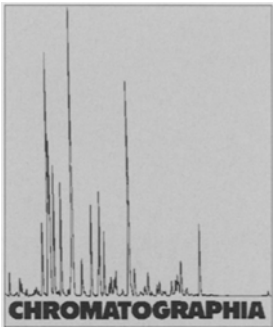
ESI-MS Response Characteristics of a Synthetic Peptide



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Key Words

Electrospray
Mass spectrometry
Synthetic peptide

Summary

ESI-MS response characteristics of a proprietary synthetic peptide are briefly studied through variation of solution composition, buffer content, liquid flow and nebulizer temperature. In accordance with Kebarle's *ion competition* theory, decreasing buffer concentration was seen to be well correlated to improved ESI-MS response. In line with general expectation, increased mobile phase organic content and heightened nebulizer temperature improved response, presumably factors promoting droplet fission/ion release via lowered droplet surface tension and added thermal energy, respectively. In contrast to expectation, added sodium chloride resulted in a modest signal increase despite concomitant formation of sodium adduct. A similar signal enhancement effect has been previously reported for quaternary ammonium halides, attributed to a thinning in the charged droplet electric bi-layer thereby promoting ion release.

Introduction

Peptide Analysis

Free peptides are essential bio-molecules implicated in numerous processes including neurotransmission, cell communication, growth and development, endocrine function, immune response, etc. Characteristics of peptide pharmaceuticals often include high target specificity, production of metabolites that are biologically inactive or which display low toxicity, and high potency. With respect to bio-analysis, LC-ESI-MS/MS techniques offer high specificity and sensitivity, rapid analysis times, and are cost effective for the high sample volumes encountered in clinical trials. However, due to the high potency of peptide drugs the corresponding low dosing levels studied place high demands on quantitative drug metabolism/pharmacokinetics (DMPK) assays, thus the optimization of detection methods to deliver maximum sensitivity is often essential. Toward improving the ESI-MS response of a proprietary synthetic peptide, the objective of this investigation is to explore solution conditions, including solution composition, buffer content, liquid flow and source temperature.

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The Electrospray Process

ESI-MS entails the analysis of solution phase ions and thus response is largely controlled by solution chemistry. Numerous detailed papers and reviews cover many current aspects of this technique and mechanism [1–4]. Very briefly, the ESI process occurs by application of a high electric field to a conductive liquid moving through a small metal capillary in the $\mu L\,min^{-1}$ range. A charge excess of the same polarity as the applied high voltage occurs in solution as counter ions are neutralized through re-dox chemistry occurring within the ES capillary walls. Electrostatic forces due to excess charge in turn overcome the emerging liquids surface tension, carrying away uniformly charged, micron sized droplets in the direction of a counter electrode placed nearby at a lower electrical potential. For most ESI-MS applications today the column effluent is pneumatically nebulized with a coaxial nebulizer gas in order to accommodate HPLC. This results in a plume of less uniformly sized, less extensively charged droplets. However, as in non-pneumatically assisted ESI, consecutive droplet fission and desolvation events occur which ultimately release free gas phase ions on a millisecond time scale. At least for moderately sized ions the ion produc-

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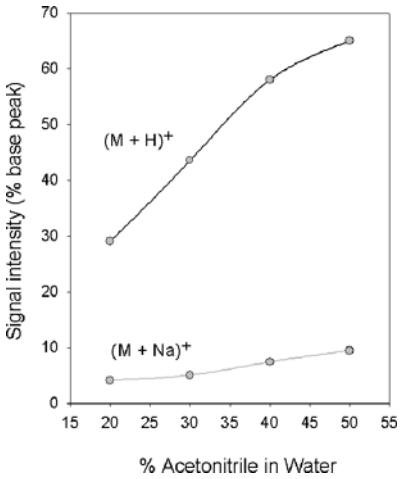

Figure 1. Peptide $(M + 1)^+$ and $(M + Na)^+$ ESI-MS responses observed while varying acetonitrile content in unbuffered water.

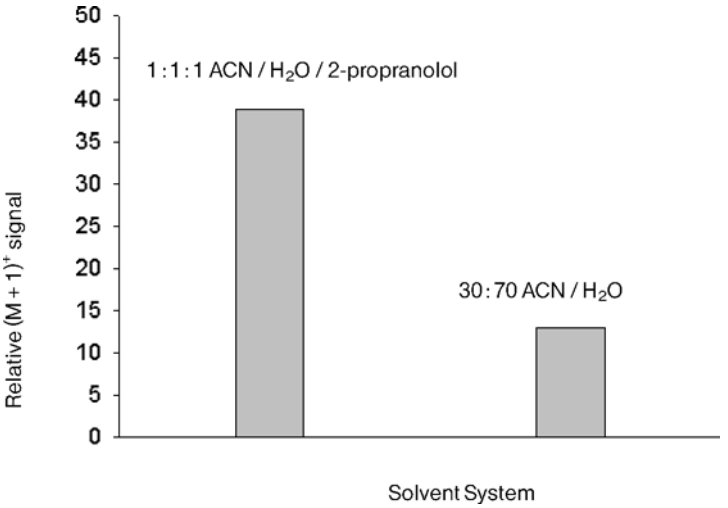

Figure 2. Peptide ESI-MS response tested with two unbufferred solvent systems. Flow 0.19 mL min^{-1}, probe temperature 150 °C.

tion mechanism is thought to be one of field emission [1–5] or so-called *"ion evaporation"* [6] formulated as an activated rate process in which ions escape from the droplet surface to the gas phase. The resulting gas phase ions are sampled by ventilation from ambient pressure through a succession of vacuum stages, wherein ions may be deliberately subjected to fragmentation prior to mass analysis and detection, thereby providing useful structural information.

Response Factors of Importance

Most authors seem to agree that the mechanism of ion release in Electrospray is not completely understood [1–6]. Performance of this technique toward the detection of a given target thus depends upon several partially understood factors both instrumental and chemical in nature. Wide experience has shown that ideally responding compounds for analysis by ESI-MS are pre-formed solution ions with high surface activities. The ESI-MS responses of peptides as a target class are probably limited in cases due to modest surface activities and/or high solvation energies in polar solvents. Solubility is one crucial chemical factor dictated by solution composition and pH. In the case of the peptide in this study, the C-terminus is chemically modified by substitution of the hydroxyl group with an NH_2 group, thus in positive mode leaving the N-terminus as the chemically active charging site through protonation. This peptide is crystallized from solution with hydrochloric acid and is handled as an HCl salt. It is thus a pre-existing ion in neutral solution. In the present study we chose to examine solution parameters including buffer/electrolyte concentration, pH, solution organic content, liquid flow and nebulization temperature with the aim of identifying conditions leading toward improved ESI-MS response.

Experimental

Mass Spectrometer

This study was carried out using a Micromass Quattro II triple quadrupole with the pepper-pot ion sampling interface. Prior to performing experiments the instrument was tuned on the target peptide signal $(M + 1)^+$ and calibrated on CsI cluster ions.

Sample Preparation and Storage

Fresh, synthetic peptide samples were prepared from a stock solution to a final concentration of 2.6×10^{-6} M. Sealed standards were stored at 5 °C. Prior to experiments standards were transferred to glass (Metric Analys, Sweden) vials using disposable glass pipettes and capped to prevent evaporation. All organic solvents (HPLC grade acetonitrile and 2-propanol) and Milli-Q water were stored in glass. Ammonium acetate (*p. a.* Merck), ammonium formate (Microselect >99%, Fluka), formic acid (puriss Kebolab) and sodium chloride (*p. a.* Merck).

Sample Introduction

Sample introduction was by syringe pump infusion using a 250 µL gas-tight syringe with a Stanford pump. A short teflon sleeve was used at the syringe tip. Microbore PEEK tubing (100 micron ID, about 1 m length) with finger-tight PEEK fittings was used to deliver sample from a 250 µl syringe to the ESI nebulizer probe.

Results and Discussion

Solution Chemistry & Liquid Nebulization

Solvent Organic Content

The presence of organic modifiers commonly used in HPLC can apparently assist in nebulized droplet fission and subsequent ion release by means of lowering liquid surface tension. Figure 1 shows the $(M + 1)^+$ response of the study peptide as well as its sodiated adduct $(M + Na)^+$ upon varying acetonitrile (ACN) content in unbuffered water at a flow of 0.19 mL · min^{-1}. In accordance with general experience it can be seen from the figure that increasing the percentage of ACN from 20% to 50% indeed results in an improved response with gains presumably leveling off at higher percentages of ACN. 2-Propanol is an organic modifier also widely used in HPLC, and is known as a means of improving ESI-MS response for example when added post-column. In Figure 2 a clear signal enhancement can be seen for 1:1:1 ACN/H_2O/2-propanol versus 30:70 ACN/H_2O.

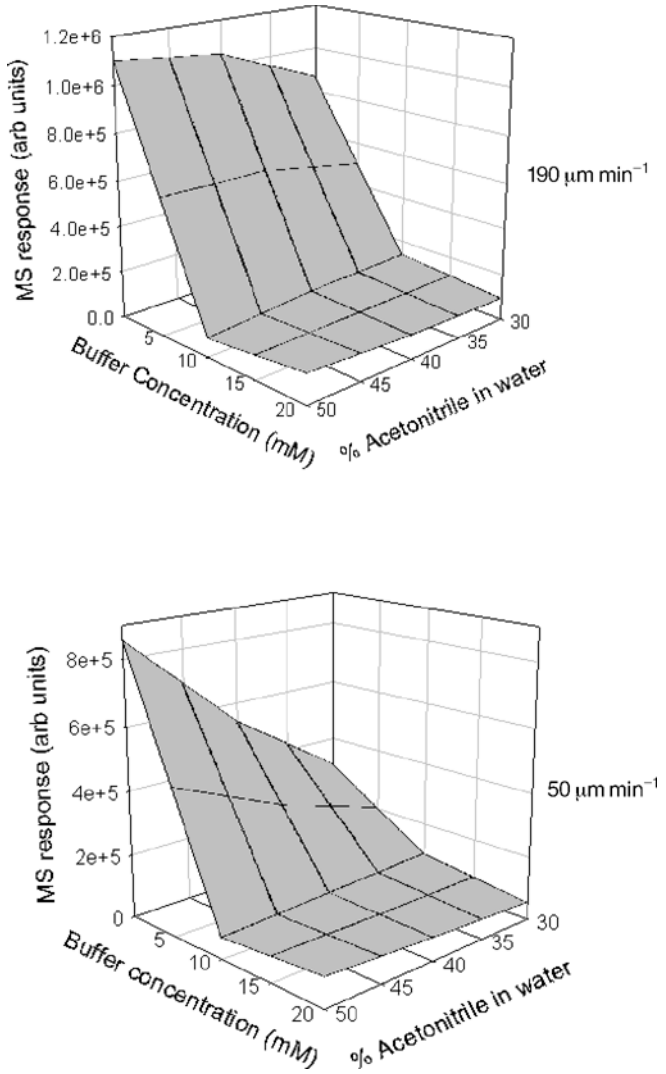

Figure 3. Peptide ESI-MS response surfaces observed upon simultaneously varying the ammonium acetate-formic acid buffer content and the acetonitrile/water composition at two flow rates. Probe temperature 150 °C.

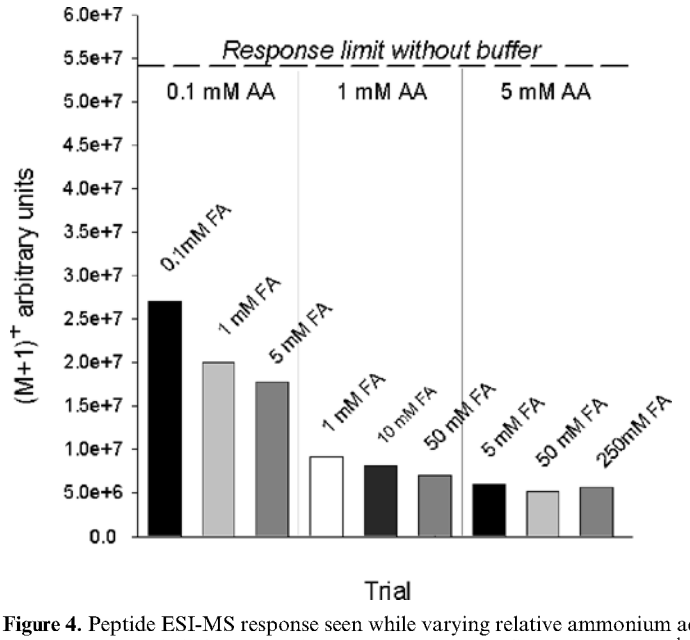

Figure 4. Peptide ESI-MS response seen while varying relative ammonium acetate-formic acid content. 30:70 acetonitrile/water, probe temperature 150 °C, flow 0.19 mL min^{-1}.

Mobile Phase Buffer Additives

It is widely known that ESI response is generally diminished by the presence of background electrolytes which are thought to suppress and compete for ionization [1, 2]. Figure 3 shows empirical ESI-MS response surfaces observed upon simultaneously varying the relative percentage of ACN and H_2O and the ammonium acetate-formic acid (1:1) buffer concentration. These experiments were carried out at two different flow rates with a constant nebulization temperature of 150 °C. In Figure 3 a response enhancement due to increased ACN content at both tested flows (50 and 190 $\mu L \cdot min^{-1}$) resembles the results obtained using the unbuffered solvents shown in Figure 1. For the flow ranges shown in Figure 3 it appears that the lower flow case (50 $\mu L \cdot min^{-1}$) may be more sensitive to increase in ACN content. The flattened portions of the ESI-MS response surfaces in Figure 3 for both of the flow conditions examined corresponds to substantial signal suppression when buffer is present at concentrations above 10 mM.

Figure 4 shows ESI-MS responses obtained upon varying both the pH and total buffer capacity. The variations include increasing both the total concentrations and relative ratios of formic acid and ammonium acetate in 30:70 ACN / H_2O with a nebulizer temperature of 150 °C. Systems tested include solutions with equimolar formic acid-ammonium acetate, solutions with a 10-fold excess in formic acid over ammonium acetate, and solutions with a 50-fold excess in formic acid over ammonium acetate. The respective pH values of these solutions are 3.75, 2.75 and 2.05, as calculated by the Henderson-Hasselbalch equation. From Figure 4 it is clear that increases in the relative amount of formic acid to ammonium acetate (i. e. decreasing pH) do not improve the $(M + 1)^+$ signal for the peptide studied. This is not surprising since the peptide is prepared as a salt and is thereby a pre-existing ion in solution. The figure thus shows that the highest available response coincides with the lowest total buffer concentration. The dashed line in Figure 4 shows the response obtained in non-buffered 30:70 ACN / H_2O at the same temperature. Thus both Figures 3 and 4 show the degree by which increasing the total mobile phase buffer concentration leads to a suppression in the target ion signal.

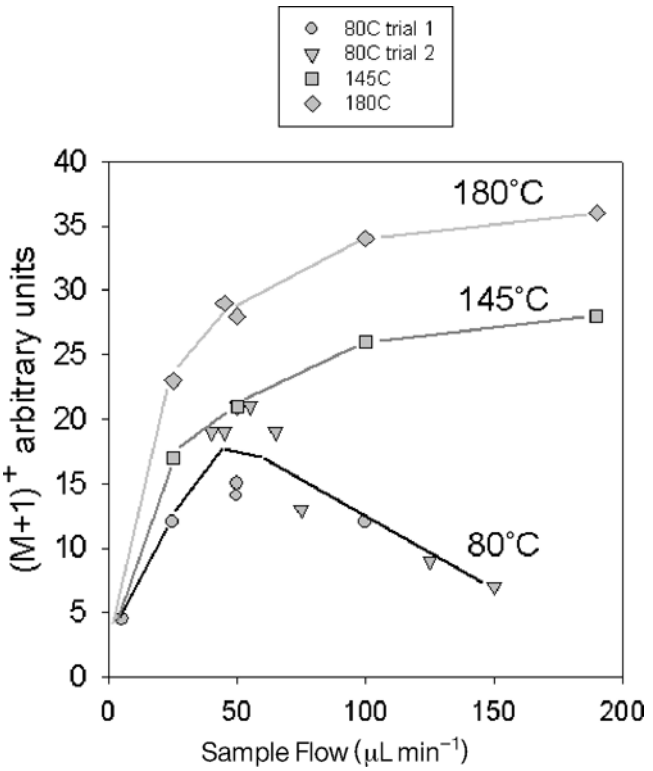

Figure 5. Peptide ESI-MS response seen while varying nebulizer probe temperature and liquid flow rate in unbuffered 50:50 acetonitrile/water.

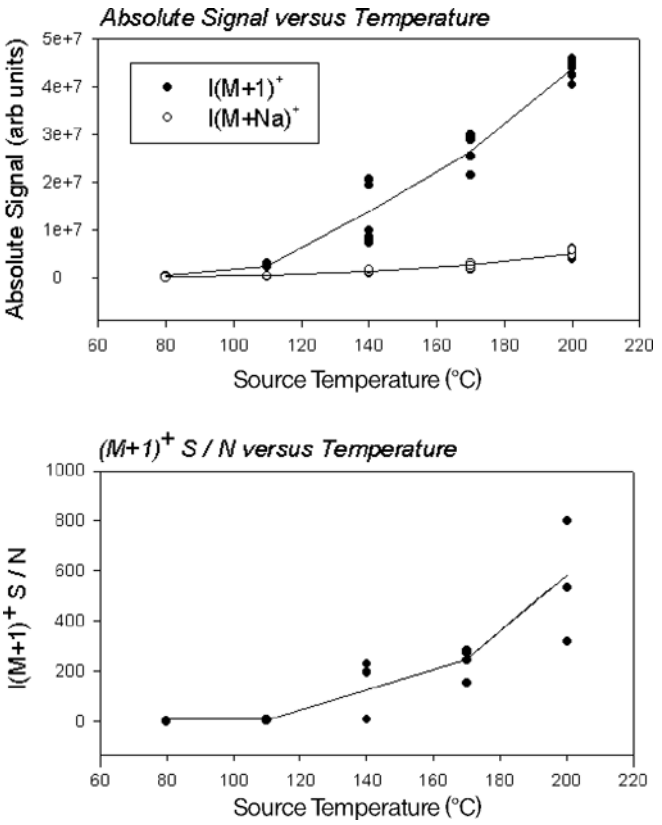

Figure 6. Peptide ESI-MS response seen upon varying the nebulizer probe temperature at 0.19 mL min^{-1} in 30:70 acetonitrile/water buffered with 20 mM ammonium acetate/formic acid. S/N is estimated from the noise observed in the full scan window surrounding $(M + 1)^+$.

Sample Flow, Source Temperature and Nebulization

Besides solvent composition, liquid flow rate and nebulization temperature also influence ESI performance. Temperature dictates the thermal energy in the ambient pressure nebulization chamber available to assist in droplet desolvation. Higher liquid flow rates obviously require more thermal energy in order to obtain satisfactory nebulization. Figure 5 shows the result of varying liquid flow at low (80 °C), moderate (145 °C) and high (180 °C) nebulizer temperatures using unbuffered 50:50 ACN/H_2O. It can be seen at the low temperature that increasing the liquid flow above 50 $\mu L \cdot min^{-1}$ causes a steadily decreasing peptide response. This most likely is a reflection of insufficient droplet desolvation. At 145 °C an increasing liquid flow is not met by signal loss, and furthermore additional signal is obtained for the same flow range compared to the lower temperature. At higher temperature even better signal response is obtained. Also seen in Figure 5 for both 145 °C and 180 °C apparent response plateaus are reached near 200 $\mu L \cdot min^{-1}$, reflecting the lack of mass flow sensitivity for which ESI-MS is generally noted.

Figure 6 shows temperature variations in 30:70 ACN/H_2O at a flow of 0.19 $mL \cdot min^{-1}$ and a buffer content of 20 mM ammonium acetate-formic acid. It was repeatedly found that the $(M + 1)^+$ signal was most intense at the maximum nebulizer operating temperature (200 °C), where $(M + 1)^+$ comprised the base peak in the full scan mass spectrum. At lower temperatures $NH_4^+(ACN)_X$ clusters dominated. Clearly the best peptide $(M + 1)^+$ signal S/N was achieved at 200 °C. The sodium adduct $(M + Na)^+$ is also shown in Figure 6, seen at approximately 10–15% the intensity of $(M + 1)^+$ over most of the studied temperature range.

Influence of Added Sodium

Sodium adducts $(M + Na)^+$ are often a product of solution contamination by background sodium. A common source of sodium is laboratory glassware. Typically sodium adducts are highly stable ions and can persist under CID conditions that virtually eliminate corresponding $(M + 1)^+$. The sodium adduct shown in Figures 1 and 6 could be easily detected in the course of investigations, so it was deemed interesting to briefly explore the sodium adduct for its analytical potential. The deliberate addition of Na^+ (as NaCl) to the solution was carried out with the aim of converting $(M + 1)^+$ to $(M + Na)^+$. Figure 7 shows the result of increasing the background sodium concentration to 100 and 500 mM on the absolute signal intensities of $(M + 1)^+$ and $(M + Na^+)$. Surprisingly, going from no sodium added to 100 mM added the absolute intensity of both $(M + 1)^+$ and $(M + Na)^+$ increased in approximately the same ratio. This was unexpected since, as noted above and as generally observed in ESI-MS, higher background electrolyte concentrations tend to have a suppression effect on the target signal. This surprising result finds support however in an unrelated study on quaternary ammonium halides [6]. The response enhancement due to the presence of background NaCl reported in that study were proposed to be due to a resultant thinning of the electric bi-layer of charged droplets thereby assisting in the release of ions into the gas phase.

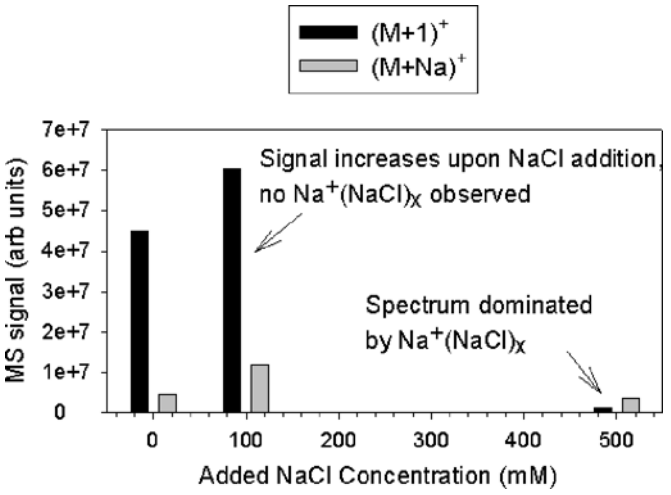

Figure 7. Effect of intentional sodium (NaCl) addition. Flow 0.19 mL min^{-1}, 30:70 acetonitrile/water, 20 mM ammonium acetate/formic acid.

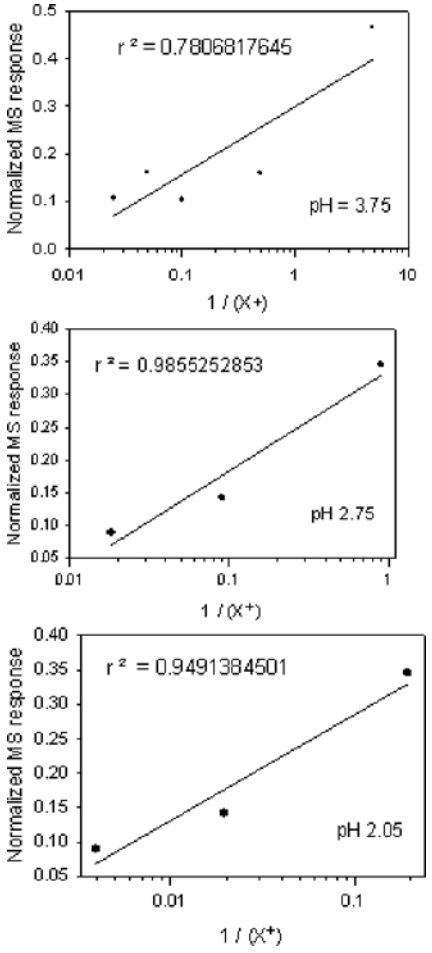

Figure 8. ESI-MS peptide $(M+1)^+$ signal response versus $1/[X^+]$ as a test of the ion competition model at three pH values. Data used as shown in Figures 3 and 4. See Eq. (1).

Ion Suppression: Practice versus Theory

The ESI-MS response of a target ion A^+ in the presence of other electrolytes X^+ has been formulated by Kebarle's ion competition model [1–2]

$$I_A^+ = \frac{f\,p\,k_A[A^+]}{(k_A[A^+] + k_X[X^+])} \qquad (1)$$

where I_{A+}: MS target ion current, f: fraction of droplet charge converted to free gas phase ions, p : MS ion sampling efficiency, k : ion release rate coefficient, (A^+) : target ion concentration and (X^+) : non-target ion concentration. In this study the peptide concentration is 2.6 micromolar, whereas the buffer when present is at a concentration at least 100-fold higher, i. e. $(X^+) \gg (A^+)$. For simplification, the correlation between buffer content and target ion signal is explored by plotting I_{A+} versus $1/(X^+)$ in Figure 8 for three pH conditions studied. Even without knowledge of the other variables in Eq. (1) it is evident from Figure 8 that the signal suppression observed due to increased buffer content appears to follow the ion competition model. However the relationship from the data shown does not appear to be linear. The unexpected, slight enhancement effect seen upon the addition of NaCl discussed above is in conflict with the ion competition model, but as noted above might be compensated in this case by other factors at the droplet surface resulting in improved ion release.[1)]

[1)] Constantopolous, T.; Jackson, G.; Enke, C. In *Proceedings of the 46th American Society for Mass Spectrometry Conference*, 1998.

Conclusions

The ESI-MS response of a proprietary peptide has been explored. As expected, it was seen that increased organic content (i. e. acetonitrile and/or 2-propanol), minimal buffer concentrations and elevated nebulization temperatures all resulted in improved response. The ESI-MS response was seen to be inversely related to buffer concentration in a manner consistent with established notions regarding ion competition effects. However, unexpectedly, additions of NaCl up to 100 mM resulted in modest signal increases, a preliminary finding that has precedents in previous work involving quaternary ammonium halide signal increases and which have been proposed to be due to a thinning in ES droplet electric bi-layers. On the basis of these results we have in practice achieved an approximatly 10-fold improvement in the limit of quantification for the study peptide by means of lowering the buffer content (15 to 2 mmol), increasing the organic content via a gradient with peptide elution at approx. 40% acetonitrile, and doubling the volume of plasma sample analyzed.

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