,32,37,39,41]

5) Formation of gas-phase ions from charged secondary droplets through a process similar to ESI[39]

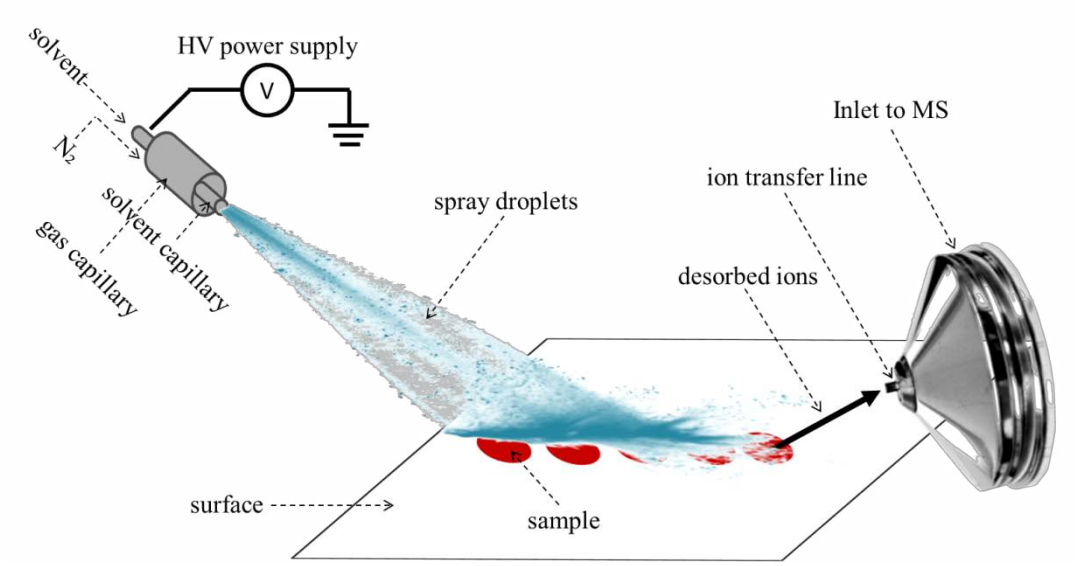

Figure 2.4. Schematic diagram of a desorption electrospray ionization (DESI) source. Electrospray solvent is sprayed from a fused-silica capillary coaxial with the sheath gas capillary that pneumatically directs the solvent to the surface of the sample. Sample is dissolved into the charged solvent and then liberated from the surface towards the mass spectrometer inlet. This process is followed by subsequent ionization.

At this point and until the sample makes it in to the mass spectrometer the size of the droplets shrink upon solvent evaporation. Free ions can form through different electrospray ionization mechanisms such as IEM, CRM and CEM as described earlier in the chapter. These steps of sample processing will be discussed in detail later in this chapter.

2.3.2. Construction of desorption electrospray ionization source

A schematic diagram of a DESI source is shown in Figure 2.4. An electrospray emitter passes through the gas capillary which is a wider capillary and coaxial to the electrospray emitter. The relative position of the electrospray emitter inside the gas capillary was reported to have significant effect on the performance and reproducibility of the DESI experiment[44].

The surface contains the analyte of interest and it can be either natural, like biological tissues, or synthetic, for when the sample needs to be deposited on to the surface. Synthetic surfaces can be from a wide range of non-conductive materials such as glass or paper and most commonly polymer surfaces like PTFE[32]. The wettability of the surface has a major effect on charge transfer and droplet behavior during DESI[45].

2.3.3. Application fields of desorption electrospray ionization

Application of DESI has been reported for a broad range of samples such as bacteria[46-49], pharmaceutical compounds[50-57], drugs of abuse[58,59], explosive and chemical warfare agents[60-64], in-plant material[65-70], forensic samples[32,71,72], separated compounds on chromatography plates[73-76], lipids profiling in biological tissues[77-87], peptides[88-90] and less frequently for proteins[42,91]. Although DESI is mostly used in analysis of smaller molecules, a few studies have reported its application for the analysis of proteins[42,91-93]. There hasn't been any description of intact protein analysis from tissues until very recently and then only for smaller proteins [94-97].

Using DESI to study proteins can offer benefits, including in situ analysis of proteins while the resulting spectrum resembles ESI in terms of ionization and yielding multiple charge states of protein[26] which delivers advantages such as higher resolution of peaks at lower m/z values, increased sequence coverage, and others as described earlier. Proteins and their complexes

embedded in biological tissues can be studied by DESI in terms of their post translational modification and their different isoforms[98] along with the ability to provide the spatial distribution of protein throughout the surface. However the use of DESI analysis for intact proteins is limited by a mass dependent loss in sensitivity of the method[91].

2.3.4 The mass dependent loss in sensitivity during DESI analysis of proteins

As the molecular mass of the protein increases, the limit of detection also increases[42](Figure 2.5).

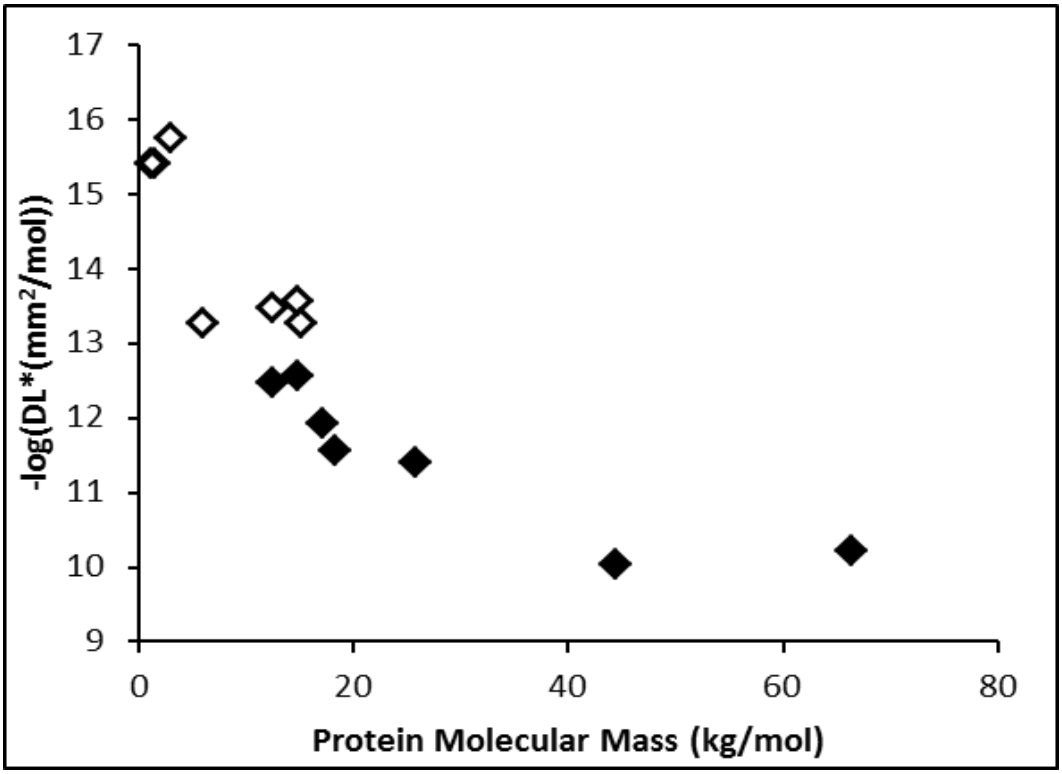

Figure 2.5. Logarithmic plot of protein DESI-MS detection limit versus molecular mass. (Reprinted with permission from Douglass, Venter, JMS,(2013) 48, 553-560.)[42]

Previously, our group developed methods to investigate different steps of DESI by isolating desorption from ionization[99]. Spray desorption collection (SDC) was used to model the

desorption process of DESI while reflective electrospray ionization (RESI) was used to study the ionization step (Figure 2.6).

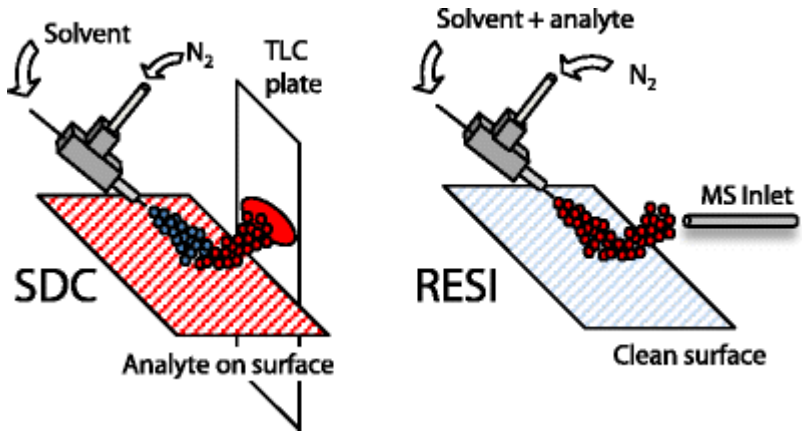

Figure 2.6. A schematic of spray desorption collection (SDC) and reflective electrospray ionization (RESI). (Reprinted from Douglass et al. JASMS. 23(11),1896-902, Copyright(2012) Springer)[99]

For the SDC experiment, a setup similar to DESI was used but instead of the analyte being sampled in the mass spectrometer, it was collected on a TLC plate or in some cases in an Eppendorf tube. The collected sample was reconstituted and then analyzed by ESI-MS. For the RESI experiment, the analyte was already dissolved in the solvent system, similarly to ESI. The mixture of sample and solvent was sprayed on a clean surface, similar to DESI. The analyte was subsequently sampled into the mass spectrometer and analyzed.

When deconstructing of DESI was used to study the steps of protein analysis by DESI, it was observed that both SDC and RESI produced similar result to ESI, without the observed loss in sensitivity with increasing mass typical of DESI. The study concluded that the mass dependent loss of signal intensity during protein analysis by DESI is due to incomplete dissolution of

protein sample and not coming from desorption or ionization of the protein[42]. Therefore, improving dissolution/extraction of proteins from solid phase into the liquid layer is the major challenge of protein analysis by DESI. Recently, successful detection of smaller proteins (up to 16kDa) from tissues by DESI imaging mass spectrometry (DESI-IMS) was reported by Towers et al. [94] where they made multiple optimizations to their DESI setup including modifying the solvent system used for analysis, acknowledging the importance of analyte solubility and addressing the poor dissolution of proteins during DESI[94].

2.4. Mechanism of desorption electrospray ionization

Studies formerly proposed that the mechanistic aspect of DESI follows a "droplet pickup" mechanism of the analyte and subsequent desolvation of the secondary droplets producing gas-phase dry ions of the analyte on their way to the inlet of the mass spectrometer[32]. Droplet pickup hypothesizes that the solid/liquid extraction starts by the wetting of the surface and formation of a micro-localized liquid layer followed by subsequent momentum transfer driven by the continuous projection of the primary droplets to this thin layer of the liquid on the surface[100]. The analyte molecules in the solid phase are extracted or dissolved into the liquid formed on the wetted surface. Production of secondary droplets is hypothesized to be due to the collision of the coming solvent jet and the liquid layer[100]. Later studies evidently showed that droplet pickup is the major driving force in the process of DESI. More detailed mechanistic description of individual steps of DESI follows.

2.4.1. Primary sprayer

For beginning the process of analysis by DESI, an electrospray solvent aiming at the surface is nebulized to produce charged micro-droplets. This solvent typically consists of an aqueous

solution but there were also reports on the use of non-aqueous solvent systems for DESI experiments[43]. The composition of the solvent of DESI is often a 1:1 MeOH:H_2O system which is slightly acidified to aid analyte protonation in positive mode. An electrical potential of several kilovolts is applied to the spray solvent and pneumatic nebulization is employed to help with desolvation. The size of the solvent droplets (primary droplets) electrosprayed from the solvent capillary can be affected by chemical factors as well as physical parameters. As for chemical parameters, the ionic strength of the solvent components[37], the ratio of organic fraction in the solvent composition[36] and the use of surfactant in the spray solvent system[101] can cause significant changes in the size of the droplets. Physical parameters include the potential applied at the tip of the solvent capillary, solvent flow rate and the size of the walls of the solvent capillary[39] that can affect the primary droplet size. Previous studies reported that the size of the majority of the droplets in the sprayed plume range between 1 to 10μm[37,39].

For the solvent droplets, the velocity by which they impact the surface of the sample can define the ionization efficiency. The velocity of the primary droplets is affected by electrical potential applied to the tip of the solvent sprayer but more importantly by the sheath gas flow rate[39]. In addition, the ionic strength of the solvent components can play a role in the primary droplet velocity[39]. It was reported that the mean velocity of the droplets arriving at the surface of the sample is about 120m/s[39].

2.4.2. Dissolution/extraction

Upon spraying the surface with the solvent, first a thin micro-localized liquid layer forms on the surface[39-41]. Formation of this liquid layer is critical for having a stable and reproducible DESI. Solubility of the analyte in the thin layer plays a major role in the extraction process of the

compounds and subsequently the performance of DESI[36,42,43]. But on the other hand, high level of sample solubility increases the chance of substrate erosion[36], which can cause sample degradation and loss of DESI signal[38]. It was shown that choosing an appropriate solvent in DESI by using octanol/water partition coefficients (K_{ow}) of analyte can create higher quality mass spectra in DESI[43]. Also increasing the organic fraction of DESI solvent system up to 90% was shown to produce smaller primary droplets which are less likely to be scattered over the surface of the sample[36]. Wettability of the surface and the contact angle of the solvent can change the erosion diameter[36]. The relation of wettability and contact angle is discussed in more detail in Chapter 3. The affinity of the analyte for the surface is also of high importance, since analytes with lower affinity can be easily blown away by the plume of primary droplets and cause a loss of signal in DESI[73]. Knowing the characteristics of surface and solvent can help in choosing the right surface that provides solvent with proper retention time and resulting better DESI response[73].

2.4.3. Desorption

The process of analyte liberation from the surface and its transfer to the mass spectrometer inlet in the form of secondary droplets emerging from the liquid layer, into which the analyte molecules have already been extracted, is known as desorption. During DESI analysis, desorption is carried out by the droplet pickup mechanism[32,40,41]. In a typical DESI setup, the impacting primary droplets, travelling at velocities around 120m/s, collide with the micro-localized thin layer of liquid that is formed on the surface. At the incident of the arriving solvent jet and liquid layer and through momentum transfer, secondary droplets are ejected from the surface toward mass spectrometer inlet[40]. Hydrodynamic simulations for the process of droplet pickup during DESI provided evidence that momentum transfer by later arriving primary micro-

droplets is responsible for formation of the analyte containing secondary droplets[40,41]. The secondary droplets are also shown to be composed of both the primary droplets and the thin liquid layer on the surface[40]. The features of the liquid thin layer can be manipulated by the physiochemical properties of the solvent as well as the surface and the flow rates of solvent and nebulizing gas[38].

Van Berkel and his group investigated the pattern of sprayed solvent on different surface materials[73,75,102]. They reported three regions that are formed on the surface by the incidence of the solvent: 1) A central region in an elliptical shape formed by high velocity solvent arriving the surface; 2) The region where the solvent impact streams away from the central impact point; 3) The area where slower moving solvent droplets impinge on the surface and form a third region outside the two other region already described[36,73,103]. It was also shown that the secondary droplets can form at the rivulets streaming away from the center of the elliptical impact region[103]. Desorption of analyte molecules form the surface was demonstrated to occur at a very close distance to the surface and within a narrow band of the desorbed material[36,39-41,102]. As discussed in section 2.4.2, SDC was developed to study the desorption process of DESI independently from ionization[99,104]. It was shown that in a DESI experiment, desorption efficiency is not affected by the applied electrical potential but can be considerably affected by hydrodynamic forces (the nebulizing gas velocity) and geometric factors (sprayer to surface distance) involved in DESI[99,105]. It was also demonstrated by using SDC that desorption efficiency unlike ionization efficiency remains the same for all the soluble species in the sample including large proteins[42].

The optimum combination of DESI parameters such as source geometry and hydrodynamic forces together can make an efficient and reasonably energetic sprayer that effectively produce secondary droplets for analysis by DESI[99].

2.4.4. Ionization

Following desorption of the charged secondary droplets from the surface, it is believed that DESI analytes undergo ionization that mechanistically are similar to ionization in ESI[32]. Despite the similarities of DESI spectrum to ESI, DESI shows a slight loss of charge states which is attributed to the collision of the charged primary droplets to the surface[32,42]. The abandoned charges can gradually build up on the surface which can affect the signal intensity[45]. DESI can be used to ionize compounds that are typically not ionizable by ESI and create spectra that resemble spectral features of corona discharge or APCI[32,105].

Ionization in DESI to a large extend relies on and proceeds with the use of a high voltage to produce charged primary droplets. Increasing the voltage supply of the electrospray emitter is shown to increase ionization efficiency of DESI[39]. The ionization efficiency of DESI is also governed by the nebulizing gas pressure. Higher gas pressure produces smaller primary droplets which can improve ionization efficiency[39]. By the application of RESI, ionization process of DESI was studied independently from desorption[99]. Using RESI for protein samples provided evidence that ionization efficiency during DESI is comparable to ESI, ruling ionization out as a reason for sensitivity loss during DESI analysis of proteins [42]. Finally the charged secondary droplets go through solvent evaporation to create ions before they are sampled into the mass spectrometer inlet.

2.5. Conclusion

The aim of this chapter was to provide detailed background information about desorption electrospray ionization and a proper understanding of the mechanistic aspect of this method prior to the following experimental chapters where DESI is used as the main method of analysis. As discussed throughout this chapter, we hypothesize that the poor dissolution of proteins is the focal point in the loss of sensitivity during DESI[42]. Different DESI parameters can be optimized to maximize the efficiency of analysis. However, DESI has some intrinsic irreproducibility due to many variable factors in the source geometry which can be mitigated to some extent by using a geometry-independent DESI source[106].

The work presented in the following chapters was done in an effort to improve protein analysis by desorption electrospray ionization through improving protein solubility. Optimizing the spray composition and modifying the solvent system composition are shown to be beneficial for improved protein analysis by DESI.

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CHAPTER III

SOLVENT SYSTEM OPTIMIZATION FOR PROTEIN ANALYSIS BY DESI-MS

3.1 Abstract

The composition of sample deposition and electrospray solvents greatly affects DESI analysis of proteins. These solvent systems interact not only with the analyte but also with the sample surface. Therefore, the choice of the sample surface is also of high importance. Here, the difference between the effect of some common depositing solvent systems, sample surfaces and electrospray solvent compositions are studied and discussed.

3.2 Effect of solvent system on DESI-MS performance

During the desorption electrospray ionization (DESI) experiment, many parameters play important roles in designing the method. One of the very crucial parameters is the composition of the solvent systems that are employed during preparation and analysis of samples and how these solvent systems interact with the substrate/surface that contains the sample[1]. The choice of a solvent system directly affects sensitivity and spatial resolution of DESI[2]. Among required optimizations for an efficient DESI performance, the solvent system optimization has a very large effect on DESI signal intensity[2,3]. In DESI experiments when the sample is on a synthetic surface, first a proper solvent system should be used to reconstitute dry sample before spotting the sample on the surface. When the sample is ready, a second solvent system is electrosprayed at it to dissolve, desorb and ionize the analyte before sampling it into the mass spectrometer. Specifically for DESI of proteins, aside from all the general considerations, the choice of a good solvent system should be made with respect to the effect of solvent on conformational changes and denaturation of proteins[4].

Commonly, aqueous solutions with varying fractions of an organic solvent are used as the desorbing electrospray solution during DESI experiments[5]. For the organic fraction of the solvent system, methanol (MeOH)[5-10] and acetonitrile (ACN)[1,11] have been widely used. Also, it is helpful to slightly acidify the electrospray solution in order to promote the protonation of the analyte molecules[1].

Similar to solvent system preparation, for preparing the sample of DESI varying percentages of MeOH[5] or ACN[1] have been employed. In a wide range of organic fractions of the solvent system, MeOH and ACN behave similarly during DESI-MS analysis, including the erosion diameter and sample removal from surfaces[2]. This similarity gives a broader selection of DESI solvent compositions for many types of analytes without sacrificing the efficiency of analysis, except for instances that other factors in the experiment limit the choice of the solvent such as materials used in the instruments that are intolerant to certain solvents (e.g. polyimide with. acetonitrile)[12]. The efficiency of a DESI experiment linearly improves when analyte solubility in the electrospray solution increases[2]. An electrospray solvent in which the compound of interest is more soluble would be a more suitable choice. This choice becomes more convoluted when it comes to protein analysis by DESI as each protein of a complex mixture may favor different systems of solvents due to their physiochemical heterogeneity[13]. For example, it has been shown that most of the time, solubility of proteins with lower molecular mass is enhanced by lowering the pH of solvent due a decrease in solvent viscosity caused by acidifying it[14]. Some solvent systems only improve efficiency of DESI for a group of proteins that share similar physical properties, such as the isoelectric point (Chapter 4)[15]. Despite the vast application of organic solvents for mass spectrometric analysis of proteins, using organic solvents may not be practical in some experiments and can cause effects such as protein denaturation and structural changes[16].

Therefore, finding a solvent system that is optimum for DESI-MS analysis of protein is a crucial step toward its successful experimental outcome.

Here we show optimization of solvent systems for solvents in depositing proteins prior to DESI experiments. Also, the effect of the depositing solvents on different types of surfaces is investigated. Optimization of electrospray solvent with different additives and varying fractions of different organic solvents is also discussed in this chapter.

3.3 Experimental details

3.3.1 Samples and surface

Equine cytochrome *c* (cyt *c*, 12.3 kDa) and bovine hemoglobin (Hgb, 15.1 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). LC-MS grade formic acid, methanol (MeOH), acetonitrile (ACN) and acetone were purchased from Fluka Analytical (CHROMOSOLV, Venezuela). Glacial acetic acid was purchased from EMD (Burlington, MA). Porous-polyethylene (PE) surface with average pore size of 15-45 μm (POR -4900) and polytetrafluoroethylene (PTFE) surface with average pore size of 10-45 μm (POR-7751) were purchased from Interstate Specialty Products (Sutton, MA). Ammonium bicarbonate was purchased from Fisher Scientific (Fairlawn, NJ)

3.3.2 Sample and solvent preparation

For DESI experiments, 3 μL droplets of protein at a concentration of 20 μM were hand pipetted onto surfaces to give an estimated surface concentration of 80 pmol/mm^2 after drying unless otherwise stated (Section 3.4.4.). Each data point is the average of scanning 10 spots of protein.

In Section 3.4.1, solutions were made with either 0.1% (v/v) formic acid or 3% (v/v) acetic acid which are commonly used concentrations for electrospray solutions.

In section 3.4.2, to investigate the effect of different electrospray solution additives, solutions were made with 0.2% (v/v) formic acid which is a recommended additive for mass spectrometric analysis of proteins[17], and 200 mM ammonium bicarbonate (NH_4HCO_3) which showed increased sensitivity for DESI of proteins (Chapter 4)[15]. More concentrated solutions of 200 mM formic - and acetic acids were also prepared for comparison between the solutions: 0.75% (v/v) of formic acid and 1.14% (v/v) acetic acid.

3.3.3 Instrumentation

A linear ion trap mass spectrometer (LTQ, Thermo Scientific, Waltham, MA, USA) was combined with a 3-dimensional translational stage (Purdue University, West Lafayette, IN, USA) for DESI analysis. The stage, that the sample surface was mounted on, was programmed to move on x and y axes relative to the fixed position of the ion-inlet of the mass spectrometer. The translation speed of the stage was set at 150 micrometers/sec controlled by WSDK motion software.

For the generation of a pneumatically-assisted solvent spray, an electrosonic spray ionization (ESSI) source was constructed in-house[18]. The outer capillary (for sheath gas) was approximately 20 mm in length with an outer diameter of 430 μm and inner diameter of 320 μm. The internal capillary (for solvent) had an outer diameter of 200 μm, and inner diameter of 75 μm. For DESI experiments, 4.0 kV spray potential was applied to the liquid junction on the stainless-steel syringe needle used to deliver the spray solvent. The spray solvent was delivered at 5 μL/min nebulized by N_2 gas at 100psi. The tip of the mass spectrometer inlet capillary was extended by 10 cm and bent at 10° to facilitate efficient ion transfer during DESI[19]. The transfer capillary temperature was set at 250°C, the tube lens was set typically at 140 V and the transfer

capillary was at 39 V. The sprayer to inlet distance was typically 4 mm, the sprayer to surface distance was 1 mm, and the incident spray angle was 55°.

Data analyses were performed by MagTran software (1.03) and the reported signal to noise (S/N) ratios were calculated for the highest intensity charge states as described by Zhang and Marshall[20].

3.4 Results and discussion

3.4.1 Optimization of the sample depositing solvent on PE and PTFE surfaces

The choice of the solution in which the DESI sample is solubilized before depositing is crucial in terms of the interaction of the solution and the sample as well as the interactions of the solution and the surface on which the sample has to be deposited. The depositing solution of protein samples can cause changes to the protein structure before its analysis by DESI and therefore affect the efficiency of analysis. The wettability of the substrate and the surface tension of the sample depositing solution can greatly affect the final surface concentration of the sample when it's dried. The contact angle of the surfaces, which is in fact a measure of surface wettability, should be considered as a key factor when considering different surfaces for an experimental method. When the solution that contains the sample is hand pipetted on a surface, the liquid sample forms small semi-spheres. These droplets exhibit a contact angle to the surface. Smaller contact angles mean that the surface has a higher attraction for the solution and the solution will be more spread over the surface (Figure 3.1). In this section, we have tested two of the commonly used surfaces, polyethylene (PE) and polytetrafluoroethylene (PTFE), to see how different fractions of organic solvents affect the final surface concentration of proteins.

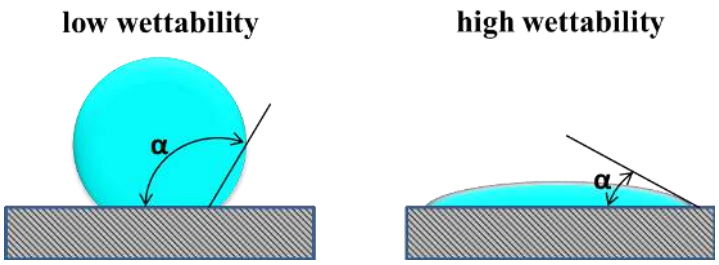

Figure 3.1. Different contact angles show the extent of surface wettability. Large angles indicate low surface wettability (left) while small angles indicate high surface wettability (right).

PE has a contact angle of 96˚ while PTFE has a higher angle 110˚ and therefore less wettability. This is in agreement with observations during sample preparation when the solution of the sample would bead up on the surface immediately after being pipetted.

To investigate the differences of protein surface concentration on PE and PTFE surfaces, a mixture of 20 μM cytochrome c was prepared in solution of 0, 25, 50, 75 and 100% of MeOH and ACN where 0% means the solution contained 100% H_2O. The mixture was then hand-pipetted as 3μL droplets on the surfaces and was let to dry either in the ambient air or in a vacuum oven at 30mmHg and room temperature. After drying the diameter of the dried droplet was measured and the surface concentration was calculated as below:

$$\frac{(sample\ droplet\ volume\ (\mu L))x(concentration\ of\ the\ sample(\mu mol.L^{-}))}{droplet\ area\ (mm^2)}$$

The surface concentrations were prepared in the pmol/mm^2 range. The measurements of protein surface concentration on PE and PTFE surface are shown in Table 3.1 and 3.2. The first row in both tables shows the measured surface concentration for when the protein was deposited out of 100% H_2O. Unlike PE, because of lower wettability of PTFE and higher contact angle of solvent droplets on it, sample droplets couldn't be easily deposited on PTFE out of water-only solution.

On the other hand, since PE has higher wettability and polar organic solvents have smaller contact angles on it, high ratios of organic solvents will be immediately absorbed upon pipetting the sample and they didn't leave any mark on the surface of PE which is indicated by "Not Available" (NA) in Table 3.1. Increasing the portion of the organic solvent decreases the surface concentration of sample on PE by decreasing the contact angle of droplet and its tendency to spread wider on the surface.

Table 3.1. Surface concentration of cytochrome c on PE surface with varying ratios of MeOH or ACN when the sample was air-dried or vacuum-dried.
"NA" indicates that the surface concentration of the sample couldn't be measured due to the sample being absorbed into the surface immediately after deposition.

PE	MeOH (surface concentration)		ACN (surface concentration)	
	air-dried	vacuum-dried	air-dried	vacuum-dried
0% (100% H_2O)	53.0	76.5	53.0	76.5
25%	26.5	34.0	21.0	26.5
50%	21.0	22.0	NA	12.0
75%	NA	14.5	NA	NA
100%	NA	NA	NA	NA

Table 3.2. Surface concentration of cytochrome c on PTFE surface with varying ratios of MeOH or ACN when the sample was air-dried or vacuum-dried. Since proteins couldn't be deposited on PTFE out of 100% H_2O, there is no data for those measurements, indicated by NA.

PTFE	MeOH (surface concentration)		ACN (surface concentration)	
	air-dried	vacuum-dried	air-dried	vacuum-dried
0% (100% H_2O)	NA	NA	NA	NA
25%	76.5	156.0	94.0	94.0
50%	76.5	94.0	94.0	76.5
75%	76.5	53.0	53.0	45.0
100%	76.5	23.5	39.0	34.0

Due to the higher contact angle of solvents on PTFE, the deposited droplets preserved their original shape of beads almost until the solvent evaporation completed, when sample was air-dried. This is why the surface concentration on PTFE remained the same for the most part. However, for higher ratios of ACN, since the solvent tends to disperse more on the surface than MeOH, the droplets are spread over a larger area and yield a lower surface concentration (Table 3.2). In addition, in all the cases, depositing the sample on PTFE gives a significantly higher surface concentration than depositing on PE. Unlike air drying, vacuum drying disturbs the bead shapes of the droplets. With vacuum drying, the evaporation occurs at higher rates and by increasing the organic ratio of the solvent, the time needed for evaporation becomes even shorter. Because of the quicker drying, vacuum dried samples are left with darker coffee rings and less sample homogeneity.

3.4.1.1 Effect of the depositing solvent on protein analysis by DESI

Air-dried cytochrome c was then analyzed by DESI-MS and desorbing it with an electrospray containing 80% ACN with 0.2% formic acid to study the effect of the depositing solvent on the efficiency of DESI analysis. Tables 3.3 and 3.4 show the signal intensity, signal intensity to surface area ratio and signal to noise ratio of these experiments for sample deposited on PE and PTFE, respectively. The attained protein signal intensity and S/N ratio on PTFE is significantly higher than on PE which is not likely to be due to the higher surface concentration of the sample on PTFE since the signal intensity to surface concentration ratios are close and sometimes even lower for PTFE. However this could be due to better sample removal from PTFE compared to PE. When the sample is deposited out of ACN, the best result on PTFE is achieved at higher percentages of ACN compared to PE. Whereas for MeOH, 50% was the best depositing solvent on both PE and PTFE.

Table 3.3 DESI analysis of cytochrome c deposited out of different ratios of MeOH and ACN on PE.

PE	MeOH			ACN		
	Intensity	$\frac{Int.}{surf.conc.}$	S/N	Intensity	$\frac{Int.}{surf.conc.}$	S/N
25%	3.81	0.14	115	4.54	0.21	118
50%	6.23	0.29	109	1.59	__	53
75%	0.66	__	40	1.63	__	71
100%	0.34	__	25	2.22	__	57

Table 3.4 DESI analysis of cytochrome c deposited out of different ratios of MeOH and ACN on PTFE.

PTFE	MeOH			ACN		
	Intensity	$\frac{Int.}{surf.conc.}$	S/N	Intensity	$\frac{Int.}{surf.conc.}$	S/N
25%	12.2	0.16	124	12.3	0.13	124
50%	18.9	0.24	170	23	0.24	160
75%	19.4	0.25	136	20.5	0.38	211
100%	8.15	0.11	118	13.7	0.35	157

3.4.2. Optimization of electrospray solvent composition

The electrospray solvent of DESI plays a key role in both ionization and desorption of sample from surface. Want et al. previously studied different combinations of some organic solvents for protein extraction and profiling by mass spectrometry[21]. From their results, we picked the best four combinations and used them as DESI spray solvent. These four solvent systems include 1) 100% MeOH, 2) 50:50 MeOH:Acetone 3) 70:30 MeOH:Acetone and 4) 70:30 ACN:MeOH. Each of these solvent systems was tested in three ways:

I) Only organic solvents and nothing else added.

II) With acetic or formic acid added.

III) With water and acetic or formic acid added.

The data for the analysis of cytochrome c by these combinations are shown in Tables 3.5-3.8. A solution of 20μM cytochrome c in water was hand-deposited on PE surface as 3μL droplets. Table 3.5 represents the signal intensity of the *highest intensity charge state* (HICS) of

cytochrome c while Table 3.6 shows the effect of these organic solvents on protein's signal to noise ratio. Tables 3.7 and 3.8 show the charge state distribution for highest intensity and *highest observed charge state* (HOCS) of cytochrome c with each spray solvent composition, respectively.

When the spray solvent composition only consisted of organic solvents (I) without any other type of fraction or additive, no protein signal was observed in the obtained mass spectrum. In fact, when the sprayer scanned through a row of droplets on the sample surface, there was no visible track of the sample removal at the foot print of DESI sprayer. This may suggest that when the spray solvent was only made up by organic fractions, protein sample wasn't easily solubilized by such solvent and remained mostly undissolved on the surface. As soon as the solution is acidified with either of acetic acid (3% v/v) or formic acid (0.1% v/v) (II), a track of sample removal from the surface as well as protein signal was observed. The exception was 70:30 ACN:MeOH with formic acid where the addition of the acid didn't help protein solubility and significant removal of the sample from the surface was not observed. With the addition of only acids to the organic solvent, in terms of protein signal to noise ratio (S/N) value, it seems that acetic acid and formic acid behave inversely (Table 3.5). While acetic acid resulted in the highest S/N of protein in the mix of 70:30 ACN:MeOH (S/N = 28), formic acid addition didn't result in any protein signal and where acetic acid addition caused the lowest protein S/N when added to MeOH (S/N = 7), formic acid addition resulted in the highest S/N by being added to MeOH (S/N = 21). Signal intensity followed a similar pattern, except in the case of acetic acid addition to MeOH which caused the highest protein signal intensity (Intensity = 14.8) while lowest S/N ratio (S/N = 7) among other organic solvent compositions (Table 3.5). Once 30% (v/v) of water was added (III) to the solution, while keeping the relative percentage of organic solvents to one another the same,

acetic acid outperformed formic acid in all the compositions in terms of both signal intensity and signal to noise ratio improvement. The difference in the performance of the two acids was the least when either of the acids was added to the solution containing 30% H_2O and 70% MeOH and became the most once added to the solution of 30% H_2O and 49:21 ACN:MeOH (70:30 relative to one another).

Addition of water provided significant increase in protein signal intensity and signal to noise ratio when used with acetic acid but when used in solutions containing formic acid, addition of water did not cause a similar increase in signal intensity or S/N ratio value. Also by addition of water to the electrospray solvent composition, the track of sample removal became much clearer since the addition of water helps with protein solubility.

In terms of protein charge state distribution, both HICS and HOCS shift to higher charge states by addition of water to the solvent systems that contained acetic acid while they remained nearly constant for formic acid containing solvents.

Overall, it seemed that the performance of formic acid, with regards to sensitivity and charging of the protein does not change much upon addition of water fraction to the organic solvent. However, acetic acid performance in sensitivity and protein charging can significantly be improved by adding water to the organic electrospray solution.

Table 3.5. Effect of solvent composition on signal intensity of cytochrome

Intensity	(I) only organic	(II) organic+acid		(III) organic+acid+H_2O	
		acetic	formic	acetic	formic
MeOH	NA	14.8	16.6	16.8	12.6
MeOH/Acetone (50:50)	NA	3.51	8.59	17.0	8.0
MeOH/Actone(70:30)	NA	7.48	4.97	18.9	6.43
ACN/MeOH (70:30)	NA	9.25	NA	33.3	2.56

Table 3.6. Effect of solvent composition on signal to noise (S/N) ratio of cytochrome c

S/N	(I) only organic	(II) organic+acid		(III) organic+acid+H_2O	
		acetic	formic	acetic	formic
MeOH	NA	7	21	40	30
MeOH/Acetone (50:50)	NA	15	14	43	17
MeOH/Actone(70:30)	NA	21	13	49	15
ACN/MeOH (70:30)	NA	28	NA	56	8

Table 3.7. Effect of solvent composition on highest intensity charge state (HICS) of cytochrome c

HICS	(I) only organic	(II) organic+acid		(III) organic+acid+H_2O	
		acetic	formic	acetic	formic
MeOH	NA	+15	+14	+15	+14
MeOH/Acetone (50:50)	NA	+13	+14	+15	+14
MeOH/Actone(70:30)	NA	+14	+14	+15	+14
ACN/MeOH (70:30)	NA	+14	NA	+15	+14

Table3.8. Effect of solvent composition on highest observed charge state (HOCS) of cytochrome c.

HOCS	(I) only organic	(II) organic+acid		(III) organic+acid+H_2O	
		acetic	formic	acetic	formic
MeOH	NA	+17	+19	+19	+19
MeOH/Acetone (50:50)	NA	+20	+20	+20	+19
MeOH/Actone(70:30)	NA	+17	+20	+20	+19
ACN/MeOH (70:30)	NA	+19	NA	+20	+19

3.4.3 Optimization of volatile additives in the electrospray solvent of DESI

A mixture of cytochrome c and hemoglobin in 50% ACN was hand-deposited on PTFE surface as 3μL droplets. The significance of using cytochrome c and hemoglobin here is that cyt c is a high pI protein, while Hgb is a low pH protein. The reason for using 50% ACN as the depositing solvent was because unlike 50% MeOH, 50% ACN does not fully denature the protein but still facilitates hand-pipetting of the protein on PTFE surface. The droplets were air-dried and the surface concentration for each protein based on the size of the dried droplet was calculated to be ~76 pmol/mm² as previously shown in shown in Table 3.2.

Formic acid at 0.2% (v/v) was used along with 200mM ammonium bicarbonate which will be shown in Chapter 4 to be beneficial for increasing signal to noise ratio of some proteins[15]. A higher concentration of formic acid, 0.75% (v/v), as well as 1.14% (v/v) of acetic acid, both equimolar to 200mM of ammonium bicarbonate, were also used as DESI electrospray additives. These four additives were separately added to electrospray solutions with varying fractions (25, 50, 75 and 100%) of either MeOH or ACN. For each solvent composition, a solution with no acidic addition was tested as a control.

Figures 3.2 and 3.3 show the effect of additives in the electrospray solution on the signal intensity of the proteins. In each of the organic solvents, MeOH or ACN, the effectiveness of additives for signal intensity of cytochrome c (Figure 3.2) was very similar to that for hemoglobin (Figure 3.3). In MeOH, 0.75% formic acid gave the highest signal intensity improvement (Figure 3.2(a) and 3.3(a)).

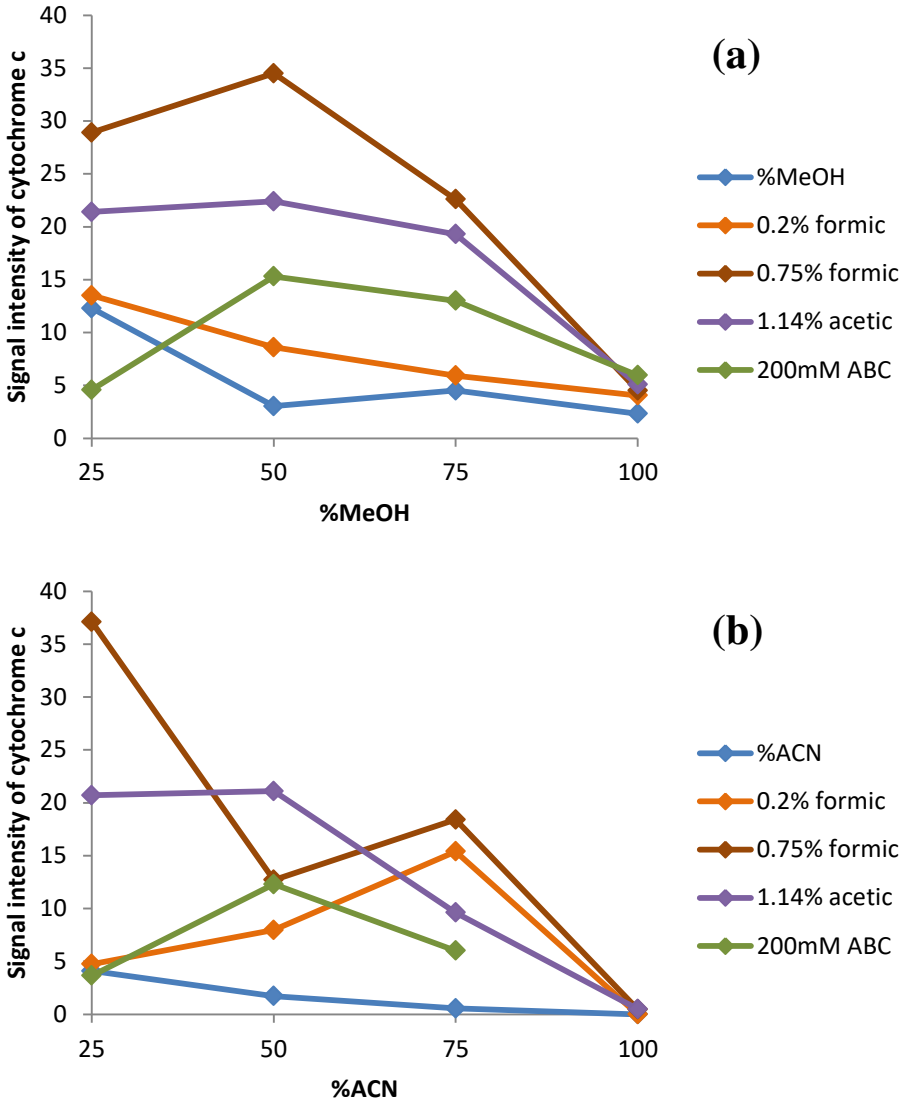

Figure 3.2. Effect of volatile additives in electrospray solvent with varying fractions of a) MeOH and b) ACN on signal intensity of cytochrome c. An electrospray solvent without any additive was used as the control (blue line).

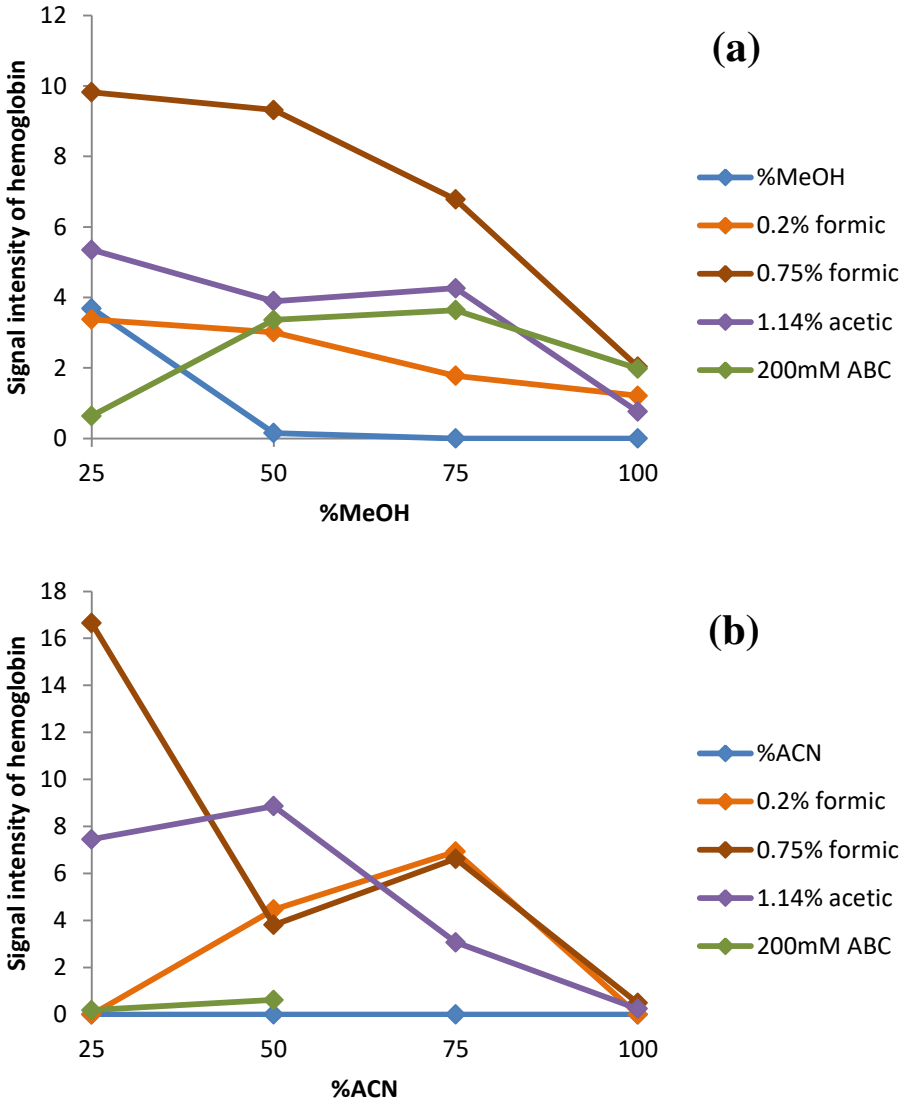

Figure 3.3. Effect of volatile additives in electrospray solvent with varying fractions of a) MeOH and b) ACN on signal intensity of hemoglobin. An electrospray solvent without any additive was used as the control (blue line).

In ACN, except for 50% ACN, 0.75% formic acid led to the highest protein signal intensity for both proteins (Figure 3.2(b) and 3.3(b)). At 50% ACN, 1.14% acetic acid took over the rest of the group for both proteins.

For both proteins, over the range of 50-100% of MeOH, the order of signal intensity improvement by the additives remained similar, going from the highest which was 0.75% formic acid to acetic acid and then ammonium bicarbonate and finally the lowest which was 0.2% formic acid (Figure 3.2(a) and 3.3(a)). In solutions consisting of ACN, all three cases with acids behave more similarly compared to solutions containing MeOH.

Figure 3.4 shows the effect of these four volatile additives at different fractions of MeOH (Figure 3.4(a)) and ACN (Figure 3.4(b)) on the signal to noise ratio of cytochrome c. As can be seen from Figure 3.4a, the performance of ammonium bicarbonate rises above other additives at lower percentages of MeOH, but starts to diminish by increasing the organic fraction. This could be attributed to the fact that the solubility of ammonium bicarbonate decreases with increasing the ratio of organic solvents in the electrospray solution. Ammonium bicarbonate began precipitatating immediately after addition to solutions with very high percentages of organic solvents. This could be one reason why it doesn't work quite as well at higher fractions of MeOH.

In the presence of ACN, Figure 3.4(b), ammonium bicarbonate stands above all other additives in improving the S/N of cytochrome c at all the times and it reaches a maximum at 50% ACN solution composition. However, since 200mM ammonium bicarbonate is only marginally soluble in 100% ACN, no mass spectrum was obtained for the last data point. At higher ratios of MeOH, Figure 3.4(a), 0.2% formic acid increases the S/N ratio of cytochrome c more than other

additives. Aside from ammonium bicarbonate and 0.2% formic acid in MeOH, all other additives didn't show a significant change over varying fractions of the organic solvents and showed similar effectiveness at different ratios of MeOH or ACN.

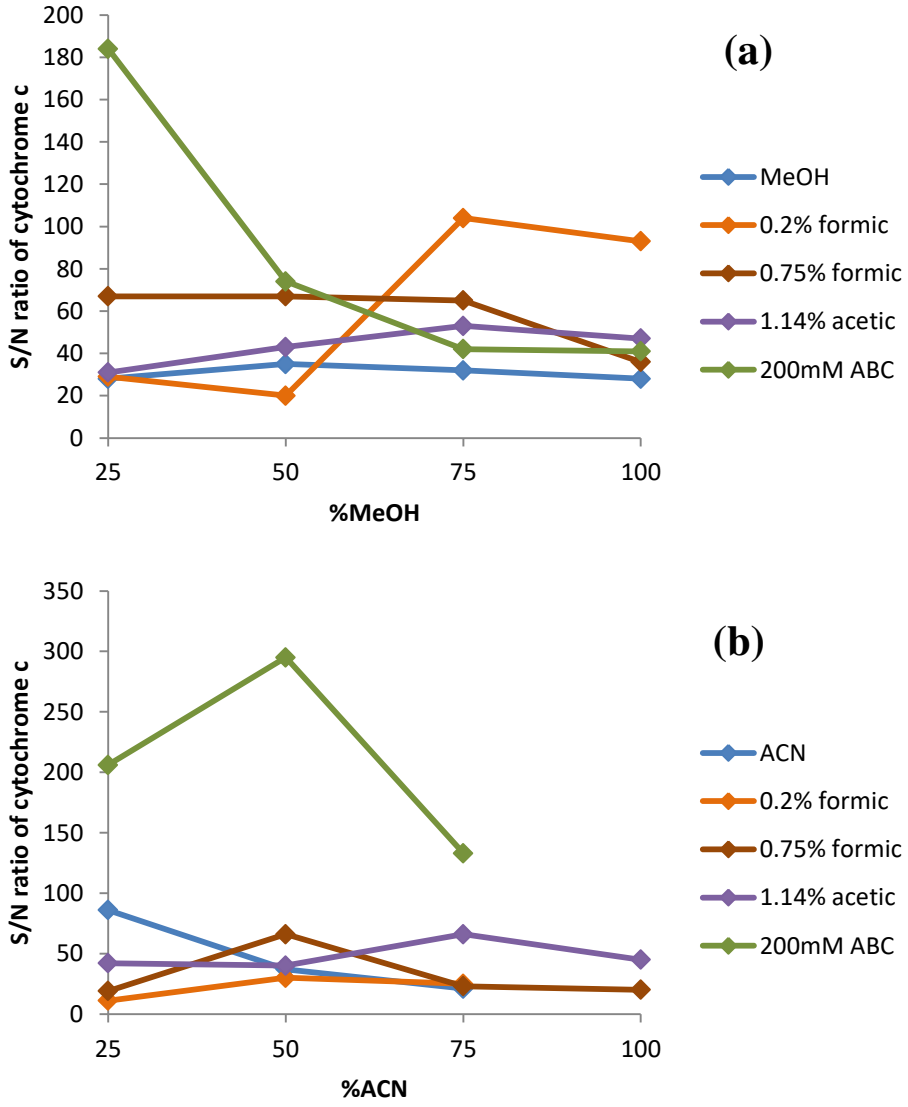

Figure 3.4 Effect of volatile additives in electrospray solvent with varying fractions of a) MeOH and b) ACN on S/N ratio of cytochrome c. An electrospray solvent without any additive was used as the control (blue line).

Figure 3.5 shows the effect of the same four electrospray additives on the signal to noise ratio of hemoglobin. As it can be seen, except for 25% and 50% MeOH, there are no control data (organic solvent without acidic additive) for hemoglobin due to difficulty of DESI analysis of this protein without the presence of additives. Unlike cytochrome c, for S/N ratio improvement of hemoglobin in both systems, MeOH and ACN, acids were more beneficial 0.75% and 0.2% of formic acid were the most effective additives respectively at lower (25-50%) and higher (75-100%) fractions of MeOH (Figure 3.5(a)).

In ACN, 1.1% acetic acid remained the most effective additive in increasing S/N at all ratios. At 75% ACN, with addition of ammonium bicarbonate to the electrospray solution, no protein peak for hemoglobin was observed, while with 100% ACN, ammonium bicarbonate was insoluble and no data was obtained. Therefore, these two data point are missing from the graph in Figure 3.5b. Also, by adding 0.2% formic acid to 100% ACN, no protein peak, neither for cytochrome c (Figure 3.4(b)) nor for hemoglobin (Figure 3.5(b)) was observed.

Overall, acidifying the electrospray solution improves the signal intensity for both proteins. For cytochrome c, which has a higher isoelectric point than hemoglobin, ammonium bicarbonate improves the S/N ratio, while the S/N ratio of hemoglobin still benefits more from addition of acids.

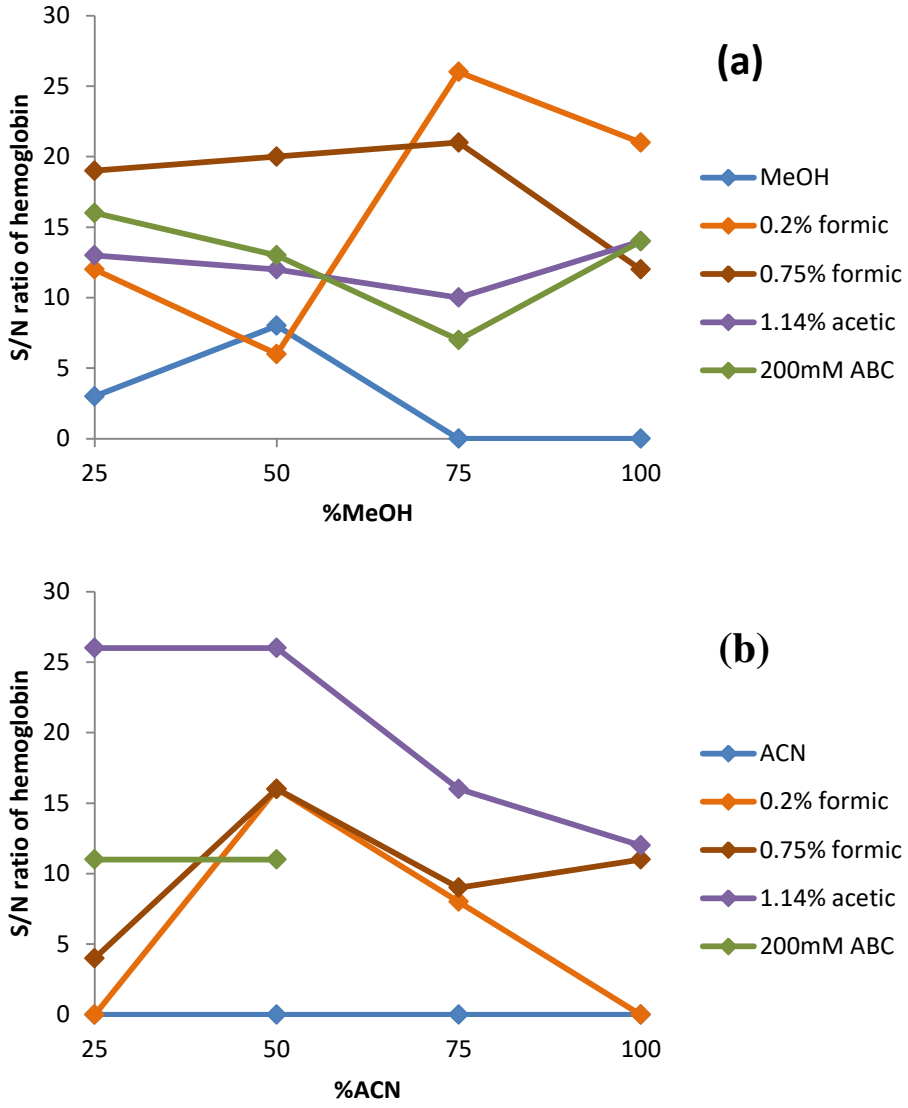

Figure 3.5. Effect of volatile additives in electrospray solvent with varying fractions of a) MeOH and b) ACN on S/N ratio of hemoglobin. An electrospray solvent without any additive was used as the control (blue line).

3.5 Conclusion

In this chapter, we show the effect of different compositions of depositing solvent as well as electrospray solvent on the performance of DESI-MS analysis. The interaction of the depositing solvent with the surface can greatly influence the results obtained by DESI analysis of proteins. It was observed that the addition of acids and/or water to organic solvent is vital for protein analysis by DESI-MS. The optimum amount of organic solvent should be separately optimized for each organic solvent as they don't follow similar patterns. The optimum amount of organic solvent can also be dependent on the type of solution additives that are used in the experiment and their solubility.

From the data shown in this chapter, we observed that PTFE yields better DESI results but its practical use for depositing proteins out of water, preserving their native state, is very limited. For the electrospray solvent, the combination of MeOH:H_2O gave the highest signal intensity and signal to noise ratio when formic acid was used in the solution while ACN:MeOH:H_2O had the best performance when acetic acid was added to the solution. The addition of volatile additives in the electrospray solvent affected signal intensity and S/N ratio of proteins differently. For both cytochrome c and hemoglobin, acids helped to improve their signal intensities. Conversely the S/N ratio of high pI proteins were improved dramatically more in the presence of ammonium bicarbonate.

It should be noted that the target of this chapter is not the analysis of one protein but to show optimization of different conditions that can be useful in the analysis of several proteins. The best depositing and electrospray solvent may vary for different proteins. The conditions should be selected to achieve satisfactory results for all the proteins in the study. However it is likely that proteins with similar physiological properties will favor similar solvent conditions.

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CHAPTER IV

THE EFFECTS OF AMMONIUM BICARBONATE ON THE ANALYSIS OF PROTEIN BY DESI-MS

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Elahe Honarvar, and Andre R. Venter,
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4.1 Abstract

The analysis of protein by desorption electrospray ionization mass spectrometry (DESI-MS) is considered impractical due to a mass-dependent loss in sensitivity with increase in protein molecular weights. With the addition of ammonium bicarbonate to the DESI-MS analysis the sensitivity towards proteins by DESI was improved. The signal to noise ratio (S/N) improvement for a variety of proteins increased between 2- to 3-fold relative to solvent systems containing formic acid and more than seven times relative to aqueous methanol spray solvents. Three methods for ammonium bicarbonate addition during DESI-MS were investigated. The additive delivered improvements in S/N whether it was mixed with the analyte prior to sample deposition, applied over pre-prepared samples, or simply added to the desorption spray solvent. The relative improvements correlated well with protein pI but not with protein size.

4.2 Protein desorption electrospray ionization mass spectrometry and its limitations

As described in earlier chapters, despite the practical utility of DESI-MS for small molecules, the method is severely limited for analysis of proteins[1]. The limitation is believed to be related to

incomplete sample dissolution during desorption resulting in the distribution of signal across protein clusters, as well as protein – protein and protein – contaminant adducts [1].

A similar problem in ESI is usually addressed through the use of spray additives, such as mass spectrometer friendly buffers to enhance the protein response [2,3] and to reduce chemical noise. For example, in 2004, Iavarone and coworkers reported improvements in sensitivity during protein analysis by ESI when volatile buffer reagents such as ammonium acetate were used[4]. Exchanging the buffer solution from nonvolatile ions to ammonium acetate or ammonium bicarbonate solutions improved the quality of protein mass spectra by removing ESI incompatible compounds [5,6].

The benefit of the addition of ammonium additives is believed to stem from the replacement of nonvolatile salts by ammonium in solutions of proteins [4,7,8]. When NH_4^+ displaces Na^+ ion in solution, it decomposes to form a proton and ammonia during the evaporation and transfer processes in ESI[9] leading to protonation of a basic site and a reduction in metal adduction. Alternatively, Iavarone et al suggested the improved signal to noise ratio (S/N) is simply caused by the precipitation of nonvolatile ions, such as Na^+ and Cl^- ions [4]. Similar to ESI, it was shown that ammonium salts can prevent formation of sodium and potassium adducts in MALDI when added to matrices during analysis of oligonucleotides[10,11]. In a study on the application of ammonium halides as co-matrices in the analysis of DNA homopolymers by MALDI, it was found that both the ammonium and halogen portions of the salt play a role in the observed signal improvements[10].

The identity of the anion also plays an important role in the charge states and intensities observed for proteins and peptides[12] when analyzed by ESI. It was found that the extent of acid molecule and cation adduction was inversely related and that this depends on the gas phase proton

affinities of the anion and the isoelectric point of the protein[13]. This dependence has been explained by the need for good matching between the gas phase basicity of the anion and apparent gas phase basicities of the cation-bearing sites on the protein[14,15].

Ammonium bicarbonate was proposed as an alternative volatile buffer for native protein analysis due to its high buffering capacity at near neutral pH[16-20]. Unfortunately, when ammonium bicarbonate was used as a buffer reagent in electrospray ionization analysis, proteins formed higher charge states, indicative of protein denaturation[18]. Hedges and coworkers suggested that when ammonium bicarbonate and proteins are present in ESI droplets, heat and the formation of bubbles act synergistically to cause protein unfolding during the electrospray process[21]. This proposed mechanism was later rejected by Cassou and Williams who showed that denaturation follows a reverse Hofmeister series, and are therefore more likely to be due to anion effects on protein stability and solubility[22].

Here, we investigate the use of ammonium bicarbonate in the analysis of protein samples by DESI-MS. Three different methods of applying ammonium bicarbonate during DESI were investigated, including addition of the ammonium bicarbonate to the DESI spray solvent, addition to the protein solution prior to deposition on the surface, and deposition on top of pre-prepared protein samples.

At the end of the chapter, we briefly discuss the effect of addition of ammonium bicarbonate to a couple of peptide samples.

4.3 Experimental details

4.3.1 Samples and surface

Equine cytochrome *c* (cyt *c*, 12.3 kDa), bovine hemoglobin (Hgb, 15.1 kDa) ovalbumin (OVA, 44.3 kDa) and bovine serum albumin (BSA, 66.4 kDa) were purchased from Sigma-Aldrich (St. Louis, MO). α-chymotrypsinogen (chtg, 25.7 kDa) was purchased from MP Biomedicals, LLC (Solon, OH). LC-MS grade formic acid and methanol (MeOH) (CHROMOSOLV, Venezuela) were purchased from Fluka Analytical. Porous-polyethylene (PE) surfaces with average pore size of 15-45 μm (POR-4900) were purchased from Interstate Specialty Products (Sutton, MA). Ammonium bicarbonate was purchased from Fisher Scientific (Fairlawn, NJ). MRFA. Met-Arg-Phe-Al, (523.65 Da) and Melittin (2846.46 Da) were purchased from Sigma-Aldrich (St. Louis, MO).

4.3.2 Sample and solvent preparation

For ESI experiments, protein was prepared at 10 μM each by dilution in 50% MeOH, unless noted differently, and salts were added at 100 mM, as previously used in work by Hedges[21] unless noted differently. Formic and acetic acids were used at 0.1% and 3% respectively.

For DESI experiments, 3 μL droplets of protein at a concentration of 20μM were hand pipetted onto surfaces to give an estimated surface concentration of 80 pmol/mm^2 after drying.

To investigate the effect of the addition of ammonium bicarbonate to protein samples by DESI, 200 mM of the salt was added into the 50% MeOH DESI desorption spray solvent, as optimized for ammonium bicarbonate. Carbonic acid solutions were prepared by bubbling CO_2 until a stable pH of 3.76 (+/-0.1) was reached, corresponding to a calculated concentration of 70 mM of carbonic acid, while formic and acetic acid were used at 0.1% and 3% respectively.

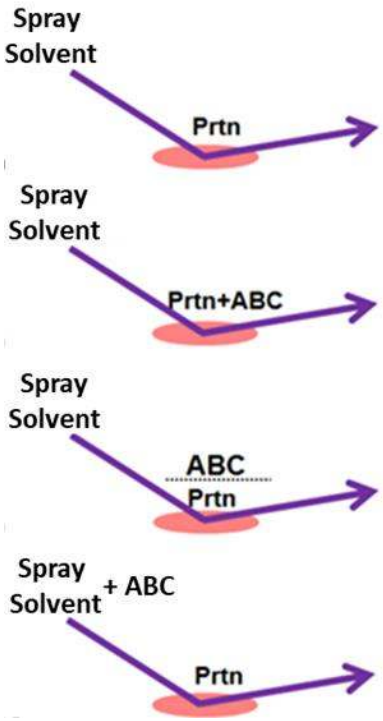
MRFA peptide sample was prepared in a 50% MeOH solution at a 5 mM concentration and melittin sample was prepared in water only at a concentration of 0.35 mM.

4.3.2.1 Ammonium bicarbonate was added to the DESI sample in one of three ways:

I. *Addition of ammonium bicarbonate to the protein mixture*. In this method, a solution containing 20 µM protein and 200 mM ammonium bicarbonate was prepared in 50% MeOH. This protein solution was then deposited on the PE surface as 3 µL droplets. After drying on the surface, the samples were analyzed with a spray solvent containing 50% MeOH.

II. *Addition of ammonium bicarbonate on top of pre-prepared protein samples*.

Here, a solution containing 20 µM of the protein was deposited on the PE surface and allowed to air dry. Then, a 3 µL solution containing 200 mM ammonium bicarbonate was deposited over the dried protein sample and left to air-dry again. The sample was then analyzed using a spray solvent containing 50% MeOH.



III. *Addition of ammonium bicarbonate to the spray solvent*. In this method, a solution containing 20 µM was deposited on the PE surface as 3 µL droplets. After drying on the surface, the sample was analyzed with a spray solvent containing 200 mM of ammonium bicarbonate in 50% MeOH.

4.3.3 Instrumentation

A linear ion trap mass spectrometer (LTQ, Thermo Scientific, Waltham, MA, USA) was combined with a 3-dimensional translational stage (Purdue University, West Lafayette, IN, USA) for DESI analysis. For the generation of a pneumatically-assisted solvent spray, an electrosonic spray ionization (ESSI) source was constructed in-house[23]. The outer capillary (for sheath gas) was approximately 20mm in length with an outer diameter of 430 µm and inner diameter of 320

μm. The internal capillary (for solvent) had an outer diameter of 200 μm, and inner diameter of 75 μm. For DESI experiments, 4.0 kV spray potential was applied to the liquid junction on the stainless-steel syringe needle used to deliver the spray solvent. The spray solvent was delivered at 5 μL/min with N_2 as nebulizing gas at 100 psi. The tip of the mass spectrometer inlet capillary was extended by 10 cm and bent at 10° to facilitate efficient ion transfer during DESI[24]. The transfer capillary temperature was set at 250°C, the tube lens was set typically at 90 V except in the case of data presented in Figure 4 were it was 140V as well as in negative ion mode which it was set at -65 V and the transfer capillary was at 20 V. The sprayer to inlet distance was typically 4 mm, the sprayer to surface distance was 1 mm, and the incident spray angle was 55°.

Data analyses were performed by MagTran software (1.03) and the reported signal to noise ratios were calculated for the highest intensity charge states as described by Zhang and Marshall[25].

4.4 Results and discussion

4.4.1. Effect of ammonium bicarbonate addition on protein charge state distribution

Similar to ESI[18], when ammonium bicarbonate was used in DESI as a volatile additive, extensive protein unfolding was observed with concurrent increase in charge states relative to those observed for 50% MeOH and formic acid. Both the *highest intensity charge states* (HICS), and *highest observed charge states* (HOCS)[26] were seen to increase even as the solution pH remained close to neutral. Figure 4.1(a-c) shows the DESI-MS spectra obtained when a protein solution was deposited from 100% water solutions. This allowed for the investigation of protein unfolding during DESI analysis and for a comparison of the relative unfolding induced by the addition of ammonium bicarbonate or the common additive, formic acid. As control, in Figure 4.1(a), an aqueous solution containing 50% methanol was used as desorption solvent. Typically, in ESI this concentration of methanol would lead to unfolding of the protein and cause an

increase in protein charge states[27,28] as can be seen in the ESI-MS analysis of cytochrome *c* in Figure 4.1(d) with a HICS near z=+14 (m/z 884) and a HOCS at z=+20 (m/z 619). However, with DESI, due to the short contact time with methanol[29], unfolding of the protein was not observed and a native state-like envelope was observed[26] with S/N=10 calculated for the HICS at z=+8 (m/z 1546).

Figure 4.1(b) and Figure 4.1(c) show the unfolding effects observed for the addition of formic acid and ammonium bicarbonate to the spray solvent during DESI. Formic acid is often added to electrospray solutions to increase charging and ionization efficiency and is also a common additive used for DESI analysis.

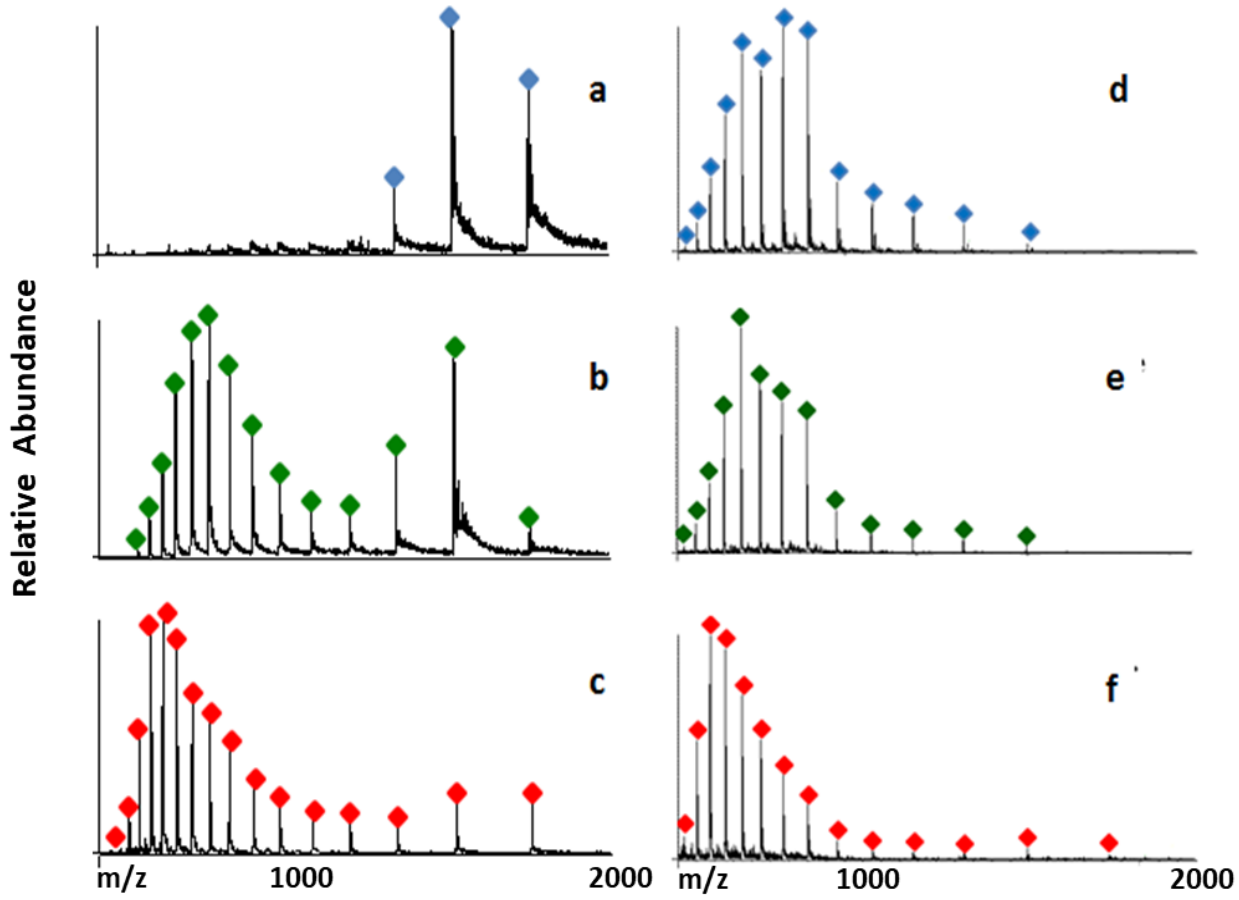


Figure 4.1. The analysis of 80pmol/mm^2 cytochrome c by DESI (left) and ESI-MS (right). For DESI experiments the sample was deposited from water and analyzed with (a) 50% MeOH (b) 0.1% formic acid (c) 200mM ammonium bicarbonate in spray solution. In ESI, samples were dissolved in (d) 50% MeOH (e) 0.2% formic acid (f) 100 mM ammonium bicarbonate before direct analysis.

Formic acid addition produced a bi-modal envelope that shows both native-like and denatured populations of cyt c, with a HICS at z=+8 (m/z 1546), a secondary HICS at z=+15 (m/z 952) and HOCS at z=+20 (m/z 619). Ammonium bicarbonate was found to be even more denaturing with HICS at z=+18 (m/z 688), a much reduced proportion of native-like charge states and HOCS at z=+22 (m/z 563).

Similarly, in ESI when formic acid and ammonium bicarbonate were added into the sample solution, as shown in Figure 4.1(e) and Figure 4.1(f) respectively, the HICS shifted to z=+16

(m/z 773) for formic acid and to z=+18 (m/z 688) for ammonium bicarbonate. Due to the high spray voltage and temperature used in these experiments the conditions are favorable for electrothermal supercharging when ammonium bicarbonate is present[18], explaining the relatively increased charging during analysis with ammonium bicarbonate additive in both ESI and DESI.

4.4.2. Effect of ammonium bicarbonate addition on S/N ratio and signal intensity of the protein

In DESI, both ammonium bicarbonate and formic acid addition also improved the signal to noise ratios measured for the observed HICS of cyt *c* for each result (Figure 4.1(b) and 4.1(c)). With the control analysis using 50% MeOH, the S/N obtained for the HICS (z=+8) was 10. The addition of formic acid resulted in a bimodal protein spectrum showing a HICS at z=+8, representative of the native–like configurations where S/N was 23, and a second HICS at z=+15 for the denatured protein population where the S/N was measured as 65. With the addition of ammonium bicarbonate for the HICS at z=+18 a S/N of 150 was observed. Ammonium bicarbonate delivered an improvement in S/N 15 times relative to the control when the samples were deposited from water, which was also more than double the improvement observed for formic acid addition.

Although ammonium bicarbonate resulted in a better S/N, the improvement in signal intensity, from 1.0 to 42 intensity units was not as pronounced as for formic acid (177 intensity units). This was likely caused by lower solubility of protein in solutions that contain high concentrations of ammonium bicarbonate, leading to less efficient droplet pickup of protein from surfaces during DESI, considering the relative positions of ammonium bicarbonate and formate in the Hofmeister series and the differences in the pH of the desorbing sprays[30]. Much less proteins was

also removed when ammonium bicarbonate was added to the spray solution as was clearly visible from observing the sprayer track on the DESI surface through the protein spots. Therefore, the improvement in S/N likely occurs due to a reduction in noise, as can be seen in Figure 4.2 as described later.

Figures 4.1(d-f) shows that the effects observed for ESI when using the same additives was different from those observed for DESI for the analysis of cyt c. For ESI the highest signal to noise ratio (S/N=305) was observed when formic acid was used while the addition of ammonium bicarbonate lead to a reduction in signal to noise ratio (S/N =188) relative to the control (S/N=196). Previously it was found that in ESI analyte intensities decrease as the ionic strength of the spray solution increases[31]. In addition, solutions such as those containing ammonium bicarbonate typically have higher surface tension, which also leads to less efficient nebulization during the electrospray ionization process. Combined, these effects lower the signal which likely leads to lower observed S/N[32].

In contrast, during a DESI analysis the inefficient ionization process due to this increased surface tension is mitigated by the reduction in droplet size that occurs when the primary droplets, produced at around 5 μm by the electrosonic spray source, collides with the surface to produce secondary droplets. These were previously measured to be reduced to around 1 μm or smaller after surface collision[29]. Protein ionization occurs from these smaller droplets according to the droplet pick-up mechanism of DESI[33], and smaller droplets are believed to be more efficient at ionization[34].

Figure 4.2 depicts deconvoluted spectra for the data presented in Figure 4.1. The amount of adduction is reduced when cytochrome c is analyzed with either additive in the spray solvent compared to the control for both DESI-MS (Figure 4.2(a)) and ESI-MS (Figure 4.2(d)). It is

typical that more adduction is experienced for proteins when analyzed with DESI relative to ESI and the degree of adduction reduction after using either additive was different with the two techniques. In DESI-MS ammonium bicarbonate (Figure 4.2(c)) reduced the extent of adduction significantly better relative to formic acid (Figure 4.2(b)), and also unlike formic acid did not cause any additional adducts that were not already present in the control. This was not the case for ESI, where ammonium bicarbonate (Figure 4.2(f)) fared poorer than either the 50% MeOH control (Figure 4.2(d)) or when formic acid was added (Figure 4.2(e)). With ammonium bicarbonate addition in ESI the deconvoluted peak was also broader than in any of the other spectra. While low resolution and low mass accuracy hindered the unambiguous identification of adducts here, common adducts appeared to be ammonia (M+17), water (M+18), sodium (M+23), potassium (M+39) or combinations of these[35]. Ammonium bicarbonate appears to mostly remove higher mass adducts such as those that could tentatively be assigned to potassium and those likely to be combinations of multiple or heavier adducting species.

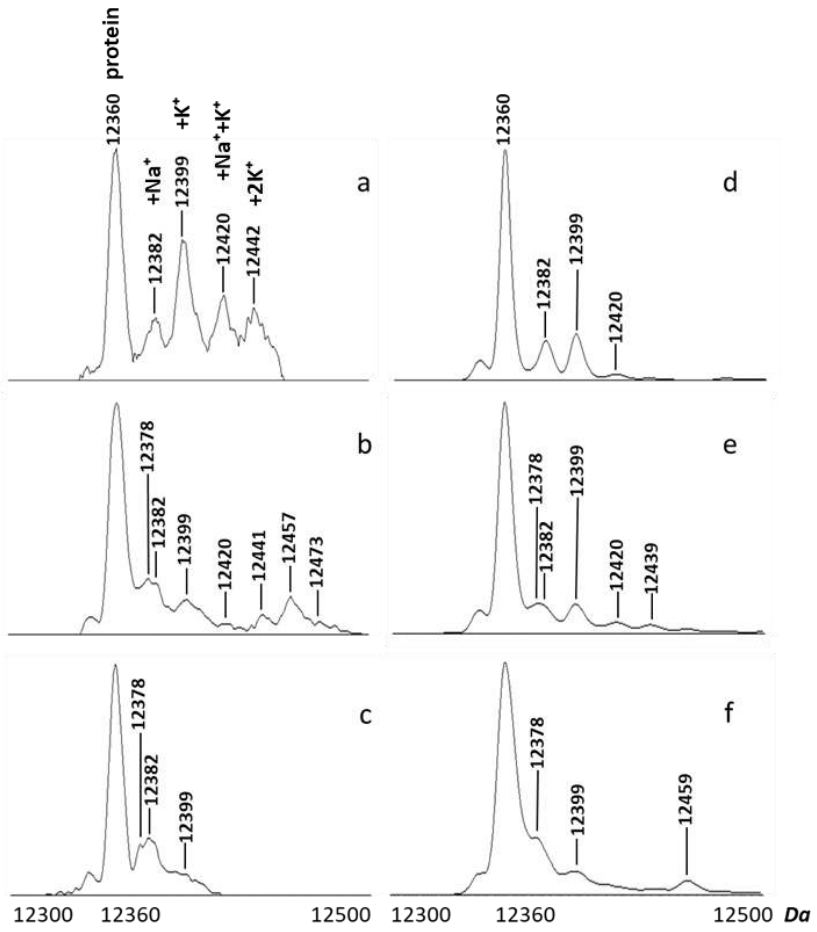


Figure 4.2. Deconvoluted spectra of cytochrome c showing different adduction forms with different solvent additives. DESI spectra of cytochrome c deposited from water and analyzed with a solution of 50% MeOH containing a) no additive b) formic acid c) ammonium bicarbonate are compared to ESI spectra of 10 μM cytochrome c prepared in 50% MeOH containing d) no additive e) formic acid f) ammonium bicarbonate.

4.4.3. Different methods of ammonium bicarbonate addition to protein

Different methods for the application of ammonium bicarbonate during DESI-MS were investigated as illustrated in Figure 4.3. These methods include addition of ammonium bicarbonate to the protein solution (I) before sample deposition on the surface (Figure 4.3(b)), (II) deposition of ammonium bicarbonate on top of deposited protein samples (Figure 4.3(c)),

and (III) addition of ammonium bicarbonate to the spray solution (Figure 4.3(d)). The sequence of protein and ammonium bicarbonate deposition as well as the results for cytochrome *c* is summarized in Table 4.1.

For these experiments, unlike those presented in Figures 4.1 and 4.2, the samples were spotted from a solution made up in an aqueous solution containing 50% MeOH and were therefore already denatured before DESI-MS analysis. This denatured control had a S/N=36 for cytochrome c on PE, which was 3.6 times higher than when the same protein was spotted from water as shown for the control in Figure 4.1. This higher S/N of the control leads to a lower apparent improvement relative to the control for ammonium bicarbonate and formic acid addition and is consistent with published observations where adduction was shown to decrease with increasing charge states[13].

Table 4.1. Signal to noise ratio values, highest observed- (HOCS) and highest intensity charge state (HICS) for the three different methods of applying ammonium bicarbonate to DESI during the analysis of 80 pmol/mm^2 cytochrome c. *Sample* indicates the solution spotted on the surface prior to analysis and *Spray* indicates the composition of the DESI solvent during analysis. ABC is ammonium bicarbonate.

Addition Method	Protein and ammonium bicarbonate distribution		S/N	HOCS	HICS
	Sample Deposition	Spray Solvent			
Control (Figure 3a)	Protein	50% MeOH	36	+18	+13
I (Figure 3b)	Protein WITH ABC	50% MeOH	174	+22	+18
II (Figure 3c)	Protein THEN ABC	50% MeOH	149	+21	+18
III (Figure 3d)	Protein	ABC in 50% MeOH	249	+20	+14

All three methods for ammonium bicarbonate addition lead to further unfolding of the protein relative to the 50% methanol control. The highest observed charge states followed the trend:

Mixed > On Top > Spray > control.

Likewise, all three methods lead to improvements in S/N relative to the control, from 4 times for mixing it with the protein prior to spotting (I) to 7 times when adding it to the spray solvent (III) relative to a 50 % MeOH control. The simplicity of adding ammonium bicarbonate to the spray solvent along with the highest resulting improvement in signal to noise ratio makes this method the most convenient implementation, especially for ambient DESI experiments from natural samples for example potentially during imaging experiments[36].

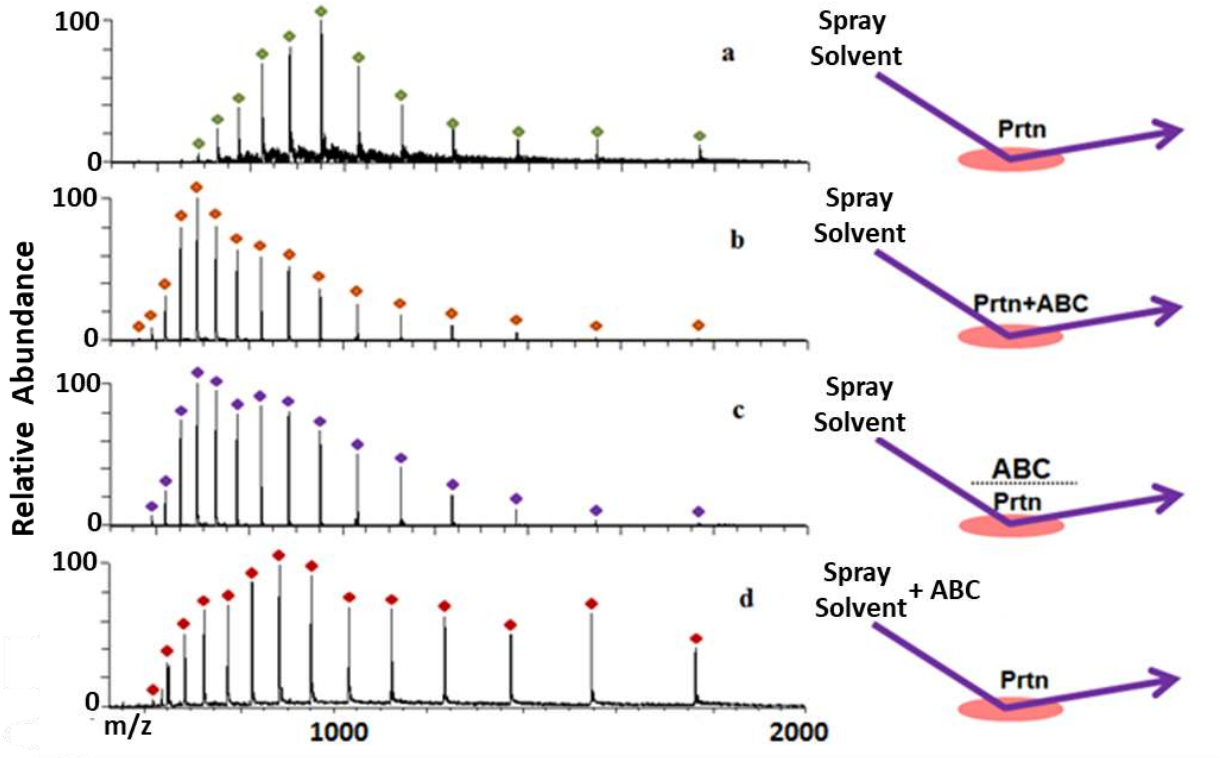


Figure 4.3. Cytochrome c was (a) deposited from 50% MeOH and analyzed with 50% MeOH, or after addition of 200 mM ammonium bicarbonate (b) to the depositing solvent, (c) deposited on-top of the previously deposited protein, or (d) only added to the spray solvent as described in Table 1.

4.4.4 The relationship of S/N ratio improvement of the protein and protein pI value

Figure 4.4 shows the S/N for formic acid and ammonium bicarbonate against protein size and pI for cytochrome *c*, hemoglobin, α-chymotrypsinogen, ovalbumin and BSA, each analyzed at a surface concentration of approximately 20 pmol/mm^2 on PE and deposited from solutions made in 50% MeOH.

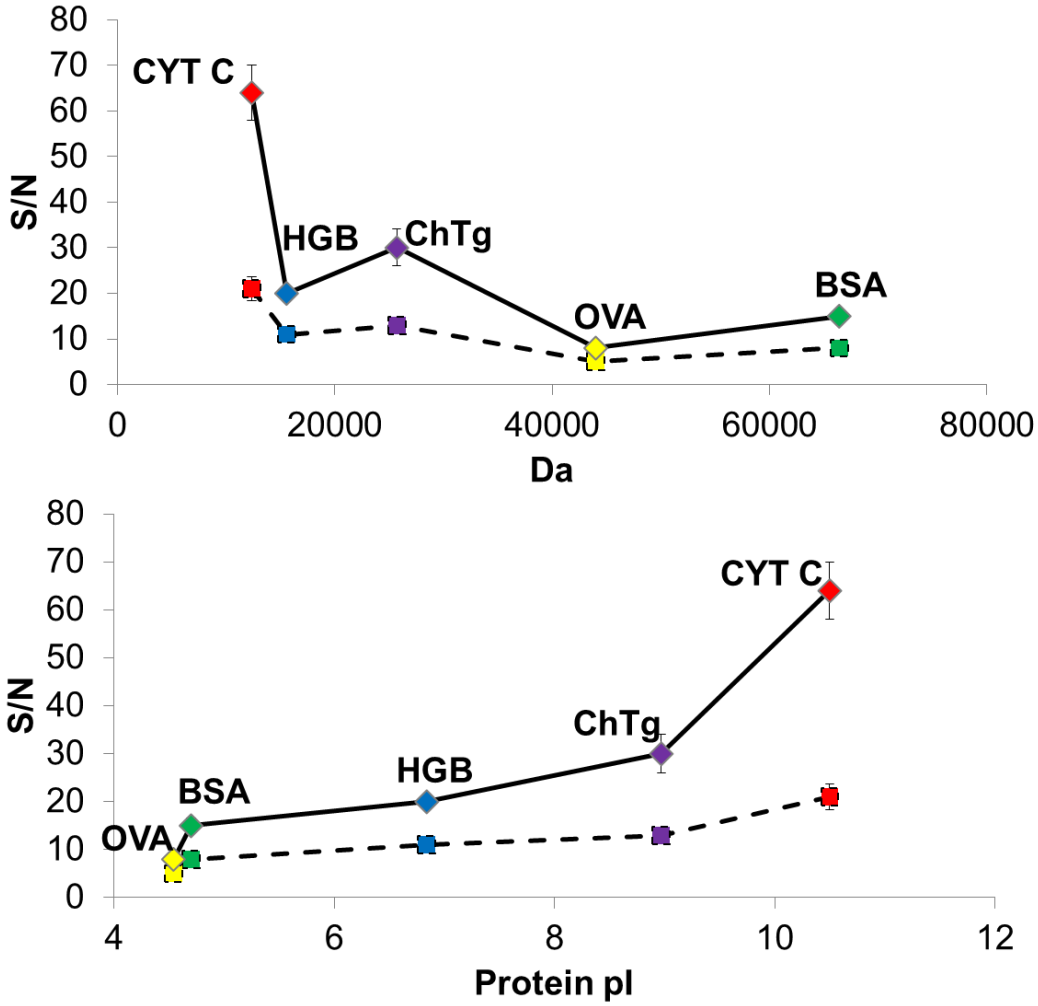


Figure 4.4. The dependence of signal to noise ratios obtained for the HICS on (a) protein size and (b) isoelectric point for the analysis of cytochrome c, hemoglobin, chymotrypsinogen, ovalbumin and bovine serum albumin deposited from 50 % MeOH onto PE by DESI-MS with the addition of ammonium bicarbonate (solid line) or formic acid (dashed line) to 50% MeOH as the spray solvent.

The addition of ammonium bicarbonate to the spray solvent improved the S/N of all proteins, with a 3.0 times greater improvement relative to formic acid in S/N for cytochrome c and 2.3 times improvement for chymotrypsinogen while S/N for hemoglobin and the albumins were enhanced by a factor of 1.8.

The stronger effect in S/N improvement by ammonium bicarbonate on cytochrome *c* and chymotrypsinogen relative to the other proteins indicate this is not a size dependent effect and other factors in protein chemistry must be important. As shown in Figure 4.4(b) a strong correlation between protein pI and S/N improvement was found. It has been shown that for lower sodium adduction the pH of the solution should to be at least 3 pH units lower than the pI of the protein[37,38]. With ammonium bicarbonate the solution pH was 7.4 and therefore cytochrome *c* (pI = 10.5) was the only protein where the difference between pI and solution pH was larger than 3 units. In contrast, hemoglobin has a pI = 6.8, which is close to the solution pH and displayed only a relatively moderate improvement.

4.4.5. Effect of ammonium bicarbonate addition to the analysis of peptides by DESI-MS

The effect of ammonium bicarbonate addition during the analysis of two peptides, MRFA and melittin, were investigated. The two peptide samples were analyzed by DESI when ammonium bicarbonate, or formic acid were used in the spray solvent. The result of analysis of these peptides analysis in both positive and negative ion modes is shown in Table 4.2 with their spectra shown in Figure 4.5, when the spray solvent contained 50% MeOH with no additive (control), formic acid (CH_2O_2) or ammonium bicarbonate (NH_4HCO_3).

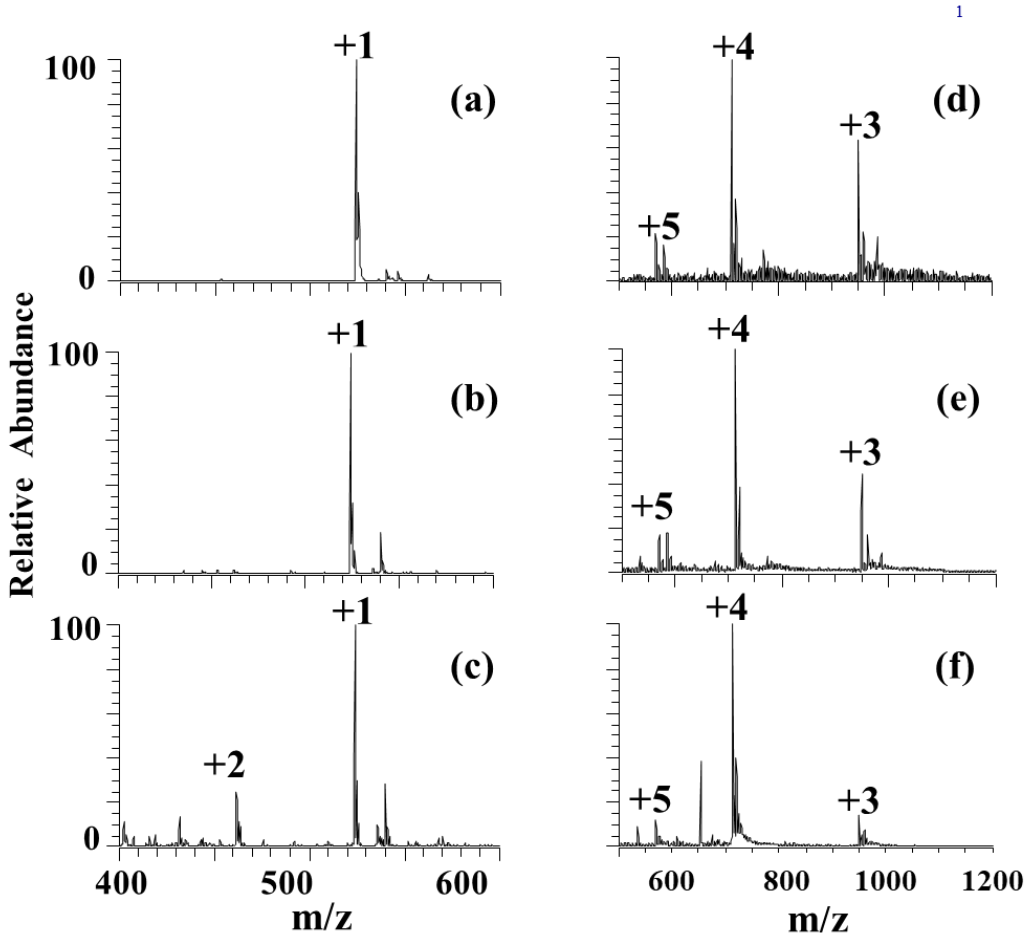


Figure 4.5 DESI mass spectra of MRFA (a-c) and Milittin (d-f), with no additive (a) and (d), with formic acid (b) and (e), with ammonium bicarbonate (c) and (f) in the electrospray solution.

In positive ion mode, the addition of ammonium bicarbonate does not increase the signal to noise ratio for neither of the peptides. Ammonium bicarbonate improves protein S/N ratio mostly through removal of potassium adducts and since such adducts are not present in these peptide samples, improvement in peptides S/N ratio was not initially expected. However, the signal intensity of melittin does increase by a factor of ~5 relative to formic acid and a factor of ~12 relative to 50% MeOH upon ammonium bicarbonate addition. Unlike proteins, observed charge states (C.S.) of peptides remain the same with all three compositions of the spray solvent.

In negative ion mode, MRFA improved both in S/N ratio and signal intensity values by a factor of ~2.0 and ~2.5, respectively, compared to the control when sprayed by the solvent system containing ammonium bicarbonate. On the other hand, melittin peaks were not observed in the negative ion mode due to the lack of aspartic and glutamic acid in the sequence of the peptide and also the fact that the C terminus in the peptide is aminated.

Table 4.2. Signal intensity, signal to noise ratio values and observed charge states (C.S.) for MRFA and melittin when no additive (control), formic acid or ammonium bicarbonate was in the spray solution.

Peptide	**Additive**	**Positive (+) Mode**			**Negative (-) Mode**		
		Intensity	S/N	C.S.	Intensity	S/N	C.S.
MRFA (pI=10.3)	**Control**	4600	2598	1,2	7.53	142	1
	CH_2O_2	3830	2428	1,2	7.97	87	1
	NH_4HCO_3	378	547	1,2	17.9	278	1
Melittin (pI=12.2)	**Control**	29.6	118	3,4,5	N.D.		
	CH_2O_2	68.2	114	3,4,5	N.D.		
	NH_4HCO_3	371	31	3,4,5	N.D.		

4.5 Conclusion

Protein analysis by DESI-MS was shown to improve with the addition of ammonium bicarbonate to the spray solvent or during sample preparation. The ammonium bicarbonate effect on S/N enhancement appears to be protein – pI dependent, which supports the role of adduct removal in its mechanism of action. Ammonium bicarbonate addition lead to protein unfolding and increased protein charge states similarly to what was previously described for ESI. This further helps to increase limits of detection since it is known that higher charge states typically carry

fewer adducts. In ambient ionization with DESI-MS, where little sample preparation is possible, increased charge states would potentially be beneficial for the identification of unknown proteins during top down proteomic experiments since increased charge states should lead to more informative fragmentation.

The addition of ammonium bicarbonate to peptides also showed improvement in signal intensity and sometimes in S/N ratio values.

It is possible that both the ammonium cation and the bicarbonate anion are involved in S/N improvement, since both ions could decompose to volatile products after replacing other non-volatile ions attached to the protein ion, such as Na^+ and Cl^-, during the ionization process. To investigate this hypothesis, additional experiments with different salts of ammonium and bicarbonate were carried out. The results for those experiments are described in Chapter 5.

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CHAPTER V

INVESTIGATING THE EFFECTS OF AMMONIUM AND BICARBONATE ADDITIVES DURING THE ANALYSIS OF PROTEIN BY DESI AND ESI

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5.1 Introduction

In the previous chapter we showed the effect of ammonium bicarbonate addition during protein analysis in DESI-MS. The observed improvements motivated us to further investigate and experiment with more ammonium salts as well as their acids in order to develop a better understanding of their effects. Since it is frequently said that DESI-MS follows a similar ionization mechanism as ESI, all the experiments that are shown in this chapter for protein analysis in DESI were compared with results obtained by ESI. While the initial motive for these experiments was to study the effects of other additives on DESI of proteins, in the process of the experiments we noticed that, surprisingly, some additives influence the analysis differently in DESI compared to ESI. Improved signal intensities with both DESI and ESI were obtained when acetic and formic acid were added into aqueous methanol spray solvents with both DESI and ESI. On the other hand, while with ESI the addition of ammonium salts into spray solutions strongly reduced both signal intensity and *S/N ratio*, with DESI signal intensity and *S/N* ratio were improved dramatically. Ammonium bicarbonate, when used with DESI, reduced the total amount of adduction and delivered excellent signal-to-noise ratios with high

intensity; however, it also denatures protein. When native state protein mass spectra are preferred, ammonium acetate would also deliver reasonable adduct removal and improved *S/N*. The amount of total adduction of individual adducting species and of all species could not be correlated with differences in either solutions pH values or with proton affinities of the anions. An obvious difference between DESI and ESI mass spectrometry is the effects of protein solubility during droplet pickup (desorption), but differences in the sizes, velocities, and composition of ionizing droplets were also discussed as important factors.

5.2 Application of solvent additives in ESI and DESI

In ESI, protein analysis can be improved with the addition of various additives during the preparation of samples for analysis. For example, the addition of organic acids (formic or acetic acid) during mass spectrometry analysis in positive ion mode facilitates protonation of compounds [1-5] and leads to higher solution conductivity resulting in increased intensity of mass spectrometric signal. Volatile, mass spectrometer friendly buffers (ammonium acetate, ammonium formate, and ammonium bicarbonate) are also commonplace in both direct and hyphenated mass spectrometric analyses[5,6]. Additives are often added for better chromatographic separation, as their ability to form ion pairs is beneficial in enhancing the peak shape and retention time [7] and for improved resolution. However, while ion-pairing reagents are advantageous for reversed-phase chromatography, their use is not always favored in mass spectrometric analysis as they can interfere with ionization and cause source fouling and contamination [8]. Addition of high concentrations of ammonium acetate to aqueous electrospray solution significantly enhances both signal intensity and S/N ratio of protein during ESI-MS analysis due to precipitation of Na^+ and Cl^- within the droplet before gas-phase protein ions are generated [9]. Use of weak chelators such as L-titrate have also been shown to

decrease levels of calcium and zinc adduction to proteins, through ion-pairing between chelator and free metal ions in the electrospray droplet prior to formation of gas-phase protein ions [10]. Millimolar concentrations of anions with low proton affinity (<315 kcal.mol^{-1}) also reduce salt adduction to protein [11]. Examples include ammonium bromide and ammonium iodide [12,13]. Chait et al. showed that higher mass adducts (molecular mass=98) are efficiently removed from proteins by addition of barium [14]. Some supercharging reagents such as m-nitrobenzyl alcohol (m-NBA) were also reported to be advantageous in reduction of protein adducts [15]. Another recent approach to reduce sodium adduction is by exposing droplets of electrospray to vapor containing volatile organic solvents [13]. Usually the performance of each additive is dependent on the experimental conditions, the characteristics of the analyte molecule and the type of mass spectrometer used [7].

Because of the similarity in ionization mechanisms, additives used in ESI are also likely to be beneficial for DESI experiments, and it is common practice to add formic or acetic acid during DESI experiments. In Chapter 4, we showed that ammonium bicarbonate improved signal intensities and S/N when added to spray solutions during the analysis of proteins [16]. It wasn't clear whether the cation or the anion of ammonium bicarbonate played a more important role in the observed improvements. Also, the effects observed for ammonium bicarbonate addition in DESI were not consistent with those in ESI. To further investigate the role of each ion as well as potential differences between ESI and DESI for protein analysis, here we compare the qualitative and quantitative effects observed in the mass spectra for the addition of a variety of organic acids and their ammonium salts.

5.3 Experimental details

5.3.1 Samples and surface

Equine cytochrome *c* (cyt c, 12.3 kDa) was purchased from Sigma-Aldrich (St. Louis, MO). Chymotrypsinogen (chtg, 25.6 kDa) was purchased from MP (Solon, OH) and myoglobin (mb, 16.9 kDa) was purchased from Protea (Morgantown, WV) LC-MS grade methanol (MeOH), formic acid, ammonium formate were purchased from Fluka Analytical (Morris Plains, NJ). Glacial acetic acid was purchased from EMD (Burlington, MA). Ammonium bicarbonate was purchased from Fisher Scientific (Hampton, NH). Ammonium acetate and ammonium fluoride were purchased from Sigma-Aldrich (St. Louis, MO). Porous-polyethylene (PE) surfaces with average pore size of 15-45 μm (POR-4900) were purchased from Interstate Specialty Products (Sutton, MA).

5.3.2 Instrumentation

A linear ion trap mass spectrometer (LTQ, Thermo Scientific, Waltham, MA, USA) was combined with a 3-dimensional translational stage (Purdue University, West Lafayette, IN, USA) for DESI analysis. For the generation of a pneumatically-assisted solvent spray, an electrosonic spray ionization (ESSI) source was constructed in-house [17]. The outer capillary (for sheath gas) was approximately 20 mm in length with an outer diameter of 430 μm and inner diameter of 320 μm. The internal capillary (for solvent) had an outer diameter of 200 μm, and inner diameter of 75 μm. For DESI experiments, 4.0 kV spray potential was applied to the liquid junction on the stainless-steel syringe needle used to deliver the spray solvent. The spray solvent was delivered at 5 μL/min with N_2 as nebulizing gas at 100 psi. The tip of the mass spectrometer inlet capillary was extended by 10 cm to facilitate efficient ion transfer during DESI [18]. The transfer capillary

temperature was set at 250°C, the tube lens was set at 90V for cytochrome c, 130 V and 180 V for myoglobin and chymotrypsinogen respectively. The ion transfer capillary was set at 20 V. The sprayer to inlet distance was typically 4 mm, the sprayer to surface distance was 1 mm, and the incident spray angle was 55°.

For ESI of cytochrome c, the sample was infused at 10 μL/min inside the IonMax source with no sheath gas applied. The same optimized tune file was used in both DESI and ESI experiments. ESI results were obtained with automatic gain control (AGC) turned on, while DESI analyses were performed with this function disabled. This is standard practice in DESI owing to the inherent instability of the ion current, compared to the higher and more stable ion currents typically obtained by ESI.

For myoglobin and chymotrypsinogen ESI, the DESI sprayer was modified and optimized to spray the sample solution toward the inlet of mass spectrometer.

Data collection was performed with LTQ 2.0 and Xcaliber 2.4 software. MagTran version 1.03 was used for protein spectra deconvolution, and the signal to noise ratios were calculated for the highest intensity charge states as described by Zhang and Marshall [19].

5.3.3 Sample analysis

For ESI experiments, protein samples were prepared at a concentration of 10 μM each by dilution in 50% MeOH and salts were added to the solutions to give a final concentration of 100 mM as optimized for ESI previously[20], unless noted differently. Formic and acetic acids were used at 0.1% and 3% (v/v) respectively to produce solutions with pH ≈ 2.5 and 2.7 respectively. Each ESI data point is the average of 3 replicates and each replicate represents the average of scans over 1.0 minute of sample infusion.

For DESI experiments of cytochrome c, 3 μL droplets of protein at a concentration of 20 μM were hand pipetted onto surfaces to give an estimated surface concentration of 20 pmol/mm^2 after drying. Each DESI data point is the average of 3 replicates and each replicate represents single pass scans of 10 consecutive spots. For myoglobin and chymotrypsinogen experiments, a sample containing 80 μM of each protein was spray-deposited in 1 mm lines on PE surface to yield an estimated surface concentration of 20pmol/mm^2. The samples were then analyzed by scanning orthogonally through the lines. Each DESI data point is the average of 3 replicates and each replicate represents the single pass scan of 5 consecutive lines. An exception was for ammonium bromide data where, because of persistent Br^- contamination to the extended ion transfer tube, only 1 replicate of 5 consecutive lines were collected and hence standard deviation could not be calculated.

To investigate the effect of the addition of ammonium salts to protein samples by DESI, 200mM of each salt was added into 50% MeOH DESI desorption spray solvent, as previously optimized for ammonium bicarbonate[16]. Carbonic acid solutions were prepared by bubbling CO_2 gas into 50% MeOH solution until a stable equilibrium pH of 3.76 (+/-0.1) was reached. This corresponds to a calculated concentration of 70 mM of carbonic acid. Formic and acetic acids were used at 0.1% and 3% respectively to produce solutions with pH ≈ 2.5.

5.4 Results and discussion

By first approximation, similar spectra are obtained for proteins analyzed by DESI-MS and ESI-MS. On closer inspection there are differences in terms of instrumental response, signal to noise ratios and the extent of adduction and protein charging. DESI and ESI also respond differently to the addition of some additives. Figure.5.1 shows the effects observed after addition of formic

acid, acetic acid, carbonic acid, and the ammonium salts of formate, acetate, bicarbonate, fluoride and bromide for both DESI (a-i) and ESI (j-r) during the analysis of cytochrome c.

5.4.1 Qualitative differences

In Fig. 5.1a the control spectrum for DESI deposited out of pure water, but analyzed with 50% MeOH, is less denatured than in the ESI result (Fig.5.1j) where the proteins is sprayed directly out of a 50% MeOH solution. This difference has been explained based on the short residence time of the protein in the denaturing solvent between desorption and ionization[21], but likely also stems from differences in solvent composition of the secondary droplets leaving the sample surface. It is plausible that the micro-localized solvent film created on the sample surface into which proteins are dissolved or extracted during DESI, would be enriched in water while the more volatile component, methanol, evaporates faster. In addition, an increase in adduction is observed for DESI compared to ESI. This reduces the observed signal intensity of the DESI spectrum by proportioning the signal over many more adducted forms.

With the addition of mass spectrometry-friendly acids such as acetic, formic, or carbonic acids in DESI and ESI both the highest observed charge states (HOCS) and highest intensity charge states (HICS) [22] shift to higher charge values compared to the controls where there were no additives in the solution. In Figure 5.1 the highest intensity charge state (HICS) in each spectrum is indicated with a blue diamond. When bimodal charge envelopes corresponding to both folded and denatured protein populations were observed, the HICS for the secondary envelope is indicated by a red dot.

The extent of denaturing induced by DESI and ESI in the control and with acetic acid appears to be similar.

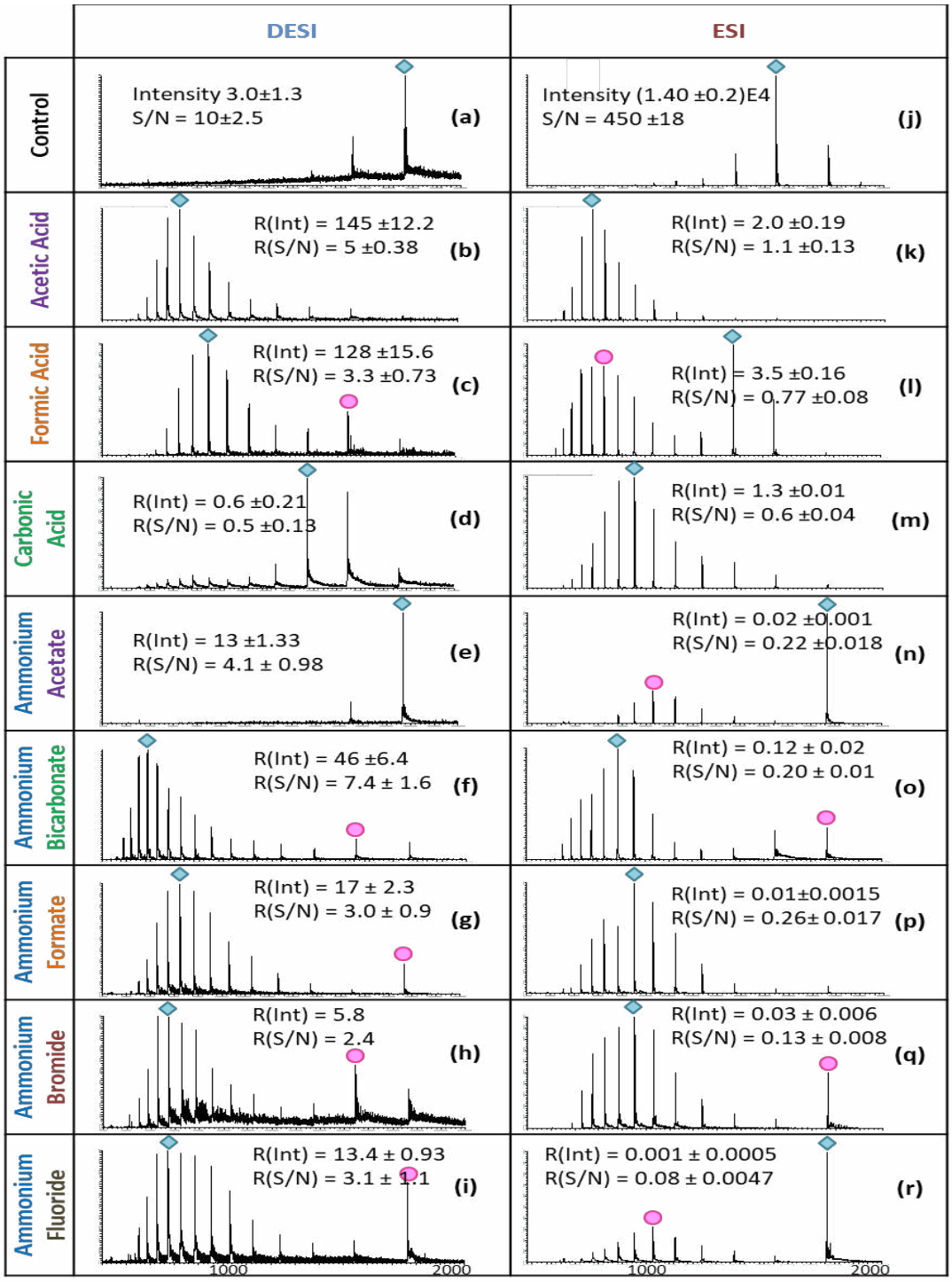

Figure 5.1. Mass spectra of different additives added to the DESI spray solvent (a-i) and to the ESI spray solution (j-r) showing the differences in the attained charge states (see Table 5.1), intensities and signal to noise ratios observed with the two ionization modalities.

However, with more volatile acid additions the HOCS in DESI is slightly lower than for ESI indicating less denaturing conditions during ionization as summarized in Table 5.1. This was especially obvious for carbonic acid where the DESI spectrum more closely resembles that of the control, suggesting the possible loss of carbonic acid during energetic surface collisions by shock-induced cavitation, which enhances the diffusion of CO_2 from the supersaturated liquid into escaping bubbles[23].

When ammonium salts were added to the DESI spray solvent, typically both the HICS and HOCS increased to higher charge states than observed for acid additions and compared to the same ammonium salts when added to ESI sample solutions. The exception here was ammonium acetate which produced native like charge states with a HICS at z=+8 for both ionization modalities. For the ammonium halide salts, mixed populations of ions in both ESI and DESI were also observed as two envelopes, one corresponding to more native like charge states (HICS=+8), as well as denatured charge envelopes with HICS at z=+17 and +18.

The effects of different cations and anions on their ability to stabilize or destabilize protein secondary and tertiary structure was first described by Franz Hofmeister and ordered according to the Hofmeister series [24]. It has been shown that the arrangement of anions in the Hofmeister series depends on the relative values of the solution pH and the protein pI [25-27]. For example, Aoki and coworkers demonstrated that while protein solubility is dependent on the pH value of the solution relative to the pI of the analyte protein, protein stability always follows a direct Hofmeister series [28]. A reversed Hofmeister series dependence on unfolding during ESI analysis of proteins was however recently described for analyses under electrothermal supercharging conditions [29].

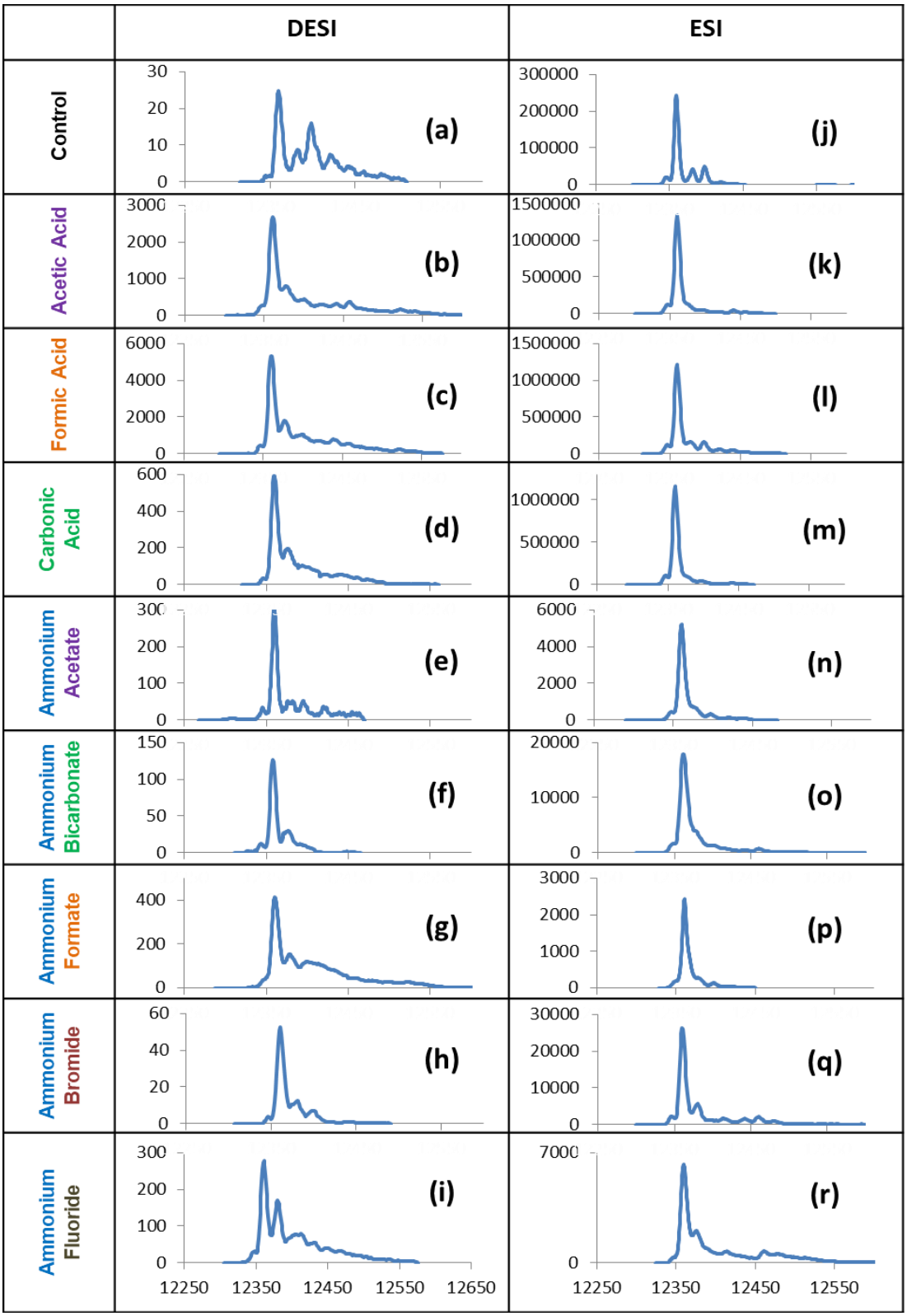

Figure 5.2. Deconvoluted spectra of different additives added to the DESI spray solvent (a-i) and to the ESI spray solution (j-r).

For the volatile anions selected for this work, all of which are in the intermediate section of Hofmeister series, no clear correlation between position in the series and HICS or HOCS was observed (Table 5.1).

5.4.2 Quantitative differences

In Fig. 1a the control spectrum for DESI containing only 50% MeOH in water shows much lower signal intensity (NL: 3.0) compared to the ESI result (NL: 1.40E4) made up in a similar solvent (Fig. 1j). These numbers are not directly comparable since with DESI-MS it is common to disable automatic gain control (AGC) due to the inherent instability of the ion current caused by the coffee ring effect and non-homogenous crystallization of sample components during preparation, and due to losses of droplet charge to the sample surface also occur during impact which can decrease ionization efficiency and in some cases can lead to irregular signal over time due to a previously described capacitive effect of non-conducting sample surfaces [30]. With ESI, AGC is enabled to limit the number of ions in the ion trap and prevent space charging effects especially when spraying from concentrated solutions. When AGC is activated for both modalities, differences in ion intensities of 3 orders of magnitude are typical (Data not shown).

One potential difference is lower concentration in the DESI analysis compared to ESI. While a direct comparison is not trivial, a simplified approximation reveals that in these experiments around 40 pmol of cytochrome c is analyzed per minute with DESI (at 20 pmol/mm^2 analyte concentration, 150 μm/sec stage scan speed, and 200 μm desorption footprint), compared to 100 pmol per minute for ESI analysis (for a 10μM solution infused at 10μL/min). This calculation assumes that with DESI the deposited protein is removed exhaustively from the surface and that ion transfer into the ionization source is equally inefficient between DESI and ESI. The fact that

signal intensities for the control experiments between DESI and ESI vary by as many as 3 orders of magnitude clearly indicates that other factors need to be considered. For example, there is likely reduction in ion sampling efficiency with DESI, since the aerodynamics of the nebulized droplet plume across the surface causes the desorption plume to fan out while it remains close to the surface as visualized by spray desorption collection (SDC) experiments using the collection of Rhodamine [31]. Finally, protein solubility into the extracting solvent of the microlocalized liquid layer would also have a dramatic effect on protein signal in DESI, as described below.

The intensities for both DESI-MS and ESI-MS were differently affected when additives were included during the analyses. To allow easy comparison of the effects that additives induce when used with either DESI or ESI, despite differences in experimental conditions such as whether AGC was ON or OFF, the observed change within each modality was compared to its own control and the relative changes were compared between the two techniques. Relative intensities ($R_{(Int)}$) for each technique were calculated by dividing the intensity of the HICS of the protein in the presence of additive by the intensity of the HICS obtained for the 50% MeOH control sample ($R_{(Int)} = Intensity_{additive} / Intensity_{control}$) obtained during the same sample set. A similar approach was followed for the calculation of relative signal to noise ratios ($R_{(S/N)}$), calculated by dividing signal to noise ratio in the presence of additive to signal to noise ratio of the control ($R_{(S/N)} = S/N_{additive} / S/N_{control}$).

For ESI, the improvements in signal intensities with acid addition were mild and increased by factors of 2.0, 3.5 and 1.3 for acetic, formic and carbonic acids, respectively (Fig. 1k-m). Despite these nominal increases in intensities, *signal to noise ratios* (S/N) were relatively unaffected for acetic acid (1.10) and even reduced for formic acid and carbonic acid by factors of 0.77 and 0.60 respectively.

For DESI, however, a dramatic improvement in signal intensity was obtained for both acetic and formic acids, increasing signal intensities by factors of 145 and 128 respectively (Figure 5.1b-c). The S/N ratios also improved with the addition of acetic and formic acids but only by factors of 5.0 and 3.3, respectively, despite the dramatic increases in intensities. By contrast, for carbonic acid S/N was reduced because of the reduced intensity and distribution of signal over an increased number of charge states. Carbonic acid also lead to unstable sprays in both ESI and DESI, and in DESI carbonic acid was likely also lost during impact of spray solvent with surface as CO_2 and H_2O so that a spectrum more similar to the control was observed (Fig. 1d).

The quantitative effects of ammonium salts of the common volatile acids (acetate, bicarbonate and formate) together with two halide salts (fluoride and bromide) were also observed to be different when used as spray solvent additives in DESI and ESI. High electrolyte concentrations are known to induce ion suppression during ESI analysis of analytes and proteins [9,32-37]. As a result, during the ESI experiments summarized in Figure 5.1n-r, addition of the ammonium salts decreased the relative signal intensities as well as relative S/N ratios. The relative intensity for the HICS of cyt c decreased by at least a factor of 8 resulting in a signal 0.12 that of the 50% methanol control sample when ammonium bicarbonate was added, and decreased even more dramatically with the other ammonium salts. S/N ratios also decreased by factors near 5 for the three volatile ammonium salts (acetate, bicarbonate and formate), and while it was previously shown that ammonium bromide could decrease sodium adduction when used as a solution additive in native protein mass spectrometry by nanospray [12] we found that under these ESI conditions, both halide salts, ammonium bromide and –fluoride, reduced S/N by a factor of almost 10.

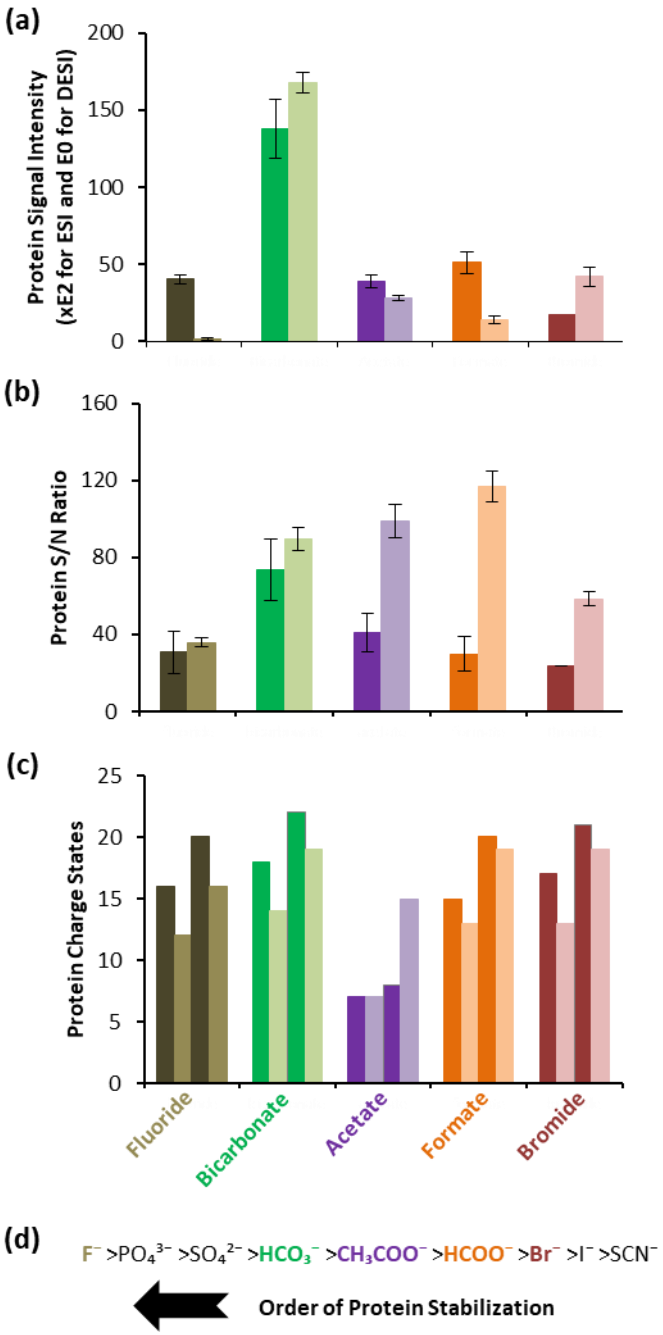

Figure 5.3. With anions arranged according to the Hofmeister series, effects on (a) protein signal intensity, (b) protein to adduct area, and (c) protein charge state distributions of HICS and HOCS for DESI (dark colors) and ESI (light colors) are shown. (d) shows the Hofmeister series.

With DESI the results were dramatically different and the addition of each ammonium salt increased both the signal intensities and S/N ratios. Ammonium bicarbonate was especially effective and increased the intensity of the HICS by a factor of 46 compared to the result for the 50% MeOH desorption spray solvent. The other ammonium salts of acetate, formate, bromide and fluoride improved the relative signal intensities by factors of 13, 17, 5.8, and 13.4, respectively (Fig. 5.1e-i).

Table 5.1 HICS (Highest Intensity Charge State) and HOCS (Highest Observed Charge State) obtained with various volatile acids and their corresponding ammonium salts during DESI and ESI analysis of cytochrome c

Charge State	HICS		HOCS	
Ionization Modality	DESI	ESI	DESI	ESI
Control (50% MeOH)	7	8	9	12
Acetic acid	15	15	19	19
Formic acid	8, 13	9, 15	18	20
Carbonic acid	9	19	13	19
Ammonium fluoride	7, 16	7, 12	20	17
Ammonium acetate	7	7, 12	8	15
Ammonium bicarbonate	8, 18	7, 14	22	20
Ammonium formate	7, 15	13	20	18
Ammonium bromide	8, 17	7, 13	21	19

These differences likely stem from the additional physical processes that occur only during the DESI analysis, such as the role of protein solubility during the DESI droplet pick-up mechanism [38]. One would expect the intensities to correlate with protein solubility and therefore to correlate with relative position of each anion on the Hofmeister series. Protein solubility has been shown to follow a direct Hofmeister series when the pH of the solution is above the pI of the protein and

a reversed Hofmeister series when the solution pH is below the protein pI [28]. Therefore it is important to also consider pH and solubility effects when selecting solvent additives. For cyt c under the experimental conditions used for DESI a reverse Hofmeister series is expected. While ammonium bicarbonate yielded exceptionally high signal intensities (Figure 5.3a), most of the other anions improved the signal by about the same amount, owing to the fact that these volatile anions are all somewhat near one another in the Hofmeister series.

While ammonium salt addition to the DESI spray solvent increased the intensity relative to the control, the improvement was not as dramatic as it was for acid additions. Even so, the S/N improvement with ammonium salt additions were relatively large considering the comparatively low improvements in signal intensities. Ammonium bicarbonate improved S/N 7.4 times compared to the control and the S/N improvements for the other ammonium salts were 4.1, 3.0, 4.4 and 3.1 for acetate, formate, fluoride, and bromide, respectively. When arranging the measured S/N in DESI with addition of ammonium salts according to the Hofmeister series, a maximum in the trend is observed for ammonium bicarbonate (Figure 5.3b), while for ESI the S/N was rather flat or mildly reduced for bicarbonate compared to acetate and formate.

The presence of monovalent cations, such as sodium or potassium, usually causes formation of adduct clusters that associate with proteins during ESI-MS experiments [14,36,39-41]. Adduct induced signal distribution, together with ion suppression [32], are two of the mechanisms by which S/N can be reduced with techniques dependent on electrospray for ion production. Better removal of these adducts prevents distribution of signal over many different cationized forms of the protein and therefore reduces mass spectral complexity.

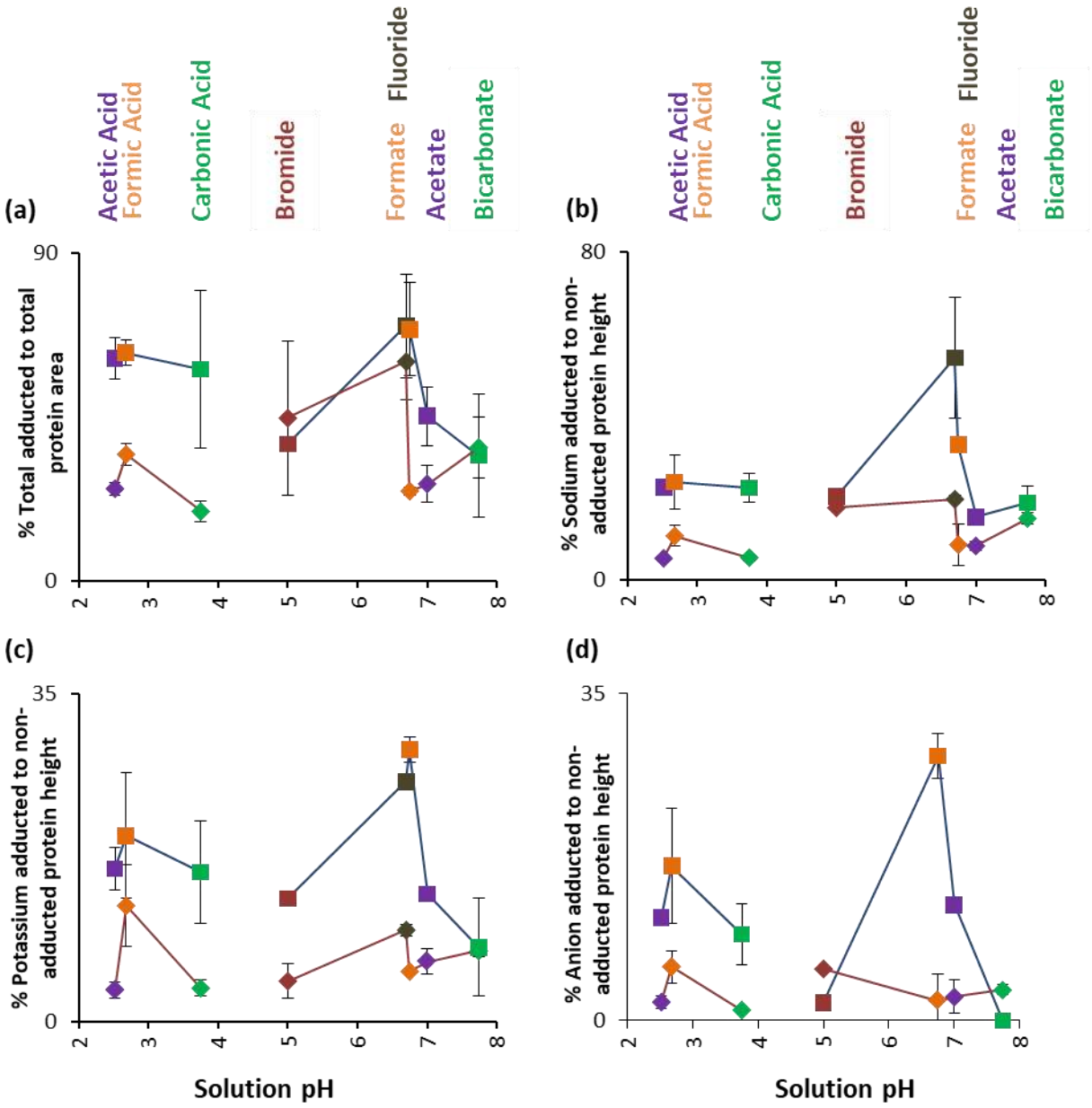

Figure 5.4. The effect of solution pH for each additive in DESI (blue line, square marker) and ESI (red line diamond marker) on relative abundance compared to non-adducted protein for (a) all adducts, (b) sodium adducts, (c) potassium adducts, and (d) anion adducts. The data shown in graphs (b–d) are calculated by protein peak heights from deconvolution spectra while in graph (a) data are calculated by protein peak areas. In (d), fluoride data are not shown due to unresolved M+18 water adduct

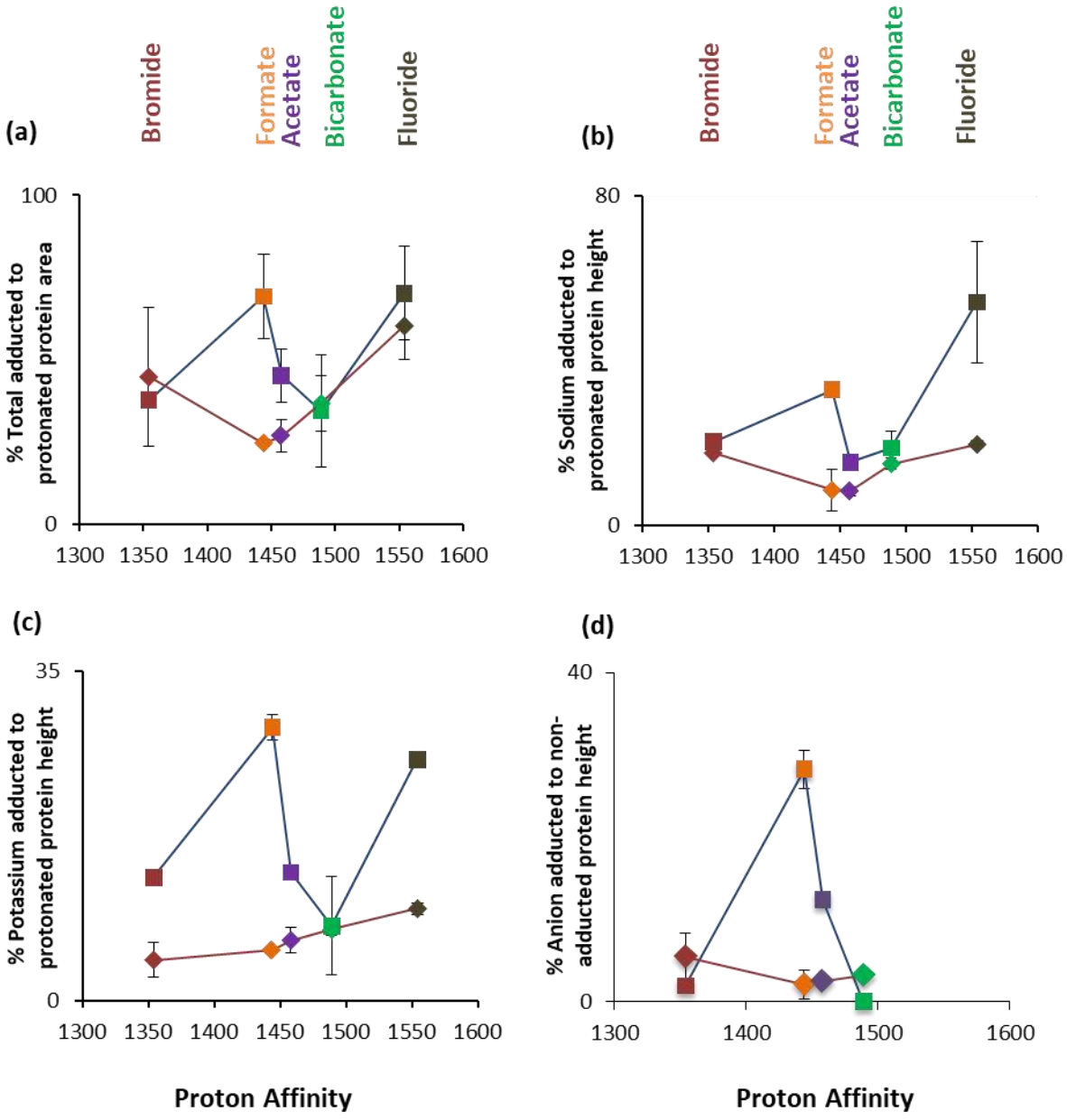

Figure 5.5. Effect of anion proton affinity for each additive in DESI (blue line, square marker) and ESI (red line diamond marker) on relative abundance compared to non-adducted protein for (a) all adducts, (b) sodium adducts, (c) potassium adducts, and (d) anion adducts. The data in graph (a) were calculated by adducted and areas; data shown in graphs (b–d) were calculated by heights from deconvolution spectra. In (d), fluoride data are not shown due to unresolved M+18 water adduct.

The extent of adduction observed in the deconvoluted protein mass spectra when each of the solution additives was used were evaluated for both DESI and ESI shown in Figure 5.2. With DESI often nearly 50% of total signal area of the protein was contained in peaks corresponding to the adducted forms of the protein, as shown in Fig. 5.4(a) and Fig. 5.5(a). The mass spectrometer available for these experiments did not have adequate resolution to definitively identify all adducting species, or to clearly separate the adduct peaks in the deconvoluted spectra. However, in an attempt to better understand the process of adduct removal by the different additives, the percent height of two common adducting species $[M+Na]^+$ and $[M+K]^+$ were normalized to the height of the deconvoluted protein peak and displayed in Figure 5.4b and 5.4c arranged by solution pH, and in Figure 5.5(b) and 5.5(c) arranged by anion proton affinity. Figure 5.4(d) and 5.5(d) show similar treatments for adduction of the anion species, except for the fluoride anion which could not be measured due to an unresolved M+18 water adduction peak in the deconvoluted mass spectrum.

As can be seen from the data in Figures 5.4 and 5.5, and as frequently commented on in the past, DESI-MS analyses of proteins are much more prone to adduction than ESI. Addition of volatile acids in DESI and ESI follow a similar pattern and acetic and carbonic acids were better at removing adducts than formic acid with both modalities. Formic acid was also less denaturing and especially the lower charge states appeared heavily adducted. It has been shown in the past that lower charge states carry more of the adduction load [12,42].

With ammonium salts, as shown in Figure 5.4, those additions which resulted in higher pH of solutions were better able to remove adducts, especially in the case of DESI. Nevertheless, all solutions had pH values more than 3 units away from the isoelectric point of the protein (cyt c, pI =10.5) which has been shown to be important consideration for cation adduct removal [36] and

previously observed to be important for DESI-MS of proteins [16] . A surprising result was the poor performance for ammonium formate in the DESI analysis, even as it produced deconvoluted spectra with the lowest amount of adduction in ESI

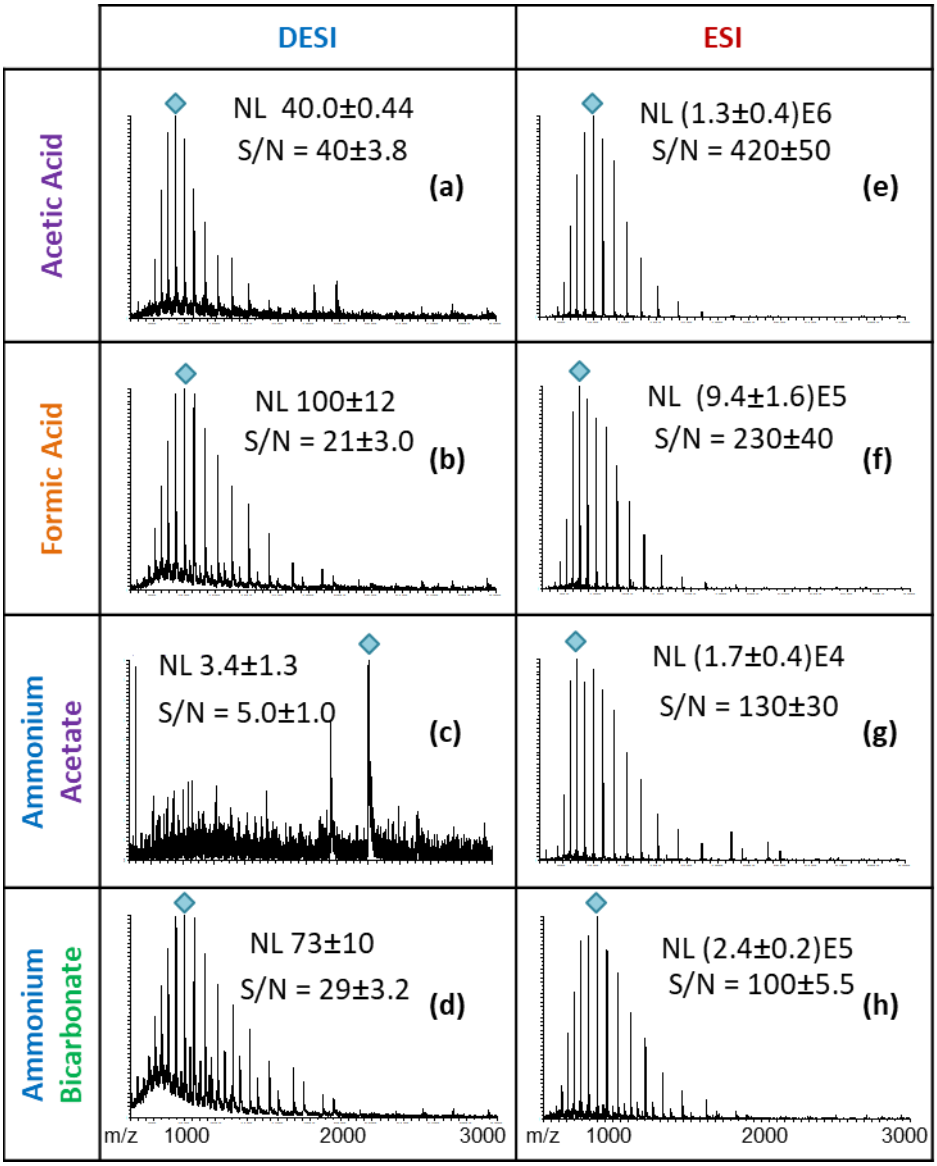

Figure 5.6. Mass spectra obtained for myoglobin with different additives in the DESI spray solvent (a–d) and ESI spray solutions (e–h) showing the differences in the attained charge states, intensities, and signal-to-noise ratios observed with the two ionization modalities. Deconvoluted spectra are available in Figure 5.8.

Figures 5.4 and 5.5 also show that additives vary in their ability to remove different cationic adducts. For example ammonium bicarbonate was slightly better at removing potassium than sodium adducts, while the reverse was true for ammonium acetate.

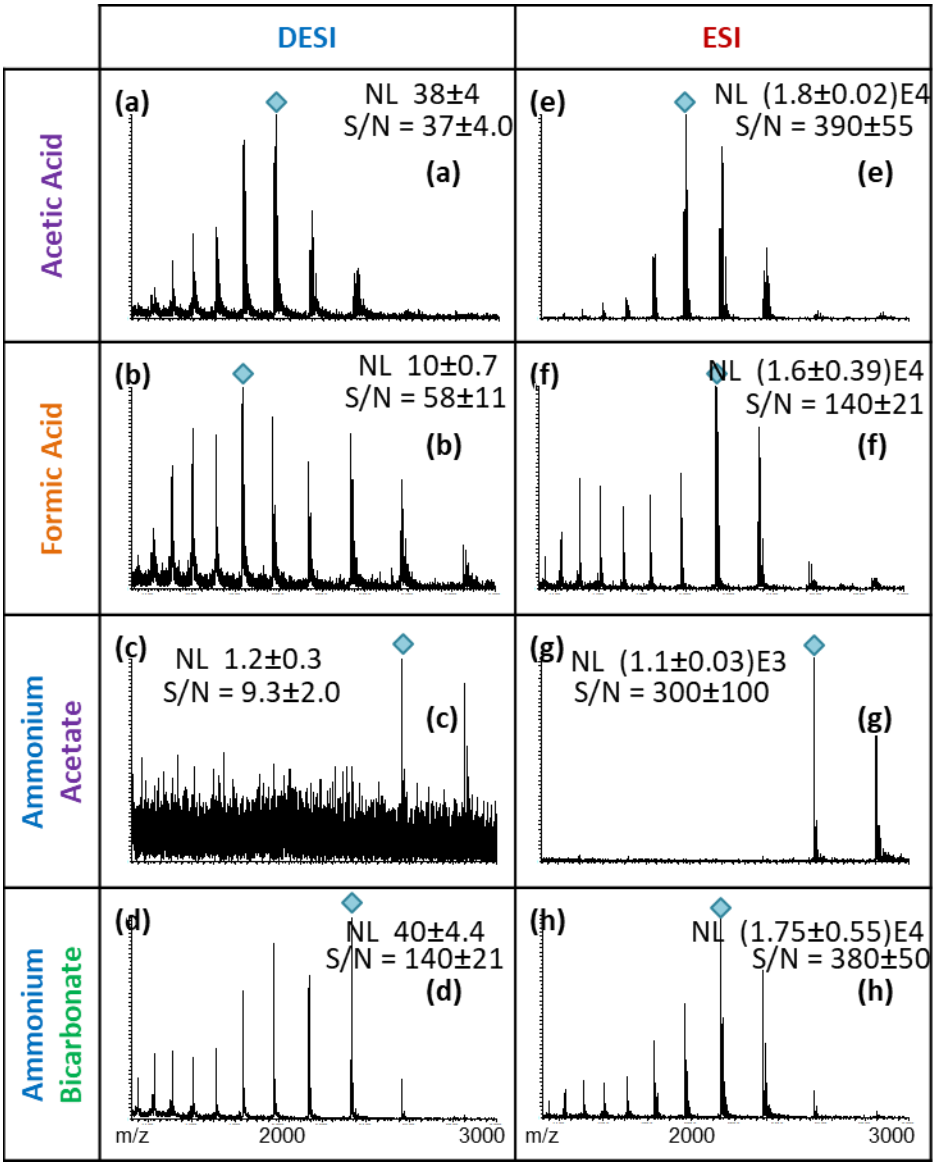

Figure 5.7. Mass spectra obtained for chymotrypsinogen with different additives in the DESI spray solvent (a–d) and ESI spray solutions (e–h) showing the differences in the attained charge states, intensities, and signal-to-noise ratios observed with the two ionization modalities. Deconvoluted spectra are available in Figure.5.9.

With increasing proton affinity in DESI, anion adduction also decreased rapidly. Ammonium bromide was better at removing cationic adducts than ammonium fluoride as previously noted in the literature [11] due to the relatively low proton affinity of the bromide anion (Figures 5.5(a) and 5.5(b)).

However, in DESI, ammonium bromide was the worst performing additive as far as signal intensity was concerned and led to a long-lasting background signal and contamination of the ion source. Bromide also did not deliver the anticipated reduction in adduction for sodium ions achieved with nano-ESI [11]. However, a difference between these results and those previously shown was that here we compared total adduction across all charge states from the deconvoluted spectra rather than that for a single charge state. Ammonium bromide produces an envelope with peaks at lower charge states and, as previously noted [12], lower charge states are likely to be more heavily adducted.

5.4.3 Effect of solvent additives on analysis of mb and chtg by DESI and ESI

Owing to differences in pI, conformation, and size of proteins, the effect of additives on different proteins may vary. Therefore to further investigate additive effects in DESI and ESI two additional proteins were analyzed: one protein with similar molecular weight to cytochrome c and a lower isoelectric point (myoglobin, pI = 6.8), and one protein with a higher molecular weight than cyt c but similar pI (chymotrypsinogen pI = 8.9). Figures 5.6 and 5.7 show the effects of additives in DESI and ESI on the mass spectra of holomyoglobin and chymotrypsinogen, respectively.

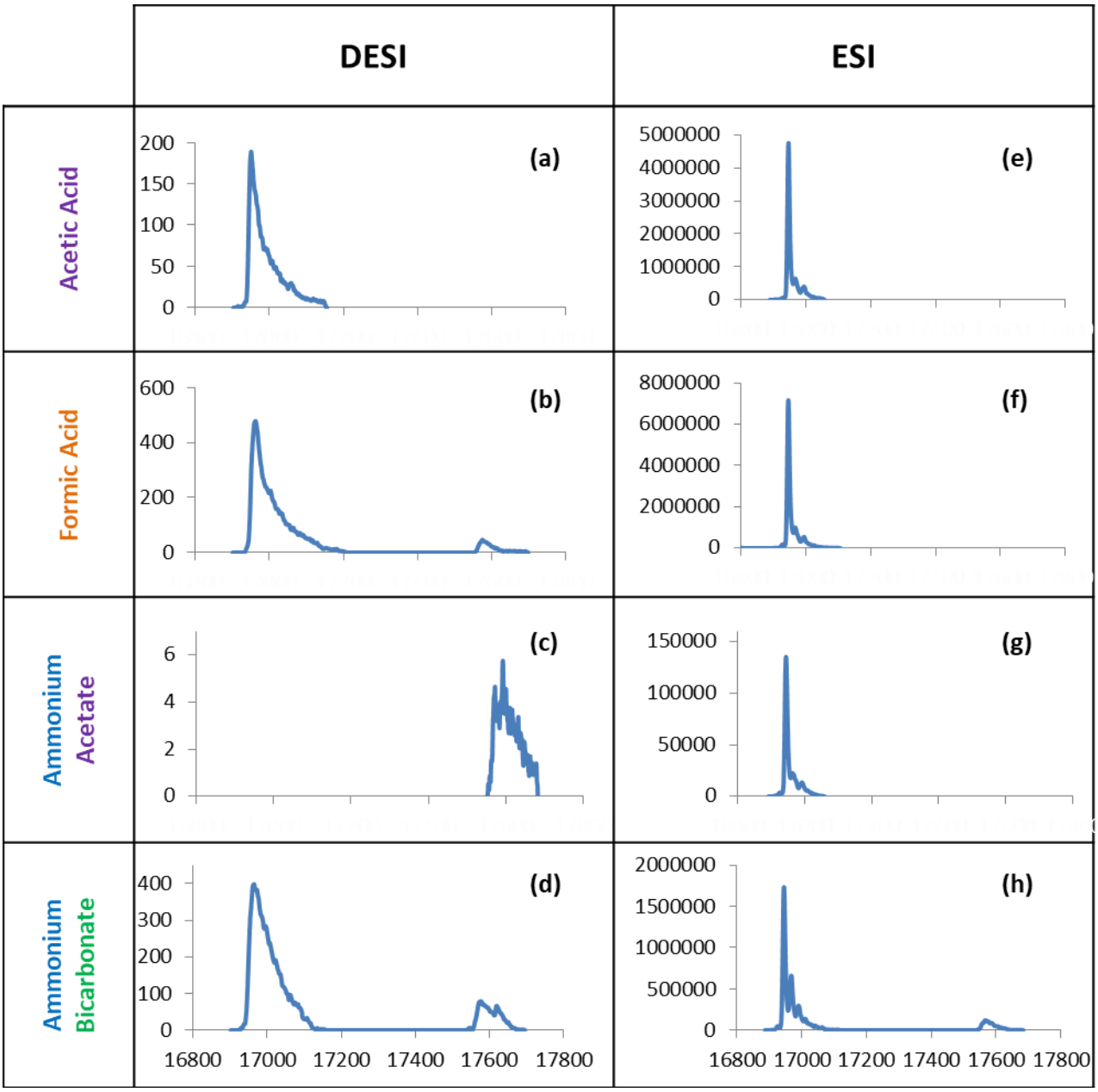

Figure 5.8. Deconvoluted spectra obtained for myoglobin with different additives in the DESI spray solvent (a-d) and ESI spray solutions (e-h)

The only additive that preserved holomyoglobin was ammonium acetate as shown in Figures 5.6 and 5.8. Ammonium acetate also produced native state like charge states and generally a low signal intensity for all proteins analyzed.

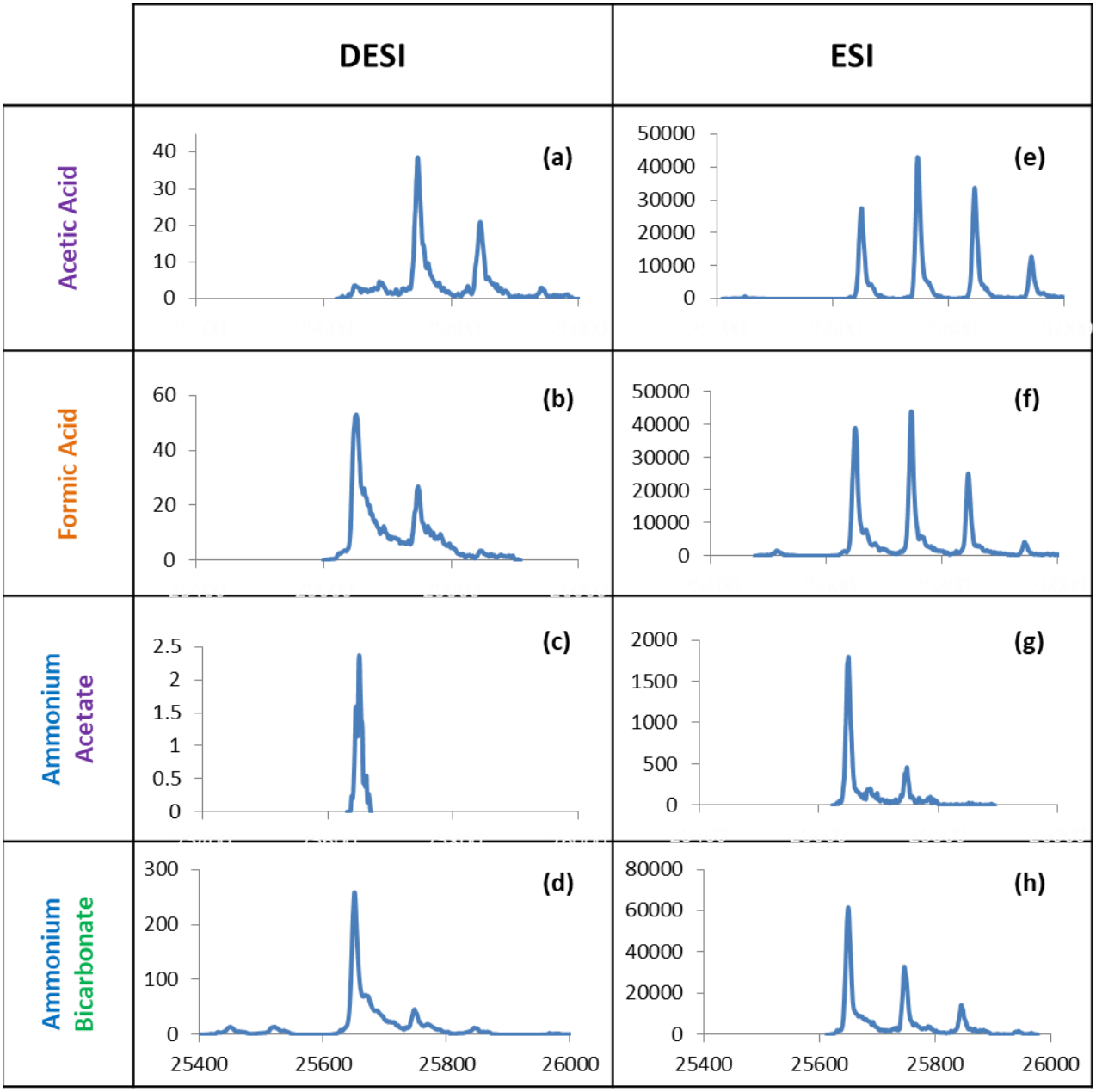

Figure 5.9. Deconvoluted spectra obtained for chymotrypsinogen with different additives in the DESI spray solvent (a-d) and ESI spray solutions (e-h)

Qualitatively, regardless of the protein analyzed or the additive investigated, the HOCS was lower in DESI compared to ESI. This is a consequence of the shorter interaction time with denaturing solution of the natively deposited protein, as described earlier. For a similar reason,

while mostly the apomyoglobin-dominant spectra was obtained for DESI when using a spray containing 50% MeOH, and formic acid or ammonium bicarbonate, some holomyoglobin peaks remain.

Regardless of the protein and in both ionization modalities, ammonium bicarbonate always caused the highest charge states compared to other additives. However, differences in adduction was observed: As can be seen in Figures 5.1 and 5.7, ammonium bicarbonate resulted in narrower peaks for each charge state of cytochrome c (cyt c) and chymotrypsinogen (chtg), as these proteins are mostly present in their protonated forms, while myoglobin (mb), with a lower pI, displayed extensive adduction with this additive.

Quantitatively, intensity values of the HICS of all three proteins studied were positively influenced by acid additions in both ESI and DESI. Similar to cyt c, chymotrypsinogen was also strongly enhanced by ammonium bicarbonate addition. The deconvoluted spectra for chymotrypsinogen, displayed in Figure 5.9, show intense and multiple adductions with an adduct having a M.W. = 98 Da in both the DESI and ESI results. This adduct was previously described by Chait et al, and is believed to be either phosphoric acid or sulfuric acid [14]. Less acidic solutions, such as ammonium bicarbonate and ammonium acetate, were better able to remove these adducts from ESI and DESI results than acidic solutions. However in both modalities loss in adduction did not compensate for the loss in signal intensity when ammonium acetate was used. When ammonium bicarbonate was used, the intensities increased as the adducts were removed in ESI by a factor of 1.6 relative to formic acid. With DESI however, the signal increased relative to formic acid by a factor of 7.5, possibly indicating the combined effects of improved desorption, as seen for cyt c, and the near complete removal of the M.W = 98 Da adducts.

Ammonium acetate consistently produced the lowest intensity for the HICS of all three proteins.

Signal to noise values were influenced by the various additives in a more complex way. In ESI acidic solutions generally produced spectra with the highest signal to noise ratios. In DESI, as shown previously[16], ammonium bicarbonate performed better for proteins with higher pI, such as cyt c and chtg, while acid additions were better suited for increasing signal to noise for proteins with low pI, represented here with mb.

A complicating factor in DESI-MS analysis is that droplet composition changes abruptly from the prepared solutions and it is anticipated that the solvent that finally interacts with the sample, and also the composition of the droplets from where ionization occurs, will be substantially different from when it was nebulized during ESI. It can be expected that the droplets arriving at the sample surface are already somewhat enriched in the less volatile solvent component and the nebulizing gas used to accelerate the colliding droplets will further increase the fraction of less volatile solvent in the surface liquid layer into which extraction takes place. In DESI the amount of excess charge on the droplets will also change through Coulombic explosions before even interacting with the analyte molecules, and further loss will occur to the non-conducting sample surface [30]. This implies that ionization initiates, after analyte desorption (droplet pickup), from droplets with fewer charges than if the analyte was already present in the spray solution. Droplets leaving the surface have been measured to be smaller than those in the pre-collision spray [21], and MD simulations have calculated these droplets to contain material from both the surface liquid layer material and later arriving desorbing droplets [43]. Changes in solvent composition, charge, size and salt concentration will cause dramatic changes in the physical properties of the secondary droplets, such as the thickness of the electrical double layer of droplets and changes in electrospray current [34]. These factors make the composition of the droplets leaving the surface

relatively unknown, and it is from these droplets that ionization occurs according to the droplet pickup model. Spray additives will also influence the many concerted processes such as extraction, desorption and ionization processes differently, and the experiments described here only measure the combined effects. Future experiments that can separate desorption effects from ionization effects such as reflective electrospray ionization (RESI) and spray desorption collection (SDC) [31] will provide additional insight into the actions of various additives used in the analysis of proteins by DESI-MS.

5.5 Conclusion

Commonly used volatile acids and their ammonium salt buffers affect the analysis of proteins differently when used in DESI and ESI.

Some of these differences come about due to the dynamically changing solvent system during the DESI process, due to changes that occur after nebulization of the solvent but prior to analyte pick up and subsequent ionization. This solvent composition effect is manifested by mild differences in protein unfolding between DESI and ESI, where typically, slightly lower charge states are obtained with DESI when volatile acids were added.

In DESI, due to influences that additives may have on the solubility of proteins, there is opportunity for competition between improvements in ionization and analyte desorption from the surface through droplet pickup. This complication is demonstrated by the reverse- versus direct Hofmeister series dependence on signal intensity that was observed for DESI and ESI, respectively. A good balance between influences on solubility and ionization may also contribute to explaining why ammonium bicarbonate is such an excellent additive to use in DESI for proteins, especially those with high isoelectric points.

The amount of total adduction of individual adducting species and of all species could not be correlated with differences in either solutions pH values, or with proton affinities of the anions.

The additive that leads to the least amount of adduction in DESI, ammonium bicarbonate, was one of the worst in ESI, while the best performing additive in ESI, ammonium fluoride, was the worst performer in DESI. Future experiments at higher resolution and where desorption is separated from ionization in DESI will further elucidate these effects.

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CHAPTER VI

IMPROVING PROTEIN ANALYSIS BY SERINE DURING DESI-MS

Adapted from:

Roshan Javanshad, Elahe Honarvar, and Andre R. Venter,

"Addition of Serine Enhances Protein Analysis by DESI-MS"

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6.1 Abstract

The addition of serine to the electrospray solvent of DESI in order to improve protein analysis was studied. We observed that there is a concentration-dependent improvement in protein signal intensity when micromolar to low millimolar concentrations of serine is combined with a suitable co-additive in DESI spray. In some cases, by addition of serine to the spraying solution of DESI, reduction in the extent of alkali metal adduction to the protein was observed. Serine appears to be an effective solvent additive for analysis of protein with high and low isoelectric points and molecular weights. However a successful combination between serine and other additives is dependent on the isoelectric point of the protein and the pH of the serine solution. The data presented in this chapter is still preliminary and the study is ongoing in our research laboratory at the time of finishing up this chapter.

6.2 Adduct removal from proteins may improve their analysis by DESI-MS

Although desorption electrospray ionization (DESI) has been widely useful in detection of small molecules, unfortunately it suffers from a mass dependent loss in sensitivity for larger proteins

analysis[1-3]. By isolating different steps in the process of DESI-MS and testing each of these steps, our group previously concluded that the low sensitivity for protein analysis in this technique comes from insufficient dissolution of the protein into the spraying solvent [4,5]. Another main reason for this shortcoming of DESI-MS is due to formation of alkali metal adducts like sodium and potassium[6,7]. As discussed in Chapter 4, we previously showed that using additives such as ammonium bicarbonate during protein analysis by DESI-MS improves S/N ratio up to 15 times compared to control solvents (50% MeOH), most likely by removing potassium adducts[8]. Removal of sodium adducts by amino acids was previously studied in native ESI-MS [9]. Clarke et al. reported that the addition of amino acids, specifically serine, into the protein solution increased signal to noise ratio (S/N) up to 4-fold. This effect was accompanied by significant reduction in sodium ion adduction. These two approaches together motivated us to try to improve the capability of DESI-MS to analyze proteins further more by combining serine and other additives to remove the most common protein adducts such as sodium and potassium adducts. To investigate this effect in DESI-MS analysis of proteins, multiple ammonium salts and formic acid were each individually combined with different concentrations of serine as solvent system additives and were tested for analysis of various proteins with both high and low molecular weights (MW) and isoelectric points (pI).

6.3 Experimental details

6.3.1 Samples and reagents

Cytochrome c (cyt c, MW: 12.3 kDa, pI:10.5), hemoglobin (hgb, MW: 15.5 kDa, pI: 6.8), α-chymotrypsinogen (chtg, MW: 25.7 kDa, pI: 8.9) and carbonic anhydrase II (CAII, MW:30 kDa, pI:4.7) were all purchased from Sigma-Aldrich (St. Louis, MO) and were used as model proteins

to provide a range of high and low molecular weights and pI values and were all used without further purification.

6.3.2 Surface and sample spraying

To deposit proteins on the PE surface a custom-made sample sprayer was used to spray protein solubilized out of water at a 5 μL/min sample flow rate when the nebulizing gas pressure was at 100 psi as the stage that carried the PE surface was moving in one direction at a 150 micrometer/sec speed. Such settings yielded average protein concentrations of 20 pmol/mm^2 for cyt c and hgb and 40 pmol/mm^2 for CAII and chtg. At least 4 lines were analyzed and averaged in each trial. For each solvent system a minimum of three trials was carried out.

6.3.3 Solution preparation

50% MeOH was used as the control solution in the desorption sprayer. To investigate the effect of serine addition on proteins, the amino acid was added into the desorption solution alongside other additives. Serine was combined into the solutions containing 0.1% formic acid, 200 mM ammonium bicarbonate and 200 mM ammonium acetate in 50% MeOH. For cyt c and chtg, serine concentrations between 1 μM to 10 mM were tested in 200 mM ammonium bicarbonate and 50% MeOH.

Both ammonium acetate and ammonium bicarbonate were added at 200 mM concentration to the desorption solution. Formic acid was added at a concentration of 0.1% (v/v). Serine was used at different concentrations ranging from 1 μM to 10 mM.

6.3.4 Instrumentation

A linear ion trap mass spectrometer (LTQ, Thermo Scientific, Waltham, MA, USA) was combined with a 3-dimensional translational stage (Purdue University, West Lafayette, IN, USA)

for DESI analysis. The stage, that the sample surface was mounted on top of it, was programmed to move in x and y axes relative to the fixed position of the inlet of the mass spectrometer. The translation speed of the stage was set at 150 micron/sec controlled by WSDK motion software. For the generation of a pneumatically-assisted solvent spray, an electrosonic spray ionization (ESSI) source was constructed in-house[10]. The outer capillary (for sheath gas) was approximately 20 mm in length with an outer diameter of 430 μm and inner diameter of 320 μm. The internal capillary (for solvent) had an outer diameter of 200 μm, and inner diameter of 75 μm. For DESI experiments, 4.0 kV spray potential was applied to the liquid junction on the stainless-steel syringe needle used to deliver the spray solvent. The spray solvent was delivered at 5 μL/min with N_2 as nebulizing gas at 100 psi. The tip of the mass spectrometer inlet capillary was extended by 10 cm to facilitate efficient ion transfer during DESI[11]. The transfer capillary temperature was set at 250°C, the tube lens was set at 130 V for cytochrome c and hemoglobin and 160 V for carbonic anhydrase II and chymotrypsinogen. The transfer capillary was at 20 V for smaller proteins and 49 V for larger proteins. Since the sample was sprayed on the surface which ended up with no "coffee ring" effect and higher homogeneity, all the analysis was performed with the automatic gain control (AGC) in the ON mode. The sprayer to inlet distance was typically 4 mm, the sprayer to surface distance was 1 mm, and the incident spray angle was 55°.

Data analyses were performed by MagTran software (1.03) and the reported signal to noise ratios were calculated for the highest intensity charge states as described by Zhang and Marshall[12].

6.4 Results and discussion

6.4.1 Optimization of solvent system additives

To study the effect of serine addition to the spraying solvent during DESI-MS analysis of proteins, different combinations of amino acid and mass friendly additives were tested. The additives included 200 mM ammonium acetate, 200 mM ammonium bicarbonate and 0.1% (v/v) formic acid all in 50% MeOH solution. Serine was added at a 1mM concentration. Cytochrome c is used in the experiment as a protein with high isoelectric point (pI=10.5) while hemoglobin was used as protein with lower pI (pI=6.8). Figure 6.1 shows the effect of serine on cytochrome c and hemoglobin when L-serine was added to spray solutions containing different additives. The percentage of mono-sodiated and mono-potassiated protein to non-adducted protein is shown as red and green labels, respectively. Controls were added to demonstrate the effects of serine separately from those of the other solvent additives. Therefore the results for the addition of each additive, the amino acid and a 50% MeOH control are shown separately for each protein.

As seen in Figure 6.1(a) By using only serine in the spraying solution of DESI, the peak intensity of the protein was reduced even lower than the protein peak intensity of control, where there was no additive in the solution, but at the same time the percentage of mono-sodiated protein to protonated protein reached its lowest value, suggesting that serine by itself was successful at removing sodium from protein. But from the low signal intensity, it is clear that the sodium removal by serine did not cause any increase in signal intensity.

When only formic acid or ammonium bicarbonate was added without the addition of serine the signal intensity of cyt c did increase and almost equal protein peak intensities were obtained. A similar intensity was also obtained when serine was used together with formic acid, indicating

that there was no improvement upon the addition of serine to the formic acid solution for cytochrome and without significant decrease in the amount of adduction to the protein. However, when serine was used together with ammonium bicarbonate this combination produced almost double the intensity of the other combinations of additives and also resulted in the lowest percentage of adducted species compared to mixtures of serine with other additives.

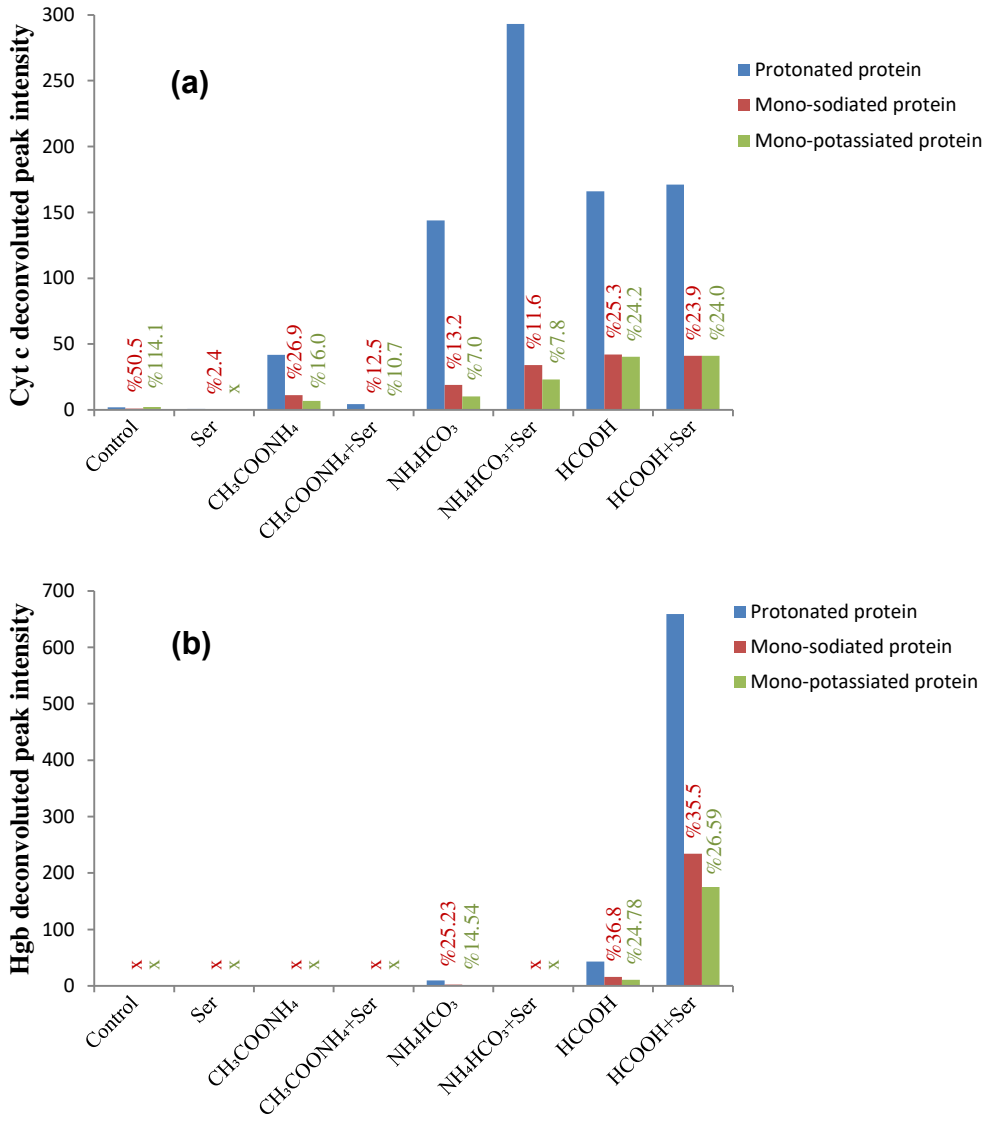


Figure 6.1. Deconvoluted protein peak intensities showing in blue, protonated peaks, in red, sodium adducted species, and in green, potassium adducted protein species for (a)

cytochrome c and (b) hemoglobin with different additives and serine. The sign "x" indicates that no peak was observed.

Addition of serine to the solution of ammonium acetate decreased the intensity of protein peak but also reduced the amount of adduction to the protein, relative to the no-additive control as well as when ammonium acetate was used alone.

All the additives compared to the control, significantly reduce the amount of sodium and potassium adduction to cytochrome c. The amount of sodium and potassium adducted protein to non-adducted protein when spraying the mixture of serine and ammonium bicarbonate to the sample became low, yet was not as low as when serine was used as the solo-additive. However the reduced adduction together with the dramatic increase in protein intensity, makes the combination of serine and ammonium bicarbonate the best solution for the analysis of a high pI protein of such as cytochrome c.

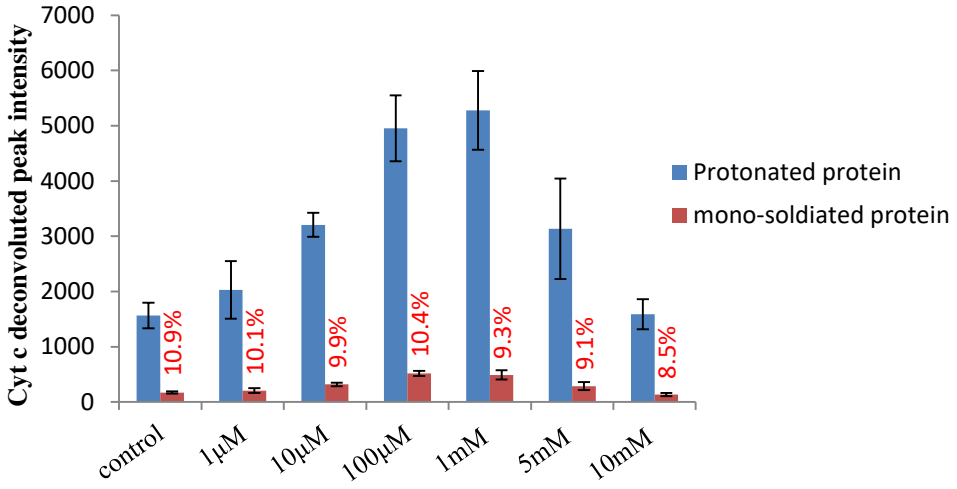
Similar experiments were conducted for hemoglobin and the results are shown in Figure 6.1(b). For this low-pI protein no protein peak was observed when the desorbing solution was 50% MeOH, or when serine, ammonium acetate with or without serine, and ammonium bicarbonate with serine was added.

By adding serine to the solution of formic acid, the protein peak height increased about 15 times compared to when spraying only formic acid. Previously, we have shown that ammonium bicarbonate is more effective at improving DESI of proteins with higher pI values[8]. Peak intensity of cytochrome c, as a protein with high pI value, increased when serine was added to the solution of ammonium bicarbonate.

For hemoglobin with a lower pI, serine became effective once it was added to the solution of formic acid. Difference in the was reported by Pan et al.[13] that the maximum signal for proteins is achieved when the solution pH is 3 units below the protein pI.

6.4.2 Serine concentration optimization

The concentration of serine in a 200 mM solution of ammonium bicarbonate in 50% MeOH was optimized for cytochrome c and chymotrypsinogen.



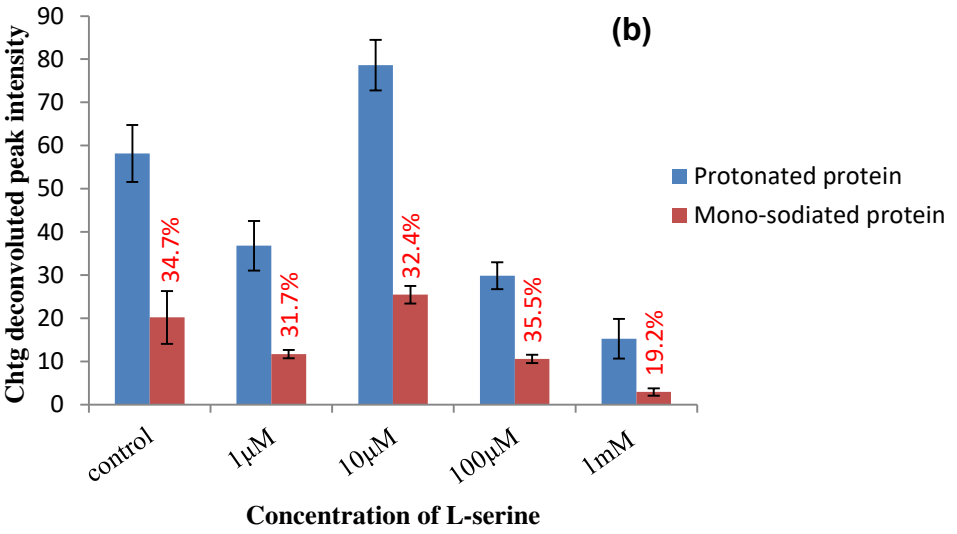


Figure 6.2. Titration of serine in a solution of 200mM ammonium bicarbonate on (a) cytochrome c and (b) chymotrypsinogen. Deconvoluted peak intensities of protonated protein and mono-sodiated protein are shown in blue and red, respectively. The percentage of sodiated to protonated protein is shown by red labels.

Figure 6.2 shows the result of serine titration on these proteins. 200 mM solution of ammonium bicarbonate in 50% MeOH was used as control and serine was added at concentrations as low as 1μM and increasing its concentration until a maximum in the protein height was observed.

In Figure 6.2(a), it can be seen that by adding serine to the solution, the protein peak height, which corresponds to integrated protein signal intensity, increases until it reaches a maximum at the concentration of 1mM serine. With additional increase in serine concentration the protein peak height decreases. This decrease is likely due to ion suppression caused by formation of serine clusters at higher concentrations.

In Figure 6.2(b), by adding serine to the solution that is sprayed to the sample of chymotrypsinogen, first the protein peak height decreases compared to the control solution but then reaches a maximum at the concentration of 10μM of serine. In both Figures 6.2(a) and 6.2(b), it can be seen that addition of serine does not cause any significant change in the percentage of sodiated to protonated protein and those percentages stay relatively the same as the control solution. Ammonium bicarbonate already removes sodium effectively and as was also seen in Figure 6.1, no additional sodium removal took place with serine relative to the amount already removed by NH_4HCO_3. Despite no further removal of adduction, the intensity of protein peak still increases. This implies that serine likely improves protein detection through an additional mechanism and likely helps protein dissolution during their DESI-MS analysis, potentially acting as a surfactant. For chymotrypsinogen the maximum in protein signal was

obtained at 10 μM. The result from the experiments in Figure 6.2 suggests that the optimum concentration of serine to increase signal intensity of different proteins may vary from one protein to another. For chtg some improvement in adduct removal can be seen when serine is added at very high concentrations (above 1 mM) but here the high serine concentration lowered protein signal intensity severely.

6.5 Conclusion

Serine is shown to improve protein analysis by DESI-MS when combined with other additives. The best additive and serine combination depends on the proteins property such as their isoelectric point (pI) and the pH of the solution that is used during DESI-MS analysis of protein. The data presented shows that the mixture of serine and ammonium bicarbonate is a good combination for high-pI protein such as cytochrome c and chymotrypsinogen while the combination of formic acid and serine works for low pI proteins such as hemoglobin and carbonic anhydrase II. For each protein, the optimum concentration of serine can be different from other proteins. While serine alone is effective at removing sodium adducts, the titration experiments indicate that the improved signal intensity with the addition of serine operates through an additional mechanism, possibly improved protein dissolution.

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CHAPTER VII

APPLICATION OF ORGANIC VAPORS FOR IMPROVING PROTEIN ANALYSIS BY DESORPTION ELECTROSPRAY IONIZATION

7.1 Abstract

In this chapter, initial and preliminary results for application of organic vapors in protein analysis by DESI-MS are shown. Electrosprayed droplets of DESI solvent were exposed to vaporized organic solvents within a spray enclosure. A setup was designed to deliver the vapors at controlled pressure to the enclosed sprayer. By introduction of ethyl acetate vapor to the protein sample, notable increase in protein signal intensity was observed. This work is an ongoing project and our research group is developing the setup, investigating various proteins and collecting data at the moment.

7.2. Introduction

As discussed in the previous chapters, improving desorption electrospray ionization for practical application in analysis of proteins, especially proteins with higher molecular weights, is a current challenge[1,2]. As shown earlier, optimizing the solvent system composition as well as using different additives in the sprayer solvent system[3,4] were beneficial towards overcoming the limitation of DESI for protein analysis. The aforementioned approaches were useful in pushing the limits for some protein samples, yet more improvement is still required to make DESI suitable for a larger number of proteins.

Formation of protein-alkali metal adducts is a common and unfavorable observation in protein analysis by mass spectrometry. Adduction usually leads to less efficient analyte ionization and decrease in signal intensity [5-9] in addition to distribution of charge among multiple adducted forms of the protein making spectrum interpretation more complicated[10].

Despite the difference in the sample phase, DESI shares most of ESI's spectral features[11]. After formation of the secondary droplets, DESI follows a similar mechanistic pathway as ESI[11]. Therefore, successful approaches in improving protein analysis by ESI can possibly be applicable to DESI as well.

In separate literature, Oldham's[12] and McLuckey's [13,14] research groups have reported the application of organic vapors in reducing protein-alkali metal adduction during ESI and nano-ESI experiments. Organic vapors such as acetonitrile, acetone, ethyl acetate and water as well as small alcohols were introduced in the atmosphere surrounding the electrospray droplets containing protein sample[12-14].

During their initial ESI experiments, a reservoir filled with the polar organic solvent was placed inside the spray enclosure housing of the ESI source[12]. Later nano-ESI experiments used a more sophisticated setup where the vapor was added to the curtain gas on its way to the nano-ESI source and controlling for the differences in vapor pressure of the individual solvents by blending vapor-entrained and clean nitrogen to create equimolar ratios [14]. The exposure of electrospray droplets to these vapors caused two main effects:

i) The charge state of multiply charged protein shifted to lower charge states (higher m/z values) and more native-like charging was observed.

ii) A considerable reduction in protein-alkali metal (Na^+, K^+, etc.) adduction was observed.

Based on experimental observations, it was suggested that the decrease in the amount of adduction and thus the improvement in the signal to noise ratio are achieved via ion evaporation of alkali metal ions from the original droplets, leaving the analyte containing droplets with a reduced concentration of alkali metal ions[14]. It is proposed that the organic vapors can reduce the

activation energy required for alkali metal ion evaporation, hence increasing the rate of ion evaporation from the electrospray droplets[14].

The aforementioned improvement in protein analysis by ESI and nano-ESI has motivated the work shown in this chapter. The effect of organic vapors such as acetone, acetonitrile, ethyl acetate, methanol and water is investigated and some preliminary data is presented.

In order to expose electrospray droplets to these organic vapors in the ambient environment of DESI, the solvent sprayer was enclosed and the vapor was delivered to the enclosure. Enclosing the DESI source was previously reported and shown to be less dependent on geometric settings when enclosed.[15,16] The design ,patented by Cooks et al., enclosed a distal portion of the sprayer that enable the DESI-active spray to be produced inside the enclosed section[16]. In the work presented here the addition of the enclosure together with the vapor entraining auxiliary nitrogen steam called for some parametric optimization of the DESI source before studying the effects of vapors. Therefore, in the discussion section, the optimization of DESI source is shown before the results of organic vapors exposure is discussed.

7.3 Experimental details

7.3.1 Sample and surface

Myoglobin (mb, 16.9 kDa) was purchased from Protea (Morgantown, WV). LC-MS grade methanol (MeOH) and formic acid were purchased from Fluka Analytical (Morris Plains, NJ). Porous-polyethylene (PE) sheets with average pore size of 15-45 μm (POR X-4900) were purchased from Interstate Specialty Products (Sutton, MA).

For myoglobin, an aqueous sample containing 80 μM of the protein was spray-deposited in 1.0mm lines on PE surface to yield an estimated surface concentration of 35 pmol/mm^2. The

sample was then analyzed by scanning orthogonally through the lines. Each DESI data point is the average of 5 sample lines.

7.3.2 Instrumentation

A linear ion trap mass spectrometer (LTQ, Thermo Scientific, Waltham, MA, USA) was combined with a 3-dimensional translational stage (Purdue University, West Lafayette, IN, USA) for DESI analysis. For the generation of a pneumatically-assisted solvent spray, an electrosonic spray ionization (ESSI) source was constructed in-house[17]. The outer capillary (for sheath gas) was approximately 20mm in length with an outer diameter of 430 μm and inner diameter of 320 μm. The internal capillary (for solvent) had an outer diameter of 200 μm, and inner diameter of 75 μm. For DESI experiments, 4.0 kV spray potential was applied to the liquid junction on the stainless-steel syringe needle used to deliver the spray solvent. The spray solvent was delivered either at 3 or 5 μL/min with N_2 as nebulizing gas either at 80 or 100 psi. The temperature of the transfer capillary which was 8 cm was set at 250°C, the tube lens was set at 160 V. The ion transfer capillary voltage was set at 20 V. The sprayer to inlet distance was typically 4 mm, the sprayer to surface distance was 1 mm, and the incident spray angle was 55°.

7.3.3 Sprayer enclosure

To expose electrosprayed droplets to organic vapors the DESI sprayer was enclosed using a 1 mL plastic pipette tip which was diagonally cut to have the bottom end of the enclosure parallel to the sample surface. This required a cut at 55˚ relative to the long side of the tube. A small notch was cut out of the bottom front of the enclosure so that the tip of the extended inlet capillary could enter into the enclosure. A piercing was made in the back which was accurately made to securely hold the vapor-delivery tubing (Figure 7.1). The enclosure covered the inlet

capillary and rested on it, but was slightly elevated off the sample surface to prevent the enclosure from distributing the sample when the sample was moved through the DESI impact zone. Figure 7.1 is illustrative of the actual setup. With the typical enclosure that was used in the experiment described in this chapter, the nebulizing capillary was closer to the sample surface.

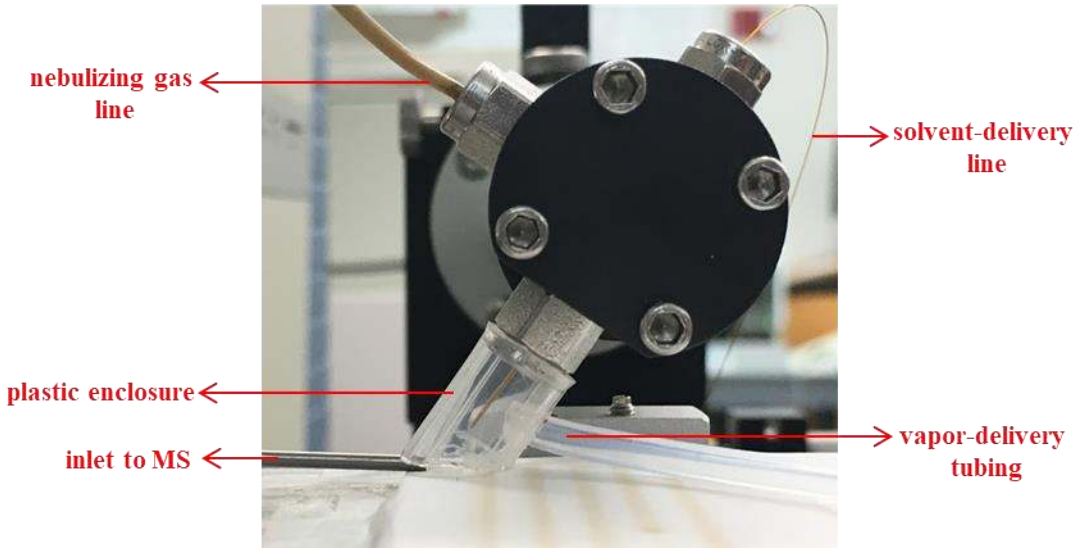

Figure 7.1. DESI sprayer enclosed with a diagonally-cut plastic pipette tip

Introduction of the enclosure together with the auxiliary nitrogen gas can cause changes to the performance of the DESI source. Therefore, parameters such as solvent flow rate, nebulizing gas pressure and the speed of the 3-D translational stage were optimized prior to the exposure of the spray solvent to the organic vapor reagents.

7.4 Optimization of DESI sprayer enclosure

To optimize DESI for the sprayer enclosure, a spray-deposited sample of myoglobin was used. Different combinations of nebulizing gas flow rate, solvent flow rate and stage speed were investigated with the sprayer both not enclosed (control) and enclosed. Nebulizing gas was set at either 80 or 100 psi while the solvent flow rate was set at 3 or 5μL/min. The sample translation

speed was set at 75, 100 or 150μm/sec. The protein sample was desorbed by DESI using a solvent containing 0.1% formic acid in 50% MeOH. Table 1 shows the signal intensity for the *highest intensity charge state* (HICS) of myoglobin with different combinations of these parameters. The same signal intensity is plotted in Figure 7.2 for easier comparison of the combinations.

Table 7.1. Optimization of nebulizing gas and solvent flow rates as well as the speed of sample translation.

N_2 *(psi)*	**solvent** *(μL/min)*	**speed** *(μm/sec)*	**not enclosed**	**enclosed**
80	**3.00**	**75**	9.0E+02	8.4E+02
		100	1.3E+03	1.2E+03
		150	2.1E+03	1.5E+03
	5.00	**75**	1.6E+03	6.2E+02
		100	1.4E+03	8.7E+02
		150	2.9E+03	7.5E+02
100	**3.00**	**75**	7.7E+02	3.0E+02
		100	9.7E+02	2.9E+02
		150	8.1E+02	4.2E+02
	5.00	**75**	6.2E+02	4.0E+02
		100	6.2E+02	3.2E+02
		150	1.4E+03	4.9E+02

Based on the attained signal intensity for the HICS of myoglobin, it seems that for both of the enclosed and not-enclosed cases using a nebulizing gas flow rate of 80psi yielded higher signal intensity than 100psi. This was irrespective of the solvent flow rate or stage translation speed. With the sprayer enclosed and the gas pressure at 80 psi, a solvent flow rate of 3 μL/min led to the highest signal intensity, while not having the sprayer enclosed 5 μL/min is the optimum flow rate.

Slowing down the sample translation speed did not improve the signal intensity but provided more data points in the chronogram for the same sample area, hence protein peaks in the mass spectrum were better resolved. However, it also increased the time required to complete each analysis.

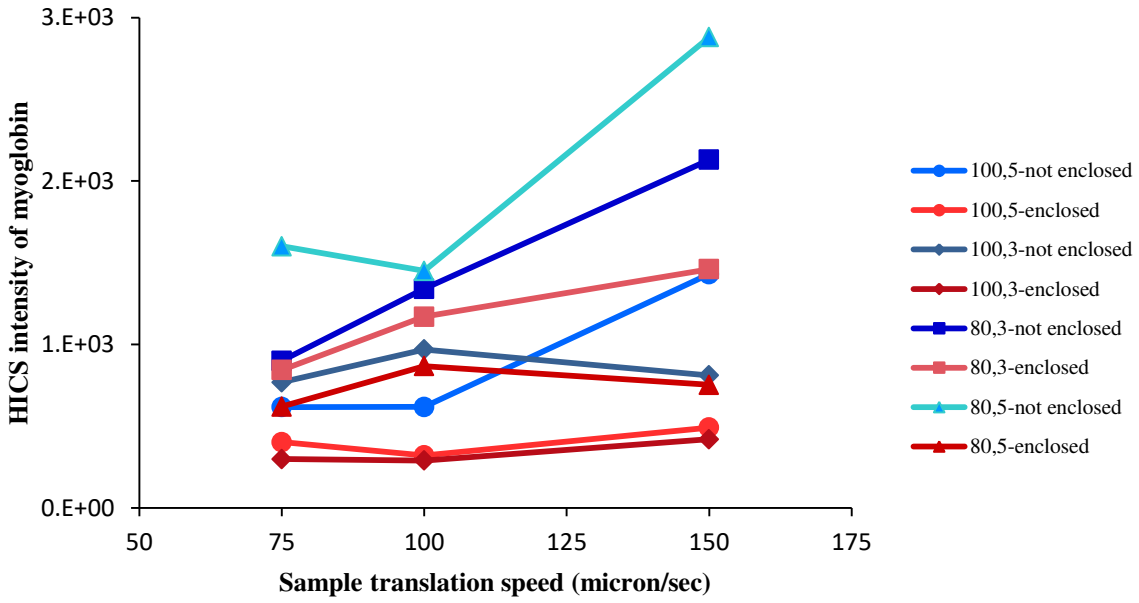

Figure 7.2 The plot of different combinations of nebulizing gas and solvent flow rates as well as the speed of sample translation. Shades of blue indicate not-enclosed while shades of red indicate enclosed conditions.

7.5 Exposing electrosprayed droplets of DESI to organic vapors

To investigate the effects of the addition of vapors of polar organic solvent to the results obtained by DESI-MS analysis of protein, a Scott ® bottle, half-filled with the solvent of interest (acetone, acetonitrile, ethyl acetate, methanol and water) was used. Into the cap of the bottle two holes were drilled through which two Teflon tubes were connected, one to deliver N_2 from the gas tank to the bottle, and the other one to deliver the solvent vapor from the bottle to the enclosure. To

measure the flow rate of the vapor entraining nitrogen, water displacement over a fixed time period was measured using an upturned glass cylinder. When the gas regulator was set at 3.0psi, the flow rate of N_2 gas entering the bottle was measured to be 1.10 L/min which corresponded to flow rates previously used by McLuckey[14]. However, our DESI setup uses a much smaller enclosure and is generally more confined than the open source conditions used for the nano-ESI source that those experiments were carried out in. This difference necessitates further optimization of the vapor flow rate for future experiments.

After optimizing nebulizing gas, solvent flow rate and stage speed in DESI when the sprayer enclosure was used, the best combination (80psi, 3μL/min and 150μm/sec) was used to test the effect organic vapors on the DESI analysis of myoglobin. The electrospray solvent of 0.1% formic acid in 50% MeOH was exposed to the organic vapors. As the control, the sample of myoglobin was analyzed while no vapor or gas was running through the enclosure to elucidate the effect of the enclosure by itself. A second control was performed using pure N_2 gas supplied to an empty bottle and then into the enclosure. The mass spectra of myoglobin after exposing the solvent droplets to the organic vapors are shown in Figure 7.3. The blue diamond indicates the HICS of myoglobin. Upon the addition of acetone, ACN and ethyl acetate the HICS of myoglobin shifts to higher charge states (z=+19 or 20). This observation is contradictory to what was previously reported on the effect of organic vapors in ESI and nano-ESI, where protein charge reduction was observed to decrease with these vapors[12-14]. Exposure of DESI solvent to the vapors of acetone, ACN and ethyl acetate induced more protein unfolding than exposure to H_2O and MeOH. In all cases only apo-myoglobin was observed, indicating substantial denaturing of the sample during the DESI analysis.

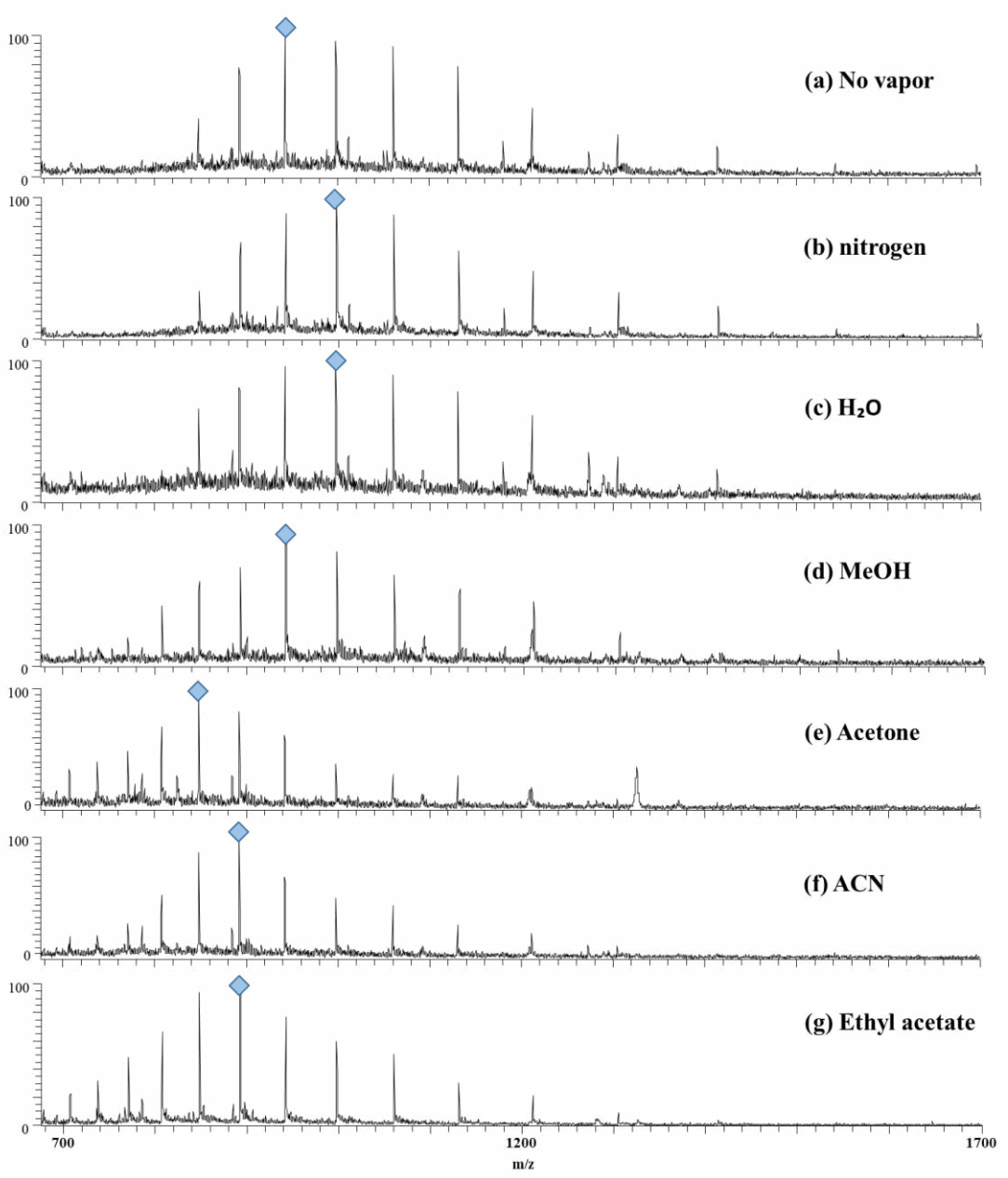

Figure 7.3. The mass spectra of myoglobin when DESI solvent was exposed to a) no vapor/gas, b) only N_2 gas, c) H_2O, d) MeOH, e) acetone, f) ACN and g) ethyl acetate within the enclosure. The blue diamond represents the HICS for apo-myoglobin.

The effect of different organic vapors on the S/N ratios and signal intensities of the HICS for apo-myoglobin is shown in Figure 7.4.

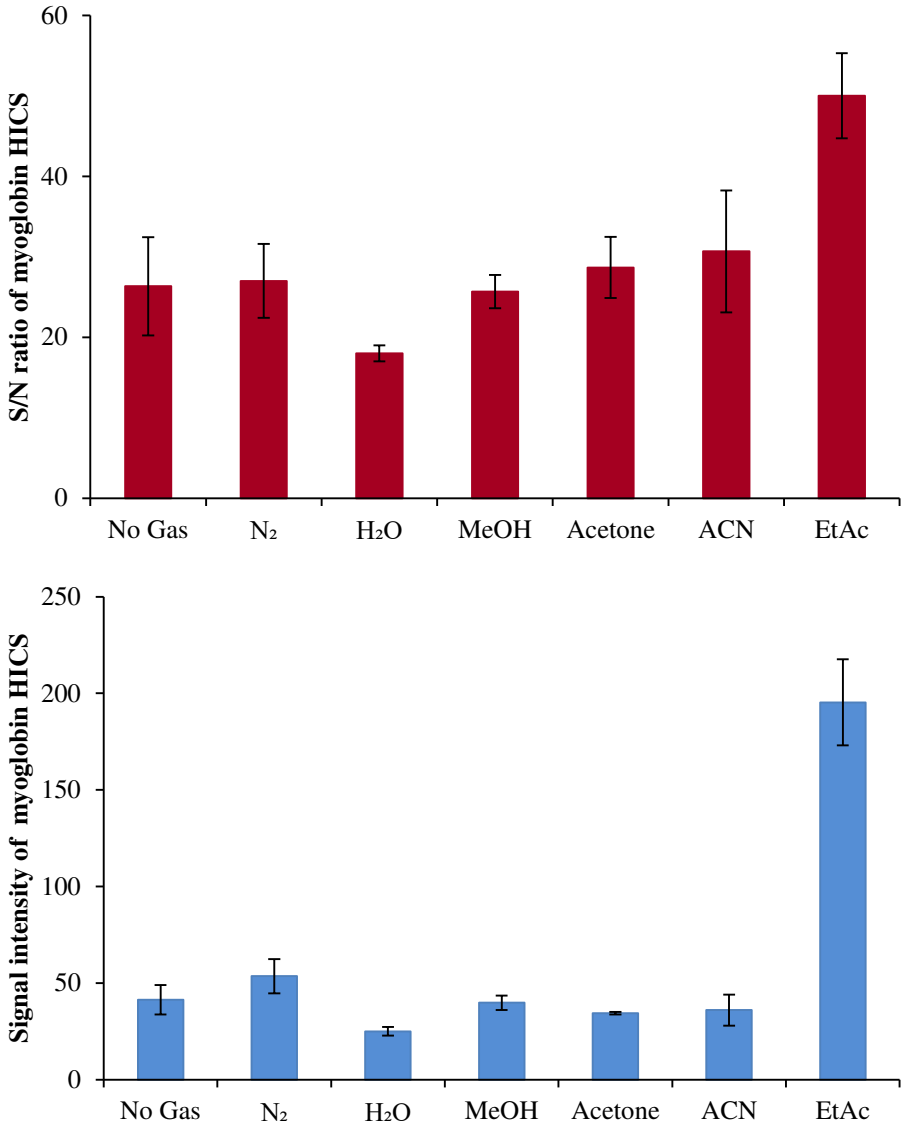

Figure 7.4. S/N ratio (top) and signal intensity (bottom) calculated for the HICS of myoglobin when the environment inside the DESI enclosure was exposed to the indicated organic vapors.

In these experiments the addition of pure nitrogen gas to the enclosure produced similar S/N ratio and signal intensity as when no gas was provided to the enclosure. H_2O resulted in the lowest S/N ratio as well as signal intensity among the organic vapors and even lower than the controls. This is interesting, since the DESI desorbing spray already contained water and

methanol, and these vapors were presumably already present in the enclosure during DESI analyses. Vapors of MeOH, acetone, and ACN did not cause any significant improvement compared to the control. Excitingly though, when ethyl acetate vapor was added into the auxiliary nitrogen flow the signal was seen to increase considerably in intensity (~5 times compared to control) and S/N ratio (~2 times) of myoglobin.

7.6 Conclusion

When organic solvent vapors were added into the enclosure during DESI analysis minor changes in the charge state distributions of myoglobin was observed. In these experiments charge states predominantly shifted to higher values. The signal intensity and S/N ratio of the protein was only increased when the vapor of ethyl acetate was added for protein analysis during by DESI analysis.

The data shown here is preliminary and this work will require further optimization and investigation better control and understanding the effects of organic vapors on the DESI analysis of proteins. Potential future experiments should include: optimization of the size and shape of the enclosure, as well as materials that it should be constructed with, geometrical considerations regarding the DESI-setup and relative position of the vapor inlet, flow rate of DESI solvent, nebulizing gas and auxiliary gases, consideration of the non-equal vapor pressures of the vapors, and other factors. Additional types of protein should also be investigated in particular the analysis of proteins with different sizes and pI-values, especially heavily adducted proteins like those in the albumin family. Finally, solvent systems that can retain the native state of the protein (e.g. ammonium acetate, etc.) in comparison with denaturing solvent systems could potentially

provide more information regarding the mechanism by which organic vapors affect protein analysis in DESI.

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CHAPTER VIII

CONCLUSIONS AND FUTURE PERSPECTIVES

Since the invention of desorption electrospray ionization mass spectrometry (DESI-MS) [1], the technique has found vast application in identifying various classes of molecules from different substrates[2]. From the beginning, small, relatively polar molecules were easily analyzable by DESI-MS but protein analysis remains much harder. While small proteins below 20kDa are easily analyzable, larger ones typically are only measurable at high concentration, making DESI of limited practical use for protein analysis. Until recently that progress has been made where still only small proteins embedded in biological tissues could be analyzed by desorption electrospray ionization imaging mass spectrometry (DESI-IMS)[3,4].

In the research described in this dissertation, I have worked towards improving protein analysis, especially for larger proteins, by DESI-MS through different approaches, focusing on methods that potentially could increase protein solubility. Mainly, my efforts were aimed at optimizing the electrospray solvent composition of DESI by using MS-friendly additives.

Although large protein analysis by DESI-MS still remains a challenge, there is no doubt that the research in the field will continue to further push back the limits and expand the functionality of DESI.

8.1 Description of the approaches followed towards improving DESI for protein analysis

The studies conducted in Chapters 3, 4, 5 and 6 described the effect of solvent composition on the DESI of proteins.

Firstly in Chapter 3, the optimization of solvent composition for the sample depositing was discussed. Then the optimization of spray solution was carried out through the use of different organic solvents with varying ratios as well as the addition of volatile compounds to DESI solvent system.

Chapter 4, proposed the use of ammonium bicarbonate in the DESI solvent and compared its effect to formic acid. Proteins with different molecular mass and isoelectric points responded differently to the addition of ammonium bicarbonate in the spraying solvent with respect to their improvements in signal to noise ratio. While some aspect of this improvements appear to be due to extensive potassium removal from proteins, similar effects were not observed in the ESI-MS analysis of similar proteins samples and solutions. This observation became the motivation to look into the effects of more additives in DESI and compare them to their effects in ESI.

The work in Chapter 5 demonstrated that ammonium salts sometimes behaved differently in DESI and ESI during the analysis of proteins. The study showed that attained improvements in DESI analysis of protein upon using different additives arise from processes that are unique to the mechanism of DESI and do not play a part in ESI, such as sample dissolution.

Chapter 6 introduced the effect of addition of serine to the electrospray solvent following a study by Clarke et al. where they suggested the application of serine to reduce sodium adduction to protein during an ESI-MS experiment[5]. The potential of their work together with the observations we reported in Chapter 4, seemed a useful combination to remove sodium and potassium adducts from proteins in a single experiment. Based on our experiments, serine did not repeat its adduct removal behavior in DESI, however, when paired with other solvent additives such as ammonium bicarbonate and formic acid it caused significant increase in signal intensity

of protein. The mechanism by which serine improves protein analysis during DESI-MS is not clear, but likely stems from the previously described ability of some amino acids to prevent protein aggregation[6].

Chapter 7 takes a different approach than previous chapters for improving DESI analysis of proteins. The chapter shows preliminary data for when vaporized organic solvents were added into an auxiliary gas flow. This study was also motivated by similar works previously performed in ESI and nano-ESI where reductions in alkali metal-protein adducts were reported [7,8]. These organic vapors didn't cause adduct removal as previously reported, however we observed a notable increase in protein signal intensity when the vapor of ethyl acetate was added into an enclosure surrounding the DESI sprayer. While the data shown in Chapter 7 is preliminary and still ongoing, this approach seems exceptionally promising.

8.2 Relative comparison of the effectiveness of the various approaches

The effectiveness of the strategies proposed throughout this dissertation was mostly dependent on the physical characteristics of each protein. Addition of ammonium bicarbonate yielded up to 15 times improvement in S/N ratio of protein. However ammonium bicarbonate was shown to be only effective for proteins with high isoelectric points. Addition of serine showed signal intensity improvements up to 15 times when accompanied by a proper solvent co-additive. For example when hemoglobin was being analyzed, serine showed effectiveness in improving protein signal intensity when added together with formic acid. The addition of vapors to the auxiliary gas flow could also cause improvements in signal intensity, approximately 5 times relative to when no vapor was applied.

The observed effects can be potentially combined to surpass the improvements reported in this work and bring DESI analysis of large proteins closer to being practical. As a start, we have demonstrated the use of ammonium bicarbonate together with serine in Chapter 6, where the combined increases obtained was. 5 times. In future additional combinations will be implemented by colleagues continuing with this project.

8.3 Future work

The strategies we described throughout this dissertation were only partially successful at achieving our goal of making DESI-MS more amenable to the analysis of larger proteins. These studies were formed by experiments that separately investigated the desorption and ionization aspects of DESI, from where it concluded that dissolution was the major reason for poor performance of larger proteins[9]. The premise of incomplete dissolution of proteins might be only one out of many reasons for limited functionality of DESI. Further improvements to DESI analysis of proteins might require different approaches where other limitations of the technique are unveiled and improved to truly make DESI a practical method of analysis for larger proteins.

Some of the strategies introduced in this dissertation describe early attempts at improving DESI for larger proteins, such as the use of vapors and serine. Further development of these two approaches will show their ultimate abilities.

The application of serine is now under further investigation in our research laboratory where the effect of L-serine and D-serine will be compared for different proteins.

For the organic vapor experiment, potential future experiments should include optimization of the size, shape and the material of the enclosure as well as its geometrical features. Proteins with different sizes and pI-values should be investigated in terms of the effect of vapors on their DESI

analysis. Studies using the previously-explained deconstruction of DESI steps into SDC and RESI will shed light on the mechanistic aspects of these improvements, such as if the ameliorating effects were due to improvements in desorption or ionization. These studies would also better explain why some of these improvements are unique to DESI and not reflected in similar ESI analyses.

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