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Ammonium Bicarbonate Addition Improves the Detection of Proteins by Desorption Electrospray Ionization Mass Spectrometry

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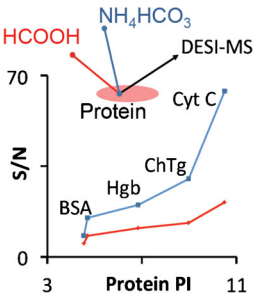

Abstract. The analysis of protein by desorption electrospray ionization mass spectrometry (DESI-MS) is considered impractical due to a mass-dependent loss in sensitivity with increase in protein molecular weights. With the addition of ammonium bicarbonate to the DESI-MS analysis the sensitivity towards 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 improvement correlated well with protein pI but not with protein size. Other ammonium or bicarbonate salts did not produce similar improvements in S/N, nor was this improvement in S/N observed for ESI of the same samples. As was previously described for ESI, DESI also caused extensive protein unfolding upon the addition of ammonium bicarbonate.

Keywords: DESI, Desorption electrospray ionization, Protein, Ammonium bicarbonate, Electrospray mechanism

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Introduction

Desorption electrospray ionization (DESI) has become a widely accepted method of analysis since its introduction in 2004 [1]. The DESI spectrum resemblance to normal electrospray ionization [2] along with little to no sample preparation requirements prior to analysis [2, 3] provide advantages relative to other ionization methods. DESI has been used for the analysis of a wide variety of sample substrates [4] such as bacteria [5–8], pharmaceutical preparations [9], [10], directly from thin layer chromatography plates [11–13], and imaging from biological tissues [14]. Applications include natural products, especially secondary metabolites [15], forensic samples [16, 17], chemical warfare agents [18–21], peptides [22], but seldom proteins [21, 23, 24].

Despite its utility for small molecules, the practical use of DESI for protein analysis is severely limited. This limitation comes about because as the mass of protein increases, the limits of detection increases exponentially [25]. It was generally believed that with increasing protein size it becomes progressively harder to desorb protein molecules from surfaces. To test this hypothesis, we developed methods to investigate desorption and ionization independently [26]. Spray desorption collection (SDC) was used as a model for desorption aspects of DESI and reflective electrospray (RESI) was used as a model for ionization after desorption occurs in DESI [27]. The experimental results of that work showed that the loss in signal intensity was not due to the physical desorption or ionization processes; rather, this mass-dependent loss in sensitivity is likely caused by incomplete sample dissolution during desorption resulting in the distribution of signal across protein clusters, as well as protein–protein and protein–contaminant adducts [25].

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 [28, 29] 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 [30]. Exchanging the buffer solution from

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nonvolatile ions to ammonium acetate or ammonium bicarbonate solutions improved the quality of protein mass spectra by removing ESI incompatible compounds [31, 32].

The benefit of the addition of ammonium additives are believed to stem from the replacement of nonvolatile salts by ammonium in solutions of proteins [30, 33, 34]. When NH_4^+ displaces Na^+ ion in solution, it decomposes to form a proton and ammonia during the evaporation and transfer processes in ESI [35] leading to protonation of a basic site and a reduction in metal adduction. Alternatively, Iavarone et al. suggested that the improved signal to noise ratio (S/N) is simply caused by the precipitation of nonvolatile ions, such as Na^+ and Cl^- [30]. 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 [36, 37]. 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 [36].

The identity of the anion also plays an important role in the charge states and intensities observed for proteins and peptides [38] 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 [39]. 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 [40, 41].

Ammonium bicarbonate was proposed as an alternative volatile buffer for native protein analysis due to its high buffering capacity at near neutral pH [42–46]. Unfortunately, when ammonium bicarbonate was used as a buffer reagent in electrospray ionization analysis, proteins formed higher charge states, indicative of protein denaturation [44]. 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 [47]. 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 [48].

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.

In order to gain some preliminary insight into the possible mechanism behind the observed enhancement in protein S/N attained by ammonium bicarbonate addition during DESI, we compared the effects of a selection of other volatile ammonium salts over a range of proton affinities. These were ammonium salts of fluoride, acetate, formate, and bicarbonate, as well as respective acid forms: acetic, carbonic, and formic acids.

Experimental

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, USA). α-Chymotrypsinogen (ChTg, 25.7 kDa) was purchased from MP Biomedicals, LLC (Solon, OH, USA). LC-MS grade formic acid, ammonium formate, and methanol (MeOH) (CHROMOSOLV, Venezuela) were purchased from Fluka Analytical. Glacial acetic acid was purchased from EMD (Darmstadt, Germany). Porous-polyethylene (PE) surfaces with average pore size of 15–45 μm (POR X-4900) were purchased from Interstate Specialty Products (Sutton, MA, USA). Ammonium bicarbonate was purchased from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate was purchased from Sigma-Aldrich (St. Louis, MO, USA).

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 [47] 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 20 μM 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 and other salts to protein samples by DESI, 200 mM of each salt was added to 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, whereas formic and acetic acids were used at 0.1% and 3%, respectively.

Ammonium Bicarbonate was Added to the DESI Sample in One of Three Ways

(1) *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 3μL droplets. After drying on the surface, the samples were analyzed with a spray solvent containing 50% MeOH.

(2) *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.

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

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 [49]. 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 [50]. 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 140 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 S/N were calculated for the highest intensity charge states as described by Zhang and Marshall [51].

Results and Discussion

Similar to ESI [44], 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) [52] were seen to increase even as the solution pH remained close to neutral. Figure 1a–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 1a, 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 [53, 54], as can be seen in the ESI-MS analysis of cyt *c* in Figure 1d 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 [55], unfolding of the protein was not observed and a native state-like envelope was observed [52] with S/N = 10 calculated for the HICS at z = +8 (*m*/*z* =1546).

Figure 1b and c show the unfolding effects observed for the addition with 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. Formic acid addition produced a bimodal envelope, showing both native-like and denatured populations of cyt *c*, observed 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 to the sample solution, as shown in Figure 1e and f, respectively, the HICS shifted to z = +16 (*m*/*z* = 773) for formic acid and to z = +18 (*m*/*z* = 688) for ammonium bicarbonate. Owing to the high spray voltage and temperature used in these experiments, the conditions are favorable for electrothermal supercharging when ammonium bicarbonate is present [44], explaining the relatively increased charging during analysis with ammonium bicarbonate additive in both ESI and DESI.

In DESI, both ammonium bicarbonate and formic acid addition also improved the S/N measured for the observed HICS of cyt *c* for each result (Figure 1b and 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 [56]. Much less protein 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 2 as described later.

Figure 1d–f show 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

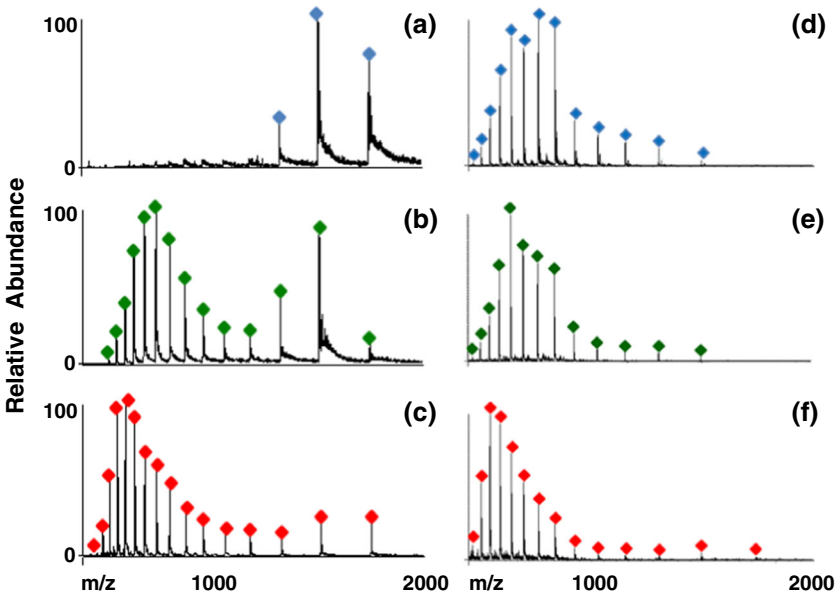

Figure 1. The analysis of 80 pmol/mm^2 cyt *c* by DESI (left) and ESI-MS (right). For DESI experiments, the sample was deposited from water and analyzed with (**a**) 50% MeOH, (**b**) 0.1% formic acid, (**c**) 200 mM ammonium bicarbonate in spray solution. In ESI, samples were dissolved in (**d**) 50% MeOH, (**e**) 0.1% formic acid, (**f**) 100 mM ammonium bicarbonate before direct analysis

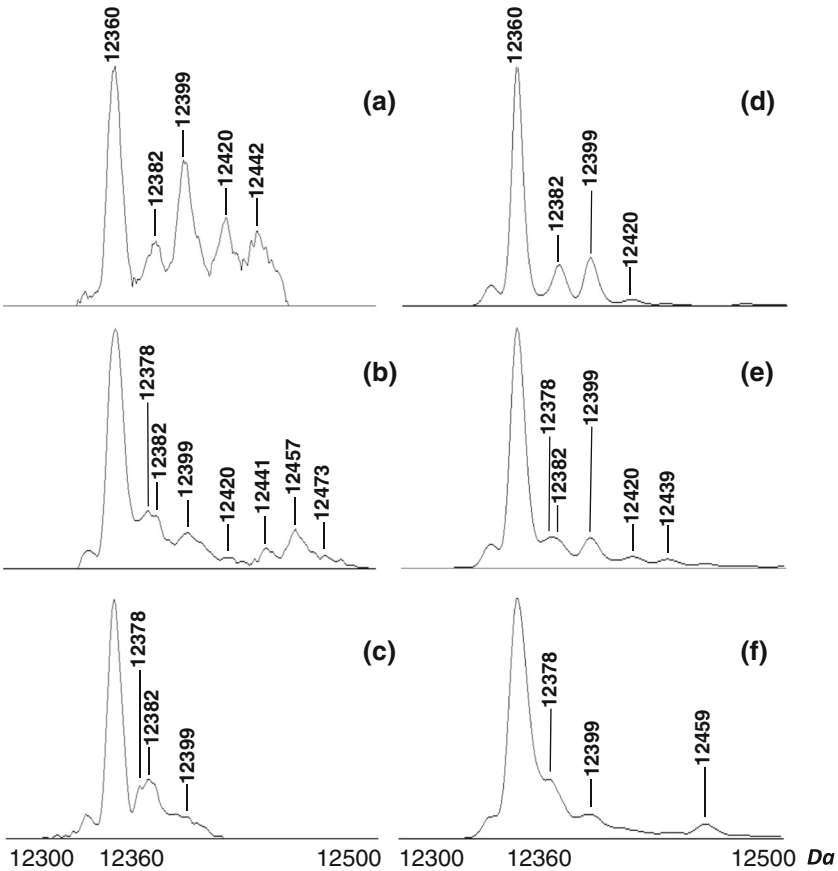

Figure 2. Deconvoluted spectra of cyt c showing different adduction forms with different solvent additives. DESI spectra of cyt *c* deposited from water and analyzed with a solution of 50% MeOH containing (**a**) no additive, (**b**) formic acid, (**c**) ammonium bicarbonate are compared with ESI spectra of 10 μM cyt *c* prepared in 50% MeOH containing (**d**) no additive, (**e**) formic acid, (**f**) ammonium bicarbonate

noise ratio (S/N = 305) was observed when formic acid was used while the addition of ammonium bicarbonate led to a reduction in S/N (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 [57]. 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 [58].

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 sample surface to produce secondary droplets. These were previously measured to be reduced to around 1 μm or smaller after surface collision [55]. Protein ionization occurs from these smaller droplets according to the droplet pick-up mechanism of DESI [21], and smaller droplets are believed to be more efficient at ionization [59].

Figure 2 depicts deconvoluted spectra for the data presented in Figure 1. The amount of adduction is reduced when cyt *c* is analyzed with either additive in the spray solvent compared with the control for both DESI-MS (Figure 2a) and ESI-MS (Figure 2d). 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 2c) reduced the extent of adduction significantly better relative to formic acid (Figure 2b), 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 2f) fared poorer than either the 50% MeOH control (Figure 2d) or when formic acid was added (Figure 2e). 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 [60]. 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.

Different methods for the application of ammonium bicarbonate during DESI-MS were investigated as illustrated in Figure 3. These methods include addition of ammonium bicarbonate to the protein solution (1) before sample deposition on the surface (Figure 3b), (2) deposition of ammonium bicarbonate on top of deposited protein samples (Figure 3c), and (3) addition of ammonium bicarbonate to the spray solution (Figure 3d). The sequence of protein and ammonium bicarbonate deposition as well as the results for cyt *c* is summarized in Table 1.

For these experiments, unlike those presented in Figures 1, 2, and 5, the samples were spotted from a solution made up in an aqueous solution containing 50% MeOH and was therefore already denatured before DESI-MS analysis. This denatured control had a S/N = 36 for cyt *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 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 [39].

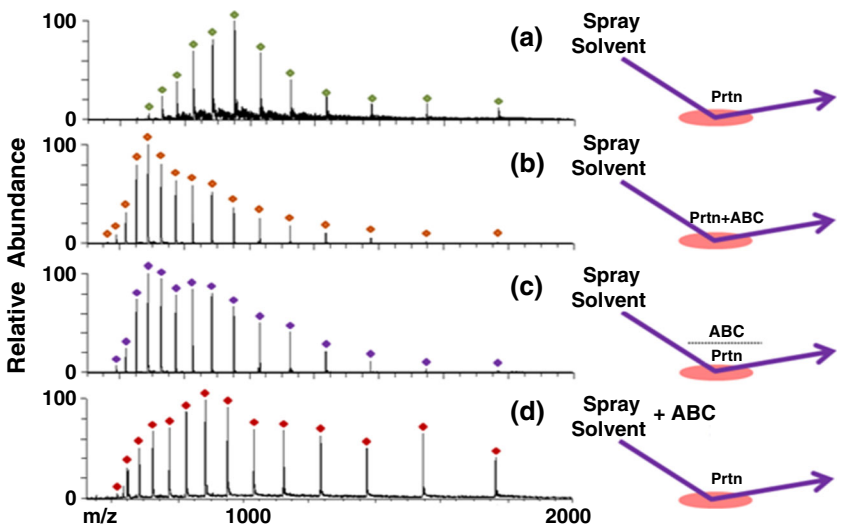

Figure 3. Cyt *c* (Prtn) was (**a**) deposited from 50% MeOH and analyzed with 50% MeOH as control. In (**b**) 200 mM ammonium bicarbonate (ABC) was added to the depositing solvent, (**c**) 200 mM ammonium bicarbonate was deposited on-top of the previously deposited protein, or (**d**) the cyt *c* sample deposited from 50% MeOH was analyzed using a spray solvent containing 200 mM ammonium bicarbonate (see Table 1)

Table 1. Signal to Noise Ratio Values, Highest Observed- (HOCS) and Highest Intensity Charge State (HICS) for the Three Different Methods of Applying Ammonium Bicarbonate (ABC) to DESI During the Analysis of cyt *c*. Sample deposition indicates the solution spotted on the surface prior to analysis and Spray solvent indicates the composition of the DESI solvent during analysis

Addition method	Protein and ammonium bicarbonate distribution		S/N	HOCS	HICS
	Sample deposition	Spray solvent			
Control (Fig. 3a)	Protein	50% MeOH	36	+18	+13
I (Fig. 3b)	Protein WITH ABC	50% MeOH	174	+22	+18
II (Fig. 3c)	Protein THEN ABC	50% MeOH	149	+21	+18
III (Fig. 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 four times for mixing it with the protein prior to spotting (1) to seven times when adding it to the spray solvent (3) relative to a 50% MeOH control. The simplicity of adding ammonium bicarbonate to the spray solvent along with the highest resulting improvement in S/N makes this method the most convenient implementation, especially for ambient DESI experiments from natural samples, for example potentially during imaging experiments [61].

Figure 4 shows the S/N for formic acid and ammonium bicarbonate against protein size and pI for cyt *c*, hemoglobin, α-chymotrypsinogen, ovalbumin, and BSA, each analyzed at approximately 20 pmol/mm^2 on PE and deposited from solutions made in 50% MeOH. 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 cyt *c* and 2.3 times improvement for chymotrypsinogen, whereas S/N for hemoglobin and the albumins were enhanced by a factor of 1.8.

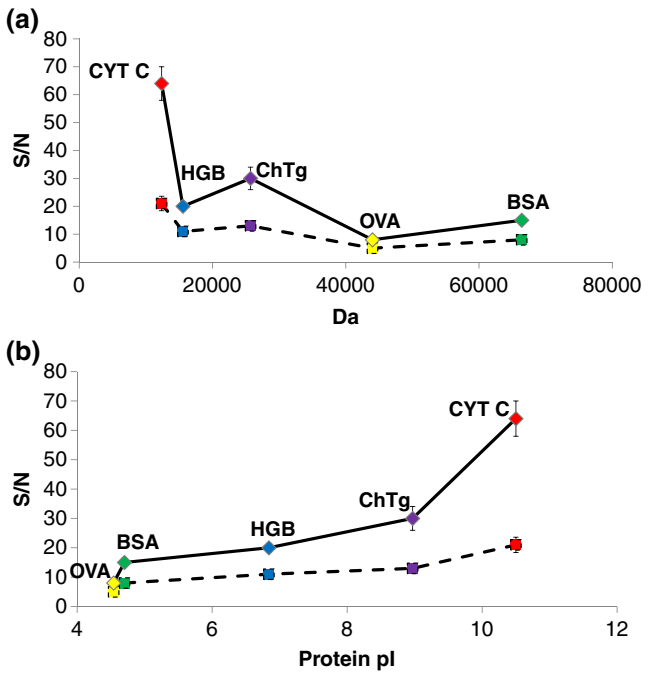

Figure 4. The dependence of S/N obtained for the HICS on (**a**) protein size, and (**b**) isoelectric point for the analysis of cytochrome *c*, hemoglobin, chymotrypsinogen, ovalbumin, and bovine serum albumin deposited from 50% MeOH onto PE by DESI-MS with the addition of ammonium bicarbonate (solid line) or formic acid (dashed line) to 50% MeOH as the spray solvent

The stronger effect in S/N improvement by ammonium bicarbonate on cyt *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 4b, 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 [62, 63]. With ammonium bicarbonate, the solution pH was 7.4 and therefore cyt *c* (pI = 10.5) was the only protein where the difference between pI and solution pH was larger than 3. In contrast, hemoglobin has a pI = 6.8, which is close to the solution pH and displayed only a relatively moderate improvement.

To further investigate the possible mechanism of improvement in S/N upon the addition of ammonium bicarbonate, different salts of the ammonium cation, including ammonium fluoride, ammonium acetate, and ammonium formate were added to the 50% MeOH DESI spray solvent. Corresponding acid forms were also investigated, namely formic acid, acetic acid, and carbonic acid.

For the ammonium salts shown in Table 2 and Figure 5, the extent of unfolding for cyt *c* deposited onto PE surfaces and analyzed by DESI-MS followed the reverse Hofmeister series described by Williams and Cassou [48] for ESI analysis of the same protein. Ammonium bicarbonate displayed the highest HICS and HOCS and mostly a denatured charge state envelope, followed by formate and fluoride for which bimodal envelopes were observed, whereas ammonium acetate, despite its position in the Hofmeister series, produced a native state-like envelope. This unusual behavior of ammonium acetate was previously described [48].

The relative change in intensities and S/N ratios obtained for the addition of each additive in the spray solvents were calculated compared with the additive-free control of 50% MeOH to account for inter-day variability in DESI-MS analysis.

While all the ammonium salts and acids increased the S/N ratios relative to the control, the highest S/N improvement was

Table 2. A Comparison of Different Additives in DESI-MS Spray Solvent. Intensities and S/N were normalized to the control to account for inter-day variability in DESI signal. Highest intensity- (HICS) and highest observed charge states (HOCS) are shown; *n* indicates the number of trails, each trail contains triplicate analyses where the average of 10 sample spots were determined. Raw data are shown in Figure 5

Additives in DESI	*n*	Rel. Int.	Rel. S/N	HICS	HOCS
No additive (control)	5	1	1	8	9
Ammonium formate	4	17 ± 7	3.0 ± 0.9	16	19
Ammonium acetate	4	13 ± 4	4.1 ± 1.0	7	8
Ammonium bicarbonate	5	46 ± 27	7.4 ±1.6	18	22
Ammonium fluoride	3	10 ± 2	3.6 ± 1.0	17	21
Formic acid	5	128 ± 77	3.3 ± 1.0	14	19
Acetic acid	3	703 ± 157	5.0 ± 1.0	15	19
Carbonic acid	3	0.6 ± 0.1	0.5 ± 0.03	9	17

obtained with the addition of ammonium bicarbonate to the DESI spray solvent. The ammonium salts generally produced similar S/N improvements as their respective acidic counterparts despite having much lower signal intensities, potentially hinting at the important role of NH_4^+ in adduct removal [42, 43], but also at the potential role of the common respective anions.

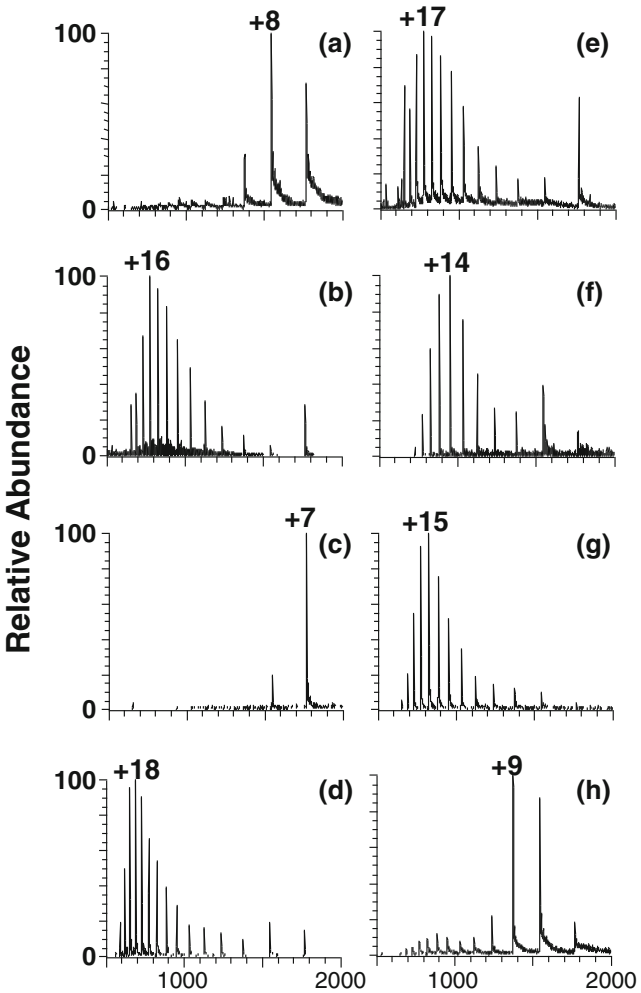

Figure 5. DESI spectra of cyt *c* deposited from water and analyzed with 50% MeOH containing (**a**) no additive (control), (**b**) ammonium formate, (**c**) ammonium acetate, (**d**) bicarbonate, (**e**) ammonium fluoride, (**f**) formic acid, (**g**) acetic acid, (**h**) carbonic acid. Data are summarized in Table 2

The outlier was carbonic acid, which reduced the signal intensity and produced a noisy result. This could be the consequence of relatively lower solution concentration of carbonic acid or that CO_2 outgassing during the spray process may have led to an unstable spray.

At a pH lower than pI, protein solubility follows a reverse Hofmeister series [56]. Protein solubility is likely important during both desorption and ionization processes in DESI-MS. According to the droplet pick-up mechanism [21], the first step during desorption is dissolution of the protein. The data in Table 2 show that the relative intensity increased from fluoride, to formate, to ammonium bicarbonate, while acetate was again the outlier here. A possible explanation for this increase in signal following the reversed Hofmeister series is increased solubility into the surface liquid layer, which has been observed to form on the sample surface during the initial dissolution stages of the DESI process. This liquid layer is removed by subsequent arriving droplets, from which ionization is believed to occur according to the droplet pickup model [64].

For the anions presented in Table 2, proton affinities are in the range of 1444–1554 kJ.mol^{-1} and increased in the order of formate < acetate < bicarbonate < fluoride. Interestingly, the S/N increased with proton affinity between formate (1444 kJ.mol^{-1}) and bicarbonate (1489 kJ.mol^{-1}) [65], but a lower S/N was obtained for fluoride (1554 kJ.mol^{-1}). The amount of sodium and acid adduction has been correlated with the proton affinities of anions present in solution during the ESI analysis of proteins, where it was shown that sodium adduction increases with the proton affinity while acid adduction decreases [39]. All the anions studied were also above the 1318 kJ.mol^{-1} transition where acid adduction has been observed to decrease for cyt *c*, while cation adduction increases with increasing proton affinity of solution additives [39]. Interestingly, a high extent of fluoride adduction was observed for the DESI analysis with ammonium fluoride in the spray solution and a S/N less than half compared with ammonium bicarbonate was obtained. Therefore, the increase in S/N could not be correlated directly with proton affinities by these experiments.

In DESI-MS, the addition of reagents to the spray solvent or applied to the sample surface could potentially influence both

the desorption and ionization processes and these effects will not necessarily be congruent. Consequently, to better understand the mechanism of enhancement in S/N achieved with ammonium bicarbonate addition, future investigations will separate the desorption and ionization processes in DESI by using spray desorption collection and reflective electrospray ionization [25, 26, 66] to further elucidate its mechanism of action.

Conclusions

Protein analysis by DESI-MS was shown to improve with the addition of ammonium bicarbonate to the spray solvent or during sample preparation. Ammonium bicarbonate outperformed other ammonium salts and related acids when added to the spray solvent. 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 such as Na^+ and Cl^- during the ionization process. The ammonium bicarbonate effect on S/N enhancement also appears to be protein–pI-dependent, which supports the role of adduct removal in its mechanism of action.

Ammonium bicarbonate addition led 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.

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