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**Effects of amino acid additives on protein solubility -  
insights from desorption and direct electrospray ionization  
mass spectrometry**

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Complete List of Authors:	Javanshad, Roshan; Western Michigan University, Chemistry Venter, Andre; Western Michigan University, Chemistry

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**Abstract**

Naturally occurring amino acids have been broadly used as additives to improve protein solubility and inhibit aggregation. In this study, improvements in protein signal intensity obtained with the addition of L-serine, and structural analogs, to the desorption electrospray ionization mass spectrometry (DESI-MS) spray solvent was measured. The results were interpreted at the hand of proposed mechanisms of solution additive effects on protein solubility and dissolution. DESI-MS allows for these processes to be studied efficiently using dilute concentrations of additives and small amounts of proteins, advantages that represent real benefits compared to classical methods of studying protein stability and aggregation. We show that serine significantly increases the protein signal in DESI-MS when native proteins are undergoing unfolding during the dissolution process with an acidic solvent system (p-value=0.0001), or with ammonium bicarbonate under denaturing conditions for proteins with high isoelectric points (p-value=0.001). We establish that a similar increase in the protein signal cannot be observed with direct ESI-MS and the observed increase is therefore not related to ionization processes or changes in the physical properties of the bulk solution. The importance of protein conformational changes during dissolution in the presence of serine is demonstrated through comparisons between the analyses of proteins deposited in native or unfolded states and by using native state-preserving and denaturing desorption solvents. We hypothesize that direct, non-covalent interactions involving all three functional groups of serine are involved in the beneficial effect on protein solubility and dissolution. Supporting evidence for a direct interaction include a reduction in efficacy with D-serine or the racemic mixture, indicating a non-bulk-solution physical property effect; insensitivity to the sample surface type or relative placement of serine addition; and a reduction in efficacy with any modifications to the serine structure, most notably the carboxyl functional group. An

alternative hypothesis, also supported by some of our observations, could involve the role of serine clusters in the mechanism of solubility enhancement. Our study demonstrates the capability of DESI-MS together with complementary ESI-MS experiments as a novel tool for understanding protein solubility and dissolution and investigating the mechanism of action for solubility-enhancing additives.

## Introduction

Understanding the mechanisms governing protein solubility and aggregation at the molecular level is of great importance to many fields including biochemistry, pharmaceutical sciences, and clinical studies.<sup>1-3</sup> Osmolytes are a diverse group of small molecules naturally selected to protect proteins against different stress factors while maintaining protein function<sup>4-5</sup> and have widespread applications in many fields.<sup>6-7</sup> Naturally occurring amino acids are amongst the osmolytes that have been studied for nearly four decades<sup>8</sup> for enhancing protein solubility and reducing protein aggregation<sup>9-11</sup>. The effect of arginine (Arg)<sup>12</sup> on suppressing protein aggregation<sup>13</sup> and the stabilizing effect of several other amino acids including glycine (Gly),<sup>14-15</sup> proline (Pro),<sup>16-20</sup> histidine (His),<sup>21</sup> alanine (Ala),<sup>22</sup> glutamic acid (Glu) - arginine mixtures,<sup>23</sup> and small amines<sup>24-25</sup> have demonstrated the ability of amino acid additives to stabilize proteins under stress conditions and to improve protein solubility by suppressing aggregation.

Despite decades of extensive studies, there is no unified theory that can explain how amino acids inhibit protein aggregation<sup>26</sup>. Progress has been made to successfully differentiate between the effects of additives on native versus unfolded or denatured structures.<sup>27</sup> Some additives help stabilize the structure of native proteins and therefore improve stability, whereas others may destabilize the native structure, or show little effect on protein structure but effectively suppress aggregation.<sup>28-32</sup> Amongst the proposed mechanisms, preferential interaction theory,<sup>33-36</sup> crowding

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3 effects<sup>37-38</sup> and/or the gap effect<sup>39</sup> have been widely studied. These proposed mechanisms have  
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5 predominantly been examined or modeled at high concentrations of additives (in  $\geq 50$  mM range)  
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7 in bulk solution by conventional biophysical techniques,<sup>31, 40-42</sup> and have not yet been studied at  
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9 micro-scale with techniques that can operate at lower quantities and concentrations.

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11 Desorption electrospray ionization mass spectrometry (DESI-MS) couples extractive-desorption  
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13 of the samples with electrospray ionization.<sup>43</sup> The analysis of sample in DESI occurs via the  
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15 droplet-pickup mechanism<sup>44</sup>, where charged droplets of solvent impact the sample, and a thin,  
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17 localized solvent layer is formed on the surface, dissolving the analytes. Progeny droplets  
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19 containing the extracted analyte are formed via subsequent droplet collisions and undergo  
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21 electrospray ionization processes.  
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27 DESI provides similar spectral information of proteins to what is obtained with ESI-MS<sup>45-47</sup> albeit  
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29 at lower intensities, and the technique especially struggles with larger proteins.<sup>45, 48</sup> Various  
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31 approaches have been developed to improve DESI analysis of proteins. For example coupling ion  
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33 mobility separation with DESI<sup>49-50</sup>, creating a very short sampling pathway<sup>51</sup> and using a pre-  
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35 wetting technique.<sup>52</sup> By deconstructing DESI and independently investigating protein desorption  
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37 and ionization, we confirmed that inefficient protein dissolution during the short time-scale of  
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39 DESI is a major contributor to the lower protein signal.<sup>48, 53</sup>  
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43 Non-specific protein aggregates in concentrated solutions or droplets, such as those generated by  
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45 electrospray are prevalent, and the formation of aggregates in both bulk solution and droplets due  
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47 to weak non-covalent interactions is well-established.<sup>54-55</sup> Aggregate numbers close to 20 were  
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49 observed in the subpopulation of droplets with apparent diameters near 220 nm and protein  
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51 concentration of 4  $\mu$ M, and the probability of higher aggregate numbers increase with higher  
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53 protein concentrations and larger droplet sizes.<sup>56</sup> Considering the thin, micro-localized solvent  
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layer on the sample surface, into which proteins are dissolved, and the size of DESI progeny droplets (average 1-4  $\mu\text{m}$ )<sup>57</sup>, aggregates can form extensively due to the high concentration of unfolding protein, resulting in protein signal reduction. The solubility of proteins in the desorption solvent and aggregate formation directly affects the efficacy of protein dissolution<sup>58</sup> in the desorbing solvent during the short DESI time-frame,<sup>59</sup> which provides a reasonable explanation of why the analysis of proteins by DESI-MS is inherently more difficult than small molecules.<sup>45, 48</sup>

Additives have been a convenient approach for improving protein solubility in DESI. We previously explored approaches such as gas-phase additives, for example vapors of ethyl acetate,<sup>60</sup> and solution-phase additives such as ammonium bicarbonate<sup>61</sup> and the amino acid L-serine<sup>62</sup> in order to enhance protein analysis by DESI-MS.

Applications of amino acid additives in ESI-MS of proteins have been explored relatively recently. The stabilizing effect of amino acids against thermal denaturation of non-covalent protein complexes in ESI-MS was reported and contributed to ion ejection and reduced columbic repulsion.<sup>63</sup> The stabilizing effect of amino acids and imidazole on protein-ligand ions against in-source fragmentation during ESI-MS has also been studied and reported as highly dependent on ESI conditions and instrumentation<sup>64</sup> and likely related to an evaporative cooling mechanism.<sup>65</sup>

It has been shown that amino acid additives, L-serine in particular, can improve protein analysis by removing sodium adducts from high NaCl concentration in the protein sample during native ESI<sup>66</sup> and DESI.<sup>62</sup> Since DESI progeny droplets follow the same ionization process as ESI droplets,<sup>44</sup> the desalting effect of L-serine, which was observed in both types of experiment, is likely related to the electrospray ionization process. On the other hand, L-serine also increased signal intensity of purified proteins in DESI-MS when combined with formic acid or ammonium bicarbonate as co-additives, but no such effect was observed in ESI-MS of similar protein

solutions, indicating that this effect is independent of electrospray ionization processes and related to protein dissolution and desorption.

With DESI-MS and ESI-MS the analysis of proteins occurs via the same ionization mechanisms, therefore, relative changes in signal between these two techniques provide a window into studying protein dissolution and desorption.<sup>48, 53</sup> With DESI, the short but controllable interaction time of proteins with denaturing solvents and additives before detection allows one the unique opportunity to study protein unfolding during dissolution, while in non-native ESI, unfolding has already occurred during sample preparation, long before protein detection. In this study we interpret relative changes in protein signal intensities as protein dissolution effects, after controlling for possible ionization differences using comparable experiments in ESI. We demonstrate that mechanistic insights into the effects of solution-phase additives on proteins can be obtained at much lower additive concentrations and protein amounts, compared to most biophysical techniques that are commonly used for this purpose.

**Experimental**

**Materials**

Lyophilized equine heart cytochrome *c* (Cyt *c*, 12.3 kDa, pI=10.3) and equine muscle myoglobin (Myo, 16.9 kDa, pI=7.4) were purchased from Sigma-Aldrich (St. Louis, MO). Proteins were at ≥95% purity and were used without further purification. BioUltra grade ammonium bicarbonate (ABC), ammonium acetate (NH<sub>4</sub>OAc), A.C.S. grade ammonium hydroxide (NH<sub>4</sub>OH), HPLC-MS grade methanol (MeOH), LC-MS grade formic acid (FA) and all amino acids (≥98%) were purchased from Sigma-Aldrich (St. Louis, MO) except N-acetyl L-serine which was purchased from Carbosynth (Berkshire, UK). Milli Q water was obtained from a Thermo-Barnstead Water

Polisher. Fused silica capillaries were purchased from Trajan Scientific (Ringwood, Australia). Porous polyethylene surfaces (PE) with average pore size of 15-45  $\mu\text{m}$  (POREX-4900) were purchased from Interstate Specialty Products (Sutton, MA). PTFE plates were purchased from Prosolia Inc (Indianapolis, IN).

### Sample preparation and solvent systems

Stock solutions of each protein were made by dissolving the lyophilized protein powder in Milli Q water to a final concentration of 800  $\mu\text{M}$  or 80  $\mu\text{M}$  depending on the desired surface concentration. Protein solutions were sprayed on the surface with a pneumatically-assisted nebulizer spray made of two coaxial fused silica capillaries similar to an ESSI sprayer.<sup>67</sup> The sprayer was orthogonally positioned at 3 mm above the surface. Nebulizing gas pressure and flow rate were 100 psi and 3  $\mu\text{l}/\text{min}$  respectively, and the stage was moved at 150  $\mu\text{m}/\text{s}$ , resulting in long homogenous protein lines deposited with  $1.1 \pm 0.1$  mm widths. The average surface concentration of these protein lines was approximately 22  $\text{pmol}/\text{mm}^2$  for all DESI experiments with the exception of spectra in Figure 1 and Figure S1-S2 which were obtained from protein lines with an approximate surface concentration of 10X higher (222  $\text{pmol}/\text{mm}^2$ ) in order to obtain clear and comparable spectra for all different solvent systems regardless of the inherently lower sensitivity of the non-acidic solvents. For PTFE slides, 3  $\mu\text{l}$  volumes of 20  $\mu\text{M}$  aqueous protein solutions were micro pipetted on the slide and dried under vacuum for 30 minutes at approximately 85 kPa. The diameter of the dried spot was approximately 1.2 mm, resulting in average protein surface concentration of 24  $\text{pmol}/\text{mm}^2$ . ESI experiments used 10  $\mu\text{M}$  protein in the appropriate solvent systems.

All DESI desorption solvent systems were made in 50% MeOH:H<sub>2</sub>O. Aqueous stock solutions of 2.0 M ammonium bicarbonate and 2.0 M ammonium acetate were used to prepare 200 mM

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3 dilutions in 50% MeOH (approximately pH 8.0 and 7.3, respectively). LC-MS grade formic acid  
4 and ammonium hydroxide were used to prepare 0.1% (v/v) formic acid and 1% (v/v) ammonium  
5 hydroxide in 50% MeOH (approximately pH 3.0 and 10, respectively). Serial dilutions from  
6 aqueous amino acid stock solutions were used to make 100  $\mu$ M amino acid in the solvent system.  
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8 The pH of aqueous solutions was measured at room temperature with a Mettler Toledo Seven Easy  
9 pH meter (Columbus, OH) equipped with an InLab Expert pH electrode. Similar to previous  
10 relevant studies,<sup>68</sup> the reported pH values were not corrected for the influence of MeOH as it was  
11 deemed inconsequential to the experiments.  
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### 21 **Instrumentation and experimental parameters**

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23 A linear ion trap mass spectrometer, LTQ (Thermo Scientific, Waltham, MA, USA) was  
24 combined with a 3-dimensional translational stage (Purdue University, West Lafayette, IN, USA)  
25 for DESI analyses. An extended ion sampling capillary with a 5 cm extension was purchased from  
26 Scientific Instruments Inc. An electrospray emitter and desorption sprayer was prepared from a  
27 Swagelok T-piece and two pieces of coaxial fused silica capillary tubing.<sup>67</sup> The outer capillary (for  
28 sheath gas) was approximately 20 mm in length with an outer diameter of 430  $\mu$ m and inner  
29 diameter of 320  $\mu$ m. The internal capillary (for solvent) had an outer diameter of 220  $\mu$ m, and  
30 inner diameter of 50  $\mu$ m. The solvent capillary extended through the T-piece and was connected  
31 to a syringe pump which delivered the solvent.  
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44 DESI sprayer incident angle was 54°. The distance between the desorption sprayer and heated  
45 extended capillary was 4 mm. The tip of the desorption sprayer was 1 mm above the surface. ESI  
46 experiments were performed under the same conditions as DESI, but protein solutions were  
47 directly sprayed towards the heated extended capillary at 5  $\mu$ L/min with 100 psi N<sub>2</sub> using the same  
48 sprayer.  
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Spray potential was set at 4.0 kV and was applied to the liquid junction of a stainless-steel syringe needle which delivered solvent at a flow rate of 5  $\mu\text{L}/\text{min}$ , with  $\text{N}_2$  as nebulizing gas at 100 psi. Capillary temperature was set at 250°C. LTQ ion optic parameters were optimized by LTQ TunePlus automatic tuning feature for each protein's highest intensity charge state (HICS) peak, using direct infusion of 5  $\mu\text{M}$  protein in each solvent system.

### **Data Analysis**

Mass spectra were collected and viewed in Xcalibur Qual Browser (2.0.7). At least three independent trials were collected for each solvent system. In each trial three protein sample lines or spots were perpendicularly scanned and averaged. Signal intensities were calculated as the average of three trials and error bars represent  $\pm$  mean standard deviation. MagTran software (1.03) was used for charge state deconvolution and calculation of integrated protein signal intensity and S/N as described by Zhang and Marshall.<sup>69</sup>

## **Results and discussion**

### **Influence of solvent composition on protein signal increase by L-serine**

Previously, we showed that the addition of low millimolar L-serine concentrations into 50% MeOH:H<sub>2</sub>O desorption solvent can improve sensitivity for protein detection by DESI-MS in the presence of 1 mM and higher concentrations of NaCl,<sup>62</sup> similar to observations reported in native ESI-MS experiments.<sup>66</sup> However, L-serine also increased signal intensity of purified proteins in DESI-MS when combined with formic acid or ammonium bicarbonate as co-additives, but no such effect was observed in ESI-MS of similar protein solutions. To investigate the role of solvent composition on the mechanism governing increased protein signal in DESI-MS with addition of L-serine, the combination of L-serine with the following four solvent systems were compared:

formic acid, ammonium acetate, ammonium bicarbonate, and ammonium hydroxide. All solvents were made in 50% MeOH:H<sub>2</sub>O and therefore 50% MeOH:H<sub>2</sub>O was treated as additive-free control. Out of the five different solvent systems, only the formic acid containing solution showed an increase in protein signal with L-serine addition for both cytochrome *c* and myoglobin. However, L-serine was also able to increase the signal intensity for cytochrome *c* in ammonium bicarbonate containing solutions, as seen in Figure 1. A complete set of representative spectra for all five solvent systems, and changes in protein signal intensities with L-serine addition, can be found in Figure S1 and Table S1.

An important consequence of changing solvent additives is the solution pH and the effect this can have on the protonation state of sample species. Serine was added into solvent systems that spanned a pH range of 3 to 10. The estimated protonation populations of serine and the protein net charge in the different pH ranges can be found in Table S2. Although the solution pH can affect serine protonation state, and therefore population ratios of different serine species in the solution, in all solvent systems except ammonium hydroxide, zwitterionic serine is the predominant form. No correlation between desorption solvent pH and increased protein signal intensities with L-serine was observed.

The other important consequence of changing solvent composition is protein conformational changes. Lower charge states, indicative of more folded protein conformations, were observed with desorbing solvents containing 50% MeOH, and when ammonium acetate or ammonium hydroxide was added into this solvent (Figure S1). In ammonium acetate and ammonium hydroxide the change in protein signal intensities with serine addition were not statistically significant ( $p$ -value>0.05) and in the additive-free solvent system the signal even deteriorated. Signal suppression in the additive-free solvent system is likely due to extensive formation of

nonspecific adducts and spreading of the protein signal over multiple peaks which causes a reduction in the signal intensity of the highest intensity charge state (HICS). As can be observed in Figure S1 (d) and (f) for myoglobin, the extensive overlapping peaks resemble non-specific adduction of small molecules to proteins<sup>70-71</sup>.

In contrast, protein signal intensities increased by a factor of  $3.8 \pm 0.1$  for cytochrome *c*, and by a factor of  $4.3 \pm 0.3$  and  $3.5 \pm 0.2$  for apo-myoglobin and holo-myoglobin, respectively, with addition of L-serine to the desorption solvent containing 0.1% formic acid (Figure 1 (c,d)). Similarly, with the addition of L-serine to 200 mM ammonium bicarbonate as desorption solvent, cytochrome *c* signal intensity increased by a factor of  $3.1 \pm 0.2$  (Figure 1 (e,f)). The signal intensity for myoglobin, on the other hand, signal increased only by a factor of  $1.2 \pm 0.2$  (Figure 1 (g,h)), which was not statistically significant ( $p\text{-value} > 0.05$ ). Extensive unfolding was also observed for both proteins, despite the near-neutral pH of the solvent system and buffering by ammonium bicarbonate. It was previously proposed that protein unfolding with ammonium bicarbonate containing solutions during ESI is likely due to protein destabilization inside the heated electrospray droplets, either because of an increase in hydrophobic surface area by bubble formation, or due to electrothermal supercharging.<sup>72-73</sup>

A comparison between the two proteins suggests that this difference could be related to protein net charge in solution. Cytochrome *c* and myoglobin are both single-chain peptides of similar size (12.3 kDa and 16.9 kDa respectively), but their isoelectric points are approximately 10.3<sup>74</sup> and 7.4<sup>75</sup>, respectively. This difference in isoelectric points results in myoglobin having a negative to near neutral net charge in ammonium bicarbonate solution, whereas cytochrome *c* has a positive net charge. Likewise, protein net charge is an important factor in determining the effect of Hofmeister series and osmolytes on protein solubility.<sup>76-77</sup>

Therefore, as evident from a comparison between the behaviors of the two proteins in the four desorbing solvent systems, a denaturing desorbing solvent and protein with a net positive charge are key factors in the mechanism governing the substantial improvement in protein signal with L-serine.

**Role of protein conformational change and serine addition timepoint**

To investigate the role of protein conformation prior to the interaction with L-serine, cytochrome *c* and myoglobin were spray-deposited out of aqueous solution (native-like conformation) or out of 0.1% formic acid solution (unfolded conformation) and analyzed with a desorption spray solvent containing 0.1% formic acid in 50% MeOH in the presence or absence of 100  $\mu$ M L-serine. As seen in Figure 2, for both cytochrome *c* and myoglobin signal intensities of proteins deposited in native conformation (top panel) increased more with serine addition than proteins already unfolded (bottom panel). The signal intensity for natively deposited cytochrome *c* and apo-myoglobin increased by a factor of  $2.6\pm0.3$  and  $3.8\pm0.8$ , respectively. Despite using 50% MeOH and formic acid as the desorbing spray solvent, holo-myoglobin signal was also observed for the natively deposited sample and increased by a factor of  $3.5\pm0.2$  with serine addition, as seen in Figure 2 (b). When the samples were deposited out of denaturing solution, serine addition achieved only mild increases in signal of  $1.3\pm0.3$  and  $1.5\pm0.4$  for cytochrome *c* and apo-myoglobin with *p*-values 0.098 and 0.042, respectively (Figure 2 (g,h)).

In the previous section we showed the importance of denaturing solvent systems for the beneficial effect of serine addition on signal intensities. The difference in relative improvements between native and unfolded protein prior to analysis further indicates that serine should be present during the unfolding process.

A similar increase in signal intensity does not occur under denaturing conditions upon the addition of L-serine when proteins are analyzed in solution using direct ESI infusion.<sup>62</sup> Figure 3 shows 10  $\mu\text{M}$  of each protein, electrosprayed out of the exact solvent systems as for the DESI results in Figure 1 (a) and (b) and Figure 2 (0.1% formic acid in 50%MeOH). The order of adding formic acid and L-serine was also alternated to measure the effect of unfolding in bulk solution in the presence of L-serine. Addition of 100  $\mu\text{M}$  L-serine to the working solution before and after protein unfolding through acidification made no meaningful difference in protein ESI signal intensity, although the changes in absolute signal intensity between adding serine first was statistically significant ( $p\text{-value}<0.05$ ) for cytochrome *c*. Adding L-serine prior to acidification in bulk solution increased the signal for cytochrome *c* by a mere 1.3 times compared to when it was not present ( $p\text{-value}=0.001$ ). On the other hand, when serine was added after formic acid addition to the cytochrome *c* solution, there was no statistically significant change compared to not having serine in the solution at all ( $p\text{-value}>0.05$ ). In the case of myoglobin, addition of serine reduced the absolute signal intensity compared to no serine present, and there was no statistical difference between adding serine before or after formic acid ( $p\text{-value}=0.56$ ), as seen in Figure 3. The lack of improvement in protein signal intensity in ESI reiterates that the improvement in protein signal intensity in DESI is likely related to the dissolution of proteins during desorption. We have previously shown that proteins desorb equally well from the surface in DESI, and poor dissolution, rather than physical desorption or ionization problems are the major contributor to poor DESI-MS signals for protein.<sup>48, 53</sup> The lack of improvement in protein signal with L-serine in ESI (Figure 3) and the difference between timescales of unfolding and dissolution to ion detection in DESI versus ESI possibly point towards affecting the kinetics of dissolution, rather than a thermodynamic effect on solubility. These observations together imply that dissolution of native proteins in denaturing

solvents are positively influenced in the presence of L-serine as a solution additive, manifested by an increase in protein signal intensity measured by DESI-MS.

**Relating DESI observations with known models for suppression of protein aggregation by amino acids**

One of the most consequential repercussions of protein unfolding is aggregation. Mechanisms of protein unfolding and subsequent aggregation can involve non-covalent weak interactions such as hydrophobic and electrostatic interactions, hydrogen bonding, Van der Waals interactions, or covalent disulfide bonds.<sup>78</sup> Many studies support the proposed mechanism that amino acid additives can improve protein solubility by suppressing aggregation of unfolded species or partially unfolded intermediates.<sup>79-80</sup> This has been demonstrated using in-vitro and in-silico studies, by NMR, X-ray spectroscopy, and crystallography, often implicating direct binding to folding intermediates.<sup>12, 41, 81-83</sup>

The exact mechanism of action for the prevention of aggregation by additives including amino acids is yet under debate,<sup>84</sup> but here we place our observations from DESI-MS and ESI-MS experiments into context of two widely studied theories on additive effects on protein solubility. *Preferential interaction theory*<sup>33-36</sup> measures changes in thermodynamic properties such as interaction of protein surface with additive and water in bulk solution. Based on this model, additives that stabilize protein such as most osmolytes, including a number of amino acids, are preferentially excluded from the protein surface and can influence protein folding equilibrium.<sup>85</sup> Other additives, such as urea and arginine that are not excluded from the surface of the protein and are weakly bound, do not stabilize native protein structure, but improve protein solubility through aggregation suppression.<sup>85-86</sup> In our data, L-serine did not shift the protein charge state distribution

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3 to those representing more folded charge states, and also destabilized myoglobin (Figure 2). Chen  
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5 *et al.* have reported similar observations in ESI-MS of myoglobin in 20 mM ammonium acetate  
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7 buffer with L-serine.<sup>63</sup> Based on these observations and according to the preferential interaction  
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9 theory, we propose that L-serine behaves as a destabilizing additive, and similar to arginine, binds  
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11 to the protein through weak interactions to suppress protein aggregation.  
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17 *The gap effect theory* differentiates between reversible or irreversible aggregation and non-native  
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19 or native aggregation and has been proposed based on results from computational models and  
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21 molecular dynamic simulations.<sup>39</sup> Based on this model, as two protein molecules associate, a gap  
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23 is formed in which an amino acid additive<sup>87</sup> is considered neither preferentially bound nor  
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25 completely excluded from the protein surface (known as a neutral crowder), while allowing water  
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27 molecules to interact with proteins. Such additives increase the free energy of protein–protein  
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29 molecules to interact with proteins. Such additives increase the free energy of protein–protein  
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31 association and slow aggregation, not because of changes in thermodynamic properties but based  
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33 on a kinetic effect on protein association during aggregation. Comparison between Figure 2 and  
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35 Figure 3 supports the hypothesis that L-serine is perhaps affecting the kinetics of protein  
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37 aggregation during unfolding which can be captured due to the short timescale of protein  
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39 dissolution in DESI compared to the time between sample preparation and analysis by direct ESI.  
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41 Recent studies have reported more evidence for direct interaction between amino acid additives  
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43 and protein<sup>42, 88-89</sup> including FTIR spectroscopy and molecular dynamic simulations that show a  
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45 direct interaction between proline and lysozyme<sup>42</sup> and X-ray crystallography study that show  
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47 binding of glycine amide to hydrophobic residues of lysozyme.<sup>81</sup> Non-covalent binding of multiple  
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49 amino acids to native and denatured cytochrome *c* in ESI-MS has also been reported.<sup>90</sup> It was  
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51 proposed that the formation of stable non-covalent complexes with these amino acids depend on  
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stable ionic interactions and successful formation of hydrogen bonds with specific charged residues of the protein, especially those with the least steric and electrostatic repulsion between amino acid additive and the protein. We also observed L-serine adducts with  $S/N > 3$  on charge states +7 to +12 at low temperatures (70°C) in both ESI-MS and DESI-MS (Figure S2). The presence of L-serine adducts on protein peaks in the denaturing solvent 0.1% formic acid in 50% MeOH provides evidence that interactions between unfolding protein and L-serine is possible. In the following sections, we further explore the possible direct interactions between L-serine functional groups and the protein.

**Investigating serine-surface interactions involved in mechanism of signal enhancement**

During the unfolding process, it is possible that the denaturing protein can bind more tightly with the surface as well as with other proteins in solution, due to exposure of a larger surface area of either hydrophobic or polar amino acids from the protein core. To evaluate the potential role of serine disrupting protein-surface interactions, the more hydrophilic PE surface, that was used for the data presented in Figure 2 (a), was replaced with the more hydrophobic surface of porous PTFE which has a different polarity and dielectric constant. As shown in Figure 4 (a), changing the identity of the surface to the more hydrophobic PTFE did not influence the extent of protein signal increase with the addition of L-serine to the solvent.

Additional evidence for eliminating surface interactions from contributing to the beneficial L-serine effect is demonstrated in Figure 4 (b). To evaluate the potential for L-serine to disrupt protein surface interactions, L-serine was added to the surface before protein deposition, after protein deposition and premixed into protein stock solution. As demonstrated in Figure 4 (b), there was no statistical difference between these different methods of serine application ( $p\text{-value} > 0.05$ ),



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3 although it could be argued that there is considerable mixing occurring on the sample surface  
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5 during the turbulent DESI analysis. Based on these observations, it is unlikely that protein-surface  
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7 interactions play a dominant role in the beneficial effect serine has on protein signal with DESI-  
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9 MS. Moreover, since similar improvements were observed with serine either in the desorbing spray  
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11 or when applied to the sample prior to desorption confirms that the improvement in signal intensity  
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13 is not due to changes in physical parameters, such as surface tension of the bulk desorption solvent.  
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17 Curiously, having L-serine in the aqueous protein stock solution prior to sample deposition did  
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19 not yield any improvement in protein signal intensity. When L-serine is mixed with the protein  
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21 stock solution prior to the analysis, similar to ESI experiments, L-serine comes into contact with  
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23 protein in bulk solution (fully hydrated protein), whereas for every other sample in Figure 4 (b),  
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25 the L-serine point of contact with protein starts with the dried protein dissolving into the desorption  
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27 spray micro-localized layer (during the hydration process). The observations from Figure 4 (b)  
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29 suggest that interaction between L-serine and the unfolding protein during the dissolution process  
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31 results in the observed increases in protein signal intensity.  
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### 37 **Investigating possible intermolecular interactions of serine with protein during solvation of** 38 **unfolding protein** 39 40

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42 After eliminating the possibility of L-serine interrupting protein-surface interactions, we  
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44 investigated L-serine-protein interactions through modifying L-serine structure, including  
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46 stereochemistry of the side chain and blocking the functional groups, as well as comparisons  
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48 between similar L-amino acids with the hydroxyl group side chain.  
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51 The least benign modification could be changing the L enantiomer to D. As Shown in Figure 5,  
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53 D-serine is not as effective as L-serine in improving protein signal and the racemic mixture of  
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serine was only slightly more effective than D-serine. This observation again suggests that the improvement in protein signal is not due to changes in the physical properties of the solvent such as surface tension, but perhaps the consequence of direct interactions between the additive and protein. The role of stereoisomer also implies that a chiral interaction is part of the interaction between protein and L-serine. Chiral recognition has been shown to play a role in weak interactions and hydrogen bonding<sup>91</sup> and also with chiral solute-water interaction in solutions.<sup>92</sup> The chirality of L-serine can potentially affect the hydrogen bonding, especially with the peptide backbone of the protein which has the greatest potential for hydrogen bonding and is sterically influenced by the chirality of the amino acids.<sup>93</sup>

Surprisingly the improvement in signal with a racemic mixture of serine was no more effective than D-serine, indicating that perhaps higher-order serine clusters are involved in the effect. Some precedent has been set for the role of clusters of both arginine and proline as protein aggregation suppressors.<sup>82, 94</sup> It is a possibility that L-serine clusters can affect protein aggregation through modulating protein-protein interactions in a similar manner. Although in our experiments the peak intensities of these higher-order serine clusters were not dominant, it is fascinating that serine, to an extent not reached by any other amino acid, forms unusually stable clusters, in particular serine octamer, with a remarkable preference for homochirality.<sup>95 96</sup> Recent studies have validated that serine clusters indeed exist not only in the gas-phase, but also in solution, although at low concentrations.<sup>97-98</sup>

In Figure 6, the importance of each functional group on L-serine in its potential direct interaction with the unfolding proteins was investigated. We systematically modified the three functional groups on the serine molecular structure and compared the effects on protein analysis of these derivatives to L-serine. For both cytochrome *c* and myoglobin, any modifications on L-serine

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3 molecular structure resulted in a reduction of improvement in protein signal. Removing the  
4 hydroxyl group from L-serine (by using L-alanine) had the least deleterious effect on signal  
5 enhancement, and still improved the signal intensity, albeit not as effectively as L-serine. The  
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7 strongest deleterious effect was observed with blocking the carboxylate group, noting the  
8 importance of this functional group in the interactions between L-serine and protein. Similarly,  
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10 FTIR and molecular dynamic simulations have also reported that the carboxyl group of proline  
11 plays a dominant role in direct proline-protein interactions.<sup>42</sup> Earlier we demonstrated that L-serine  
12 is much more effective in improving protein signal intensity of unfolding protein when the protein  
13 has a net positive charge (Table S2). One could speculate that the interaction between the  
14 negatively charged carboxyl group on the zwitterionic serine and positive charges on the protein  
15 contributes to the mechanism of protein dissolution improvement.  
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28 The observation that all three functional groups of L-serine are important in the beneficial  
29 interaction with protein during dissolution can support one of two hypotheses: Zwitterionic L-  
30 serine could potentially have a direct interaction that involves all 3 functional groups interacting  
31 with positively charged unfolding proteins; Alternatively, homochiral serine clusters might be at  
32 play, as evidenced by lack of efficacy of racemic serine mixtures, and the potential for any  
33 structural changes to also affect clustering. To elucidate the exact points of interactions on protein,  
34 detailed investigations with complementary techniques such as molecular modeling is needed.  
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## 47 **Conclusion**

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49 We demonstrated the application of DESI-MS combined with ESI-MS as a novel approach for  
50 probing the mechanism of solubility-enhancing additives such as amino acids by using L-serine as  
51 a model additive. The effects of L-serine on signal intensity were investigated by using five  
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different native-state preserving and denaturing solvent systems, changing the protein conformation prior to interaction with the additive, and measuring changes in protein signal during unfolding in the bulk solution before and after addition of L-serine, versus unfolding during dissolution. These results were interpreted at the hand of existing models of amino acid action on protein solubility. Our results indicate that protein-surface interaction interruption is unlikely to contribute to improvements in protein signal enhancement. We hypothesize that L-serine potentially influences protein-protein interactions by acting as a destabilizing neutral crowder and by suppressing aggregation when it is present during unfolding of proteins carrying a net positive charge in solution. Our observations could be explained by a possible direct, noncovalent, chiral, three-pronged interaction between L-serine and the protein. Alternatively, it is possible that serine clusters could be involved in the dissolution enhancing effect.

DESI-MS studies provide a novel perspective for understanding the mechanisms governing the effects of additives on protein dissolution, solubility, and aggregation. Complementary DESI-MS and direct ESI-MS experiments under controlled conditions allow differentiating between thermodynamic and kinetic effects of additives on solubility and dissolution. Integration of other complimentary tools such as ion mobility and delayed desorption with DESI-MS will enable time-resolved analysis of protein dissolution processes and aggregate measurement in the presence of additives. In addition to providing new insights into mechanisms of different additive effects, even at low concentrations, this novel perspective can be a useful tool for rapid development of additives important for protein chemistry and its applications, such as protein therapeutics and formulation.

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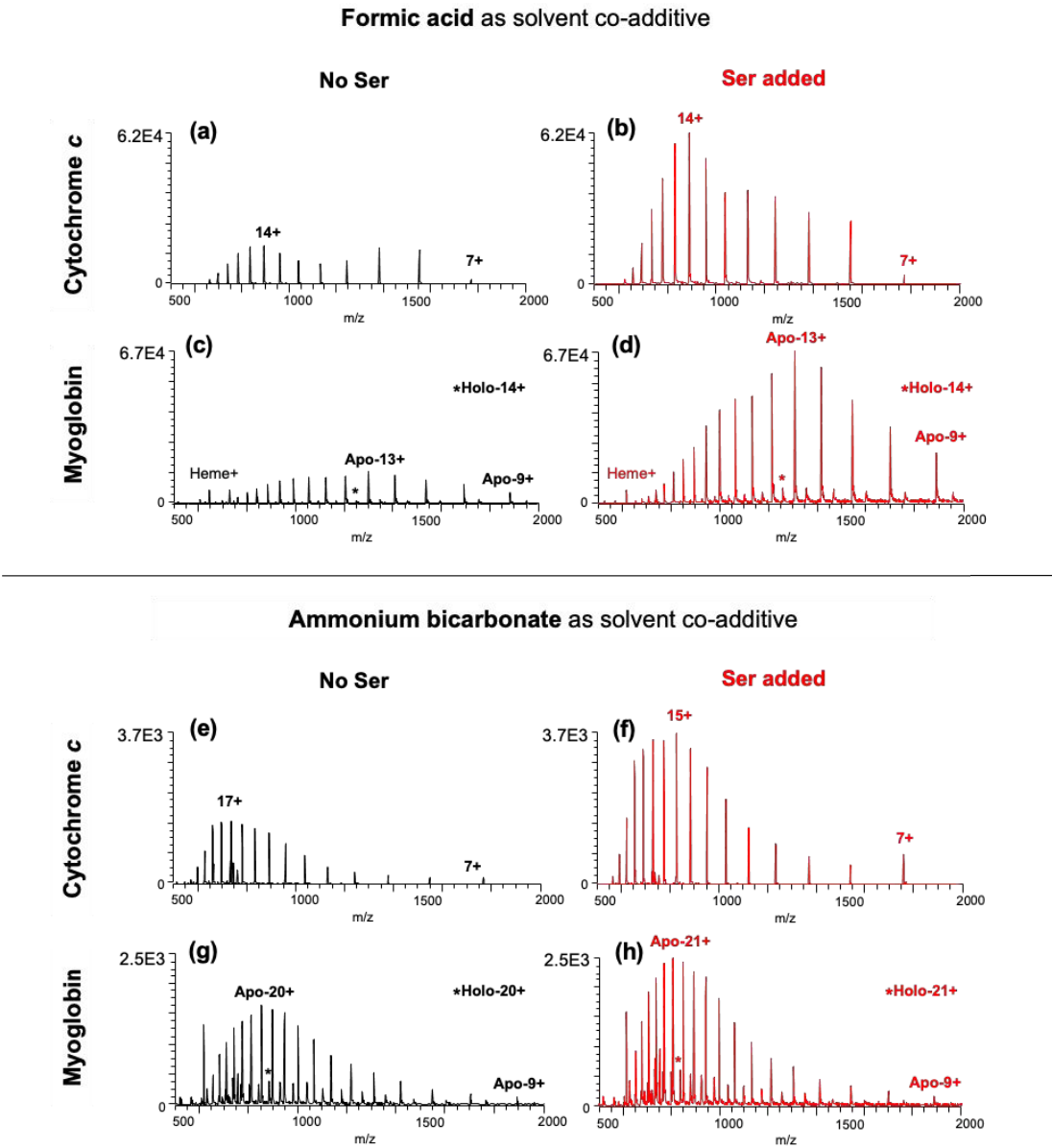
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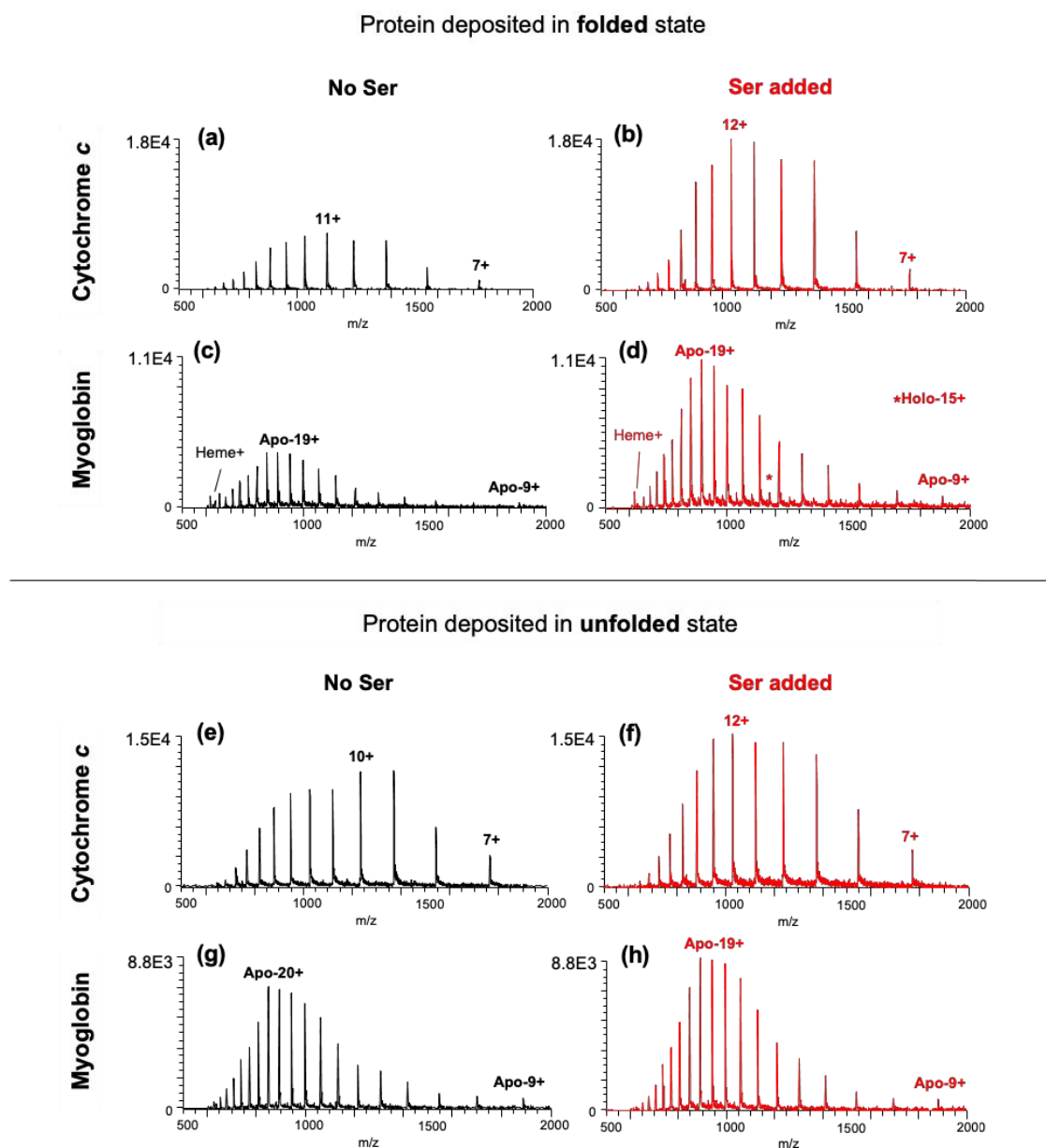
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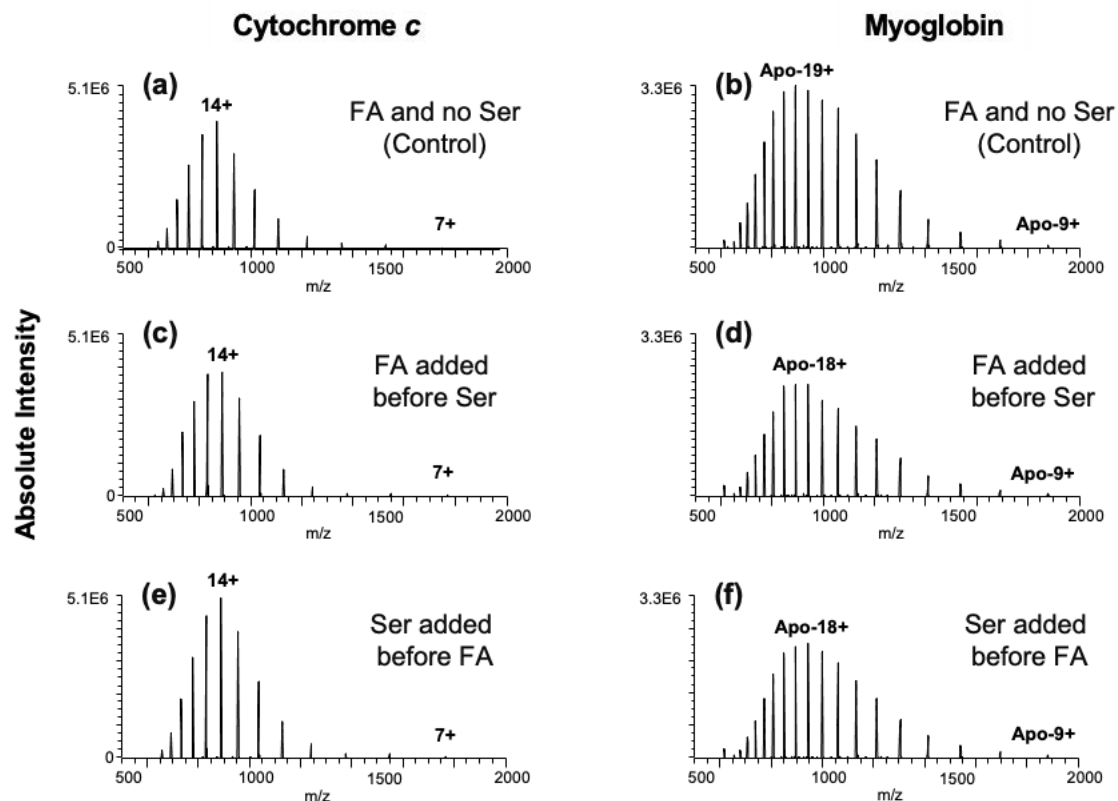
**Figure 1.** Representative DESI-MS spectra of natively deposited cytochrome *c* and myoglobin analyzed without L-serine (shown in black) and with 100  $\mu$ M L-serine (shown in red). (a), (c) 0.1% formic acid in 50% MeOH without L-serine, and (b), (d) with 100  $\mu$ M L-serine. (e), (g) 200 mM ammonium bicarbonate in 50% MeOH without L-serine, and (g), (h) with 100  $\mu$ M L-serine. The absolute intensities reported are the average intensity of the highest observed charge state (HICS) for the protein in each desorbing solvent system.



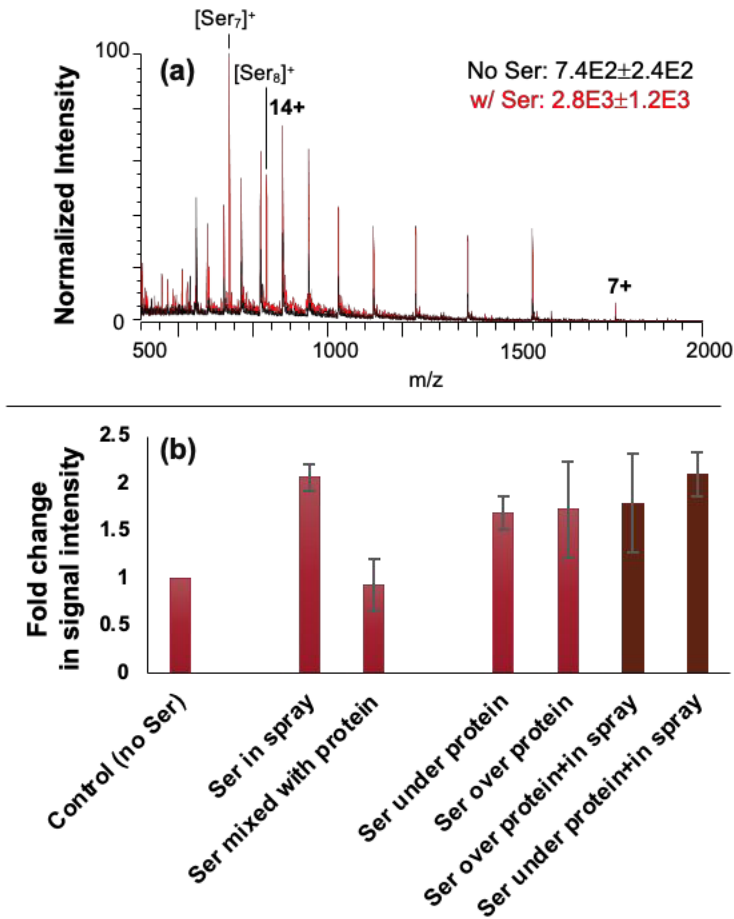
**Figure 2.** Representative DESI-MS spectra of cytochrome *c* and myoglobin deposited from (a-d) aqueous solution vs. (e-h) acidic solution, analyzed with 0.1% formic acid in 50%MeOH (shown in black) and with 100  $\mu$ M L-serine added (shown in red). The intensities are scaled to the average intensity of highest observed charge state (HICS) for each protein when analyzed with 100  $\mu$ M L-serine. This signal change was not statistically significant for denatured-deposited cytochrome *c*

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(p-value>0.05) and only marginally significant for denatured-deposited myoglobin (p-value 0.0420).

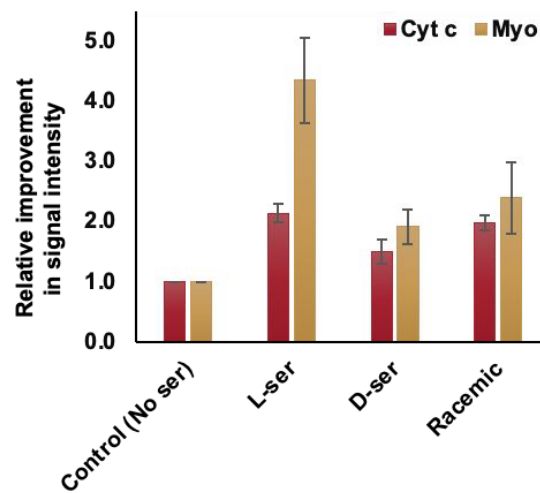


**Figure 3.** Representative ESI spectra of cytochrome *c* and myoglobin unfolding in bulk solution containing (a), (b) 0.1% formic acid only, (c), (d) containing 0.1% formic acid and 100  $\mu$ M L-serine with formic acid added first followed by serine after mixing to the protein solution, and (e), (f) containing 0.1% formic acid and 100  $\mu$ M L-serine with L-serine added first to the protein solution followed by formic acid.

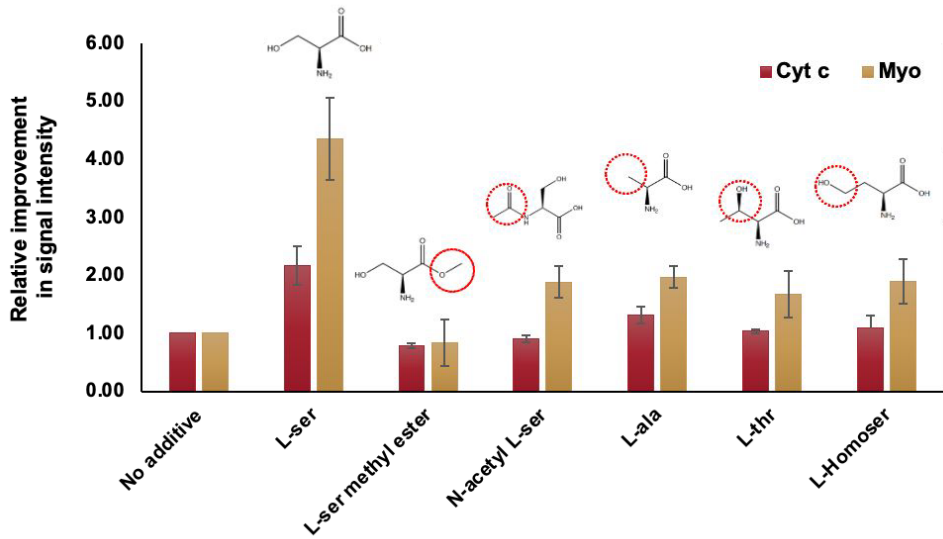


**Figure 4.** (a) Representative DESI-MS spectra of aqueous cytochrome *c* deposited on PTFE slides and analyzed with 0.1% formic acid in 50% MeOH (shown in black), and with 100  $\mu$ M L-serine (shown in red). The reported intensities are the average intensity of the highest intensity charge state (HICS) for cytochrome *c*. The equivalent PE data is found earlier in Figure 2 (a). (b) Signal intensity of cytochrome *c* on PE analyzed with 0.1% formic acid in 50% MeOH (control) and 100  $\mu$ M L-serine in different places during the DESI-MS analysis.





**Figure 5.** Comparison between serine enantiomers and the racemic mixture when used as solvent additives for analysis of natively deposited proteins with 0.1% formic acid in 50% MeOH.



**Figure 6.** L-serine derivatives with systematically altered functional groups as additives and their effect on signal intensities of natively deposited cytochrome *c* and myoglobin with 0.1% formic acid in 50% MeOH. The modifications are highlighted in red circles and include methylation of the hydroxyl group, amidification of the primary amine of serine, a secondary versus primary hydroxyl or amine, and moving the hydroxyl group further away.

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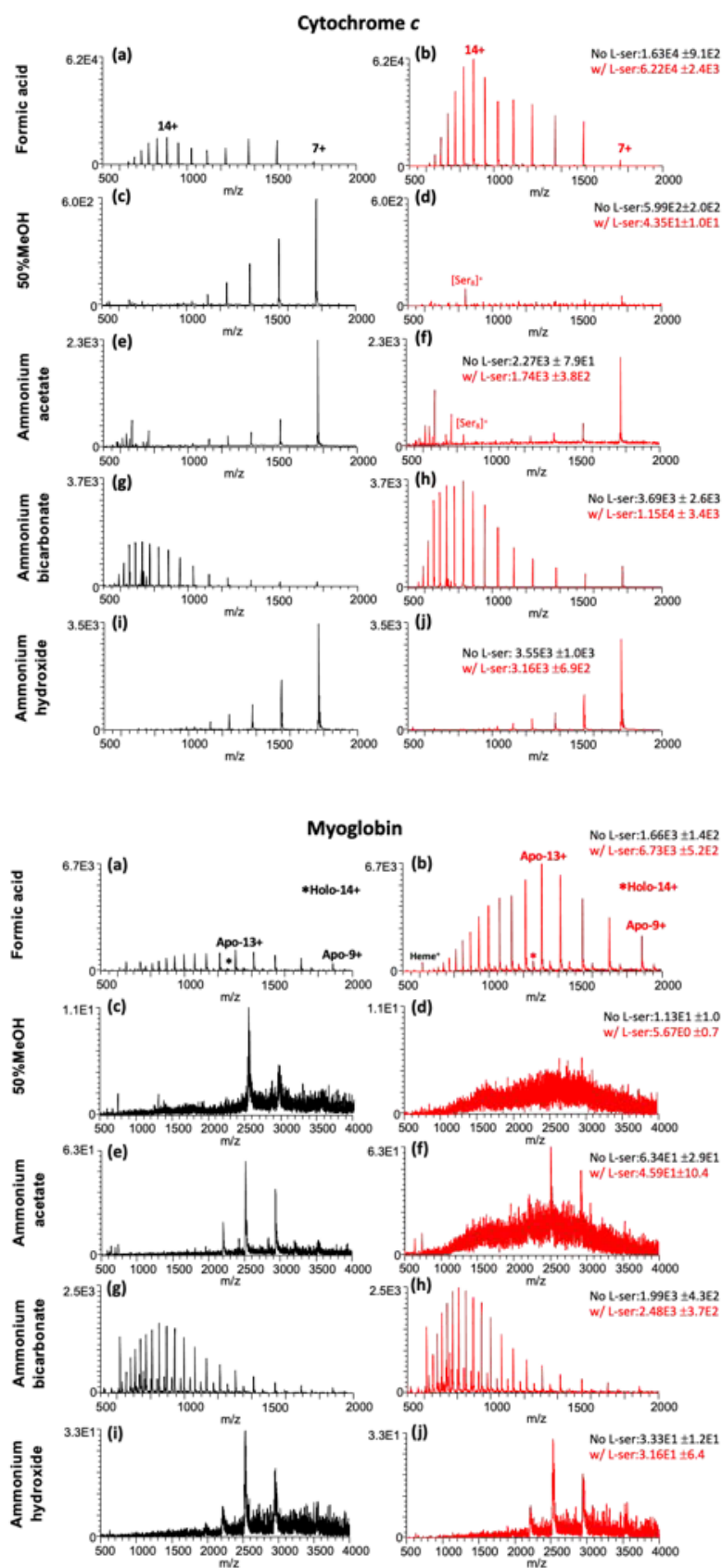
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**Supporting Information**

Roshan Javanshad, Andre R. Venter\*

Department of Chemistry, Western Michigan University, Kalamazoo, MI 49008-5413

\*Corresponding author: Andre Venter, Department of Chemistry, Western Michigan University, Kalamazoo, MI, 49008-5413, +1-269-387-2420, [andre.venter@wmich.edu](mailto:andre.venter@wmich.edu)



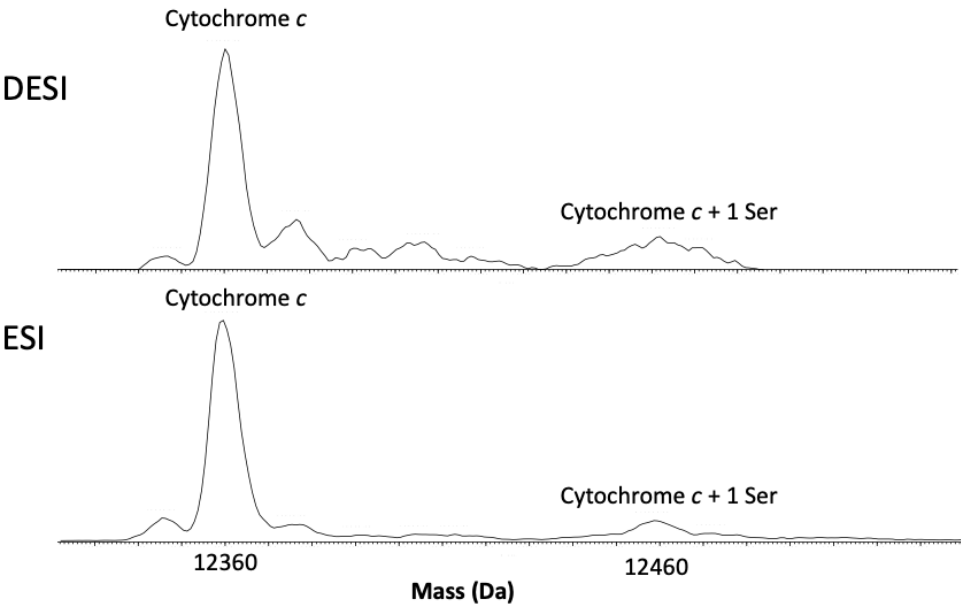
**Figure S1.** Representative DESI-MS spectra of natively deposited cytochrome *c* and myoglobin analyzed with five different desorption solvent systems with 100  $\mu$ M L-serine (shown in red) and without it (shown in black) arranged by increasing desorption solvent pH. (a), (b) 0.1% formic acid in 50% MeOH. (c), (d) 50% MeOH. (e), (f) 200 mM ammonium acetate in 50% MeOH. (g), (h) 200 mM ammonium bicarbonate in 50% MeOH. (i), (j) 1% ammonium hydroxide in 50% MeOH. The intensities reported are the average intensity of the highest observed charge state (HICS) of the protein in each solvent system with L-serine (in red) and without it (in black). Statistically significant improvement in signal intensity were observed for cytochrome *c* (b) and (h) and myoglobin (b) (p-value < 0.001).

**Table S1-** Improvement in signal intensity for each protein with addition of L-serine to the five different solvent systems.

	pH	Average fold change Cyt c HICS Intensity	Average fold change Cyt c Integrated Intensity	Average fold change apo-Myo HICS Intensity	Average fold change Apo-myo Integrated Intensity	Average fold change holo-Myo HICS Intensity	Average fold change Holo-myo Integrated Intensity
Formic acid	2.99	3.8±0.1	3.1±0.4	4.3± 0.3	3.7± 0.2	3.5± 0.3	3.31±0.3
50% MeOH	5.75	0.1± 0.02	0.4±0.02	N/A	N/A	0.5± 0.05	N/A
Ammonium acetate	7.32	0.8± 0.2	0.7±0.1	N/A	N/A	0.7±0.2	N/A
Ammonium bicarbonate	7.97	3.1± 0.2	3.1±0.3	1.4± 0.2	1.2± 0.2	1.3± 0.2	1.3± 0.2
Ammonium hydroxide	10.02	0.9± 0.2	0.8±0.2	N/A	N/A	0.9± 0.2	N/A

**Table S2.** Approximate net charge on protein and serine in different solvent systems.

<b>Cytochrome c</b>						
<b>Solvent</b>	<b>Intensity Fold Change</b>	<b>≈pH</b>	<b>Protein Net Charge (pI 10.3)</b>	<b>Serine Population</b>	<b>pH-pKa<sub>1</sub> (2.21)</b>	<b>pH-pKa<sub>2</sub> (9.15)</b>
<b>Formic acid</b>	3.1	3	+	60-70% zwitterion 30-40% positive	0.79	
<b>50% MeOH</b>	0.8	6	+	100% zwitterion	3.79	
<b>Ammonium acetate</b>	0.8	7	+	99% zwitterion 1% negative		-2.15
<b>Ammonium bicarbonate</b>	3.1	8	+	92-94% zwitterion 6-8% negative		-1.15
<b>Ammonium hydroxide</b>	0.9	10	-	10-20% zwitterion 80-90% negative		0.85
<b>Myoglobin</b>						
<b>Solvent</b>	<b>Intensity Fold Change</b>	<b>≈pH</b>	<b>Protein Net Charge (pI 7.4)</b>	<b>Serine Population</b>	<b>pH-pKa<sub>1</sub> (2.21)</b>	<b>pH-pKa<sub>2</sub> (9.15)</b>
<b>Formic acid</b>	3.7	3	+	60-70% zwitterion 30-40% positive	0.79	
<b>50% MeOH</b>	0.5	6	+	100% zwitterion	3.79	
<b>Ammonium acetate</b>	0.7	7	-	99% zwitterion 1% negative		-2.15
<b>Ammonium bicarbonate</b>	1.2	8	-	92-94% zwitterion 6-8% negative		-1.15
<b>Ammonium hydroxide</b>	0.9	10	-	10-20% zwitterion 80-90% negative		0.85



**Figure S2.** Representative deconvoluted spectra of cytochrome *c* shows presence of L-serine adducts on cytochrome *c* peaks that were detected at low temperature (70°C) in DESI and ESI with 0.1% formic acid in 50% MeOH with 100 µM L-serine as desorption solution and working solution respectively.