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The Influence of Mixed Solvents Volatility on Charge State Distribution of Peptides During Positive Electrospray Ionization Mass Spectrometry

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Abstract : Understanding the mechanisms that control and concentrate the observed electrospray ionisation (ESI) response from peptides is important. Controlling these mechanisms can improve signal-to-noise ratio in the mass spectrum, and enhances the generation of intact ions, and thus, improves the detection of peptides when analysing mixtures. The effects of different mixtures of aqueous: organic solvents (25, 50, 75%; v/v): formic acid solution (at pH 3.26) compositions on the ESI response and charge-state distribution (CSD) during mass spectrometry (MS) were determined in a group of biologically active peptides (molecular wt range 1.3 - 3.3 kDa). The ESI response is dependent on type of organic solvent in the mobile phase mixture and therefore, solvent choice affects optimal ion intensities. As expected, intact peptide ions gave a more intense ESI signal in polar protic solvent mixtures than in the low polarity solvent. However, for four out of the five analysed peptides, neither the ESI response nor the CSD were affected by the volatility of the solvent mixture. Therefore, in solvent mixtures, as the composition changes during the evaporation processes, the pK_b of the amino acid composition is a better predictor of multiple charging of the peptides.

Keywords : ESI-MS response, charge-state distribution (CSD), neuropeptides, solvent volatility

Introduction

Electrospray ionisation (ESI) mass spectrometry is widely used for the study of peptides and proteins due to its unsurpassed sensitivity and the ability to form multiply charged ions. Although ESI mechanisms are still being debated, it is well established that the observed ESI response (i.e. charge state (*z*) and intensity of ions), is affected by instrumental parameters including, sheath gas flow rate,[1] electrospray source geometry,[2] the internal energy of the ions as well as their velocity when leaving the droplets (desorption model).[3] Other non-instrument parameters are analyte concentration, solvent and analyte basicity[4-6], solvent viscosity or 'volatility' which is governed by solvent surface tension,[7,8] gas phase basicity solution[9] and pH.[10] Further, properties such as number and type of amino acids and the conformation of the proteins[4,10,11] in solution play important roles in determining the maximum obtainable charge state for peptides.

For the analysis of peptides by ESI-MS the solvent contributes to several processes: in very general terms, 1) the analytes needs to be in solution prior to ionisation; 2) the solvent facilitates charge stabilisation in solution; and 3) the solvent evaporation from charged ESI droplets causes droplet shrinkage and splitting of droplets. The latter is reviewed by Kebarle and Verkerk.[12] As the initial solvent conditions define the starting conditions, it is important to develop a deeper understanding of the solvent effects on peptide ESI responses. Solvent polarity affects the observed charge state distribution during the ESI process.[13,14] Specifically, a higher dielectric constant solvent will shift the charge-state distribution towards higher z (lower *m/z*) values due to the increased ease of dissociation, and hence, polar solvents are more effective in stabilizing multiply charges whereas lower-polarity solvents will disfavour dissociation and counter ions will have an increased propensity to remain attached to one charge site.[13,14] In addition, during the solvent evaporation process, the most volatile solvent preferentially evaporates, resulting in a solvated analyte ion in which the solvation is enhanced in the least volatile component. Ridge and co-workers proposed that the maximum charge state of an ion should then be determined by the gas-phase basicity of the least volatile solvent.[15] If this was the case, then there

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should be no effect on the maximum charge state due to the addition of the various solvents that are more volatile than water and conflicting data has been reported in literature. For example, Iavarone and co-workers showed that addition of even small amounts of solvents that have higher gas-phase basicities result in significant shifts in both the maximum charge state and charge state distributions to a lower charge.[16] They explained this effect to the propensity of the solvent to remove protons preferentially from the higher charge state ions. This can occur either by gas-phase collisions with "naked" analyte ions or it can occur in the ion desolvation process, whereby the solvent molecule removes a proton as it evaporates.

These results clearly demonstrate that solvents which are more volatile than water do influence both the maximum charge state and the charge state distributions observed in electrospray ionisation. However, their investigations[15,16] involved the use of pure solvents rather than mixtures. To test this, here, we added different organic solvents in varying proportions to water:formic acid mixtures, typical of LC-MS mobile phases, and assessed the ESI response for a number of peptides**.** The commonly used ESI solvents methanol and acetonitrile are similar with respect to surface tension, volatility, and dielectric constant (Table 1). The most important difference is that methanol is a protic (polar) solvent, while acetonitrile is aprotic (polar). Because of the protic nature of methanol, negative ions are more strongly solvated in methanol than in acetonitrile, that is, the protonated form of the acid should be favoured in acetonitrile and the deprotonated form in methanol. In acetonitrile-water mixtures, the water increases the protic nature of acetonitrile. To be able to compare 'like-for-like' we introduced *iso*-propanol (IPA or 2-propanol) to our studies. IPA is a protic solvent with a boiling point (bp) similar to that of acetonitrile**.**

Experimental

Chemicals and reagents

Prolactin-releasing peptide fragment 12-31 (PrRP, 2272.57 Da), corticotropin releasing factor fragment 6-33 (CRF, 3220.66 Da), calcitonin gene-related peptide fragment 8-37 (CGRP, 3125.6 Da), vasoactive intestinal peptide (VIP, 3325.8 Da) and Substance P (SP; 1,347.71 Da) were selected on the basis of similar molecular mass (number of amino acid residues 20 to 30) to avoid complications with mass-dependent transmission in the mass spectrometer.[17] The general characteristics of the peptides; molecular mass, amino acid sequence, expected (based on pK_b at pH 3.26), actual number of multiple protonations are shown in Table 2. PrRP fragment 12-31(2272.57 Da; 20 amino acids), CRF fragment 6-33 (3220.66, 28 amino acids), CGRP fragment 8-37 (3125.59 Da; 30 amino acids), VIP (3325.80 Da; 28 amino acids) and SP (1347.71, 11 amino acids) were used as purchased (Sigma-Aldrich, Gillingham, UK). Solvents and reagents were of HPLC grade; water, acetonitrile (MeCN), methanol (MeOH) and isopropanol (IPA) (Rathburn Chemicals Ltd, Walkerburn, UK), and formic acid (FA) (WRI, Fontanay sous Bois, France) were also purchased.

ESI-Q ToF mass spectrometry

The mobile phase was delivered at a constant flow rate of 4 μL/min by a nano-LC pump (Agilent 1200 Nano-LC, Waldbrome, Germany) equipped with a six-port injection valve. Samples (1 μL) in triplicate were injected into the eluent stream.

For each injection, a total of 3.2×10^{-10} moles were introduced into the eluent stream for each peptide. Measurements were implemented using a hybrid Q ToF mass spectrometer with a Z-spray ESI interface (Q ToF Ultima API; Waters, Milford, MS, USA). The following conditions were used for ESI-Q ToF/MS analyses; the nitrogen desolvation gas was set at a flow rate of 300 L/hr, desolvation temperature at 120°C, nebulizer gas flow rate at 60 L/hr, and the source temperature was 90°C. The capillary and cone voltages were 2.50 kV and 28 V, respectively. Acquisition of mass spectral data for the peptides was performed by scanning the instrument in the *m/z* range of 450-1,000. The most intense multiple protonated ions were identified.

Results and Discussion

ESI-MS Response

The ESI mass spectra of CGRP, VIP and CRF were all dominated by the quadruply $[M+4H]^{4+}$ charge state i.e. CGRP (*m/z* 782.206, Figure 1), CRF (*m/z* 806.489) and

Table 1. Some physical characteristics of the solvents used. All organic solvents are miscibility with water. *Chemical Polarity: Relative permittivity $\varepsilon_r(\omega)$ or Dielectric constant at 20°C, the term dielectric constant is still commonly used, but has been deprecated by standards organizations [23].

Solvent	Boiling pt °C	Chemical Polarity*	Surface tension nM/m (25°C)	Volatility ranking
Acetonitrile (MeCN)	81.6	37.5 (25°C)	28.7	2
Methanol (MeOH)	64.6	33	22.1	1(most volatile)
iso-propanol (IPA)	88.2	18.0	23.3	3(least volatile)
Water	100	80.10	72.1	

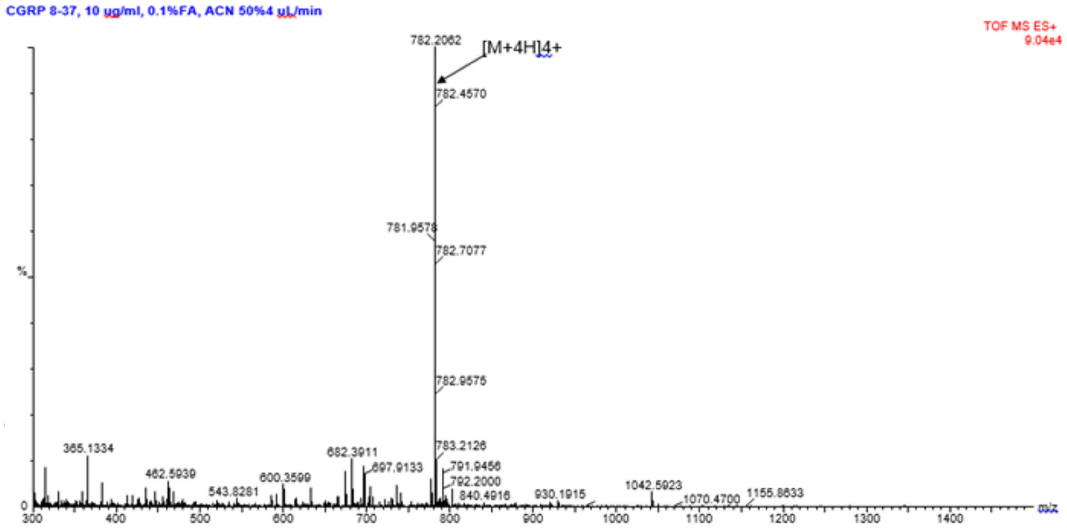

Figure 1. Typical ESI mass spectrum of the peptide CGRP (concentration 3.2×10^{-6} mol/mL; 1 μL injection volume, mobile phase flow rate 4 μL/min, and composition H_2O:MeCN (1:1; v/v) + 0.1% FA, pH 3.26). The quadruply protonated ($[M+4H]^{4+}$) ion is indicated.

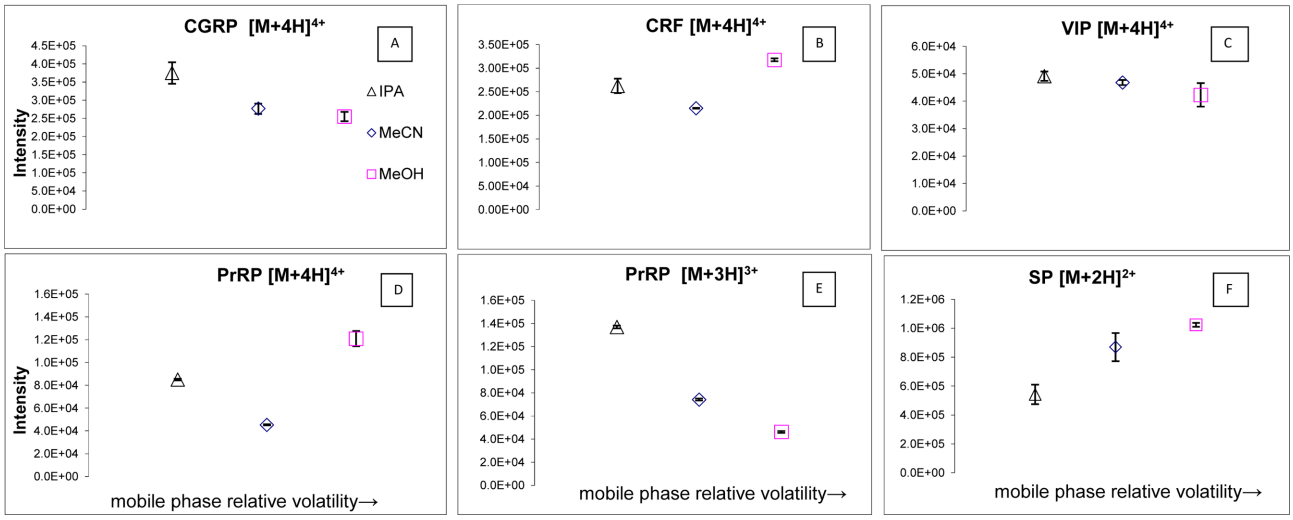

Figure 2. ESI response of CGRP (A), CRF (B), VIP (C), PrRP (D and E) and SP (F) in different mobile phase compositions (25, 50 and 75%; v/v IPA, MeCN or MeOH). Each point indicates the mean ion intensities on n (*m/z*) (n=3).

VIP (*m/z* 832.786) regardless of the choice of organic solvent (MeCN, MeOH and IPA) or composition (25, 50 or 75%v/v) of the mobile phase. Similarly, for the smallest of the peptides analysed, SP, the doubly charged species $[M+2H]^{2+}$ (*m/z* 674.883) dominated regardless of the type of organic solvent (MeCN, MeOH and IPA) or composition (25, 50 or 75%, v/v) of the mobile phase.

In contrast, the ESI mass spectra of PrRP ions showed that the triply charge state $[M+3H]^{3+}$ (*m/z* 758.956) or the quadruply charge state (*m/z* 569.477) dominates in the mass spectrum depending on the solvent used; in 25, 50 and 75%, v/v MeOH, the $[M+4H]^{4+}$ ion dominated whereas in 25, 50 and 75%, v/v MeCN and in IPA the $[M+3H]^{3+}$ dominated. This clearly demonstrates that the dominant charge state of PrRP can be controlled by the nature of the organic solvent in the mixture. The effect, however, cannot be ascribed to the organic solvent being protic (MeOH, IPA) or aprotic (MeCN) or solvent dielectric constant. Rather, it indicates the importance of the relative volatility of the mobile phase mixture when analysing certain peptides (Figures 2a-f): In the high boiling point organic solvents (IPA, 88.2°C and MeCN, 81.6°C) the $[M+3H]^{3+}$ dominated. In contrast, in the lower boiling point solvent (MeOH, 65°C) the $M+4H]^{4+}$ species

Table 2. Characteristics of the peptides. [a]Based on pKb values at pH approx. 3.26 (i.e.: R: 12.48, D:3.65, E: 4.25, H 6.0, K: 10.53, N-term ~ 9 and C-term ~2)[24]

Peptide (Mol wt, Da)	Amino acid composition	Predicted charge state (solution)[a]
CGRP 8-37 (3, 125.59)	*COOH*-VT**HR**LAGLLS[10] **R**SGGVV**K**NNF[20] VPTNVGS**K**AF30**NH$_3^+$**	5
CRF 6-33(3,220.66)	*COOH*-ISL*D*LTF**H**LL[10] **R***E*VLEMA**R**AE[20] QLAQQAS**H**[28]**NH$_3^+$**	4
SP (1,347.71)	*COOH*-**RPK**PQQFFGL[10]M**NH$_3^+$**	2
PrRP 12-31 (2,272.57)	*COOH*-TP*D*INPAWYA[10]**SRGIRPVGRF**[20]**NH$_3^+$**	3
VIP (3,325.8)	*COOH*-**HS***D*AVFT*D*NY[10] T**RLRK**QMAV**K**[20] **K**YLNSILN[28]**NH$_3$**	6

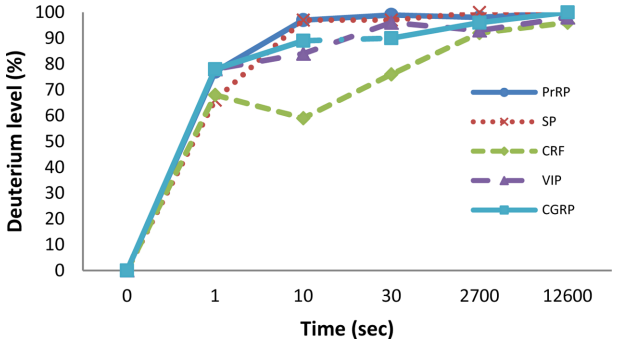

Figure 3. Time evolution profiles of the H/D exchange for PrRP, SP, CRF, VIP and CGRP.

dominated. This means that higher charge states (lower *m/z*) dominate in more volatile solvent mixtures, Figure 2d and e. This effect most likely is attributed to a more rapid desolvation of the droplets: MeOH (in H_2O and FA) evaporate faster than droplets with IPA and MeCN (in H_2O and FA) because of their higher vapour pressure. In line with our observations, Iavarone and co-workers also reported that ions were observed to deprotonate when small amounts of solvent more volatile than water were added to the droplets.[16,18] They rationalised the observation in terms of solvent composition and number of gas phase collisions affecting the proton transfer reaction in the ionisation process. Effects on CSD induced by the electrospray conditions cannot be ruled out and the use of a nano- ESI source would minimize/remove this effect. Though, the solvent mixture composition changes during the ESI evaporation process making the true composition upon ion emission unknown.

For the other peptides (CGRP, CRF, SP, VIP), the solvent environment did not result in an enhancement of the charge state observed in the ESI spectrum. Hence, solvent volatility, by itself, was not sufficient to affect overall charge state.

It is worthy to note that the observed ESI signal of CRF, PrRP and partly CGRP and VIP were more intense when these peptides were analysed in a mobile phase with IPA or MeOH (polar protic solvents). In these solvent mixtures, the ion intensities ($[M+3H]^{3+}$ and $[M+4H]^{4+}$) were significantly higher for PrRP and CRF ions at all solvent compositions, and for CGRP ions at 25% IPA, $[M+3H]^{3+}$ at 50 and 75%, v/v IPA, and for VIP ($[M+4H]^{4+}$and $[M+5H]^{5+}$) at 25%, v/v. However, a more intense signal was obtained for SP in MeCN (75%, v/v), for VIP in MeOH (75%, v/v) and in IPA, less intense ESI responses (25 and 50% IPA) was generated. This effect was observed in all repeated experiments.

The higher charge states in the more volatile solvent mixtures (MeOH compared with MeCN) could also be ascribed to the differences in solution structure: Though the behaviour of peptides is different in solvent from that in the gas phase, it has been shown that gas phase structure is influenced by solution structure.[19,20] Circular Dichroism (CD) and Hydrogen-Deuterium (H/D) exchange results showed that although the solvent choice induced significant changes in conformational preference, all structures produce similar charge states, independent of conformation. This is in line with previously reported observations for proteins. H/D exchange MS and CD data showed that the peptides exist in open conformation in water except for CRF which appears to adopt some helical structure in water (H/D data, Figure 3). This however, does not affect the observed charge state (base peak $[M+4H]^{4+}$) which can still be predicted based on the pKb of the amino acids in the peptide chain.

For PrRP the observed charge states, $[M+3H]^{3+}$ and $[M+4H]^{4+}$, in the ESI mass spectra were clearly affected by the choice of organic solvent in the mobile phase. Interestingly, studies by Sterner and co-workers conclude that the maximum charge state might be more dependent on the proton affinity of the less volatile solvent[15]; there is a large loss of solvents before the small droplets containing the peptides are formed.

The peptides GGRP, VIP and CRF all showed characteristic $[M+4H]^{4+}$ ions in the mass spectrum. Based on the pK_b of ionisable side chains in these peptides, the predicted number of charges on the peptides in solution (at pH 3.26) should be 5 for CGRP, 6 for VIP and 4 for CRF (Table 2); hence other factors may be equally important. In addition to our observation, many studies have shown that there is little correlation between charge state distribution in solution and the charge state distribution observed in the mass spectrum.[16,21]

Solvent effects on ESI response are of major importance when using solvent gradients in peptide LC-MS separation. Therefore, multiple charging of these peptides could be

controlled by other parameters, notably, pK_b of the amino acid side chain in the peptide molecule, than conformation, solvent or amino acid composition at the determined pH of 3.26.

Conclusions

Changes in solvent composition affect the rate of evaporation of the droplets during the ESI process, the relative gas-phase basicity, the surface tension of these droplets and possibly the orientation of the peptides within the droplet. Here, we examined the effect of mobile phase composition and volatility on the ESI response (CSD and total ion intensity) of five peptides. The study of charge state provides some unique information that cannot be accessed by other analytical means.

Although the gas phase structure is influenced by solution structure, the exact composition of the solution upon ion emission may not be known. And therefore, controlling the CSD of these peptides at the set pH cannot be determined by solvent composition, i.e., organic solvent composition has little of no influence on CSD of the peptides studied. Still, pH and pK_b values are relatively good predictors of the dominant charge state.

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