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RESEARCH ARTICLE

Comparing the Effects of Additives on Protein Analysis Between Desorption Electrospray (DESI) and Electrospray Ionization (ESI)

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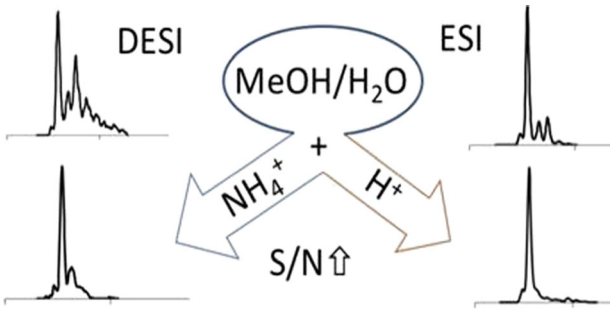

Abstract. It is frequently said that DESI-MS follows a similar ionization mechanism as ESI because of similarities usually observed in their respective mass spectra. However, practical use of DESI-MS for protein analysis is limited to proteins with lower molecular weights (< 25 kDa) due to a mass-dependent loss in signal intensity. Here we investigated commonly used volatile acids and their ammonium salt buffers for DESI-MS analysis of protein. 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 and *S/N*, with DESI signal intensities and *S/N* 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.

Keywords: Ambient ionization, DESI, Desorption electrospray ionization, Protein, Ammonium bicarbonate, Electrospray, Ionization, Cytochrome *c*, Myoglobin, Chymotrypsinogen

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Introduction

With ambient ionization mass spectrometry, most of the sample preparation takes place in a concerted fashion, immediately during the analysis, and in close proximity to the ionization process [1]. Desorption electrospray ionization mass spectrometry (DESI-MS) was one of the first ambient ionization techniques to be developed [1–4]. In DESI, sample processing is usually an extraction, dissolution, or dilution of material present on a sample surface [5].

During DESI, solvent is emitted as droplets from a solvent capillary held at a high field of several kilovolts relative to the atmospheric inlet of the mass spectrometer. The droplets are transported at high velocity [6] by a nebulizing gas toward a surface where the sample is present in the solid or liquid phase. When the collision of solvent droplets with the surface takes place, a thin film of solvent forms on the surface into which the analyte is extracted or dissolved. Analytes are then picked up by subsequent high velocity droplets during collision, according to the widely accepted droplet pickup mechanism [2, 4, 6–8]. After the solvent-surface incident, droplets with reduced velocities and decreased droplet sizes leave the surface at low

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angles of around 10° [6]. Fluid dynamic simulations have indicated that these droplets contain liquid originating from the substrate surface as well as from the incident droplet [6]. This departure of the analyte from the surface solubilized in liquid droplets is often referred to as desorption in ambient ionization literature. The small analyte-containing charged droplets that are transported almost parallel to the surface are assumed to be the dominant part of the analyte that is sampled into the mass spectrometer [6, 9].

Mass spectra of compounds generated by DESI present similarities to the spectra obtained when those compounds are analyzed by ESI [3]. Therefore, analyte ionization in DESI is believed to follow the same mechanism as in ESI [10]. After formation of secondary droplets during the droplet pickup mechanism of DESI, they undergo solvent evaporation and yield gas-phase ions by one of the generally accepted ESI mechanisms such as ion evaporation for small molecules, or charge residue, or chain ejection for biopolymers [10–13].

While DESI has found wide-ranging application in the detection of small molecules such as lipids [14–16], explosives [3, 17–19], metabolites [20, 21], and pharmaceuticals [21, 22], from both synthetic surfaces and biological tissues, it has found limited use for protein analysis due to a loss in sensitivity with increasing mass of proteins [23, 24]. This is unfortunate as the ability to analyze large biopolymers by mass spectrometry is one of the key advantages enabled by ESI [25]. This mass-dependent loss in signal sensitivity has been attributed to adduction which causes noisy spectra with low and distributed protein ion signal [24]. This is in contrast to the high tolerance for salts reported for the analysis of small molecules [26]. Many proteins also display nonspecific dimers or perhaps even multimers, shifting protein envelopes outside of the mass range of the analyzer, potentially due to poor dissolution of the sample during the DESI analysis [24].

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 [27–31] 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 [31, 32]. 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 [33] 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 [34]. 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 [35]. Use of weak chelators such as L-tartrate has 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 [36]. Millimolar concentrations of anions with low proton affinity (< 315 kcal mol^{-1}) also reduce salt adduction to protein [37]. Examples include ammonium bromide and ammonium iodide [38, 39]. Chait et al. showed that higher mass adducts (molecular mass = 98) are efficiently removed from proteins by addition of barium [40]. Some supercharging reagents such as *m*-nitrobenzyl alcohol (*m*-NBA) were also reported to be advantageous in reduction of protein adducts [41]. Another recent approach to reduce sodium adduction is by exposing droplets of electrospray to vapor containing volatile organic solvents [39]. 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 [33].

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. We showed recently that ammonium bicarbonate improved signal intensities and *S*/*N* when added to spray solutions during the analysis of proteins, in a manner not consistent with its effects in ESI [42]. To further investigate potential differences between ESI and DESI for protein analysis, we now compare the qualitative and quantitative effects observed in the mass spectra for the addition of a variety of organic acids and their ammonium salts.

Experimental

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 (myo, 16.9 kDa) was purchased from Protea (Morgantown, WV). LC-MS grade methanol (MeOH), formic acid, and 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 X-4900) were purchased from Interstate Specialty Products (Sutton, MA).

Instrumentation

A linear ion trap mass spectrometer (LTQ, Thermo Scientific, Waltham, MA, USA) was combined with a three-dimensional translational stage (Purdue University, West Lafayette, IN, USA) for DESI analysis. For the generation of a pneumatically assisted solvent spray, a standard electrosonic spray ionization (ESSI) source was constructed in-house [44]. 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 [45]. The transfer capillary temperature was set at 250 °C, the tube lens was set at 90 V for cytochrome *c*, and 130 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°.

With ESI, the sample was infused at 10 μL/min 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.

Data collection was performed with LTQ 2.0 and Xcalibur 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 [46].

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 [43], unless noted differently. Formic and acetic acids were used at 0.1% and 3% (*v*/*v*), to produce solutions with pH ≈ 2.5 and 2.7, respectively. Each ESI data point is the average of three replicates and each replicate represents the average of scans over 1.0 min 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 three 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 20 pmol/mm^2. The samples were then analyzed by scanning orthogonally through the lines. Each DESI data point is the average of three replicates and each replicate represents the single pass scan of five consecutive lines. An exception was for ammonium bromide data where, because of persistent Br^- contamination to the extended ion transfer tube, only one replicate of five 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, 200 mM of each salt was added into 50% MeOH DESI desorption spray solvent, as previously optimized for ammonium bicarbonate [42]. 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.

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 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*.

Qualitative Differences

In Fig. 1a, the control spectrum for DESI deposited out of pure water and analyzed with 50% MeOH was less denatured than in the ESI result (Fig. 1j) analyzed when protein was dissolved in 50% MeOH. This difference has been explained based on the short residence time of the protein in the denaturing solvent between desorption and ionization [6] but likely also stems from differences in solvent composition of the secondary droplets leaving the sample surface. It is plausible that the microlocalized 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) [47] shift to higher charge values compared to the controls where there were no additives in the solution. In Fig. 1, the 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. 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 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

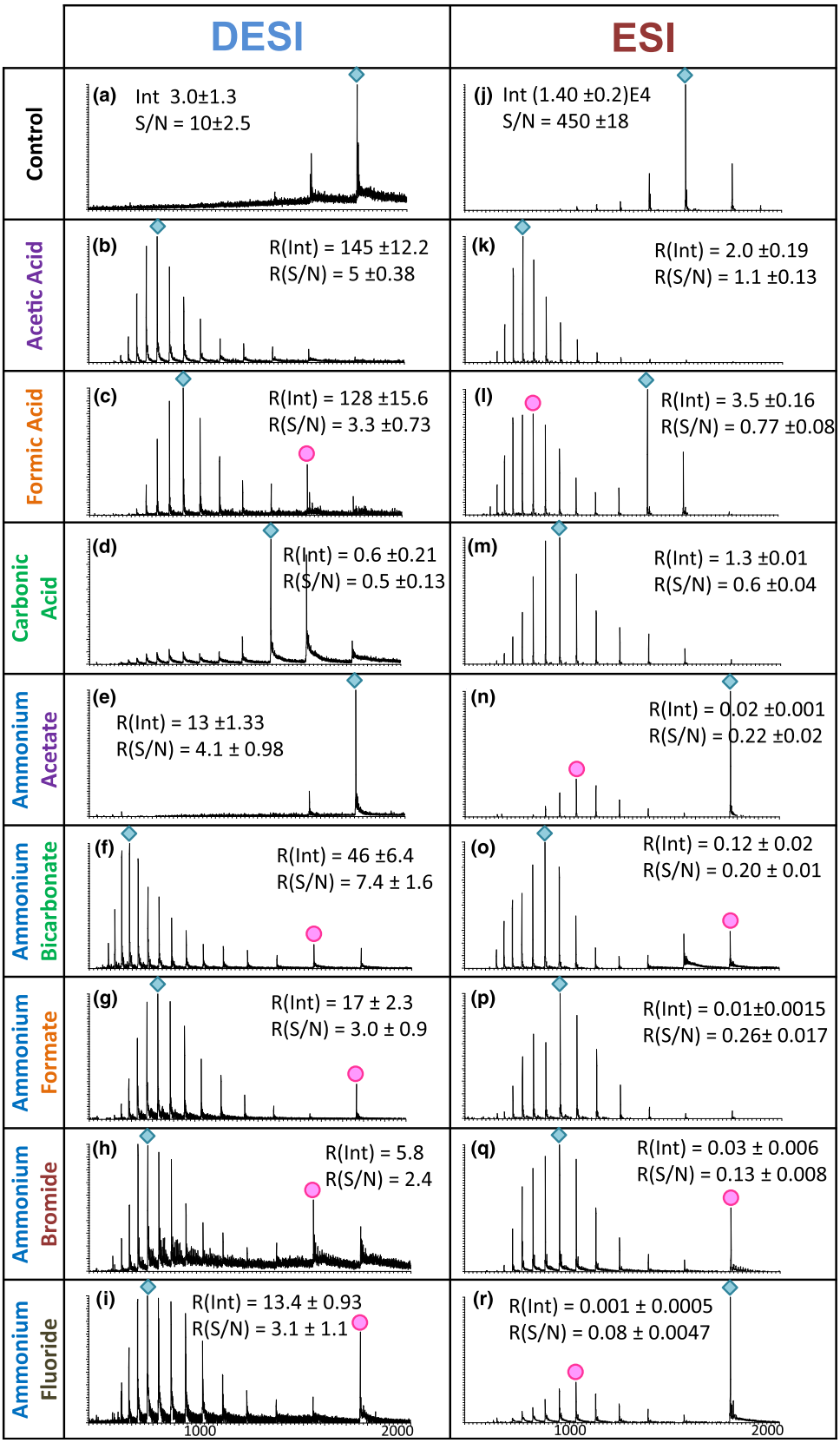

Figure 1. Mass spectra of different additives added to the DESI spray solvent (**a–i**) and to the ESI spray solution (**j–r**) showing the differences in the attained charge states (see Table 1), intensities, and signal-to-noise ratios observed with the two ionization modalities. Deconvoluted spectra are available in Fig. S1

Table 1. Highest intensity charge state (HICS) and highest observed charge state (HOCS) 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

diffusion of CO_2 from the supersaturated liquid into escaping bubbles [48].

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 were first described by Franz Hofmeister and ordered according to the Hofmeister series [49]. 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 [50–52]. 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 [53]. A reversed Hofmeister series dependence on unfolding during ESI analysis of proteins was however recently described for analyses under electrothermal supercharging conditions [54]. 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 1).

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 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. 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 [55]. 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 an order 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/s stage scan speed, and 200 μm desorption footprint), compared to 100 pmol/min for ESI analysis (for a 10 μM solution infused at 10 μL/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 an order 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 [9]. 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 ($R_{(Int)} = \text{Intensity}_{additive} / \text{Intensity}_{control}$) 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 ($R_{(S/N)} = S/N_{\text{additive}} / S/N_{\text{control}}$).

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 (Fig. 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 leads 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 [35, 56–61]. As a result, during the ESI experiments summarized in Fig. 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 [38], we found that under these ESI conditions, both halide salts, ammonium bromide, and fluoride, reduced *S*/*N* by a factor of almost 10.

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. 1e–i).

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 pickup mechanism [1]. 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 [53]. 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

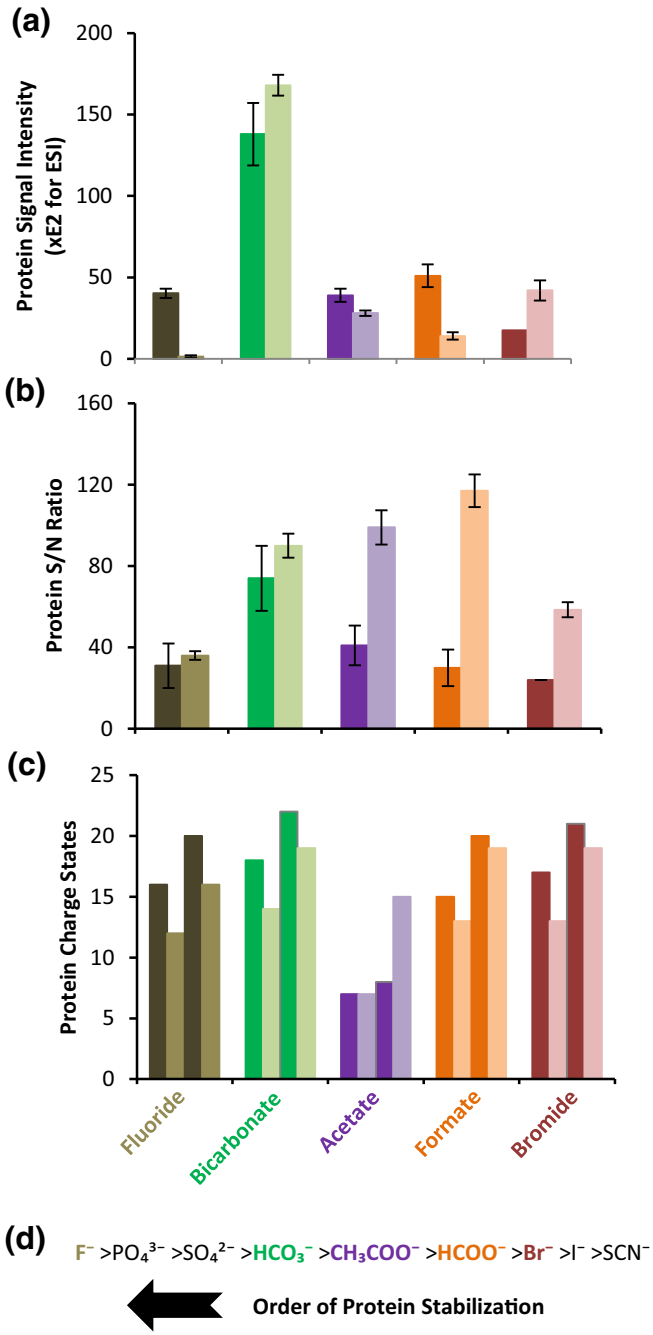

Figure 2. With anions arranged according to the Hofmeister series, effects on (**a**) protein signal intensity, (**b**) protein to adduct area, and (**c**) protein charge state distributions of HICS and HOCS for DESI (dark colors) and ESI (light colors) (**d**) the Hofmeister series

yielded exceptionally high signal intensities (Fig. 2a), most of the other anions improved the signal about the same, 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 was 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 (Fig. 2b), 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 [40, 60, 62–64]. Adduct-induced signal distribution and ion suppression [56] 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.

The extent of adduction observed in the deconvoluted protein mass spectra when each of the solution additives was used was evaluated for both DESI and ESI (Fig. S1). 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 Figs. 3a and 4a. 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 $[M+K]^+$ was normalized to the height of the deconvoluted protein peak and

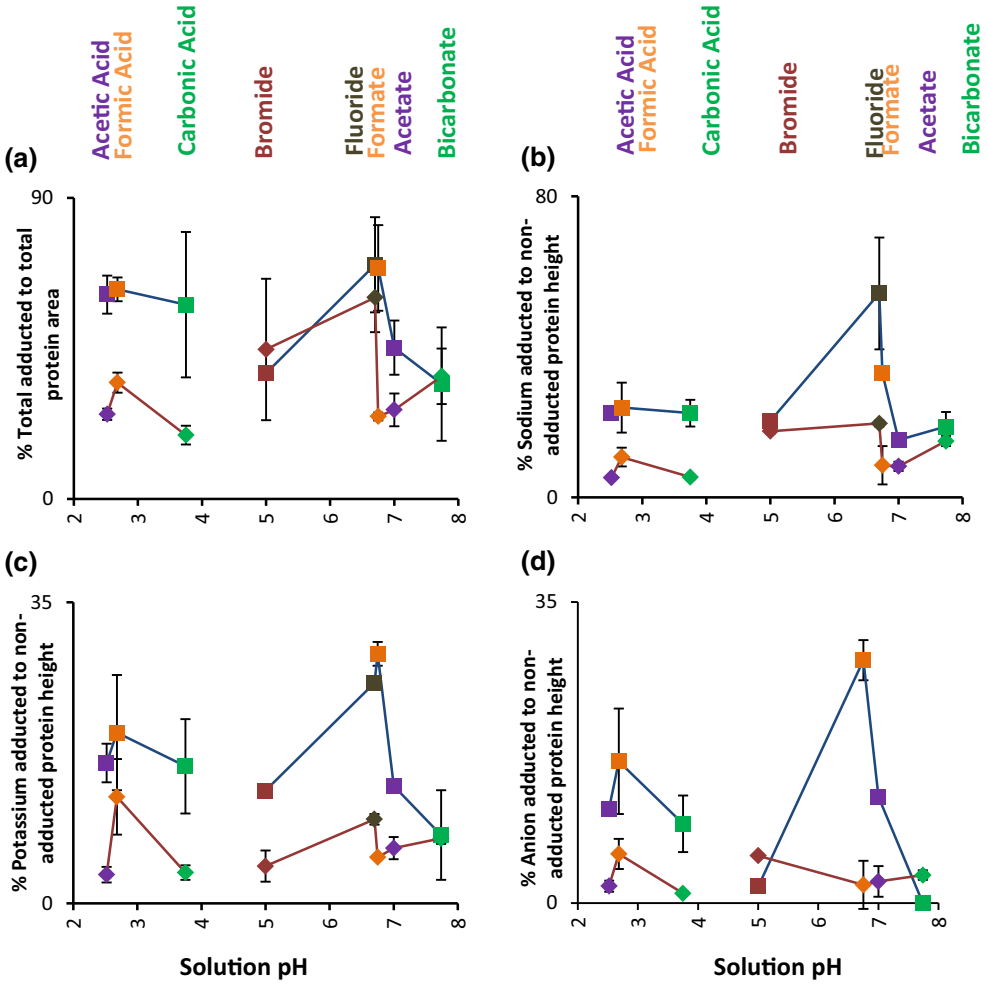

Figure 3. The effect of solution pH for each additive in DESI (blue line, square marker) and ESI (red line diamond marker) on relative abundance compared to non-adducted protein for (**a**) all adducts, (**b**) sodium adducts, (**c**) potassium adducts, and (**d**) anion adducts. The data shown in graphs (**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

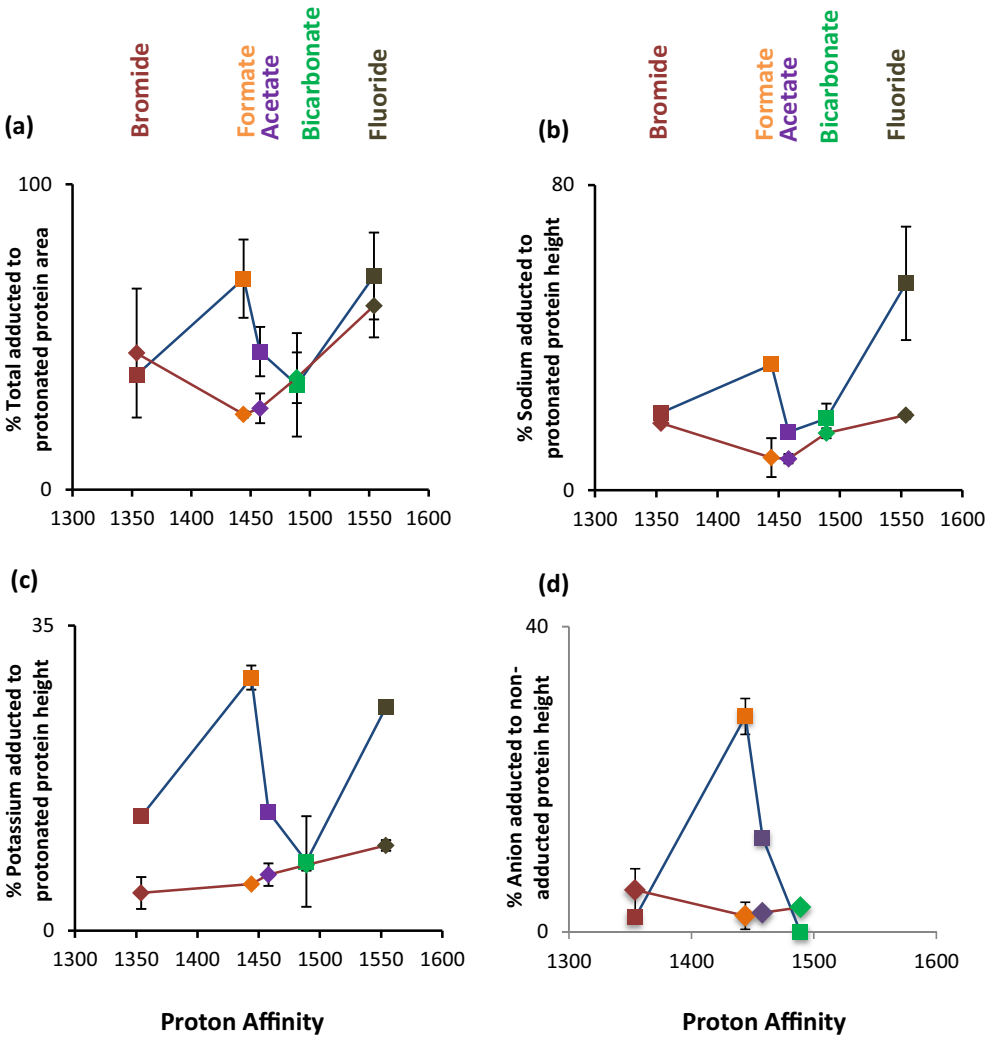

Figure 4. Effect of anion proton affinity for each additive in DESI (blue line, square marker) and ESI (red line diamond marker) on relative abundance compared to non-adducted protein for (**a**) all adducts, (**b**) sodium adducts, (**c**) potassium adducts, and (**d**) anion adducts. The data in graph (**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

displayed in Fig. 3b, c arranged by solution pH and in Fig. 4b, c arranged by anion proton affinity. Figures 3d and 4d 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 Figs. 3 and 4, 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 follows 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 [38, 65].

With ammonium salts, as shown in Fig. 3, 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) which has been shown to be important consideration for cation adduct removal [60] and previously observed to be important for DESI-MS of proteins [42]. 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. Figures 3 and 4 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.

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 [37] due to the relatively low proton affinity of the bromide anion (Fig. 4a, 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 [37]. 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 [37], lower charge states are likely to be more heavily adducted.

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 and 6 show the effects of additives in DESI and ESI on the mass spectra of holomyoglobin and chymotrypsinogen, respectively.

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 were obtained for DESI when using a spray containing 50% MeOH, and formic acid or ammonium bicarbonate, some holomyoglobin peaks remain. The only additive that preserved holomyoglobin was ammonium acetate as shown in Figs. 5 and S2. Ammonium acetate also produced native state like charge states and generally a low signal intensity for all proteins analyzed.

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 were observed: As can be seen in Figs. 1 and 6,

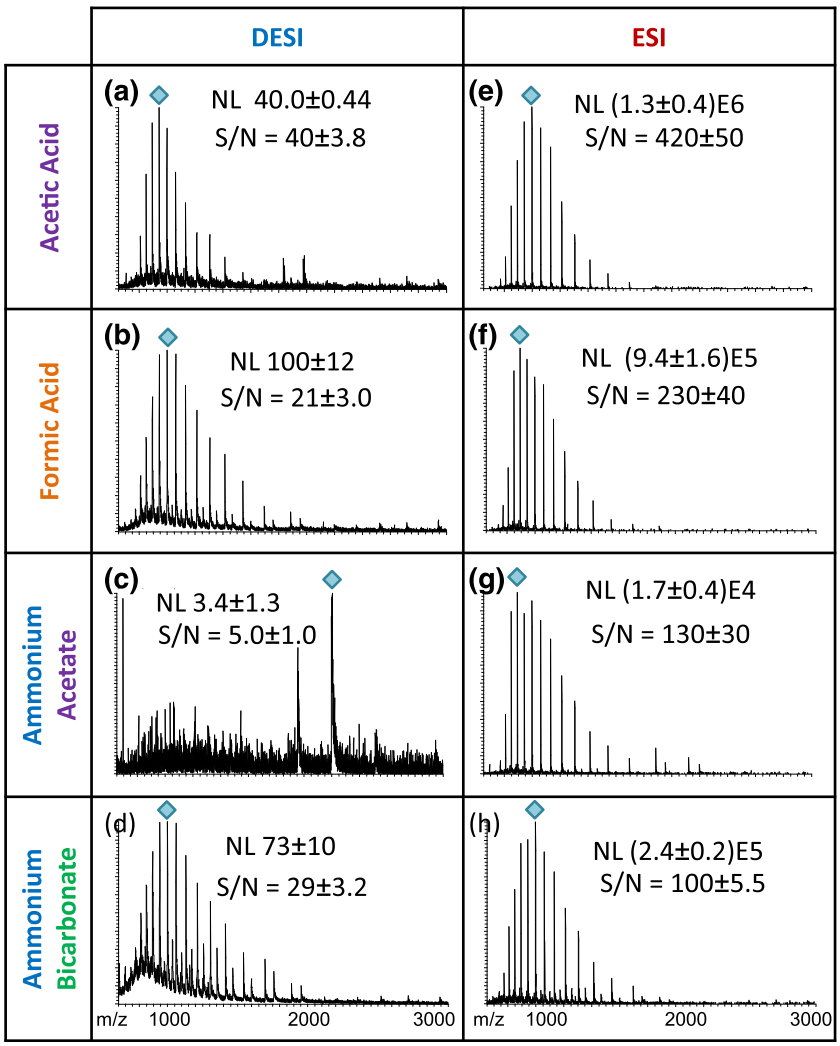

Figure 5. Mass spectra obtained for myoglobin with different additives in the DESI spray solvent (**a**–**d**) and ESI spray solutions (**e**–**h**) showing the differences in the attained charge states, intensities, and signal-to-noise ratios observed with the two ionization modalities. Deconvoluted spectra are available in Fig. S2

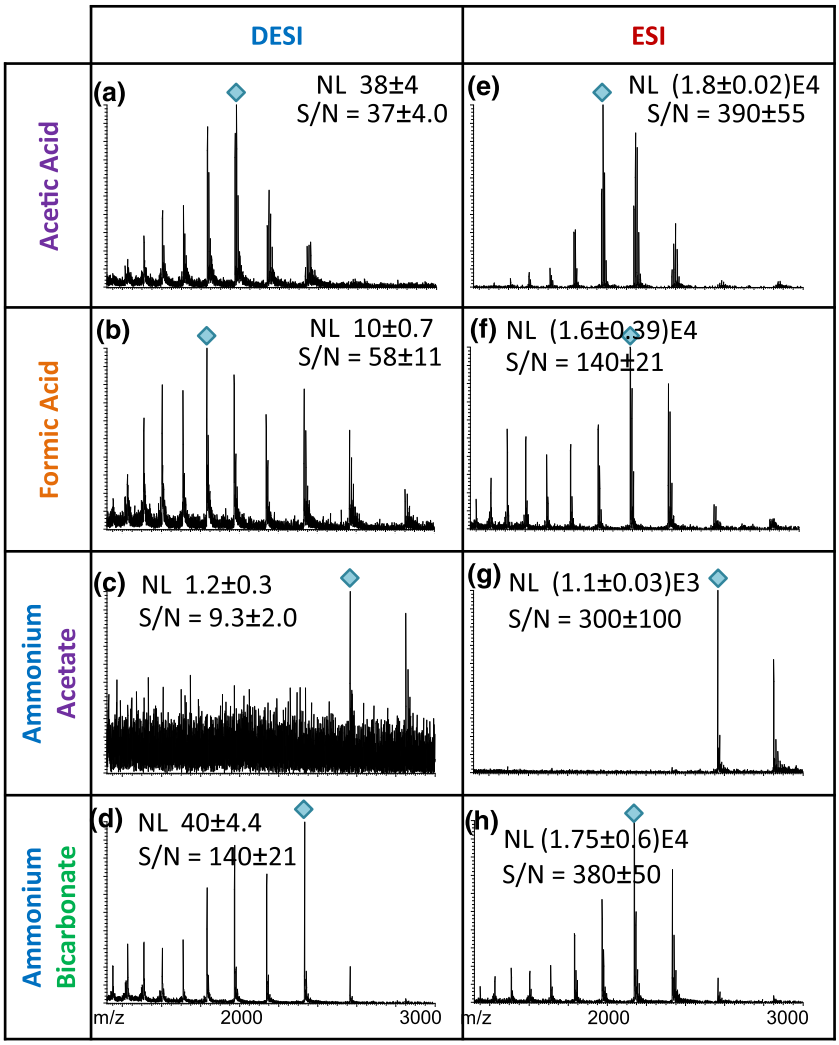

Figure 6. Mass spectra obtained for chymotrypsinogen with different additives in the DESI spray solvent (**a**–**d**) and ESI spray solutions (**e**–**h**) showing the differences in the attained charge states, intensities, and signal-to-noise ratios observed with the two ionization modalities. Deconvoluted spectra are available in Fig. S3

ammonium bicarbonate resulted in narrower peaks for each charge state of cyt *c* and chtg, as these proteins are mostly present in their protonated forms, while myoglobin (myo), 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 (Fig. S3) 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 [31]. Less acidic solutions of 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 [42], 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 myo.

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 Columbic explosions before even interacting with the analyte molecules, and further loss will occur to the non-conducting sample surface [55]. 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 [6], and MD simulations have calculated these droplets to contain material from both the surface liquid layer material and later arriving desorbing droplets [7]. 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 [58]. 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) [9] will provide additional insight into the actions of various additives used in the analysis of proteins by DESI-MS.

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 pickup 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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