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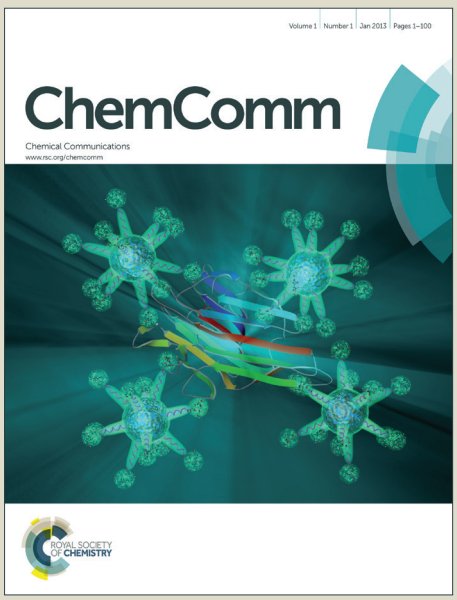

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Enhancing the performance of LC-MS for intact protein analysis by counteracting the signal suppression effects of trifluoroacetic acid during electrospray

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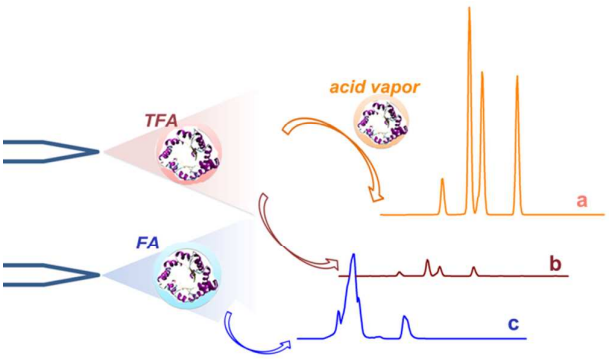
We develop an acidic vapor assisted electrospray ionization strategy within an enclosed electrospray ionization source to counteract the ion suppression effects caused by trifluoroacetic acid. The mass spectrometry signal intensity of intact proteins was improved 10 times and the number of valid signals for *E. coli* intact protein sample was improved 96% by using this strategy.

High performance liquid chromatography coupled with mass spectrometry (LC-MS) is one of the most important techniques for high efficient analyses of many types of biological samples, such as peptides and metabolites.[1] Although the LC-MS technology is greatly developed in the recent years, LC-MS analysis of intact proteins is still a big challenge due to their large size, poor solubility and poor ionization efficiency, which always lead to poor LC separation resolution and low MS detection sensitivity.[2]

Trifluoroacetic acid (TFA) is an ideal ion-pair additive in the mobile phase of LC separation to significantly improve the intact proteins separation selectivity, resolution and peak shape due to its excellent ion pairing and solvating characteristics.[3] However, TFA is not compatible with ESI-MS detection due to the ion-pair adducts result in serious signal suppression in MS detection.[4] Many strategies, such as low concentration of TFA,[5] high temperature for the inlet of MS capillary,[6] improved in-source collision induced dissociation (CID),[7] and post-column electrophoretic mobility control,[8] were developed to alleviate the TFA ion-pair suppression effects in ESI-MS detection. Apffel et al counteracted the deleterious effects of TFA by post-column addition of propionic acid and 2-propanol,[9] which would result in extra analytes dilution and re-mixing, and greatly reduce the LC separation resolution and MS detection sensitivity, especially in the nanoflow LC-MS system.[10] Therefore, utilizing TFA containing mobile phase is still a challenge for biological samples, which greatly limits the improvement of LC separation resolution and the final analysis performance, especially for intact proteins.

In this study, a novel acidic vapor assisted electrospray ionization strategy was developed by doping organic acidic vapor (formic acid (FA), acetic acid (AA) or propionic acid (PA)) into an enclosed ESI source (CEESI source, Haochuang Biotech.[11]) to counteract the TFA ion-pair suppression effects in LC-MS analyses using TFA containing mobile phase (Scheme 1, Figure S1). The average MS peak intensity of intact proteins was feasibly improved about 10 times with PA vapor assistance, and the chromatographic resolution of LC separation was averagely enhanced 3 times and the average half-peak width ($W_{0.5}$) has decreased 36% compared to FA containing mobile phase. Furthermore, the number of valid signals within the full mass spectra (MS1) was improved 96% for complex intact protein sample extracted from *E. coli*, and the MS peak intensity of 44 randomly selected intact proteins was enhanced 7 times by using our new strategy.



Scheme 1. Schematic diagram of the acid vapor assisted electrospray ionization strategy. The TFA (a, b) or FA (c) containing mobile phases for LC separation were sprayed into the enclosed ESI chamber (CEESI source, Haochuang Biotech.) at a flow rate 300 nL/min. Signal suppression caused by TFA ion-pair effects was counteracted by the acidic vapor assistance within the enclosed ESI source (a). Orbitrap XL (Thermo) was utilized in all of our experiments.

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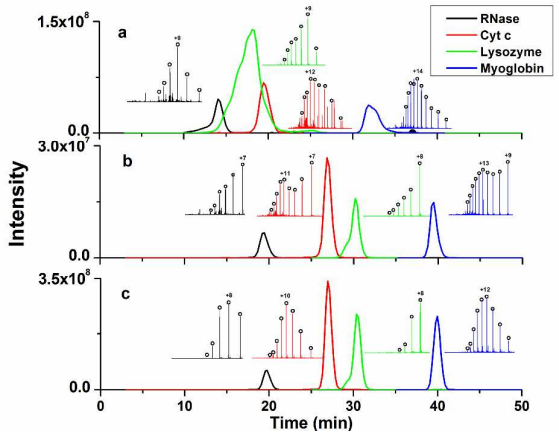

Figure 1. Extracted ion chromatograms and charge state distribution of four standard proteins with 0.1% FA (a) and 0.05% TFA (b) in mobile phase without acidic vapor, and 0.05% TFA in mobile phase with PA vapor assisted electrospray ionization (c).

Firstly, the mixture of four standard proteins, RNase, Cyt c, lysozyme and myoglobin, were utilized in LC-MS analyses to investigate the influence of different mobile phases in LC separation and different acidic vapors within the enclosed ESI source. Since FA is the most widely utilized mobile phase additive in current LC-MS analysis, we compared the mobile phases with 0.1% FA and 0.05% TFA in LC-MS analyses of intact proteins, respectively (Figure 1a and b). It was observed the LC separation selectivity, resolution and peak shape were all significantly improved by using the TFA containing mobile phase, which was consistent with previous reports.[4b] The chromatographic resolution was averagely enhanced 3 times (Table S1), resulting in baseline separation of the four intact proteins, in contrast to the RNase, Cyt c and lysozyme were seriously co-eluted by using the FA containing mobile phase. Further, the peak width at half peak height ($W_{0.5}$) averagely decreased 33% by using TFA containing mobile phase (Table S2), which indicated the LC separation peak capacity was significantly improved. However, we also observed the MS peak intensity of the intact proteins averagely decreased 62% due to the TFA ion-pair suppression, comparing with the FA containing mobile phase.

Then, three organic acids vapors, FA, AA, and PA were doped into the gas phase of the enclosed ESI chamber, respectively, to investigate the influence on TFA ion-pair suppression. It was indicated that all types of the acidic vapors within the enclosed ESI chamber can significantly improve the MS signal intensity of the intact proteins (Figure 2, Table S3). The signal intensity was averagely improved 3, 8 and 10 folds by utilizing FA, AA and PA vapors, respectively. Further, the LC-MS analyses using TFA containing LC mobile phase also exhibited much higher signal intensity than the FA containing mobile phase when the AA and PA assisted electrospray were applied, and signal intensity was averagely improved by 3 and 4 times, respectively (Figure 2). On the other hand, the peak shape and peak width ($W_{0.5}$) were nearly not influenced by the acidic vapor within the enclosed ESI chamber. Compared to the FA containing mobile phase, the $W_{0.5}$ of intact proteins obtained by using TFA containing mobile phase exhibited 34%, 34% and 36% decrease for FA, AA and PA vapors assisted electrospray, respectively (Figure 1b and c, Table S1). The PA vapor

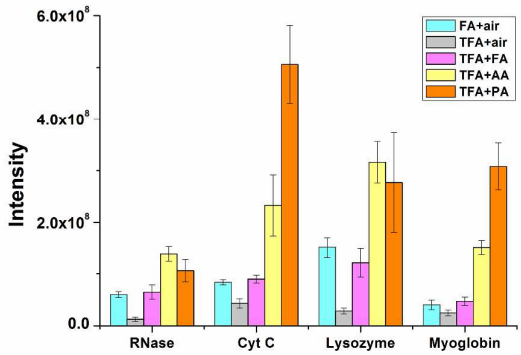

Figure 2. Signal intensity of the four standard proteins in LC-MS analyses (n=3) with different acid additives in the LC mobile phase and different acidic vapors within the gas phase of enclosed ESI source. "FA+air" means 0.1% FA in the LC mobile phase and no acidic vapor in the ESI chamber. "TFA+air", "TFA+FA", "TFA+AA" and "TFA+PA" means 0.05% TFA in the LC mobile phase and air, FA, AA or PA vapor in the ESI chamber, respectively.

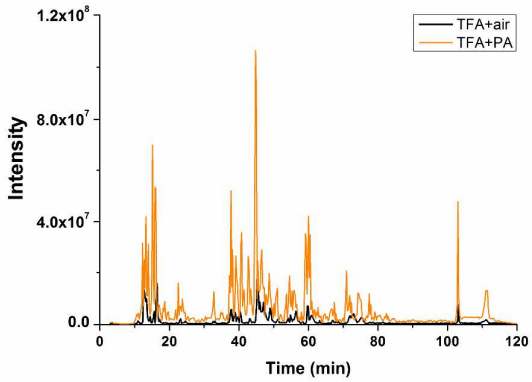

Figure 3. Base peak chromatograms of LC-MS analyses of complex intact protein samples extracted from *E. coli* by using TFA containing mobile phase with (orange) and without (black) PA vapor assisted electrospray ionization.

assistance exhibited the best performance in counteracting the TFA ion-pair suppression effect and enhancing the signal intensity, and it was applied in all of the following experiments.

The influence of LC liquid phase additive and ESI acid vapor on protein charge state distribution (CSD) was further investigated (Figure 1 and Table S4). The average abundance weighted charge states (q_{ave}) of four intact proteins were slightly shifted to lower charge states due to the anionic effect of TFA within the liquid phase.[12] The PA vapor doped into the enclosed ESI source not only enhanced the signal intensity of the intact proteins, but also made the CSD much narrower, which might be related to the pH and proteins conformation changes within the electrospray droplets.[13]

We analyzed the intact protein samples extracted from *E. coli* (strain K12) by using the TFA containing mobile phase and PA vapor assisted electrospray. Consistent with the above results, the PA vapor assistance could significantly improve the signal intensity of MS detection for complex intact proteins (Figure 3). We extracted the multiple charged isotopic mass clusters from the MS1 of LC-MS analyses with 10 Da mass tolerance and 10 min retention time tolerance (mass>5000 Da, intensity>10^5), and 3650 ± 148 (n=3) and 1867 ± 403 (n=3) valid signals were feasibly obtained with and without PA vapor assistance, respectively (S2). Thus the acidic vapor improved the number of valid signals 96%. After database

searching through ProSight PC 3.0, the numbers of characterized intact proteins were 78 ± 3 (n=3) and 57 ± 2 (n=3) with and without PA vapor assistance, respectively, due to the relative low efficiency of the MS for intact protein characterization. Then, we investigated the peak intensity of extracted ion chromatograms (XICs) of randomly selected protein individuals to determine the MS signal enhancement ratio with the PA vapor assistance. Finally, the enhancement ratios were ranged from 2 to 16 times with average value of 7 times for 44 randomly selected intact proteins (Table S5). Therefore, the performance of LC-MS for complex intact protein analyses was also significantly enhanced by using the TFA containing LC mobile phase and PA vapor assisted electrospray.

The LC-MS analysis of the same intact protein sample was also performed by using the FA containing mobile phase and 63 ± 0 (n=3) intact proteins were identified. 36 protein individuals were randomly extracted to compare the peak shape and half-peak width obtained by using TFA or FA as liquid phase additive (Table S6, Table S7). It was observed 75% of the extracted intact proteins exhibited much narrower peak shapes in the LC-MS analysis using TFA containing mobile phase against FA containing mobile phase. The average half-peak width was decreased 17% with the TFA additive. The XICs of three groups of adjacent intact proteins were extracted to exhibit the improvement of selectivity and resolution in LC separation using TFA additive, and these proteins that seriously co-eluted in FA containing mobile phase were all baseline separated (Figure S2).

The mechanism for counteracting the TFA ion-pair suppression and enhancing the MS signal intensity by using the acidic vapor assisted electrospray may be attributed to the following two reasons. Firstly, the abundant acidic vapor within the gas phase can dissolve into the droplets during electrospray to competitively combined with the TFA anions due to the TFA is more volatile, which significantly counteracts the ion-pair effects of TFA during the electrospray process.[4a] Secondly, the acidic vapor can also improve the analytes ionization efficiency as described by Li et al.[14] We compared the enhancement ratio of MS signal intensity for complex *E. coli* intact proteins by using FA and TFA containing mobile phases with and without PA vapor assistance, respectively (Figure S3). Finally, the signal intensity was averagely enhanced 3 and 7 times for the experiments with FA and TFA containing mobile phase, respectively. Thus, both of the above two reasons contribute to the enhancing of MS signal intensity in intact proteins analyses.

Compared with peptide and metabolites, the capability of LC-MS for intact proteins analyses is greatly lagged due to the extremely complex biophysical and chemical properties of intact proteins. Improving the LC separation resolution and MS detection sensitivity for intact proteins is still a great challenge. TFA is recognized as an ideal mobile phase additive to significantly improve the LC separation selectivity, resolution and peak shape. However, it is excluded out of current LC-MS analysis because of the TFA ion-pair effects will seriously suppress the MS detection signals. In this study, we developed a novel acidic vapor assisted electrospray ionization strategy within an enclosed ESI source, and we demonstrated the TFA ion-pair suppression effects is successfully counteracted as the MS signals were improved 10 times for the standard intact proteins and 7 times for complex intact proteins extracted from *E. coli*. Furthermore, the LC separation resolution was enhanced 3 times with 36% decrease in half-peak width compared to FA containing mobile phase.

In conclusion, we develop an acidic vapor assisted electrospray ionization strategy for LC-MS analysis of intact protein samples by using TFA containing mobile phase. As the signal suppression effects of TFA was successfully counteracted during electrospray, both high LC separation resolution and high MS detection sensitivity were successfully achieved for intact proteins analyses. This strategy is simple, stable and reproducible, and providing a promising way for high performance intact protein analyses by LC-MS.

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Notes and references

The authors declare no competing financial interest.

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