Original Article

Influence of Solvent Composition and Surface Tension on the Signal Intensity of Amino Acids in Electrospray Ionization Mass Spectrometry

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Abstract

The influence of solvent composition and surface tension upon the signal intensity of deprotonated molecules [M – H]+ in electrospray ionization mass spectrometry (ESI MS) was evaluated using alanine (Ala), threonine (Thr) and phenylalanine (Phe) differing levels of hydrophobicity. The surface tension of ESI solution was varied by changing the ratio of the organic solvents methanol (MeOH) and acetonitrile (MeCN) in water (H2O). In ESI MS, the signal intensity of all the amino acids increased by decreasing the surface tension in two solutions, H2O/MeOH and H2O/MeCN. The use of H2O/MeCN was more favorable for the signal intensity of analytes than that of H2O/MeOH. A smaller vaporization enthalpy of MeCN compared to MeOH was proposed as one of the most plausible reasons. The order of the signal intensity of amino acids was Phe>Thr>Ala in relation to the order of hydrophobicity of amino acids. It can be practically concluded that the use of solutions with lower surface tensions and lower vaporization enthalpies results in higher signal intensities in ESI MS.
INTRODUCTION

Electrospray ionization mass spectrometry (ESI MS) has been widely used for quantitative and qualitative analysis of various organic compounds such as amino acids, peptides, natural products and synthetic chemicals. Its combination with liquid chromatography, a methodology known as liquid chromatography mass spectrometry, has been almost exclusively used for the quantification of trace analytes, because of its incomparable sensitivity and selectivity \(^1\)\(^-\)\(^5\)). However, the major drawback of ESI MS is that it is strongly governed by the physicochemical properties of analytes and solvents, which makes it difficult to use as an absolute quantification method. To solve this issue and/or to understand the phenomena, a great number of studies have been reported \(^6\)\(^-\)\(^18\)).

Regarding the solvent, Ikonomou et al reported that an increase in volume ratio of methanol to a solvent system of water/methanol increased the signal intensity of protonated cocaine in ESI \(^8\)). Zhou and Hamburger also reported that the signal intensity of organic compounds increased by increasing the volume ratio of organic solvent in water/methanol and water/acetonitrile systems \(^9\)). They have explained that the increasing signal intensity is due to the increase in production efficiency of small droplets generated from the Taylor cone caused by the decrease in surface
tension of the solution system in ESI MS. The droplet size can be represented by the function of surface tension in a solvent as follows\textsuperscript{10}.

\[ R \propto (\rho V_f \gamma)^{1/3} \]  \hspace{1cm} (1)

where \( R \), \( \rho \), \( V_f \) and \( \gamma \) represent the droplet diameter, density, flow rate and surface tension of solvent, respectively.

The first plausible mechanism of gas-phase ion generation was reported by Kebarle and Verkerk\textsuperscript{11}, which consists of major two steps after the generation of parent droplets from the Taylor cone. The proposed two steps are (a) shrinkage and fission of the charged droplets by the solvent evaporation and (b) production of the gas-phase ions from the charged droplets. The process of repeated droplet fissions of the parent droplets leads to smaller progeny droplets. The fission of droplets occurs when the repulsion of the surface excess charge on the surface of the droplet overcomes the surface tension of the droplet. The gas-phase ions are produced from the charged droplets only when the droplet size is very small. Overall, it is obvious that the surface tension of parent droplets has a significant impact on the production of smaller droplets, thereby the ESI efficiency.

Besides the surface tension of droplets, solvent pH is known to affect the ESI signal intensity. For example, Liigand et al.\textsuperscript{12} have surveyed the influence of analyte
pKa and solvent pH on ESI signal intensity. Twenty-eight analytes having different pKa and logP were tested under acidic and neutral aqueous solvent conditions (pH 2.1-7.0). In general, the ionic dissociation of analytes in a solution is determined by its pKa and the solution pH. Acidic functional groups such as carboxylic acid are dissociated into a pair of a negatively charged ion and one proton at the pH greater than their pKa, whereas basic amino groups are protonated at the pH lower than their pKa. Dissociation and protonation produce charged species which are less hydrophobic but will readily become gas-phase ions. To simplify the interpretation by cancelling the influence of solution pH and pKa in this study, neutral amino acids, which are zwitterions of those functional groups, were chosen as model analytes. The pI values of the analytes are shown in Table 1. The net charge of those molecules becomes zero at the neutral pH, which allowed us to omit the difference of ionization efficiency among the analytes, and to avoid the use of pH modifiers.

It should be also noted that the nature of hydrophobicity or surface activity of analytes strongly affect the signal intensity in ESI MS. Cech and Enke \(^{13,14}\) and Tang and Kebare \(^{15,16}\) have reported that more hydrophobic analytes give higher signal intensity in ESI MS, because such analytes tend to exist on the surface layer of ESI droplets. One of the authors has reported that the signal intensity in ESI MS can be
explained by dividing the total ion yield flux $J_i$ into two terms, namely ionization efficiency $I_i$ and vaporization flux $J_v$ \(^{17,18}\), as follows.

$$J_i = I_i J_v$$ \hspace{1cm} (2)

This equation means that the signal intensity is governed by the thermochemical natures of the analyte and solvent, such as gas-phase basicity and acidity for $I_i$, and the physical properties of the analyte and solvent, such as hydrophobicity, surface tension and vaporization enthalpy for $J_v$.

In this report, we examined the influence of the solvent composition and surface tension of the solution and the hydrophobicity of the analytes upon the signal intensity in ESI MS. The purpose of this study was to empirically reveal the influence of hydrophobicity of analytes and surface tension of solutions on ESI signal intensity. As the first step, neutral amino acids, alanine (Ala), threonine (Thr) and phenylalanine (Phe), were chosen as model analytes. The ESI signal intensities were monitored in the negative-ion mode to probe the significance of surface activity. By design, the influence of other factors that may alter the ionization efficiencies $I_i$ were excluded because all the test analytes share a common twitter ion structure. We anticipated that the interpretation of complex ESI ionization processes would be
simplified by focusing on the difference of non-ionic sidechains. To fully understand the processes, the surface tension of the solution was varied by changing the volume ratios of the organic solvents in water/methanol and water/acetonitrile systems.

(Table 1)

MATERIALS AND METHODS

Reagents and Sample Preparation

Alanine, threonine and phenylalanine were purchased from the Peptide Institute (Minoh, Osaka, Japan). Acetonitrile, methanol and water were purchased from Wako Pure Chemicals (Osaka, Japan). All the solvents were LCMS grade. All the reagents were used without further purification. Each amino acid was dissolved into two solvent systems, water/acetonitrile and water/methanol at 1 μM concentration. Each solvent was prepared at volume ratios of 0, 10, 30, 50, 70, 100%. The concentration of the analytes for surface tension measurements was 1 μM, representing the typical concentration for ESI measurements.

Mass spectrometry
Mass spectrometric measurements were performed on a LCMS-8050 triple-quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) coupled with a Nexera HPLC system (Shimadzu, Kyoto, Japan). The instrument parameters were as follows: injection volume was 4 μL, flow rate of the flow-injection analysis solvent was 0.2ml/min, the sample cooler temperature was 25℃, rate of N₂ drying gas was 10L/min, the rate of N₂ nebulizing gas was 3L/min, and capillary voltage was –3.0kV for the negative ion detection mode. The signal intensity was monitored for m/z values of deprotonated molecules [M · H]⁻ (m/z 88 for Ala, m/z 118 for Thr and m/z 164 for Phe) in selected ion monitoring mode. The signal intensities were estimated from the peak areas of selected-ion chromatograms on flow injection analysis (triplicated measurement).

**Surface tension**

The surface tensions of the bulk solutions with and without analyte were measured by the Wilhelmy plate method with a model DCAT 21 tensiometer (Dataphysics, Germany). Bulk solution in a glass bottle of 30mL was stored in a thermostatic bath at 25℃ overnight. The value of surface tension was estimated from the pulling force of the test solution when the bottom part of the plate barely
touched the surface of the liquid. At this point, the liquid wets up against the plate, and surface tension of bulk solution acted along the periphery of the plate, trying to pull the plate into the bulk solution. This pulling force was measured by a microbalance. One measurement took 5-10 min to reach the surface tension equilibrium. The surface tension measurement was repeated 3 times per sample and the averaged values were used for evaluation.

RESULTS AND DISCUSSION

Influence of solvent composition upon the signal intensity of amino acids

Here we employed negative-ion mode to estimate the signal intensity of amino acids, because positive-ion mode in ESI MS often detects adducted cations such as [M+Na]^+, [M+K]^+ and [M+NH₄]^+, as well as [M+H]^+. The signal intensities of deprotonated amino acids [M – H]⁻ were obtained with two different solvent systems H₂O/MeOH and H₂O/MeCN, by changing the volume ratio of the organic solvent (Figure 1). The volume ratio of organic solvent to water was varied from 10 to 70 %. The relative standard deviations (RSD) of the signal intensity were in the range of 0.3%~16%.
The signal intensity of analytes increased by raising the volume ratio of organic solvents in both solvent systems. In both solvent systems, the order of signal intensity of amino acids was Phe > Thr > Ala, which is consistent with the order of hydrophobicity by means of the Bull and Breeze (B&B) hydrophobicity index \(^{19}\), as shown in Table 1. Furthermore, the use of H\(_2\)O/MeCN as a solvent system showed higher signal intensity than when H\(_2\)O/MeOH was used. The results obtained above indicate that the signal intensity depends on the volume ratio of organic solvent, organic solvent used and the hydrophobicity of the analyte.

We used the B&B hydrophobicity index for our investigation. The B&B hydrophobicity index is based on the measured surface tension values of each amino acid in 0.1M NaCl aqueous solution. The hydrophobicity based on the B&B index is known to well represent the surface activity of an analyte and has been used to discuss the hydrophobicity of amino acids. Although the octanol-water partition coefficient, log\(P\), is also frequently used as an index of the hydrophobicity of amino acids, no apparent correlation with ESI signal intensities was found in our system.
It should be noted that thermochemical properties of Ala, Thr and Phe, such as the isoelectric point (pI), gas-phase acidity (GA) and gas-phase basicity (GB) do not differ much from each other, as shown in Table 1. From this, it is reasonable to assume that the ionization (deprotonation) efficiencies \( I_i \) in Eq. 2 of Ala, Thr and Phe are nearly equal to each other. Therefore, the difference in the signal intensity showed in Figure 1 could be explained by the vaporization flux \( J_v \) in Eq. 2. As already reported, both positive- and negative-ion yields of peptides can positively be correlated to the B&B hydrophobicity index \(^{17}\). This positive correlation between the signal intensity and analyte hydrophobicity can be explained by which analyte with greater hydrophobicity exists with a higher concentration in the surface layer of the ESI droplets \(^{15,16,19}\). The analytes existing in the surface layer are more favorable for vaporization than those existing in the interior of the ESI droplets. The reason why the rise in the volume ratio of the organic solvent results in the increased signal intensity can be explained by the fall in surface tension of the solvents \(^{9}\). The lower surface tension of the solvents may result in smaller droplets, as is understood from Eq. 1. The formation of small droplets is favorable for the solvent and analyte molecules to evaporate from the surface of the ESI droplets.

The influence of a droplet size on signal intensities was estimated by changing the
flow rate. The flow-injection analysis of 1μM Ala solution using two solvent systems (10% and 70% MeCN) was conducted at different flow rates (0.05, 0.1, 0.2, 0.5 mL/min). The peak areas for Ala (from extracted ion chromatograms for m/z 88) decreased as the flow rate increased in both solvents (data not shown). Provided that the ion-transfer efficiency inside the vacuum region was constant at those flow rates, smaller peak areas at higher flow rates would be explained by the size difference of ESI droplets.

Another important factor for the vaporization efficiency of the droplets is the vaporization enthalpy of the solvent used. The higher signal intensity in the use of H₂O/MeCN solution compared to H₂O/MeOH may be due to the lower vaporization enthalpy of acetonitrile compared to methanol, as shown in Table 2. For example, Wilhelm et al. reported that solvents having high evaporation efficiencies produced small charged droplets and ascribed it to the solvent vaporization enthalpies. According to Fenn, the enthalpy required to evaporate the solvent from the charged droplet was provided by the gas to which the charged droplets were exposed. Although the importance of enthalpy is obvious from the literature, a more thorough investigation will be needed to reveal its significance on overall ESI processes.
**Influence of solvent composition and analyte upon the surface tension of solution**

Figure 2 shows the influence of the volume ratio of organic solvent in two solution systems (H$_2$O/MeOH and H$_2$O/MeCN) upon the surface tension of the solution with and without analytes. The surface tension values obtained in this study were consistent with the previous report $^{29}$. The RSD of the surface tension on triplicate analyses were less than 4%. In both solution systems, the surface tension decreased steeply as the volume ratio of the organic solvent to water went from 10% to 40%, and the decrease in surface tension was less steep at 40-100% organic solvent ratios.

(Figure 2)

The influence of analytes (Ala, Thr and Phe) on the surface tension of the solution was examined with an usual analyte concentration of 1 $\mu$M. All the analytes had little effect on the surface tension under the concentration of 1 $\mu$M, as shown in Figure 2. This may be due to the fact that the number of solvent molecules on the surface of the solution is much larger than that of analyte molecules. Assuming that 1 $\mu$ M analyte molecules are uniformly dispersed in pure water, the number of water molecules is about 380 times
greater than that of analyte molecules. This suggests that 1 \( \mu \) M amino acids do not greatly disrupt the hydrogen bonding network of the surface of the solutions used.

Interestingly, the surface tension of \( \text{H}_2\text{O}/\text{MeCN} \) more steeply decreased with the ratio of organic solvent than that of \( \text{H}_2\text{O}/\text{MeOH} \) at ratios of less than 50%, as shown in Figure 2. Furthermore, the surface tension of \( \text{H}_2\text{O}/\text{MeCN} \) was lower than that of \( \text{H}_2\text{O}/\text{MeOH} \) in ratios of organic solvent which were under 50%. Regardless of the surface tension, neat acetonitrile was lower than that of neat methanol (Table 2). This may be due to the endothermic and exothermic processes occurring from the mixing of an aprotic acetonitrile \(^{23}\) and a protic methanol \(^{24}\) with water, respectively. The endothermic property of the heat of mixing in the \( \text{H}_2\text{O}/\text{MeCN} \) system suggests that acetonitrile molecules disrupt the hydrogen-bonding network of water molecules, while the exothermic property of \( \text{H}_2\text{O}/\text{MeOH} \) forms a stronger solvation network in the solution system. The disruption of the hydrogen-bonding network in water with acetonitrile may result in the decrease of the surface tension of the \( \text{H}_2\text{O}/\text{MeCN} \) solution, and it seems to be favorable for the evaporation of solvent and analyte molecules from the surface of ESI droplets.

(Table 2)
Influence of the surface tension upon the signal intensity

As described above, it was suggested that the signal intensity is governed by several factors such as the hydrophobicity of the analytes, the ratio of organic solvent to water (solvent composition), the heat of mixing protic (MeOH) and aprotic (MeCN) solvents, and the vaporization enthalpy of the solvents. The surface tension of the solution and the hydrophobicity of analytes are particularly essential properties governing the signal intensity, as shown in Figure 3, made from Figures 1 and 2. Figure 3 clearly shows that the signal intensity increases with the fall in surface tension of the solution and with the increase in the hydrophobicity by means of the B&B index of amino acids. In both solvent systems, the signal intensity of Ala was least affected by the reduction of the solvent’s surface tension. The plausible reason is its low B&B index (+2.55), which must force most of Ala molecules to stay inside the solvent droplets in the tested solvent systems.

It is of interest to note that in Figure 3 the signal intensity steeply increased with the fall in surface tension at values lower than 35 mN/m. The surface tension of 35 mN/m in the solution used here approximately corresponds to the ratio of organic solvents with 50% or higher to water, as seen in Figure 2. This indicates that the use of the ratio of
50% or higher acetonitrile composition to water is favorable for obtaining higher signal intensity in a given analyte.

(Figure 3)

CONCLUSION

The influence of several factors upon the signal intensity of deprotonated amino acids \([M - H]^−\) was evaluated using amino acids alanine (Ala), threonine (Thr) and phenylalanine (Phe) with differing levels of hydrophobicity and a common isoelectric point (pI). The decrease in surface tension of two different solutions made of H₂O/MeOH and H₂O/MeCN increased the signal intensity of all the amino acids in both solution systems. The surface tension of the solution systems was changed together with the changing of the solvent composition, i.e., the ratio of organic solvent to water. The use of H₂O/MeCN as a solution was favorable for the signal intensity of analytes used compared to H₂O/MeOH, although the surface tension of neat acetonitrile was larger than that of neat methanol. The reason why the use of H₂O/MeCN is favorable for signal intensity may be due to the fact that the vaporization enthalpy of acetonitrile is smaller than that of methanol. In both solution systems, the order of the signal intensity of amino acids
was in compatible with the order of hydrophobicity by means of the B&B index, i.e., Phe>Thr>Ala. Considering the common twitter ionic state, \( \text{NH}_3^+\text{Ca}(-\text{X})\text{COO}^- \), and the pI values of the amino acids used (Table 1), the ion yields \( \mathcal{J} \) of deprotonated molecules \([M – H]^–\) of Ala, Thr and Phe here are governed by the vaporization flux \( \mathcal{J}_v \) in the phenomenological equation (2). In fact, the factors described in this paper, such as the hydrophobicity of analytes, surface tension and the vaporization enthalpy of solvents, can be related to droplet formation and vaporization. It can be practically concluded, therefore, that the use of solutions with lower surface tensions and lower vaporization enthalpies results in higher signal intensity in ESI MS of any given analytes.

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Legends for Figure and Table

Figure 1. Signal intensities for deprotonated molecules [M–H]\(^{-}\) of alanine, threonine and phenylalanine with two solvent systems of (a) H\(_2\)O/MeOH and (b) H\(_2\)O/MeCN. The RSD of the signal intensity were in the range of 0.3%~16%.

Figure 2. Influence of solvent composition of ESI solutions of (a) H\(_2\)O/MeOH and (b) H\(_2\)O/MeCN with and without amino acids. The RSD of the surface tension on triplicate analyses were less than 4%.

Figure 3. ESI signal intensities of the deprotonated molecules [M – H]\(^{-}\) of alanine, threonine and phenylalanine against the surface tension of ESI solution systems of (a) H\(_2\)O/MeOH and (b) H\(_2\)O/MeCN.

Table 1. Properties of amino acids used, molecular mass (M\(_r\)), isoelectric point (pI), a measure of hydrophobicity (B&B, kJ/mol), gas-phase acidity (kJ/mol) and gas-phase basicity (kJ/mol).

Table 2. Surface tension and vaporization enthalpy for solvent used for ESI MS.
Table 1. Properties of amino acids used, molecular mass (Mr), isoelectric point (pI), a measure of hydrophobicity (B&B, kJ/mol), gas-phase acidity (kJ/mol) and gas-phase basicity (kJ/mol).

| Amino acid | Mr  | pI*1 | B&B*2 | GA*3 | GB*4 |
|------------|-----|------|-------|-------|------|
| Ala        | 89.1| 6.00 | +2.55 | 1430  | 864.1|
| Thr        | 119.1| 5.60 | +1.21 | 1388  | 886.3|
| Phe        | 165.2| 5.48 | -6.36 | 1418  | 888.0|

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Table 2. Surface tension and vaporization enthalpy for solvent used for ESI MS.

| Solvent            | Surface tension (mN/m) *1 | Vaporization enthalpy (kJ/mol) *2 |
|--------------------|---------------------------|----------------------------------|
| Water (H$_2$O)     | 72.0                      | 44.0                             |
| Methanol (CH$_3$OH)| 22.6                      | 38.3                             |
| Acetonitrile (CH$_3$CN) | 30.0                    | 33.8                             |

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Fig. 1. Signal intensities for deprotonated molecules $[\text{M} - \text{H}]^-$ of alanine, threonine and phenylalanine with two solvent systems of (a) H$_2$O/MeOH and (b) H$_2$O/MeCN. The RSD of the signal intensity were in the range of 0.3%~16%.

Fig. 2. Influence of solvent composition of solutions of (a) H$_2$O/MeOH and (b) H$_2$O/MeCN with and without amino acids. The RSD of the surface tension on triplicate analyses were less than 4%.
Fig. 3. Signal intensities of the deprotonated molecules [M – H]⁻ of alanine, threonine and phenylalanine against the surface tension of solution systems of (a) H₂O/MeOH and (b) H₂O/MeCN.