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RESEARCH ARTICLE

Influence of Alkylammonium Acetate Buffers on Protein–Ligand Noncovalent Interactions Using Native Mass Spectrometry

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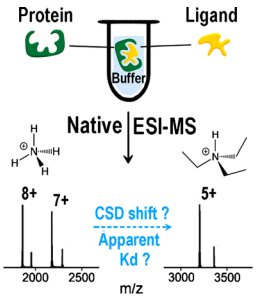

Abstract. We investigate the influence of three volatile alkylammonium acetate buffers on binding affinities for protein–ligand interactions determined by native electrospray ionization-mass spectrometry (ESI-MS). Four different types of proteins were chosen for this study. A charge-reduction effect was observed for all the cases studied, in comparison to the ions formed in ammonium acetate solution. When increasing the collision energy, the complexes of trypsin and the ligand were found to be more stable when sprayed from alkylammonium acetate buffers than from ammonium acetate. The determined dissociation constant (K_d) also exhibited a drop (up to 40%) when ammonium acetate was replaced by alkylammonium acetate buffers for the case of lysozyme and the ligand. The prospective uses of these ammonium acetate analogs in native ESI-MS are discussed in this paper as well.

Keywords: Noncovalent interactions, Alkylammonium salts, Charge reduction, Native mass spectrometry

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Introduction

The most widely used buffers for native electrospray-mass spectrometry (ESI-MS) are aqueous ammonium acetate (AmAc) and ammonium bicarbonate, since both of them are volatile and evaporate readily during the ESI process. Various studies used buffer concentrations in the range of 5 mM to 1 M [1, 2]. Biophysical studies have shown that quaternary protein structures can often be preserved under these conditions [3–5]. Trialkylamine–ammonium buffers, for example, are sometimes used in ESI-MS, too [6, 7]. How these other buffers perform in native ESI-MS has not been well researched and is of interest in this context.

Many researchers employed triethylammonium acetate (TEAA) as an additive for charge reduction [8–10]. Marchand and Gabelica have used trimethylammonium acetate (TMAA) to replace AmAc to provide physiological ionic strength, while suppressing the nonspecific K^+ adducts on DNA G-quadruplexes [6]. Lemaire et al. have shown the benefits of the use of triethylammonium bicarbonate (TEAB) in analyzing protein and protein complexes. The low charge states observed in the mass spectra improve the separation of ions arising from macromolecular species of close molecular weights. Moreover, the multiply charged heme/myoglobin complex ions generated in a TEAB solution are significantly more stable than those formed in ammonium bicarbonate and acetate solution [11].

However, to the best of our knowledge, how these buffers influence the protein–ligand noncovalent interactions and apparent binding strengths measured by native ESI-MS have not been reported. With this in mind, the effects of three volatile alkylammonium acetate buffers on native ESI-MS measurements of four different protein–ligand complexes were investigated in this study.

Experimental

Chicken egg white lysozyme (Lys), bovine pancreas trypsin (Try), bovine β-lactoglobulin A (BLA), pefabloc TH (PB), bovine carbonic anhydrase II (CAII), ammonium acetate

Electronic supplementary material The online version of this article (doi:10.1007/s13361-016-1526-6) contains supplementary material, which is available to authorized users.

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(AmAc), trimethylammonium acetate (TMAA), and triethylammonium acetate (TEAA) were purchased from Sigma-Aldrich (Buchs, Switzerland). Tri-*N*-acetylchitotriose (NAG_3) was purchased from Carbosynth Ltd. (Berkshire, UK), chlorothiazide (CTA) from Enzo Life Science (Lausen, Switzerland), lauric acid (LA) from Acros Organics (Geel, Belgium), and diethylammonium acetate (DEAA) from TCI (Tokyo, Japan).

Data were acquired using a Waters Synapt G2-S HDMS quadrupole-time-of-flight mass spectrometer (Waters, Manchester, UK) with a Nanolock Spray ionization source. The instrumental parameters were carefully tuned to ensure gentle detection conditions. A capillary voltage of 0.8–1.2 kV was used, and the cone voltage was kept at 40 V. The trap and transfer collision energies (CE) were 4–10 V and 2 V, respectively. The pressures in the trap and transfer regions were 7.0×10^{-3} mbar and 6.7×10^{-3} mbar, respectively. The trap DC bias was 3 V. The source temperature was maintained at 30 °C. The pH of all the solutions ranged from 6.7 to 6.9.

The determination of K_d is based on a direct ESI-MS approach that has been described in detail elsewhere [12]. The following equations were used:

$$R = \frac{[\mathrm{P \bullet L}]_{eq}}{[\mathrm{P}]_{eq}} = \frac{\sum_n I(\mathrm{P \bullet L^{n+}})}{\sum_n I(\mathrm{P^{n+}})}$$

$$K_a = \frac{1}{K_d} = \frac{R}{[\mathrm{L}]_0 - \frac{R}{1+R}[\mathrm{P}]_0}$$

where $[P]_0$ and $[L]_0$ are the initial protein and ligand concentrations, respectively. The ratio (R) of the protein–ligand complex (P • L) to bare protein (P) peak areas were calculated for each spectrum, all charge states (n) were taken into account. It is assumed that the ionization efficiencies for the bare protein and the complex are equal, which allows the use of the ratios of the peak areas of the free protein over the complex instead of their concentrations [13].

Results and Discussion

Lysozyme–NAG_3

Lysozyme is a well characterized and thoroughly studied protein, which catalyzes the hydrolysis of β-1,4-glycosidic linkages in some gram-positive bacterial walls [14]. The dissociation constants for lysozyme–NAG_3 were measured in aqueous AmAc, DEAA, TMAA, and TEAA. A concentration of 20 mM was used for each type of buffer. The concentrations of the protein and ligand were fixed at 5 μM for all the measurements.

The 7+ and 8+ charge states for bare and ligand adducted lysozyme corresponding to the "native" state were observed in AmAc. When DEAA, TMAA, and TEAA were used as ESI electrolyte, a significant charge decrease was observed. In DEAA and TMAA, the dominant charge state was 6+, whereas most of the free protein and complex ions carried 5+ charges in TEAA (Figure 1). The apparent K_d values measured in these ammonium acetate analogs are lower than that obtained in AmAc. For example, the K_d value dropped from 16.20 ± 0.40 μM to 9.87 ± 0.55 μM, a decrease by more than 40% as the buffer was changed from AmAc to TEAA.

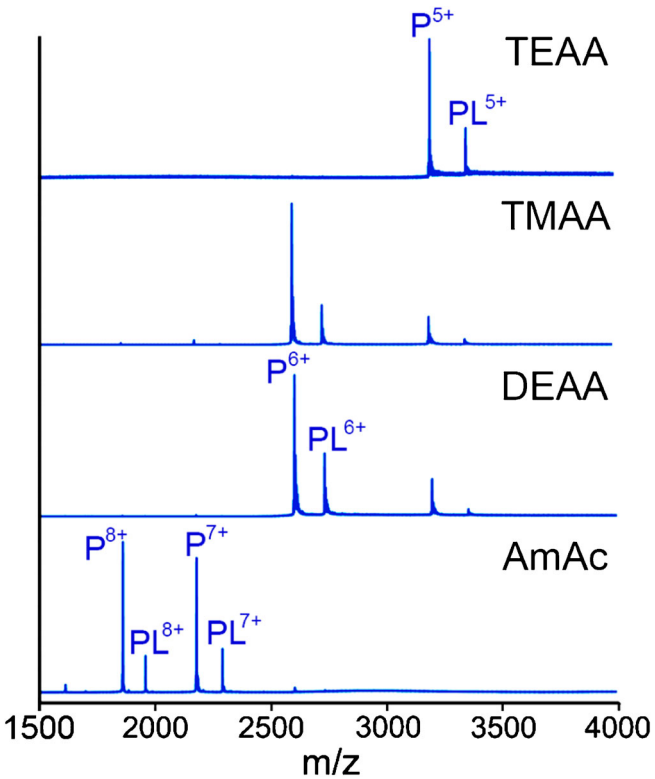

Figure 1. Nano-ESI spectra of 5 μM lysozyme (P) in complex with 5 μM NAG_3 (PL) sprayed from 20 mM AmAc, DEAA, TMAA, and TEAA

Lower charge states will be less affected by collision-induced dissociation (CID) in the ion source. In other words, a lower charge state in DEAA, TMAA, and TEAA should contribute to the decrease of the apparent K_d values of the complexes [15]. This is confirmed by tandem MS experiments. Noncovalent complex ions with different charges $[\text{lysozyme–NAG}_3]^{n+}$ were selected as precursor ions. CID MS/MS was performed by changing the trap CE value to accelerate the ions to collide with collision gas. When a trap CE value of 20 V was used, 48% of $[\text{lysozyme–NAG}_3]^{8+}$ and 43% of $[\text{lysozyme–NAG}_3]^{7+}$ precursor ions were fragmented in AmAc, whereas only about 10% of $[\text{lysozyme–NAG}_3]^{6+}$ ions selected in DEAA and TMAA were found to dissociate. No product ions were detected for the $[\text{lysozyme–NAG}_3]^{5+}$ ions in TEAA. Further increasing the trap CE to 40 V led to the full dissociation of 8+ and 7+ charged precursor ions, whereas 16% of intact $[\text{lysozyme–NAG}_3]^{6+}$ and 65% of intact $[\text{lysozyme–NAG}_3]^{5+}$ were detected after the collision (Supplementary Figure S1).

Isothermal titration calorimetry (ITC) experiments were also carried out in these four buffers. The K_ds measured by this solution-based method are shown in Supplementary

Figure S2, showing no considerable differences within these four situations. The ITC results not only agree with the assumption that the stabilization of the protein–ligand interaction is due to the lower charges, but also suggest that the use of 20 mM alkylammonium buffers does not affect the actual K_d in solution.

Trypsin–Pefabloc

Trypsin is a serine protease that cleaves peptide chains selectively. The benzamidine moiety of the inhibitor is a key structure for the competitive inhibition of trypsin-like enzymes. It mimics the protonated side-chain of the basic amino acids arginine and lysine, which is hydrolyzed by these enzymes [16].

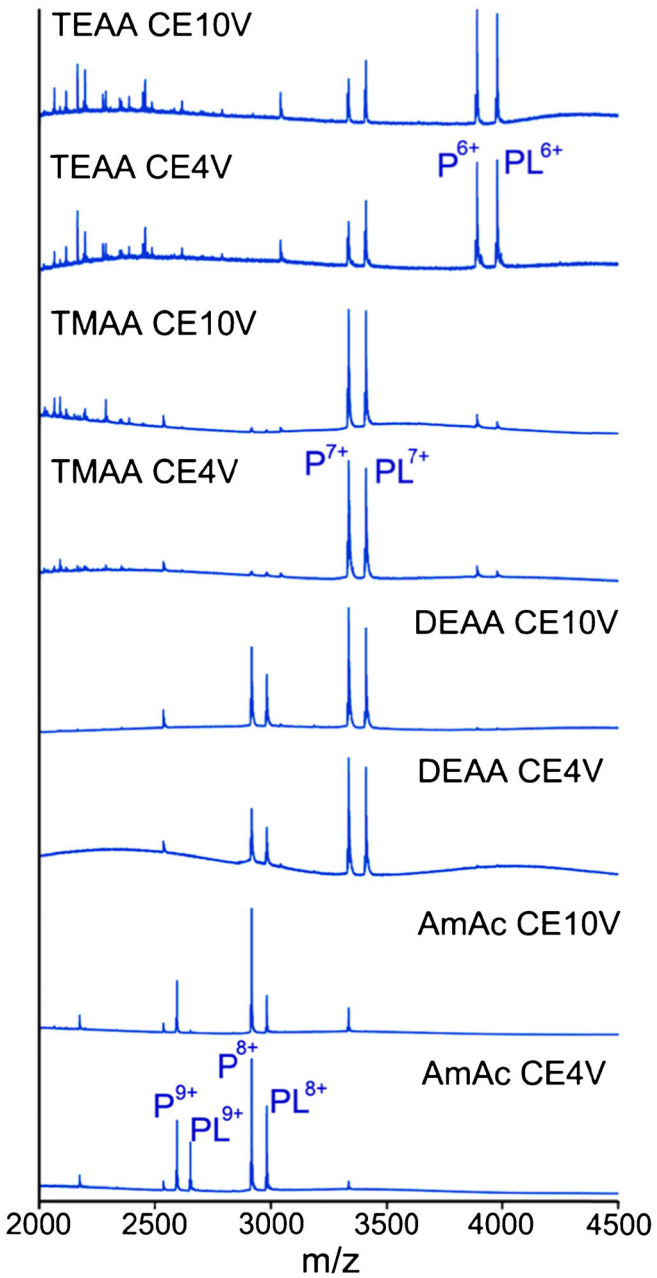

Figure 2. Nano-ESI spectra of 5 μM trypsin (P) in complex with 5 μM pefabloc (PL) sprayed from 20 mM AmAc, DEAA, TMAA, and TEAA. Data were acquired with collision energy (CE) of 4 or 10 V

Compared with AmAc, the use of DEAA, TMAA, and TEAA as ESI buffer seems to increase slightly the binding affinities of the ligand toward trypsin. In 20 mM AmAc, as the CID energy was increased from 4 V (default value) to 10 V, the K_d increased dramatically, almost 3-fold, indicating the in-source dissociation of the complex (Figure 2). However, the complexes sprayed from DEAA, TMAA, and TEAA were much less sensitive to the collisional energy. The measured K_ds did not show obvious differences under these two collision energy conditions. In other words, the complexes formed with alkylammonium acetate buffers seem to be more stable than those obtained with AmAc.

In solution, the trypsin–benzamidine complexes are believed to be stabilized predominantly by an ionic interaction [17]. However, it has been proven that protonated ions of these types of complexes exhibit low gas-phase stabilities and are prone to dissociate in the ion source. Similar stabilization effect for trypsin–benzamidine complexes were reported by Klassen and by Oldham, with imidazole and neutral MeCN vapor, respectively [18, 19]. This may arise from enhanced evaporative cooling of the protein–ligand complex ions in the ion source. As the nonspecific adducts between the salts and trypsin–ligand complex should be kinetically less stable compared with specific trypsin–ligand complex after loss of the nonspecific interactions in the gas phase, the internal energy of trypsin–ligand ions in the source should be lowered. The loss of the bound alkylammonium ions and alkylammonia molecules on the complex takes away more energy, thereby stabilizing the complex. Another plausible explanation for this stabilization effect is the proton stripping effect of these "high-basicity buffers" because lower charge states take up less energy per collision and, therefore, there is less collisional dissociation of the complex in the ion source.

Carbonic Anhydrase II–Chlorothiazide

Carbonic anhydrase catalyzes the hydration of carbon dioxide to carbonic acid. It has a divalent zinc ion that is a noncovalently attached cofactor and essential for catalytic activity [20].

The apparent K_d values measured in AmAc, DEAA, TMAA, and TEAA were found to be very similar. However, when the buffer concentration was raised from 20 to 50 mM, the measured K_d values increased by nearly 2-fold for all four buffers investigated. This obvious trend can be readily observed from the mass spectra (Figure 3). As discussed in an earlier publication from our laboratory [2], this is likely due to the interaction of the acetate ions with the catalytic Zn(II) center of CAII. The increasing concentration of acetate ions shields the binding sites, thus decreasing the binding affinity. It is noteworthy that the four different cation ions did not have any observable influence on the affinities of the complexes.

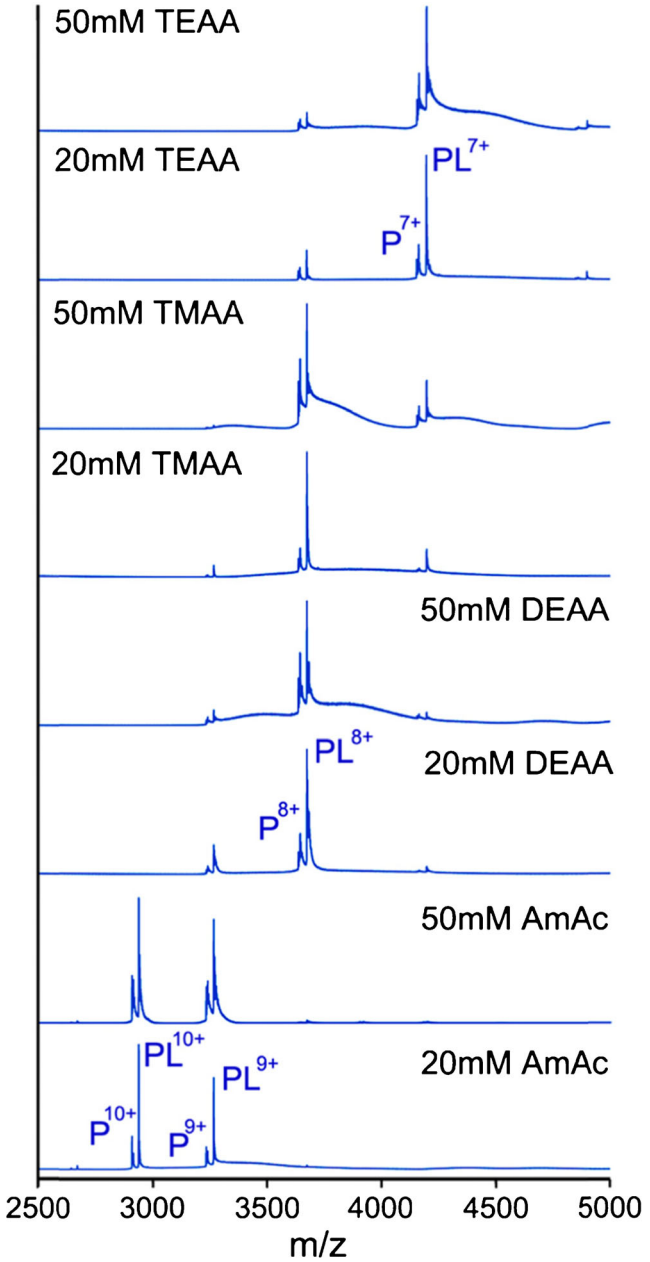

Figure 3. Nano ESI mass spectra of 5 μM carbonic anhydrase II (P) in complex with 25 μM chlorothiazide (PL). Data were acquired with 20 and 50 mM buffer concentrations

β-Lactoglobulin A–Lauric Acid

β-Lactoglobulin, the major whey protein in bovine milk, is an extensively studied protein and is known to bind hydrophobic ligands including fatty acids. The BLA–LA complex is mainly stabilized by hydrophobic interactions in the calyx, which is a β-barrel sandwich built by eight β-strands with a central cavity [21, 22]. The hydrogen bonds between the ligand's carboxyl group and BLA also contribute to the stability of the complex. The binding affinities for BLA (5 μM) in complex with LA (25 μM) were evaluated with the four buffers, both in positive and negative ion modes. The results showed that the K_d values measured in both positive and negative modes are higher than the reported values obtained in the liquid phase, suggesting part of the complex ions were dissociated. In addition, in-source dissociation was prevalent when the measurements were performed in positive ion mode for all the cases, which is in good agreement with the literature [12]. However, the apparent K_d measured in different buffers did not exhibit significant differences (Supplementary Figure S3), which is probably due to hydrophobic interactions mainly stabilizing the complex.

Effect of Buffer Cation on the Charge-State Distribution

The most obvious common feature of the mass spectra acquired in positive ion mode for every system studied is that the use of DEAA, TMAA, and TEAA leads to a reduction in charge state in comparison with the ions formed in AmAc solution (Supplementary Figure S4).

Lemaire et al. had reported similar results for seven proteins that a prominent decrease of the average charge state was observed when TEAB was used in the solution instead of ammonium bicarbonate [11]. As suggested by the combined charge residue field emission model (CCRFEM), the lower charging of protein ions formed from solutions with alkylammonium salts could be a result of cation evaporation from the ESI droplet, lowering the overall charge available for the protein [23, 24]. Salts with low solvation energies carry away some of the droplet surface charge, resulting in fewer charges available to the macromolecule. It is also known that the addition of chemical species with a high gas-phase basicity reduces the charge on electrosprayed proteins. The substantially higher gas-phase basicities of triethylamine (TEA), trimethylamine (TMA), and diethylamine (DEA) than ammonia should also account for this.

In general, the order of gas-phase basicities among different degrees of substitution of amines is tertiary > secondary > primary, where amines with the same substituent groups are compared. Increasing the size of the alkyl group increases the basicity as well. It has also been pointed out that DEA and TMA have very similar gas-phase basicities [25]. In our case, the relative order of gas-phase basicities among the reagents we used was TEA (951 kJ/mol) > DEA (919 kJ/mol) ≈ TMA (918 kJ/mol) > ammonia (819 kJ/mol) [26]. This also explains why TEAA decreased the charge states of proteins so prominently. However, for the systems investigated in this paper, TMAA exhibits a less prominent charge-reducing effect than DEAA.

The Influence of Alkylammonium Buffers on Nano ESI-MS Determined K_d Values

It is clear that the type and concentration of the buffers can affect the stability, solubility, and function of proteins through nonspecific interactions. Both the cation and the anion of a salt in solution contribute to the stability of a protein, although anions tend to have a more significant effect than cations [27].

Table 1. Calculated Dissociation Constants (K_d) for Four Protein–Ligand Systems Determined by the Direct native ESI-MS Method

Buffer	Dissociation Constant K_d (μM)			
	Lys–NAG_3	Try–PB	CAII–CTA	BLA–LA
AmAc, 20 mM	16.20 ± 0.40	3.88 ± 0.19	2.63 ± 0.14	46.92 ± 0.85
DEAA, 20 mM	11.20 ± 1.10	3.62 ± 0.25	2.88 ± 0.21	50.32 ± 1.80
TMAA, 20 mM	13.34 ± 0.44	3.53 ± 0.31	3.07 ± 0.15	46.34 ± 0.50
TEAA, 20 mM	9.87 ± 0.55	2.66 ± 0.26	2.94 ± 0.23	42.33 ± 2.21

The dissociation constants derived from native ESI-MS measurements for all the systems are summarized in Table 1, for a buffer concentration of 20 mM. The nonspecific alkylammonium adducts on protein–ligand complex are more readily lost in the gas phase, taking away internal energy and thereby stabilizing the complex in some cases [28]. In addition, owing to the charge reducing effect of the alkylammonium buffers, the complexes sprayed from these buffers carrying lower charges may also be less sensitive to in-source dissociation [11].

If high concentrations of the buffers are used, there is a possibility that larger alkyl substituted ammonium ions will lead to steric hindrance between the binding partners, whereas in the low millimolar range, the alkylammonium cations are not expected to exhibit a big influence on the interactions between proteins and ligands.

The Analytical Use of the Alkylammonium Buffers in Native MS

When alkylammonium-based salts are used as buffers, their volatility renders them ESI-friendly. Various alkyl ammonium acetate buffers were investigated (for example, dipropylammonium acetate, dihexylammonium acetate, tetraethylammonium acetate, etc.), but were found to produce extensive cationic adducts with the proteins, which renders the interpretation of the mass spectra difficult. In comparison, DEAA, TMAA, and TEAA were found to more readily evaporate and leave the naked protein in the gas phase, producing a high abundance of protonated molecular ions with no alkylammonium cation adducts.

Finally, the charge reduction effect of these basic salts renders them interesting for analytical applications. The benefits of charge reduction lay mainly in reducing intra- and intermolecular Coulomb repulsion within a protein ion complex, potentially affording a more native-like and stable species. Lower charge states may also be desirable when studying mixtures of proteins or protein complexes of close molecular weights. It helps to improve the separation of ions arising from macromolecular species of close masses and, thereby, overlapping peaks could be better resolved. On the other hand, the disadvantage of the charge reduction is the need of mass spectrometers with a wide mass range. Most of the time, lower charge states carry more adducts, which may result in difficulties in interpreting the data. It should be possible to find a compromise to determine a suitable charge state without losing too much signal.

Conclusions

In this study, it has been shown that the native-state conformations and the protein–ligand interactions are maintained in the gas phase with aqueous DEAA, TMAA, and TEAA buffers, as well as AmAc.

The apparent K_d values determined by native ESI-MS are different when different buffers were used. For the trypsin–pefabloc interaction, the alkylammonium buffers exhibited a pronounced stabilization effect on the complex than when using AmAc. The measured K_d values decreased by up to 40% when AmAc was replaced by alkylammonium acetate buffers for the lysozyme–NAG_3 system. In the case of CAII and BLA, the K_d measured in AmAc, DEAA, TMAA, and TEAA did not differ much. A charge-reducing effect was observed for all the systems studied in positive ion mode, which is of great analytical interest. These three volatile buffers are not only ESI-friendly but also give clear mass spectra without too many adducts. In some cases, introducing these buffers in ESI solution can result in protein–ligand complex ion intensities that better reflect their binding ratios in solution through charge reduction and enhanced evaporative cooling. All in all, they should be good choices to substitute AmAc in native MS.

Acknowledgments

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