Protonation in Electrospray Mass Spectrometry: Wrong-Way-Round or Right-Way-Round?

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The term "wrong-way-round ionization" has been used in studies of electrospray ionization to describe the observation of protonated or deprotonated ions when sampling strongly basic or acidic solutions (respectively) where such ions are not expected to exist in appreciable concentrations in solution. Study of the dependence of ionization of the weak base caffeine on the electrospray capillary potential reveals three distinct contributors to wrong-way-round ionization. At near-neutral pH in solutions of low ionic strength, protonation of caffeine results from the surface enrichment of electrolytically produced protons in the surface layer of the droplets from which ions are desorbed. For solutions made strongly basic with ammonia, gas-phase proton transfer from ammonium ions can create protonated caffeine. These two mechanisms have been discussed previously elsewhere. For solutions of high ionic strength at neutral or high pH, the data suggest that discharge-induced ionization is responsible for the production of protonated caffeine. This mechanism probably accounts for some of the wrong-way-round ionization reported elsewhere. (J Am Soc Mass Spectrom 2000, 11, 961–966) © 2000 American Society for Mass Spectrometry

Protonation is arguably the most important means of ionization in positive ion electrospray mass spectrometry (ES MS) [1, 2]. Nevertheless, questions remain concerning the correlation between observed gas-phase ions and the acid/base equilibria of bulk sample solutions [3–9]. Because ES MS samples preformed ions (including those formed through electrochemical reactions [10]), it is reasonably anticipated that both signal intensities and ion charge states should be sensitive to changes in solution pH. However, numerous researchers investigating different types of samples (including proteins [6, 11], peptides [9], and amino acids [5, 12]) have observed behavior inconsistent with expected bulk solution chemistry. For example, Boyd and co-workers [5] reported that intensities of protonated amino acid signals varied only about threefold when spraying solutions with pH ranging from 3 to 11, even though the corresponding equilibrium concentrations should vary by several orders of magnitude over this pH range. Similarly, many experimental results have shown that the charge distribution in ES MS is surprisingly insensitive to changes in the pH of the sample solution [9] (except when conformational structure is altered [13]), although it has been proposed that the roughly Gaussian charge distributions observed in ES protein spectra reflect solution acid/base equilibria [7, 14]. For example, protonated ions of lysozyme with up to +9 charges were detected by ES MS from a basic solution (pH $\sim$ 10) [6] in which extensive protonation would not be expected. Conversely, deprotonated ions of myoglobin with maximum charge of -17 were observed from an acidic solution (pH $\sim$ 3.5) [6]. The underlying charging phenomena were systematically studied by Wang and Cole [9] using some small peptides as model samples (so that conformational effects could be excluded). They found that the intensity ratios between doubly and singly protonated ions in ES mass spectra were only quite weakly dependent on the solution pH. Such nonequilibrium charging phenomena have collectively been dubbed "wrong-way-round" electrospray mass spectrometry by Boyd and co-workers [5].

The source of ionizing protons is a key question for understanding the processes that lead to production of gas-phase protonated molecules from solutions of high pH. Several mechanisms have been proposed. For example, protons can be generated by solvent oxidation in positive ion ES [3, 12, 15, 16]; the resulting decrease in the pH of electrosprayed solutions has been confirmed using laser-induced fluorescence [16]. Fenn [2] proposed that such effects may be amplified if ions are desorbed from small and highly charged droplets resulting from solvent evaporation and Rayleigh subdivi-

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sions. However, because ES currents are generally below 10^{-6} A [17, 18], this mechanism cannot significantly affect the pH of buffered or strongly basic solutions [5]. This is not to say that there might not be *regions* of low pH in inhomogeneous electrosprayed droplets. For example, Gatlin and Tureček [15] suggested that excess charges (e.g., protons) are confined within a thin surface layer of the droplet rather than homogeneously distributed in the bulk of the droplets, so that the *local* acidity of the droplet surface can be 3–4 orders of magnitude higher than that of the bulk solution. They and others [5] have invoked this charge localization to help explain observation of otherwise anomalously high charge. Siu and co-workers [7] suggested an alternative explanation for "excess" protonation from high-pH solutions containing nitrogen bases such as ammonia and triethylamine. They invoked charge transfer from protonated amines to neutral analyte molecules in the gas phase. This mechanism has been further investigated by other researchers [5, 12, 19]. However, Boyd and co-workers obtained strong signals from protonated analytes even when solutions were modified to pH $>$ 11 using tetramethylammonium hydroxide (TMA·OH). Protonated ions from such a solution can arise neither by a titration effect involving electrolytically produced protons, nor by gas-phase proton transfer reactions involving a protonated base. The work presented here will provide an alternative explanation for this wrong-way-round phenomenon by careful examination of the ES ionization of caffeine.

Experimental

Mass Spectrometry

Mass spectra were obtained with a Micromass (Manchester, UK) Quattro II triple quadrupole mass spectrometer equipped with a standard ES probe and interface. The source temperature was 80 °C. The flow rates of nebulizing gas and drying gas (boil-off from liquid N_2) were 20 and 400 L/h, respectively. The capillary voltage was varied from 0.5 to 4.0 kV, while the counterelectrode voltage was held constant at 0.5 kV. The probe was modified to allow monitoring of the ES emitter current by connecting a Keithley 600A electrometer (Keithley, Cleveland, OH) in series with the high voltage emitter power supply line. Leakage currents in this configuration were found to be less than ~2 nA at 4 kV (by operating the probe with no solution flowing). Solutions were infused at a flow rate of 5 μL/min using a Harvard Model 22 syringe pump. Mass spectra were acquired over a range of mass-to-charge (m/z) from 20 to 750 using the first analyzer ($Q1$), normally in the multichannel accumulation (MCA) mode (12 scans summed in 30 s). Data were background subtracted using a solvent blank and generally averaged from triplicate spectra (36 scans total). The instrument was tuned using a 2 μM Glu-fibrinopeptide (GFP) peptide solution and was maintained at unit mass resolution (FWHM $\simeq$ 0.7) throughout the scanned range. The cone voltage was set at 20 V except where noted.

For tandem MS experiments, the resolution of $Q1$ was decreased (FWHM = 3.5) for better sensitivity. Roughly 5×10^{-4} torr of argon was used as the collision gas in $q2$, and the collision voltage was 1 V. Fragment ions were resolved with $Q3$ at unit mass resolution.

Chemicals and Sample Preparation

Caffeine and arginine hydrochloride (reagent grade, 99%) were purchased from Sigma (St. Louis, MO) and used as received, as was decyltrimethylammonium bromide (DTMA·Br, reagent grade, 99%; TCI America, Portland, OR). Stock solutions (2 mM) were prepared using 50/50 (v/v) deionized water (purified with a Milli-Q HPLC water purifier; Millipore; Bedford, MA)/methanol (HPLC grade; Fisher, Pittsburgh, PA). Analytical samples were prepared by dilution of the stock solutions to the desired concentration (0.25–5 μM) with water, so that the final methanol concentration was $\leq$0.2%. Modifiers (NaCl and NaOH from Sigma; formic acid and aqueous ammonia from Acros Division, Fisher Chemical, Pittsburgh, PA) were all reagent grade and used as received.

Results and Discussion

Protonation of Caffeine in ES MS

From the pK_b of caffeine (14.15 [20]), it can be estimated that the equilibrium concentration of protonated caffeine (MH^+) is only ~1.4×10^{-13} M in a 2 μM neutral aqueous solution. This is far below the typical limit of detection for species in ES MS (~10^{-9} M) [17]. Nevertheless, there is a strong signal for MH^+ (at m/z 195) in the ES spectrum acquired from a 2 μM aqueous caffeine solution at pH 6.8 (Figure 1a). Indeed, the MH^+ signal intensity for caffeine is only about 10-fold lower than that for a 2 μM aqueous solution of arginine (m/z_{MH^+} = 175; pK_a ~ 12.48, or pK_b = 1.52 [21]) at pH 2.8 (adjusted with formic acid; Figure 1b), despite the fact that the expected equilibrium concentrations in bulk solution differ by 7 orders of magnitude! The m/z values differ by only 20; mass discrimination in the quadrupole analyzer cannot account for the "anomalously" high caffeine (or low arginine) signal. Differences in ion desorption efficiency are also unlikely to account for the signals; these efficiencies usually differ by less than 20-fold at low concentration ($<5 \times 10^{-6}$ M), even for compounds greatly different in size and solvation [17].

The model of Gatlin and Tureček [15] offers an alternative explanation based on the high acidity of the ES droplet surface. As a result of droplet evaporation and subdivision, both caffeine and arginine will become more concentrated (by a factor f) in the final droplets before ion emission, i.e., $C_{caffeine} = f\,C^0_{caffeine}$, $C_{arginine}$ =

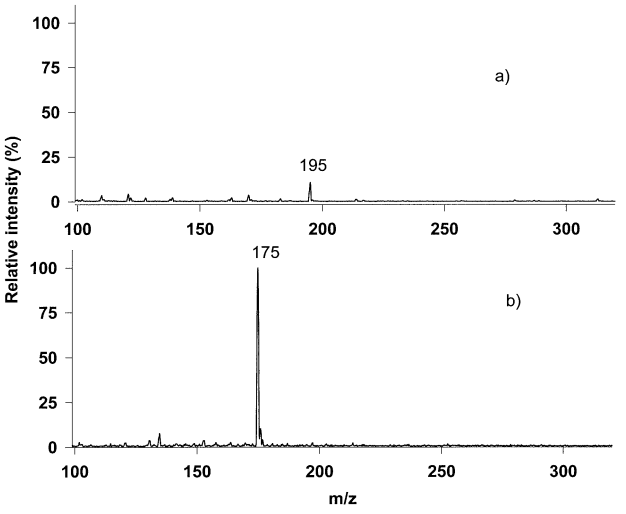

Figure 1. Positive ion ES mass spectra acquired from (**a**) a 2 μM aqueous caffeine solution at pH ~ 6.8 and (**b**) a 2 μM aqueous arginine solution at pH ~ 2.8 (adjusted with 0.2% v/v formic acid). Both spectra are normalized to the base peak (*m/z* 175) for (**b**). Capillary voltage: 3.0 kV.

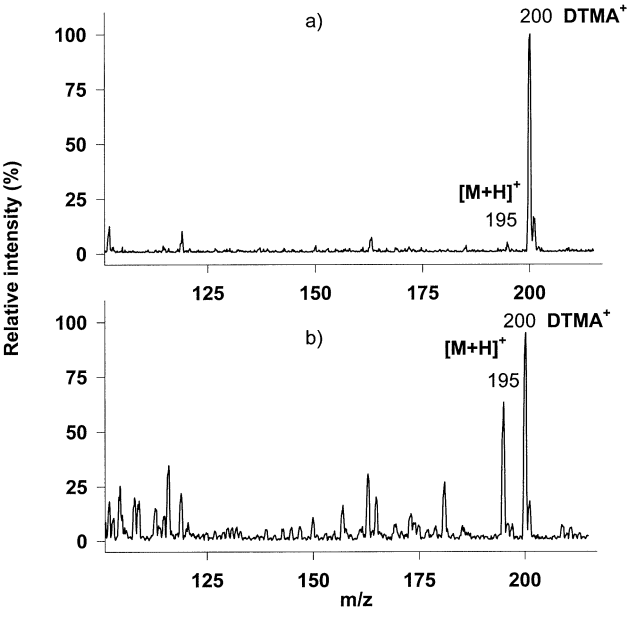

Figure 2. Positive ion ES mass spectra acquired from an aqueous solution containing 5 μM caffeine and 0.25 μM decyltrimethylammonium (DTMA) bromide. Capillary voltage (**a**) 1.0 kV and (**b**) 3.0 kV. Both spectra are normalized to their respective base peaks. The absolute base peak intensity in (**b**) is about 5.7 times higher than in (**a**).

$f\,C^0_{\text{arginine}}$, where C refers to formal concentrations, and the superscript 0 refers to the initial bulk condition. Furthermore, if arginine is fully protonated (as it should be at pH 2.8), the actual concentration of protonated arginine ($[MH^+]_{\text{arginine}}$) will be equal to C_{arginine}. Finally, if $C^0_{\text{caffeine}} = C^0_{\text{arginine}}$, it can be shown by substitution from the K_b expression for caffeine that

$$\frac{[MH^+]_{\text{caffeine}}}{[MH^+]_{\text{arginine}}} = \frac{fC^0_{\text{caffeine}}\,\dfrac{K_{b,\text{caffeine}}}{[OH^-] + K_{b,\text{caffeine}}}}{fC^0_{\text{arginine}}} = \frac{K_{b,\text{caffeine}}}{[OH^-] + K_{b,\text{caffeine}}} \quad (1)$$

From eq 1 (and K_w) it can be estimated that the surface proton concentration ($[H^+]_s$) must be ~0.16 M to achieve the observed 1:10 ratio of protonated caffeine:protonated arginine. Can this concentration reasonably be achieved in an electrospray droplet? According to the model of Iribarne and Thomson [22], a droplet must typically reach a radius of 8 nm with ~70 excess elementary charges for ion evaporation to occur. If these 70 charges are all protons (a reasonable possibility if spraying unbuffered pH-neutral solutions; *see below*), and if the excess protons are all constrained to a surface layer with $[H^+]_s = 0.16$ M, it can be estimated that the thickness of that layer will be ~1.2 nm. This is slightly less than the diameter of a hydrated proton (~1.8 nm [23]); if the surface layer thickness expands to 1.8 nm, the droplet radius must decrease to ~6.5 nm to maintain $[H^+]_s = 0.16$ M with just 70 excess protons. This is only slightly smaller than the Iribarne value; it therefore seems feasible that emission could occur from a sufficiently acidic surface layer *provided that all surface excess charges are protons*. This in turn could explain why so many compounds can be protonated in ES MS.

Effect of Electrolytic Production of Protons

The bulk concentration of electrolytically produced protons $[H^+]_e$ will be equivalent to the total excess charge $[Q]$ if there is 100% current efficiency and there are no subsequent reactions of the protons generated. For typical ES conditions (current $I = 50$ nA and flow rate $V_f = 5$ μL/min), $[H^+]_e = \sim 6 \times 10^{-6}$ M [16]. In basic or buffered solutions, these protons will rapidly react, so that high surface acidity is unlikely. For strongly acidic solutions, the pH change due to $[H^+]_e$ is negligible. For an unbuffered solution near neutral pH, the final proton concentration $C_{H^+} \simeq C^0_{H^+} + [Q]$, where $C^0_{H^+}$ is the initial concentration of protons in bulk solution. Thus, increasing $[Q]$ (e.g., by increasing the capillary voltage, V_c) should increase the availability of protons and the surface acidity. For a binary mixture solution wherein one analyte is basic and the other is an intrinsic ion, the increasing acidity should affect only the intensity of the basic analyte ion. Figure 2a, b compare positive-ion ES spectra for such a mixture [5 μM caffeine and 0.25 μM decyltrimethylammonium (DTMA) bromide] acquired at capillary voltages V_c = 1.0 and 3.0 kV, respectively. The spectra are normalized to facilitate comparison; the absolute intensity of the base peak in Figure 2b is actually ~5.7 times higher than that in Figure 2a. More importantly, it is clearly evident here and in Figure 3 that the intensity *ratio*

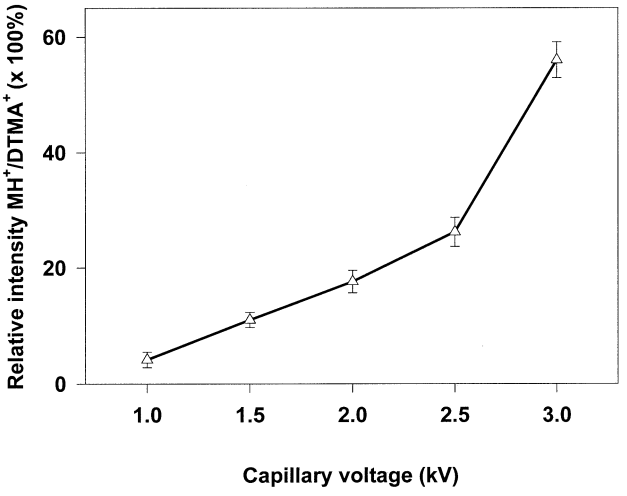

Figure 3. Intensity of MH^+ from caffeine relative to $DTMA^+$ as a function of capillary voltage in positive ion ES MS. Solution composition was the same as in Figure 2. Error bars represent ±one standard deviation for triplicate measurements.

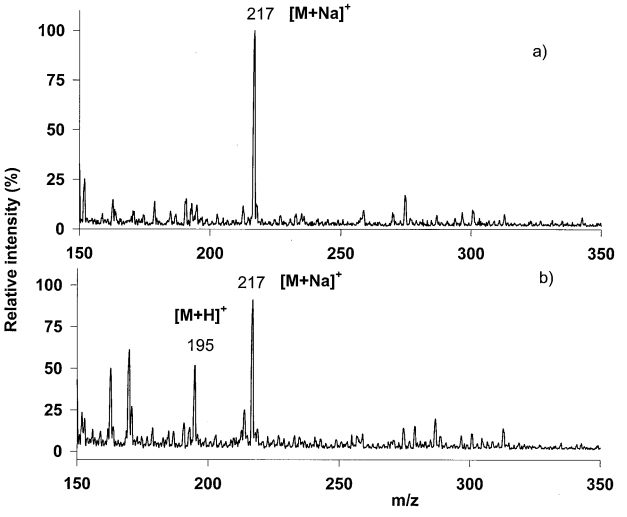

Figure 4. Positive ion ES mass spectra acquired from a 2 μM aqueous solution of caffeine at pH 10.5 adjusted with NaOH. Capillary voltage: (**a**) +2.5 kV and (**b**) +3.0 kV.

$MH^+/DTMA^+$ increases significantly with increasing capillary voltage (thus increasing [Q]). Some of the increase may be due to the onset of a discharge (*see below*), as evident from the greater number and intensity of background ions in Figure 2b. Nevertheless, the strong dependence of relative ion response on V_c clearly complicates the potential application of ES MS as a quantitative probe of solution chemical compositions.

Wrong-Way-Round or Right-Way-Round Ionization?

Although enhanced surface acidity can account for the "excess" MH^+ observed in unbuffered, near-pH-neutral solutions, it is primarily the observation of MH^+ from strongly basic solutions for which ionization can be said to be "wrong-way-round." At pH 10, electrolytically generated protons will be quickly consumed by the excess (~100 μM) base, so that the ~6 μM $[H^+]_e$ mentioned above will be inadequate to significantly affect the pH. The cation of the added base (e.g., NH_4^+ if the solution pH was adjusted using NH_4OH) will become the predominant excess cation in the surface layer instead of H^+. Due to the very weak basicity of caffeine ($K_b = 7 \times 10^{-15}$ as compared to NH_3 $K_b = 1.7 \times 10^{-5}$), solution-phase protonation of caffeine even in the droplet surface layer is unlikely. Instead, the strong signal for MH^+ ions observed from caffeine solutions adjusted to pH 10.5 with NH_4OH (spectrum not shown) is probably due to gas-phase ion–molecule reactions (IMR; eq 2) or collision-induced dissociation (CID) of $[M + NH_4]^+$ ions (eq 3) [7, 12]:

$$NH_4^+(g) + M(g) \xrightarrow{\text{IMR}} NH_3(g) + MH^+(g) \quad (2)$$

$$(NH_3\text{–}H^+\text{–}M)(g) \xrightarrow{\text{CID}} NH_3(g) + MH^+(g) \quad (3)$$

The *possibility* of contributions from eq 3 was confirmed by observing a strong $[M + NH_4]^+$ signal (*m/z* 212; 66% abundance relative to the MH^+ base peak) when the cone voltage was lowered to 5 V (from the 20 V default). However, for either reaction to be thermodynamically favored requires that caffeine be a stronger gas-phase base than NH_3. The gas phase basicity (GB) for caffeine is not available in the literature, so the relative GB's were assessed by tandem MS. Under gentle collision conditions (1 V offset and low collision gas pressure), $[M + NH_4]^+$ dissociated virtually quantitatively to protonated caffeine, suggesting that GB(caffeine) > $GB(NH_3)$, so that proton transfer from NH_4^+ to neutral caffeine is thermochemically favored [24].

What about the case where there is no abundant proton source in either the condensed or gas phase? This would be the case, for example, in a solution adjusted to high pH with NaOH instead of ammonia. As evident in Figure 4, the pervasive MH^+ ion from caffeine is evident even in this case, provided that V_c is adequate. Even at the V_c of Figure 4b, however, the emission current is only about ~120 nA (Figure 5), so the resulting production of protons (~7 μM) cannot significantly alter the solution pH. The data of Figure 5 provide the answer. The appearance of abundant MH^+ ions coincides with a sudden, substantial increase in emission current; these ions evidently derive from the onset of corona discharge. This conclusion is corroborated by the near identical appearance of curves D and E of Figure 6. Whether from ~0.3 mM NaOH (at pH 10.5) or 0.32 mM NaCl (pH ~6.7), sodiated caffeine ions are dominant at low and moderate V_c, suggesting that the final droplets from these two solutions are enriched

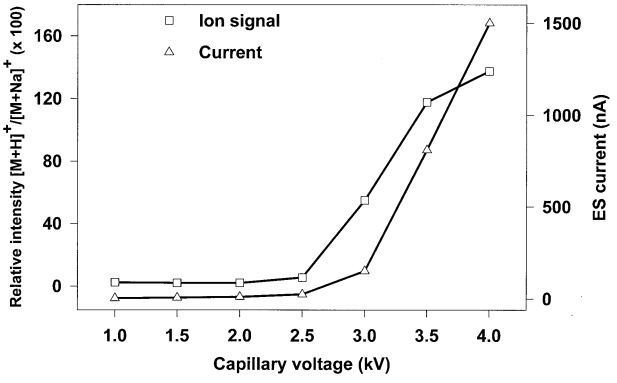

Figure 5. Intensity of MH^+ relative to MNa^+ ions from caffeine obtained from a 2 μM aqueous caffeine solution at pH 10.5 (adjusted with NaOH) as a function of capillary voltage in positive ion ES MS. Emission current is indicated on the right-hand *y* axis. Zero values are offset slightly for clarity.

in excess Na^+ ions. In both cases, protonated ions appear upon the onset of discharge. According to a suggested mechanism for atmospheric pressure chemical ionization (APCI) [25–27], ionization of air molecules (e.g., N_2 and O_2) induced by the discharge leads (via a complex reaction series [27]) to production of proton–solvent cluster ions (e.g., H_3O^+) that act as the main reagent ions for gas-phase protonation of analyte molecules. Because GB(H_2O) = 165 kcal/mol < GB(NH_3) = 196.4 kcal/mol [28], and GB(NH_3) < GB (caffeine) (as established in the collision-induced dissociation experiments described above), proton transfer from H_3O^+ (generated by APCI) to neutral caffeine molecules is a thermodynamically favored reaction. Although direct evaporation of neutral caffeine molecules from ES droplets may be unlikely due to limited volatility, caffeine molecules can be brought into the gas phase through desorption of sodium adducts (i.e., MNa^+) or as caffeine-solvent clusters in the final small droplets, so that removal of the last solvent molecules in the droplets should lead to the production of neutral caffeine. Therefore, proton transfer reactions may occur by paths like the following two:

$$H^+(S)_n(g) + MNa^+(g) \rightarrow MH^+(S)_{n-l}(g) + Na^+(S)_l(g) \rightarrow MH^+(g) \quad (4)$$

$$H^+(S)_n(g) + M(g) \rightarrow [MH^+(S)_n]^*(g) \rightarrow MH^+(g) + nS(g) \quad (5)$$

where $H^+(S)_n(g)$ denotes a protonated solvent cluster generated by APCI. Similar processes probably account for Boyd's observation of protonated amino acids from solutions made basic with tetramethylammonium hydroxide; his ES current (~1 μA) was certainly adequate to include a contribution from a discharge. [An alternative (suggested by a reviewer) that discharge ions may be incorporated into the droplets cannot be ruled out, but seems unlikely in light of the coulombic repulsions likely to exclude acidic species from positive droplets, and the large excess of such incorporation needed to "titrate" the excess base.]

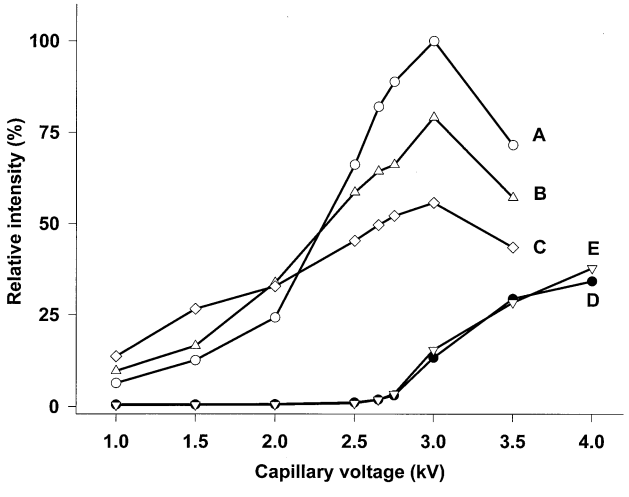

Figure 6. Intensities of MH^+ ions from caffeine obtained from various solutions as a function of capillary voltage in positive ion ES MS. 2 μM aqueous solutions were at (**A**) pH 10.5 adjusted with NH_4OH; (**B**) pH 6.8 without modifier; (**C**) pH 2.8 adjusted with formic acid; (**D**) pH 10.5 adjusted with ~3 mM NaOH, and (**E**) pH 6.8 with addition of 3.2 mM NaCl. All intensities are normalized to the highest intensity in curve (**A**).

It is informative to compare the behavior of the discharge-related MH^+ with that from the other solutions (curves A, B, and C of Figure 6). For each of the latter curves, the relative abundance of MH^+ first increases with increasing capillary voltage, then decreases as the voltage continues to increase above about 3.0 kV. The initial increase in curve A is probably due to an increase in the efficiency of the reactions of eqs 2 and (especially) 3 as the collision energy increases. For curve B, the initial increase probably arises from an increasing contribution from electrolytically generated protons. Curve C may derive from effects analogous to those in curve A (eqs 2 and 3), although no clusters could be detected in this case, even at reduced cone voltage. Existence of such clusters may account for the surprising observation that addition of formic acid actually suppresses formation of MH^+ for caffeine at high capillary voltage; further study is needed to explain this effect. In all cases, the eventual decrease in MH^+ coincides roughly with the onset of discharge, suggesting a degradation in ES performance, possibly due to the onset of multijet electrospray [19, 29] and/or space charge effects.

It is concluded that ionization in ES MS is strongly governed by the chemistry in the droplet surface layer. Observation of MH^+ ions from strongly basic solutions is a result of right-way-round ionization, but through gas-phase chemical ionization with precursors either present in solution (e.g., NH_4^+) or induced by corona discharge. Presumably, deprotonated ions from acidic solutions in negative-ion ES MS may be generated

analogously by basic gas-phase species (e.g., OH^- or methoxide) generated in negative-ion discharges [30, 31]. Such processes appear to be more complex (involving inter alia radicals from radiation damage to solvent molecules) and less well-characterized than their positive-ion analogies.

Acknowledgments

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