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Positive and negative analyte ion yield in matrix-assisted laser desorption/ionization revisited

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ABSTRACT

The most commonly accepted model for the formation of analyte ions in MALDI-MS assumes a primary ionization of the matrix e.g., by photoionization, leading among others to stable protonated and deprotonated matrix ions, respectively. Peptide and protein ions are then formed by secondary proton transfer reactions in the expanding plume. This model had been checked experimentally by comparing the yield of positive to negative ions of three peptides (Bradykinin, Angiotensin I and Fibrinopeptide A) and six matrices (α-cyano-4-hydroxycinnamicacid (CHCA), 2,5-dihydroxybenzoicacid (DHB), 6-aza-2-thiothymine (ATT), 4-nitroaniline (4-NA), 2-amino-5-nitro-4-picoline (ANP), 5-aminoquinolione (5-AQ)), differing in gas-phase basicity by about 100 kJ/mole [M. Dashtiev, E. Wäfler, U. Röhling, M. Gorshkov, F. Hillenkamp, R. Zenobi, Int. J. Mass Spetrom. 268 (2007) 122]. The data have been revisited for a more general and in-depth analysis. Model predictions are presented for a wide range of experimental parameters, in particular for ranges of the gas-phase basicity and acidity of analyte and matrix and for different molar ratios of analyte to matrix as well as the yield of primary matrix ions. It is shown that the observed ion yields cannot be explained by any single and consistent set of parameters. It is concluded that the existing simple model needs be modified to fully explain the experimental findings. Such modifications should primarily address the formation of negative matrix and analyte ions.

1. Introduction

In 2007 Dashtiev et al. [1] published a study comparing the yield of positive to negative ions in MALDI-MS. Three different peptides, spanning the range from acidic to basic (Fibrinopeptide A, Angiotensin I and Bradykinin) and six matrices (α-cyano-4-hydroxycinnamicacid (CHCA), 2,5-dihydroxybenzoicacid (DHB), 6-aza-2-thiothymine (ATT), 4-nitroaniline (4-NA), 2-amino-5-nitro-4-picoline (ANP), 5-aminoquinolione (5-AQ)), spanning the range of gas-phase basicities (GB) of ca. 800–900 kJ/mol were tested. The results were compared to theoretical predictions based on the equilibrium model for charge transfer between matrix and analyte ions in the laser ablation plume, as originally published by Ehring et al. and later refined in a series of publications by several authors [2]:

$$(M + H^+) + A \overset{K^+}{\rightleftarrows} (A + H^+) + M \quad (1)$$

$$(M - H^-) + A \overset{K^-}{\rightleftarrows} (A - H^-) + M \quad (2)$$

where M stands for matrix and A for analyte.

Note that these reactions were originally suggested based on the matrix ions observed in the spectra [2a]. They do not, however, imply that the initial concentrations of $(M+H^+)$ and $(M-H^-)$ be equal. Also, for some of the matrices the dominant matrix anion is $(2M-H^-)$ rather than $(M-H^-)$. This should not change the basic proton transfer reactions systematically, as discussed further down.

K^+ and K^- are the equilibrium constants for the two reactions.

$$K^+ = \exp\left\{-\frac{[(GB(M) - GB(A)]}{RT}\right\},$$

$$K^- = \exp\left\{-\frac{[(GB(A - H^-) - GB(M - H^-)]}{RT}\right\} \quad (3)$$

$$\frac{[A + H^+]}{[A - H^-]} = \frac{[M + H^+]}{[M - H^-]} \exp\left\{-\frac{[(GB(M) + GB(M - H^-)]}{RT}\right\} \times \exp\left\{\frac{[GB(A) + GB(A - H^-)]}{RT}\right\} \quad (4)$$

For a given peptide this reduces to:

$$\frac{[A + H^+]}{[A - H^-]} = \text{const.} \frac{[M + H^+]}{[M - H^-]} \exp\left\{-\frac{[(GB(M) + GB(M - H^-)]}{RT}\right\} \quad (5)$$

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Table 1
Approximate solutions of the coupled reaction equations for selected parameter ranges. For all parameter ranges $K^+ \gg 1$; $x_A^0; x_{M+H^+}^0; x_{M-H^-}^0 \ll 1$.

	$x_A^0 \gg x_{M+H^+}^0; x_{M-H^-}^0$	$x_A^0 \ll x_{M+H^+}^0; x_{M-H^-}^0$	$x_A^0 = x_{M+H^+}^0 + x_{M-H^-}^0$
$K^- \gg 1$ approximations: $K^+ x_{M+H^+}^0; K^- x_{M-H^-}^0 \gg 1;$ $K^+ x_A^0;\ K^- x_A^0 \gg 1$	$x_{A+H^+} = \frac{K^+ x_{M+H^+}^0}{1+K^+ x_A^0} x_A^0$ $x_{A-H^-} = \frac{K^- x_{M-H^-}^0}{1+K^- x_A^0} x_A^0$ $\frac{x_{A+H^+}}{x_{A-H^-}} = \frac{K^+ x_{M+H^+}^0}{K^- x_{M-H^-}^0} \frac{1+K^- x_A^0}{1+K^+ x_A^0} \approx \frac{x_{M+H^+}^0}{x_{M-H^-}^0}$	$x_{A+H^+} = \frac{K^+ x_{M+H^+}^0}{K^+ x_{M+H^+}^0 + K^- x_{M-H^-}^0} x_A^0$ $x_{A-H^-} = \frac{K^- x_{M-H^-}^0}{K^+ x_{M+H^+}^0 + K^- x_{M-H^-}^0} x_A^0$ $\frac{x_{A+H^+}}{x_{A-H^-}} = \frac{K^+ x_{M+H^+}^0}{K^- x_{M-H^-}^0}$	$x_{A+H^+} = x_{M+H^+}^0$ $x_{A-H^-} = x_{M-H^-}^0$ $\frac{x_{A+H^+}}{x_{A-H^-}} = \frac{x_{M+H^+}^0}{x_{M-H^-}^0}$
$K^- \ll 1$ approximations: $K^+ x_{M+H^+}^0 \gg 1$ $K^- x_{M-H^-}^0 \ll 1$	$x_{A+H^+} = \frac{K^+ x_{M+H^+}^0}{1+K^+ x_A^0} x_A^0$ $x_{A-H^-} = K^- x_{M-H^-}^0 x_A^0$ $\frac{x_{A+H^+}}{x_{A-H^-}} = \frac{K^+ x_{M+H^+}^0}{K^- x_{M-H^-}^0} \frac{1}{1+K^+ x_A^0} \approx \frac{x_{M+H^+}^0}{x_{M-H^-}^0} \frac{1}{K^- x_A^0}$	$x_{A+H^+} = \frac{K^+ x_{M+H^+}^0}{1+K^+ x_{M+H^+}^0} x_A^0 \approx x_A^0$ $x_{A-H^-} = \frac{K^- x_{M-H^-}^0}{1+K^+ x_{M+H^+}^0} x_A^0 \approx \frac{K^-}{K^+} \frac{x_{M-H^-}^0}{x_{M+H^+}^0} x_A^0$ $\frac{x_{A+H^+}}{x_{A-H^-}} = \frac{K^+ x_{M+H^+}^0}{K^- x_{M-H^-}^0}$	$x_{A+H^+} = x_{M+H^+}^0$ $x_{A-H^-} = K^- (x_{M-H^-}^0)^2$ $\frac{x_{A+H^+}}{x_{A-H^-}} = \frac{x_{M+H^+}^0}{K^- (x_{M-H^-}^0)^2}$
$K^- = 1$	$x_{A+H^+} = \frac{K^+ x_{M+H^+}^0}{1+K^+ x_A^0} x_A^0$ $x_{A-H^-} = x_{M-H^-}^0 x_A^0$ $\frac{x_{A+H^+}}{x_{A-H^-}} = \frac{K^+ x_{M+H^+}^0}{1+K^+ x_A^0} \frac{1}{x_{M-H^-}^0} \approx \frac{x_{M+H^+}^0}{x_{M-H^-}^0} \frac{1}{x_A^0}$	$x_{A+H^+} = \frac{K^+ x_{M+H^+}^0}{1+K^+ x_{M+H^+}^0} x_A^0 \approx x_A^0$ $x_{A-H^-} = \frac{K^- x_{M-H^-}^0}{1+K^+ x_{M+H^+}^0} x_A^0 \approx \frac{1}{K^+} \frac{x_{M-H^-}^0}{x_{M+H^+}^0} x_A^0$ $\frac{x_{A+H^+}}{x_{A-H^-}} = \frac{K^+ x_{M+H^+}^0}{x_{M-H^-}^0} = \frac{K^+ x_{M+H^+}^0}{K^- x_{M-H^-}^0}$	$x_{A+H^+} = x_{M+H^+}^0$ $x_{A-H^-} = (x_{M-H^-}^0)^2$ $\frac{x_{A+H^+}}{x_{A-H^-}} = \frac{x_{M+H^+}^0}{(x_{M-H^-}^0)^2}$

Table 2
Measured and estimated gas-phase basicities and gas-phase acidities of some MALDI matrices and analytes.

Compound	GB(M) (kJ/mol)	GB(M−H⁻)[a] (kJ/mol)	Refs.[c]
5-aminoquinoline	n.a. (>850[b])	n.a. (1350[a,b])	
2-amino-5-nitro-4-picoline	n.a. (>850[b])	n.a. 1350[a,b])	
p-nitroaniline	834	1407	[7,8,9]
6-aza-2-thiothymine	n.a. (835[b])	n.a. (1335[a,b])	
2,5-dihydroxybenzoic acid	822[c]	1329[c]	[9,10,11]
α-cyano-4-hydroxy-cinnamic acid	809[c]	1309[a]	[11]
Glycine	852	1402	[7,12]
Lysine	951	1383	[5,12]
Arginine	1006	1359	[5,12]
Bradykinin[b]	>1025	<1329	[1], This work
Angiotensin I[b]	n.a. (<1000)	n.a.	This work
Fibrinopeptide A[b]	n.a. (<975)	n.a.	This work

n.a. = not available.

[a] GB(M−H⁻) = GA(M); for matrices, for which values for GB(M−H⁻) are not known, they were derived from the GB(M) values by addition of 500 kJ/mol (R. Knochenmuss, personal communication).

[b] Estimated values: where only values for the proton affinity PA are reported, the GB was derived by subtraction of 33 kJ/mol.

[c] Most up-to-date values, some of them corrected due to recent changes in the basicity scale, see http://webbook.nist.gov/chemistry/.

Experimentally a relatively constant ratio was observed for all three peptides and the tested matrices with GBs scanning a range of ca. 100 kJ/mol. The authors concluded that these experimental results did not agree with the theoretical predictions of a substantial decrease of the ratio of positive to negative ions with increasing matrix basicity and that the gas-phase proton transfer model and/or some of the assumptions typically made, needed to be refined or modified or even reconsidered. One of the assumptions leading to that conclusion was that the ratio $[M+H^+]/[M-H^-]$ remains constant throughout the plume development process. As will be shown further down, this assumption is not necessarily justified. In 2008 Knochenmuss published a reinterpretation of the experimental results [3] pointing out that if one considers limiting reagents and employs mass balance equations, the experimental results could, in fact, be compatible with the model predictions under certain assumptions. In the following it will be shown that, while pointing in the right direction, the analysis by Knochenmuss is still flawed in some cases and incomplete in others and that a more detailed analysis of the model and the data lead to a more complex interpretation. In particular, it will be shown that the theory predicts very different values for the ion ratio, depending on the original composition of the MALDI plume and that several of the decisive parameters are not sufficiently well known to make unambiguous predictions. The square brackets in Eqs. (4) and (5) symbolize concentrations. However, in the case of an expanding plume, and also for the purpose of direct comparison with [3] the relevant quantities are presented as mole fractions x_i, throughout the rest of this paper.

2. Theory

Knochenmuss has set up two independent equations for the mass balance of the positive (Eq. (6) in reference [3]) and negative ions, but neglected the fact that the two reactions are more or less strongly coupled, because they both use the same pool of matrix and analyte neutrals. As shown below, this can result in massively wrong predictions, depending on the relative concentration of the analyte to the original laser generated matrix ions. The correct mass balance equations of the coupled reactions can be derived to read:

$$K^+ = \frac{[A+H^+][M]}{[M+H^+][A]} = \frac{x_{A+H^+}\{1+x_{A+H^+}+x_{A-H^-}-(x^0_A+x^0_{M+H^+}+x^0_{M-H^-})\}}{\{x^0_{M+H^+}-x_{A+H^+}\}\{x^0_A-x_{A+H^+}-x_{A-H^-}\}} \quad (6)$$

$$K^- = \frac{[A-H^-][M]}{[M-H^-][A]} = \frac{x_{A-H^-}\{1+x_{A+H^+}+x_{A-H^-}-(x^0_A+x^0_{M+H^+}+x^0_{M-H^-})\}}{\{x^0_{M-H^-}-x_{A-H^-}\}\{x^0_A-x_{A+H^+}-x_{A-H^-}\}} \quad (7)$$

x_A and x_M are the mole fractions of analyte and matrix, normalized to the total of all constituents, $\sum_i x_i = 1$. x_{M+H^+}, x_{M-H^-}, x_{A+H^+}, x_{A-H^-}, are those of the matrix and analyte positive and negative ions, respectively. x^0_A, $x^0_{M+H^+}$, and $x^0_{M-H^-}$ are the initial mole fractions of the analyte and the matrix ions in the sample and plume. Equilibrium theory assumes a sufficiently dense and well-mixed MALDI plume [4]. In contrast to the non-coupled equations, these are coupled quadratic equations (in x_{A+H^+} and x_{A-H^-}), which cannot be solved in closed form. Approximate solutions can be derived, however, for the yield of x_{A+H^+} and x_{A-H^-}, as well as their ratio for certain parameter ranges. The results of these calculations are listed in Table 1 along with the approximations used to derive them.

Some of the relevant parameters can be taken from the literature or can be inferred from other molecules to a reasonable degree of accuracy. Their values are listed in Table 2. Unfortunately, some of the most relevant parameters are not known to the necessary degree of accuracy. This holds in particular for the gas-phase basicity of the deprotonated peptide anions (equal to the peptide's gas-phase acidity GA). The gas-phase basicity of all measured neutral peptides is significantly larger than that of all tested matrices. As a result $K^+ \gg 1$ holds for all cases of interest here. However, based on the gas-phase acidities of amino acids (e.g., Gly, 1402 kJ/mol; Lys, 1383 kJ/mol; Arg, 1359 kJ/mol [5], see Table 2) one could argue that the difference between the values of GB(M−H⁻) and GB(A−H⁻) can be positive or negative. The former was tacitly assumed by Knochenmuss. The other not sufficiently well known parameter is the concentration of the initially laser generated matrix cations and anions. Values in the range of 10^{-3} to 10^{-5} have been determined for a number of different MALDI matrices [6], and values for LDI and SIMS experiments are in a similar range. It is, most probably also safe to assume that the initial matrix ion yield is somewhat different for different matrices and will also increase with increasing laser fluence. The only known parameter in the samples investigated in [1] is the 10^{-3} initial ratio of analyte to matrix neutrals, a value which was chosen in order to detect positive and negative ions for all tested combinations of matrices and analytes. For MALDI-MS of peptides this is not excessive, but at the high end of concentrations typically used; for proteins a concentration of 10^{-5} would be more typical.

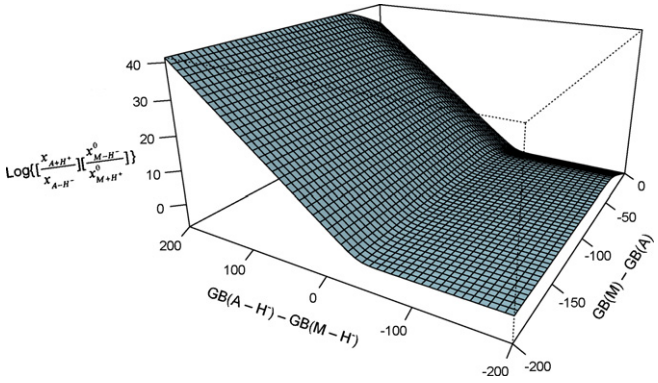

Fig. 1. Plot of the ratio of positive to negative analyte ions as a function of the difference of gas-phase basicities of the matrix and the analyte neutrals and anions, scaled to the ratio of the initial matrix cations and anios. $x^0_A = 10^{-3} \gg x^0_{M+H^+}; x^0_{M-H^-}$.

Because of the uncertainty about the magnitude of these parameters and the as yet unresolved contradictions in the interpretation of the experimental findings, the model predictions have been derived for several different parameter ranges of different relative initial concentrations of analyte neutrals to matrix ions as well as for positive and negative differences of the gas-phase basicities between matrix and peptide anions. K^- is, therefore, allowed to range from very small to very large numbers. Different from the assumptions in [1,3], the ratio of the initial mole fractions of matrix ions $x^0_{M+H^+}/x^0_{M-H^-}$ is kept as a free parameter to retain more generality.

For the special case of $x^0_A \gg x^0_{M+H^+}; x^0_{M-H^-}$ the two reactions are both limited by the amount of available charged matrix molecules of either polarity. In this case the mole fractions of x_A and x_M stay almost constant and equal to their initial values x^0_A and x^0_M throughout and the reactions can, to a first order, be considered non-coupled. The ratio of x_{A+H^+}/x_{A-H^-} can, therefore to a good approximation be derived from the non-coupled equations as derived by Knochenmuss [3]. The result is shown in Fig. 1, which is essentially identical to Fig. 3 of [3] with the added parameter range for $K^- \leq 1$. Plotted is actually the ratio $\left(x_{A+H^+}/x_{A-H^-}\right) \times \left(x^0_{M-H^-}/x^0_{M+H^+}\right)$ to facilitate interpretation for unequal mole fractions of the initial matrix ions. In the range of $K^- \leq 1$ the results also depend on the initial mole fraction of the neutral analytes in the sample. $x^0_A = 10^{-3}$ was chosen to conform to the experimental situation in [1].

For the special case of $x^0_A \ll x^0_{M+H^+}; x^0_{M-H^-}$ only a small fraction of the initial $x^0_{M+H^+}$ and $x^0_{M-H^-}$ gets consumed, so their ratio stays nearly constant throughout the plume expansion and the ratio of x_{A+H^+}/x_{A-H^-} is given by the ratio of the equilibrium constants K^+/K^-. This is the scenario that was assumed by Dashtiev et al. in reference [1]. The results of the ratio $\left(x_{A+H^+}/x_{A-H^-}\right) \times \left(x^0_{M-H^-}/x^0_{M+H^+}\right)$ for this special case and the appropriate ranges of K^+ and K^- are plotted in Fig. 2. Clearly this result differs dramatically from Fig. 2 of reference [3], where x^0_A was assumed to have a value of 10^{-3} and $x^0_{M+H^+}$ to be equal to 10^{-2}.

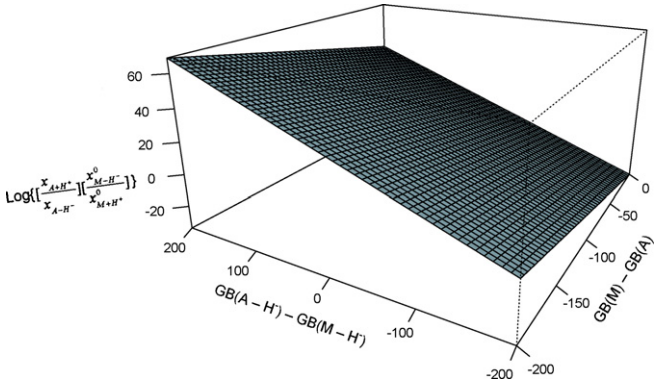

Fig. 2. Plot of the ratio of positive to negative analyte ions as a function of the difference of gas-phase basicities of the matrix and the analyte neutrals and anions, scaled to the ratio of the initial matrix cations and anions. $x^0_A = 10^{-3} \ll x^0_{M+H^+}; x^0_{M-H^-}$.

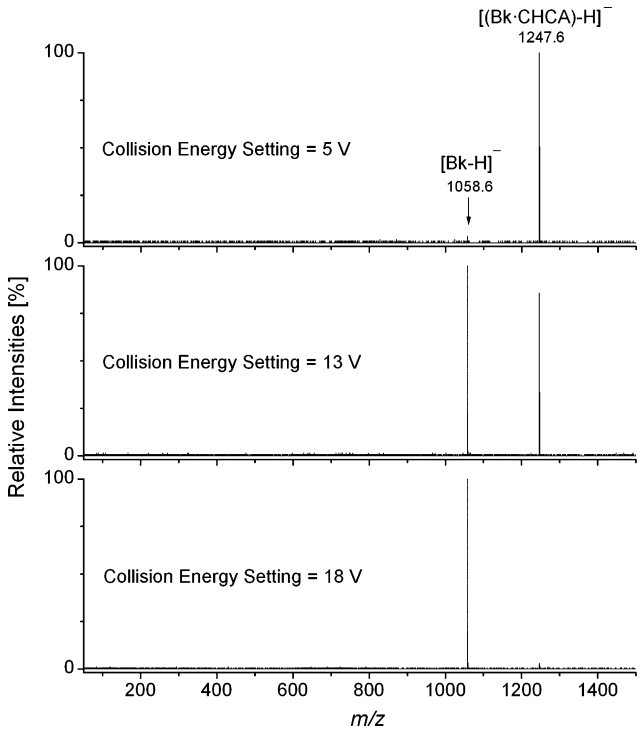

Fig. 3. 5 μM Bradykinin (Bk, monoisotopic MW 1059.6) and 20 μM α-cyano-4-hydroxycinnamic acid (CHCA, monoisotopic MW 189.0) were mixed in a methanol/water (2:1, v/v) solution. A nanoelectrospray ion source (NanoMate model 100; Advion Bioscience, Ithaca, NY) was used for ion production from the mixture, and negative ion mode mass spectra were recorded on a hybrid quadrupole time-of-flight mass spectrometer (Q-TOF Ultima; Waters/Micromass Ltd., Manchester, UK). For MS/MS, the ion at m/z 1247 corresponding to the Bk-CHCA complex was selected in the quadrupole. MS/MS spectra were obtained using different collision energy settings, as shown in the Figure. For low collision energies, the cluster/complex remains intact, whereas for medium and high collision energies the cluster/complex dissociates exclusively into deprotonated Bk ions and neutral CHCA (not detected).

3. Discussion

For a comparison of the model predictions to the experimental results assumptions about the limiting magnitude of the relevant parameters have to be made. Even for Fibrinopeptide as the most acidic peptide analyte and 5-aminoquinoline as the most basic matrix used in [1] the difference in gas-phase basicities is still estimated to be ca.−50 kJ/mol. It is therefore certainly safe to assume that for all cases investigated here $K^+ \gg 1$. Also, for all realistic MALDI applications $x^0_A; x^0_{M+H^+}; x^0_{M-H^-} \ll 1$. Moreover, given the value of $x^0_A = 10^{-3}$ for all experiments and the best available estimates of $x^0_{M+H^+}; x^0_{M-H^-} \leq 10^{-3}$ the approximate solutions of the mass balance equations for $x^0_A \geq x^0_{M+H^+}; x^0_{M-H^-}$ should be appropriate. K^- cannot be calculated directly, because the value for the gas-phase basicity of the analyte anion ($A-H^-$) (= gas-phase acidity of A) is not known. Based on the fact that the GB(A) exceeds the GB(M) even of the most basic amino acids Arg and Lys (Table 2),

one might, at first sight, expect that for all three of them GB(A−H$^-$) would also be larger than GB(M−H$^-$), resulting in a $K^- \ll 1$.

The general trend of this equilibrium constant can, however be checked with a simple dissociation experiment. Using ESI of a mixture of matrix and peptide, the complex (A+M−H$^-$) can be isolated and subjected to CID. This was done for complexes of Bradykinin with ATT, 2,5-DHB, and CHCA. The combination of CHCA with Bradykinin represents the most critical case, i.e., the most acidic matrix with the basic Bradykinin (Fig. 3). For all matrices and for a range of collision energies below those generating fragments of A or M, only A−H$^-$ was observed, supporting the notion that GB(peptide − H$^-$) < GB(M−H$^-$) and $K^- \gg 1$. In other words, the A−H$^-$ appear strongly favored over the (M−H$^-$) ions in reaction (2). This is contrary to the expectation mentioned above.

For the ratio of positive to negative analyte ions to be independent of the gas-phase basicities of the matrix as observed experimentally and explained by Knochenmuss [3], the model calculations not only require that GB(A−H$^-$) < GB(M−H$^-$), i.e., $K^- \gg 1$, but also that $K^-.\ x^0_{M-H^-} \gg 1$. This sets the estimate for the limit of K^- to 10^4–10^6, depending on the assumed yield of primary $x^0_{M-H^-}$ ions (10^{-3} to 10^{-5}), or a corresponding difference of gas-phase basicities of minimally −50 to −80 kJ/mol, assuming a temperature of 700 K. It should then be noted that for this case the approximate solutions listed in Table 1 predict a ratio of negative matrix to analyte ions of

$$\frac{x_{M-H^-}}{x_{A-H^-}} = \frac{x^0_{M-H^-} - x_{A-H^-}}{x_{A-H^-}} = \frac{1}{K^- x^0_A}$$

a number much smaller than 1. Experimentally the predicted very low ratio of negative matrix to analyte ions is, however not observed. For all combinations of peptides and matrices tested, this ratio is above three, in the majority of cases it is ten or larger. Examples for this can be seen in Figs. 3–5 of reference [1]. In addition, to the best knowledge of the authors a matrix suppression effect by negative peptide ions, predicted by the model, has not been observed experimentally in a systematic way. This is in strong contrast to the positive ion mode for which a vanishingly small matrix ions signal is predicted for the case of $x^0_A \geq x^0_{M+H^+}$ as well and indeed observed as the well documented "matrix suppression" effect. Actually the model would predict a nearly complete suppression of matrix signals for all combinations of analyte and matrix for which K^+ is substantially larger than one, i.e., the gas-phase basicity of the analyte exceeds that of the matrix. In reality, small (compared to the analyte signal) positive matrix-ion signals are usually observed. The most likely explanation is that matrix ions near the rim of the plume will not encounter enough collisions with analyte neutrals for the reaction to attain equilibrium. This interpretation is supported by the fact that matrix suppression in positive ion mode can also depend on the details of the sample preparation and sample morphology, as was observed early on in one of the authors' (F. H.) group. Similar considerations also apply to the data plotted in Fig. 7 of reference [1]. If K^+ and K^- are both much larger than one, the ratio x_{A+H^+}/x_{A-H^-} should for all matrices be independent of the basicity of the peptide in contrast to the common assumption that the yield of positive analyte ions should increase with the basicity of the peptide. Given the large uncertainties of the experimental values and the fact that the trend was observed for only two of the six matrices tested, the validity of the trends shown in that figure may well be questionable. Note that this consideration applies only for the special case of a single peptide of given basicity in any given sample, as was the case for the experiments discussed here. For samples containing several peptides of different basicity, as is the case e.g., in peptide mapping of proteins after protease digest, competition will result in signal strengths reflecting the respective basicities, at least if $\sum_i (x^0_{A_i}) \gg x^0_{M+H^+}$ holds for all peptides.

In summary, some of the experimental results are compatible with the model predictions under certain, reasonable assumptions for the relevant parameters but others are in strong contradiction to the model predictions applying the same parameter set.

As shown in Table 1, very different ratios are predicted by the model for the case of $\sum_i (x^0_{A_i}) \ll x^0_{M+H^+}; x^0_{M-H^-}$. In this case the ratio of x_{A+H^+}/x_{A-H^-} should indeed show the strong dependence on the gas-phase basicities of the matrix as postulated in reference [1]. It is quite possible that this condition would be fulfilled for a molar analyte to matrix ratio in the sample of $\leq 10^{-5}$, as is typically used for the analysis of larger proteins. Unfortunately, this case could not be tested by a quantitative experiment for the chosen set of matrices and peptides. For a analyte-to-matrix ratio of substantially below 10^{-3} no analyte signals above noise were observed in the negative ion mode for most of the matrices, which at least is in qualitative agreement with the model, because K^+ should be considerably larger than K^-, even if $K^- > 1$ and provided $x^0_{M+H^+}$ and $x^0_{M-H^-}$ are of comparable magnitude.

4. Conclusions

The model is based on the mechanism of a secondary formation of analyte ions of either polarity as described by the reactions (1) and (2). Reaction equilibria are assumed, which should be reasonable for the bulk of the expanding plume [4].

The experimental observation that the ratio of positive to negative analyte ions is essentially independent of the gas-phase basicity of the matrix is predicted by the model under the additional assumption that $x^0_A \gg x^0_{M+H^+}; x^0_{M-H^-}$ and $K^- \gg 1$. The observed ratio of negative matrix to analyte ions of typically about 10 can, however, not be easily rationalized under theses assumption.

Several scenarios can be considered in view of this observation. First, it could be imagined that a very different distribution of M−H$^-$ anions exists in the plume, such that equilibrium is attained for the positive but not the negative ions. However, this appears highly unlikely. Second, the mole fractions of M−H$^-$ and M+H$^+$ must not necessarily be equal, as was assumed in references [1,3]. Because all experiments involved desorption from dielectric substrates, only charge separation can be induced by the laser exposure. However, the balance of positive and negative charges only applies to the total charge. As has been described in [1] and in earlier literature [13] the majority of charge of either polarity resides indeed in the chemical noise clusters rather than identifiable matrix or analyte ions. Clear evidence of the decay of such clusters was also presented in [1]. One could assume that the molar fraction of $x^0_{M-H^-}$ greatly exceeds that of $x^0_{M+H^+}$ such that $x^0_A \gg x^0_{M+H^+}$, but $x^0_A \ll x^0_{M-H^-}$ and a dominating fraction of the matrix anions would be left after all analyte neutrals are consumed. However, in this case the ratio of positive to negative analyte ions should have been substantially less than 1, because of the increased likelihood of an analyte neutral to collide with a matrix anion as compared to matrix cations. This again is not observed experimentally. Third, the secondary proton transfer to form the analyte anion might involve matrix species other than the M−H$^-$ anion. In that scenario, the gas-phase basicity of these matrix species would have to exceed that of the peptide anions, whereas that of the M−H$^-$ would stay below, as one might assume at first sight, based on the values for the amino acids. Such other species might involve e.g., the M−2H$^-$ radical or the 2M−H$^-$ anions, sometimes observed in matrix spectra. However, their signals are not observed for all matrices even without any analytes in the sample and, if present, usually with a minor intensity. Matrix anion fragments have also been suggested as proton acceptors. At least

for sinapic acid as a prototype acidic matrix, the basicities of the dimer and of typical fragment anions have, however been shown to be smaller than those of the ($M-H^-$) matrix anion [4]. It should also be noted, that the anion spectra of pure matrices typically show only limited fragmentation, certainly less than those of the corresponding cation spectra. As another explanation one could assume that structurally different $M-H^-$ anions of different basicities are generated, considering that the starting species are, most probably, single photoelectrons. Recently some evidence has been found for the generation of $M-H^-$ carbanions, besides phenolate or carboxylate anions in certain matrices [14]. These should indeed have a very large basicity. However, in all of the latter cases the experimental findings for the CID dissociation of the $A+M-H^-$ complexes are very difficult to rationalize.

Given the experimental evidence as presented in [1] and discussed above, it seems that particularly, but not exclusively the formation of negative ions must be reconsidered. In fact, the pathways to primary negative charges in the MALDI plume and the nature of the initial negative ions ($M^{\bullet -}$; $M-H^-$ and possibly other species) have not been well studied. In the literature two pathways for the formation of primary matrix ions are discussed. The first assumes a photo-ionization of the matrix requiring at least the pooled energy of two photons. In this scenario a radical M^+ ion and a photoelectron are generated upon exposure of the sample to UV-A laser radiation, followed by reactions resulting in relatively stable even electron and radical matrix anions [2a]. The second assumes proton disproportionation in matrix complexes or clusters, comprising matrix or matrix as well as analyte molecules [15]. The former is particularly interesting, because it involves the reaction of electrons with the matrix or possibly even the analytes. Relatively strong photoelectron signals have indeed been observed for a 2,5-DHB matrix, even for matrix desorption from a dielectric to avoid interference by electron emission from a metal substrate [16]. In view of the above-mentioned CID dissociation experiments, electron capture of complexes or small clusters involving analyte and matrix species would be of particular interest.

It was the purpose of this publication in conjunction with references [1,3] to point out that there exist rather basic contradictions between the experimental findings and the simple equilibrium proton transfer model from matrix to analyte in the expanding plume. The presented experimental results do not carry further and cannot unambiguously predict a modified or different model. More and more sophisticated experiments need to be done to further our understanding of the ion formation processes.

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