Quantitative reproducibility of mass spectra in matrix-assisted laser desorption ionization and unraveling of the mechanism for gas-phase peptide ion formation

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Introduction

Matrix-assisted laser desorption ionization (MALDI) is widely used for mass spectrometry of biological molecules. Even though the mechanism for gas-phase ion formation in MALDI is not well established, it is generally accepted that the whole process can be divided into two steps. In MALDI of many analytes such as peptides to be dealt with in this work, the second step is thought to involve proton transfer from a protonated matrix ([M + H]⁺) to an analyte (A), i.e. [M + H]⁺ + A → M + [A + H]⁺.

Unlike the second step, the mechanism for the first step, i.e. the formation of gas-phase primary ions ([M + H]⁺), is not well understood. The cluster ion model[4,6] and the exciton pooling model[4,7] are the attempts to explain the first step. From very weak cluster ion signals in MALDI of peptides, we suggested that they might be unimportant as intermediates for the primary ion formation or might disintegrate rapidly. The latter case is indistinguishable from preformed ion emission, i.e. the laser-induced emission of ions that are already present in a solid sample into the gas phase. The exciton pooling model is an elaboration to accommodate multiphoton ionization of a matrix molecule in solid phase – laser fluence commonly adopted in MALDI is too low to induce multiphoton ionization of gas-phase molecules.[4] If [M + H]⁺ is generated by laser-induced ionization, the total number of gas-phase ions should increase with the laser fluence. Even though ion signals in a single-shot spectrum generated from a laser spot increase rapidly with the fluence, this is due to the ablative process rather than the ionization.[2,8,10]

In the study of the efficiency of gas-phase ion formation in MALDI,[8] we found that the total number of gas-phase ions generated from an entire sample was independent of the laser fluence, in disagreement with the hypothesis of laser-induced ionization. We also observed that the total number was independent of the peptide concentration in the solid sample.[8] That is, it was the same as that from a pure matrix sample. The fact that the increase in the total number of analyte-derived ions is matched by the decrease in that of matrix-derived ions is consistent with the general consensus that [M + H]⁺ is the main primary ion in MALDI of peptides and that the peptide ion, [A + H]⁺, is produced by the matrix-to-peptide proton transfer.[11,12]

Recently, we observed that MALDI spectral patterns obtained by repetitive irradiation of a spot on a sample varied systematically as the laser shot continued.[13] That is, the extent of fragmentation for [M + H]⁺ and [A + H]⁺ decreased whereas the peptide-to-matrix ion abundance ratio increased as the shot continued. Lowering of the temperature in the early matrix plume, Tearly, indicated by the decrease in ion fragmentation, was found to occur because the sample-to-target thermal conduction became more efficient as the irradiated spot got thinner.[13,14] Temperature lowering was also consistent with...
the increase in the peptide-to-matrix ion abundance ratio because the matrix-to-peptide proton transfer was exothermic. An important finding in the study was that spectral patterns were similar regardless of the experimental condition when $T_{\text{early}}$ was the same. Also important was that the second step in the gas-phase ion formation, i.e. the matrix-to-peptide proton transfer, was in quasi-equilibrium at the temperature of $T_{\text{early}}$.

Subsequently, we attempted to extract information on the mechanism for gas-phase primary ion ($[M + H]^+$) formation from temperature-selected MALDI with $\alpha$-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) as matrices - CHCA and DHB are the two most popular matrices. In the course of this effort, we came across empirical rules concerning ion abundances at the single-shot spectrum level, not at the entire sample level observed previously. The most important of these rules was that temperature-selected MALDI spectra were quantitatively reproducible, i.e. not only the spectral pattern but also the ion abundances (intensities) became reproducible upon temperature selection. The results and their implication on the mechanism for gas-phase ion formation are presented in this paper.

**Experimental**

The homebuilt MALDI time-of-flight instrument and its operation were reported previously. The light source was a 337-nm output from a nitrogen laser (MNL100, Lasertechnik Berlin, Berlin, Germany). The threshold laser pulse energies were 0.30 and 1.4 μJ/pulse for CHCA-MALDI and DHB-MALDI, respectively. For the signal-to-noise ratio to improve, spectral data from every ten laser shots on a spot were summed. Then, the results at the same shot number interval from 20 different spots were summed. The method to evaluate the number of ions in each peak was reported previously. We also made the ion transmission correction, 28.3 and 35.0% for the instruments used for CHCA- and DHB-MALDI works, respectively, and 62% entrance to microchannels in MCP. Then, the numbers of ions at the source exit were larger than those detected by a factor of 5.7 and 4.6 for CHCA- and DHB-MALDI, respectively.

**Sample preparation**

Peptides $Y_6$, $Y_5K$, $Y_5R$, YLYEIAR and YGGFL were purchased from Peptron (Daejeon, Korea). Creatinine, histamine, CHCA and DHB were purchased from Sigma (St. Louis, MO, USA). Aqueous solution of an analyte(s) was mixed with water/acetonitrile solution of CHCA or DHB. In MALDI of $Y_5K$, 1.0 μl of the CHCA/analyte solution containing 0–10 pmol of $Y_5K$ and 25 nmol of CHCA was loaded on the target and vacuum dried. One microliter of the solution used in the study of MALDI of the mixture contained 1.0 pmol each of $Y_5K$, $Y_5R$, YLYEIAR, YGGFL, creatinine and histamine and 25 nmol of CHCA. Sampling for DHB-MALDI of $Y_6$ was carried out in two steps. In each step, 1 μl of a solution containing 0.0, 1.0 or 10 pmol of $Y_6$ and 50 nmol of DHB was loaded and vacuum dried.

**Results and discussion**

**Temperature determination**

We began our study of peptide ion dissociation kinetics by analyzing time-resolved photodissociation data. The method led to a simpler technique to determine the rate–energy relation, $k(E)$, and the temperatures in the early and late plumes from the precursor ion survival probabilities at the source exit and at the detector. In particular, the temperature in the early plume, $T_{\text{early}}$, which was estimated to allow kinetic explanation for the in-source decay (ISD) of peptide ions, was a measure of the internal energies of peptide ions. Hence, it is a property that is an average over the time span of ISD. If we accept that a matrix plume undergoes expansion cooling, $T_{\text{early}}$ would be lower than the temperature of the newly formed, or very early, plume. In a more recent study, we presented data relating $T_{\text{early}}$ to the $[M + H – H_2O]^+$-to-$[M + H]^+$ abundance ratio in a MALDI spectrum, where $M$ denotes the matrix used, either CHCA or DHB. Even though $T_{\text{early}}$ thus estimated might suffer from unknown systematic errors, this is not a problem when it is used to compare the spectra associated with the same temperature.

**Laser-induced ionization versus laser-induced ablation**

Depending on the hypothesis concerning the role of photo-excitation in MALDI, models on the mechanism for primary ion ($[M + H]^+$) formation are divided into two groups. In the first group, to be called laser-induced ionization, ionization occurs in an excited electronic state(s) of the matrix accessed directly or indirectly by photo-excitation. In the second group, excitation by laser is simply a way of supplying thermal energy to the matrix, thereby inducing material emission – to be called ablation – from the solid sample. Photo-excitation provides thermal energy for ablation in the first group of models also. The exciton pooling and preformed ion emission are popular models belonging to the first and second groups, respectively.

Different roles of the photo-excitation in the two groups of models suggest that it might be possible to determine which group is appropriate by studying the fluence dependence of ion signals in MALDI. In this regard, Derrick et al. made an interesting observation that the threshold laser pulse energies measured for the cation and anion of a matrix and those of an analyte were the same. The result might be taken as evidence supporting the second group of models. However, Knochenmuss noted that the result could be reconciled with the first group of models with the postulation that ions formed in the solid sample at laser fluence lower than the threshold for ablation were not released into the vacuum.

**Fluence dependence of ordinary MALDI spectra**

Before we present the data, let us infer the predictions of the two models on the fluence – this is proportional to the laser pulse energy when the spot size is fixed – dependence of ion signals in MALDI, without and with temperature selection. Step-by-step accounts for the fluence dependence in ordinary MALDI, i.e. without temperature selection, are as follows. Under the first group of models, in the early plume, the total abundance of matrix-derived ions will initially increase with the fluence more rapidly than or as rapidly as analyte-derived ions do. In contrast, under the second group of models, the relative increase will be the same for all the species. In both cases, the matrix-to-analyte proton transfer reaction will approach a quasi-equilibrium after a while, resulting in the analyte-to-matrix ion abundance ratio of $c([A + H]^+)/c([M + H]^+) \propto [c(A)/c(M)]$. At this stage, the total abundance of matrix-derived ions, and that of analyte-derived ions also, would be larger at higher fluence. In the final step, we must take into account the fact that the effective temperature in the early plume ($T_{\text{early}}$) goes up as the fluence increases. This will shift the
quasi-equilibrium of the second step to the left, i.e. the total number of analyte-derived ions will decrease whereas that of matrix-derived ones will increase, if the matrix-to-analyte proton transfer is exothermic as in MALDI of peptides. Overall, the following changes in ion abundance are expected as the fluence increases, regardless of the model: (1) the total abundance of the ionic species, i.e. the sum of matrix- and analyte-derived ions, will increase, (2) the total abundance of matrix-derived ions will increase, (3) the total abundance of analyte-derived ions may increase or decrease.

So that the preceding inference can be tested, three sets of MALDI spectra were taken by repetitive irradiation of samples containing 3 pmol Y5K in 25 nmol CHCA using two, three and four times the threshold pulse energy. From each set, spectra obtained from the first 20 shots on fresh spots were averaged. The results are shown in Fig. 1. In the case of matrix-derived ions, their total abundance increased with the pulse energy. The increase was particularly significant for [CHCA+H−CO2]+. In the case of analyte-derived ions, the abundance of [Y5K+H]+ decreased with the pulse energy, whereas that of the immonium ion Y increased, indicating that the effective temperature got higher as pulse energy increased. The total numbers of analyte-derived ions were 680, 1600 and 2300 ions per pulse at two, three and four times the threshold pulse energy, respectively. The total numbers of ions generated at these pulse energies, i.e. the sum of the matrix-derived and analyte-derived ions, were 4000 (0.17), 9700 (0.16), and 15000 (0.15) ions per pulse, respectively. The numbers in parentheses are the fractions of the analyte-derived ions in the total. Even though the decrease in the analyte fraction with the increase in laser pulse energy might be consistent with the exothermic nature of the CHCA-to-peptide proton transfer reaction, we do not render any significance to these numbers because the errors involved are too large.

We also carried out similar measurement for samples containing 20 pmol of Y6 in 100 nmol of DHB, using two, two and a half and three times the threshold energy. As can be seen from the spectra in Fig. 2, the general trend for the fluence dependence in DHB-MALDI of Y6 is similar to that in CHCA-MALDI of Y5K. Specifically, the relative abundances of fragment ions got larger at higher fluence both for the matrix and the analyte. The total number of ions increased with the fluence also, from 4200 (0.26) ions per pulse at two times the threshold to 9700 (0.23) ions per pulse at three times the threshold. The results from the measurements for CHCA-MALDI and DHB-MALDI are consistent with the three changes predicted earlier. However, the result does not provide any new insight on the mechanism for primary ion formation.

Fluence dependence of temperature-selected MALDI spectra

Now, let us compare MALDI spectra measured with different fluorences but selected for the same Tearly−Tearly associated with each spectrum obtained from a spot with constant laser fluence decreases as the shot continues because the sample gets thinner. Under both models, with Tearly kept the same, the total amount of materials ablated will be nearly the same regardless of the fluence. Under the first group of models, the participation of laser-induced ionization means that the concentrations of ionic species in the plume will increase with fluence. However, the analyte-to-matrix ion abundance ratio will not be affected by fluence if the matrix-to-analyte proton transfer is in quasi-equilibrium at the same Tearly. In contrast, under the second group of models, neither the analyte-to-matrix ion abundance ratio nor the absolute abundances of ions will be affected by fluence, as long as Tearly is kept the same. That is, we can determine which of the two groups of models is appropriate by checking whether the total abundance of ions will increase with fluence or not in the temperature-selected MALDI spectra. To find out, we calculated Tearly for each spectrum in the aforementioned sets of spectra and selected a spectrum with a particular Tearly from each set. We would like to mention that the ranges for Tearly in CHCA-MALDI accessed at two, three and four times the threshold were 860–910, 860–950 and 870–980 K, respectively. Similar data

Figure 1. MALDI spectra of 3 pmol Y5K in 25 nmol CHCA were taken with (a) two, (b) three and (c) four times the threshold pulse energy. In each case, spectra obtained from the first 20 shots on fresh spots were averaged. Ion signals represent the raw outputs of the detection system, i.e. they were not normalized. Y5, b3, y4, y5 and Y are the fragment ions of [Y5K+H]+ generated by in-source decay, whereas those marked with asterisks are the fragment ions generated by post-source decay.

Figure 2. MALDI spectra of 20 pmol Y5 in 100 nmol DHB were taken with (a) two, (b) two and a half and (c) three times the threshold pulse energy. In each case, spectra obtained from the first 20 shots on fresh spots were averaged. Ion signals represent the raw outputs of the detection system, i.e. they were not normalized. a2, b2, n (n = 2–5), y3, y4, y5 and Y are the fragment ions of [Y5+H]+ generated by in-source decay, whereas those marked with asterisks are the fragment ions generated by post-source decay.
in DHB-MALDI at two, two and a half and three times the threshold were 770–805, 770–810, 775–815 K, respectively. Three CHCA-MALDI spectra of Y5K with T_{early} of 900 ± 5 K are shown in Fig. 3. The overall patterns of the three spectra are nearly the same, as found in our previous study. Three DHB-MALDI spectra of Y6 with T_{early} of 800 ± 5 K shown in Fig. 4 display excellent similarity also. The results are consistent with the hypothesis that the second step of ion formation in MALDI is in quasi-equilibrium. More important here is that the absolute abundances of the corresponding ions are similar regardless of the fluence when T_{early} is kept the same. In fact, the total numbers of ions in the CHCA-MALDI spectra at T_{early} of 900 ± 5 K obtained with two, three and four times the threshold pulse energy were similar, 3400 (0.18), 3900 (0.17) and 3900 (0.17) ions per pulse, respectively. We observed a similar trend at other temperatures also (in Supporting information). Similarly, the total numbers of ions in the DHB-MALDI spectra at T_{early} of 800 ± 5 K obtained with two, two and a half and three times the threshold were 3800 (0.26), 3900 (0.25) and 3600 (0.28), respectively. The results are consistent with the second group of models for the gas-phase primary ion formation in MALDI.

Previously, we showed four normalized temperature-selected MALDI spectra of Y5R in CHCA to demonstrate that the overall patterns of the spectra were unaffected by the sample amount, laser pulse energy and laser wavelength when temperature selection was made. We plotted the same data in Fig. 5, this time without normalization. It is to be noted that the four spectra are almost the same. The total numbers of ions in spectra 5(a), 5(b), 5(c) and 5(d) were 16000 (0.26), 14000 (0.25), 14000 (0.23) and 15000 (0.25) ions per pulse, respectively, also consistent with the second group of models for the gas-phase primary ion formation in MALDI.

So far, we have presented data showing that a MALDI spectrum strongly correlates with, or is virtually determined by, T_{early}. We have also suggested that the laser-induced ablation, not laser-induced ionization, is responsible for the gas-phase ion formation in MALDI. In the laser-induced ablation model, the amount of the materials ablated by a laser pulse and the average temperature of the initial plume will be determined by the temperature of the irradiated surface. With the density and temperature of the initial plume specified, the physical (density and temperature) and chemical (ISD, proton transfer, etc.) evolution of the plume will also be specified. In particular, the overall MALDI spectrum and T_{early} will be specified, resulting in a strong correlation between the two.

Figure 3. MALDI spectra of 3 pmol Y5K in 25 nmol CHCA taken with (a) two, (b) three and (c) four times the threshold pulse energy. In each case, the spectra associated with T_{early} of 900 ± 5 K were selected and averaged. Ion signals represent the raw outputs of the detection system, i.e. they were not normalized. See the caption of Figure 1 for the information on fragment ions.

Figure 4. MALDI spectra of 20 pmol Y6 in 100 nmol DHB taken with (a) two, (b) two and half and (c) three times the threshold pulse energy. In each case, the spectra associated with T_{early} of 800 ± 5 K were selected and averaged. Ion signals represent the raw outputs of the detection system, i.e. they were not normalized. See the caption of Figure 2 for the information on fragment ions.

Figure 5. Reproduction of Figure 3 of Bae et al. without normalizing ion signals. The spectrum with T_{early}, near 968 K was selected from each MALDI spectral set for samples with Y5R : CHCA = 1 : 8300 obtained under four different experimental conditions denoted as (# picomoles of Y5R, # nanomoles of CHCA, laser pulse energy in unit of the threshold, laser wavelength in nanometer). (a) 71–90 shot number range of (3, 25, ×6, 337), (b) 51–70 shot number range of (3, 25, ×4, 337), (c) 101–120 shot number range of (4.2, 35, ×6, 337), (d) 31–50 shot number range of (3, 25, ×6, 355).
Mechanism for the generation of primary ions

As mentioned earlier, Derrick et al.[10] observed that the threshold laser pulse energies for the generation of gas-phase cations and anions of a matrix and those of an analyte were the same and suggested that ablation was the initial step in MALDI. In our previous study on the efficiency of gas-phase ion formation in MALDI, we found that the total number of gas-phase ions generated from an entire sample was independent of laser pulse energy.[8] Finally, in this work, temperature-selected single-shot MALDI spectra – averaged over some shots to be rigorous – obtained at different laser pulse energies have been found to be quantitatively similar. Taken together, the three results make a very strong case against laser-induced ionization as the mechanism for the generation of primary ions in MALDI.

In our previous study,[8] on the efficiency of gas-phase ion formation in MALDI, we derived three models for gas-phase ion formation by modifying the cluster ion model of Karas et al.[5,6] and the exciton pooling model of Knochenmuss[7] such that they became compatible with our experimental results. In retrospect, the effort was not quite fruitful because we were unaware that the matrix-to-analyte proton transfer was in quasi-equilibrium and that we could treat \([M + H]^+\) as the only primary ion for practical purposes. The situation became clearer after a subsequent study.[13] Before going further, we will ignore model 3 proposed in the previous work[8] because it is simply an expression of the occurrence of the proton transfer reaction in the second step.

When an anion is formed together with a cation, Coulomb attraction between these ions is an obstacle to the generation of free gas-phase ions.[23] In a previous study,[8] we noted that the equilibrium constant for the charge separation of an ion pair, \(A^-B^+ \rightarrow A^- + B^+\), would be very small. Two models, models 1 and 2, were proposed to circumvent this problem, both of which were modified versions of the cluster model of Karas et al.[5,6] In model 1, gas-phase ions are generated by the emission of dielectrically screened preformed ions. In model 2, material emission occurs in the form of ion pairs such as \([A + H]^+ [M - H]^–\), and the reaction between an ion pair \([M + H]^+ [M - H]^–\) generates \([A + H]^+ [M - H]^–\). Under the current understanding that \([M + H]^+\) is the main primary ion and that \([A + H]^+\) is generated by the matrix-to-analyte proton transfer, model 2 becomes obsolete. Hence, among the original three models, only model 1 remains, ‘the gas-phase primary ions (\([M + H]^+\)) are generated by emission of dielectrically screened pre-formed ions (\([M + H]^+\)) in the solid sample’. We would like to emphasize that models 1-3 were modified versions of the cluster ion model and the exciton pooling model. That is, there was no guarantee from the beginning that they were the only models worthy of scrutiny. Furthermore, it will be shown later that the only survivor of the three, i.e. model 1, has its own weakness.

In the aqueous solution of CHCA, \([M + H]^+\) will be generated together with \([M - H]^–\) by autoprotolysis of neutral matrix molecules.

\[
M + M \rightarrow [M + H]^+ + [M - H]^–
\]

Small fractions of these ions may remain in the solid sample and get emitted by laser-induced ablation. In fact, \([M - H]^–\) is the main matrix-derived anion in the negative ion spectrum of CHCA-MALDI,[24,25] whereas the corresponding anion appears together with \([M - 2H]^–\) with comparable abundances in DHB-MALDI.[26] The forward reaction, charge separation, will be highly endothermic and hence will have a low probability to occur in the gas phase, whereas the backward reaction, recombination of a cation with an anion, will readily occur. At this point, it is important to note that the matrix-to-analyte proton transfer is in quasi-equilibrium in the early plume.[13] If the pressure in the early plume is high enough to assure quasi-equilibrium for the proton transfer reaction, the same would be the case for the autoprotolysis. Autoprotolysis may occur in the solution, in the selvedge and in the early plume. However, the reaction occurring in the early plume, if it is in quasi-equilibrium, will be the most important because it will determine the concentrations of \([M + H]^+\) and \([M - H]^–\) in the early plume – \(c([M + H]^+)/c(M) = 10^{-7}\) or less in CHCA-MALDI.[8] Even if dielectric screening helps the release of preformed ions into the gas phase, the numbers of ions thus released might have nothing to do with the numbers of the same ions present in the early plume if quasi-equilibrium is established for the gas-phase autoprotolysis. Then, the quasi-equilibrium in the gas-phase autoprotolysis, rather than model 1, might determine the number of the primary ions generated by MALDI.

Earlier, we mentioned that strong Coulomb attraction between cations and anions was an obstacle in the preformed ion emission.[23] The same will be the main argument against the autoprotolysis model – charge recombination is part of the reverse reaction for the autoprotolysis. In this regard, we would like to emphasize that similar amounts of cations and anions are present in the plume. Also to be emphasized is that even if gas-phase ions are generated by a mechanism other than gas-phase autoprotolysis, such as laser-induced ionization, the final amounts of the ions detected will be limited by the efficient charge recombination. That is, no model for the primary ion formation is free from the limitation imposed by charge recombination. This suggests either that the number of ions generated by MALDI is smaller than speculated or that the equilibrium constants for autoprotolysis are larger than might be roughly estimated. In fact, we observed that the ion yields in MALDI of peptides were much smaller than generally thought.[8] More reliable studies are needed in this respect. Finally, we would like to mention that there might be alternative thermal explanations for the generation of the primary ion, which we are searching for.

Total number of gas-phase ions generated by a single laser shot

In our previous study of the ionization efficiency in CHCA-MALDI of peptides, we found that the total number of gas-phase ions generated from an entire sample was independent of the laser fluence.[8] In this work, we observed a similar but more powerful rule applicable to temperature-selected single-shot spectra, for both MALDI with CHCA and DHB. That is, the total number of gas-phase ions generated by a single laser shot was independent of the laser fluence, when temperature was kept constant. As mentioned earlier, this empirical rule supports the laser-induced ablation as the role of laser but not the laser-induced ionization. Another rule observed in our previous study was that the total number of gas-phase ions generated from an entire sample was independent of the analyte concentration.[8] This is also expected for temperature-selected single-shot spectra because the amount of material ablation is kept the same when temperature is fixed and because the decrease in the abundances of matrix-derived ions is matched by the increase in those of peptide-derived ions. To check this, we measured sets of CHCA-MALDI spectra by repetitive irradiation of three samples, each containing 0.10, 1.0 and 10 pmol of Y3K in 25 nmol CHCA, and selected one from each set.
with $T_{\text{early}}$ of 900 $\pm$ 5 K. Three spectra thus obtained are shown in Fig. 6 together with the spectrum for pure CHCA at the same $T_{\text{early}}$. As the amount of the peptide in the sample got larger, the abundances of peptide-derived ions increased whereas those of the matrix-derived ions decreased. The total numbers of gas-phase ions generated by a single laser shot calculated from these spectra are listed in Table 1. It is obvious that the total number is independent of peptide concentration, i.e. it is the same as that generated from pure CHCA. This, in turn, suggests that the total number of gas-phase ions generated by a single laser pulse will be the same regardless of the analyte, when temperature selection is made. To check this, we obtained MALDI spectra of 0.1 pmol each of Y5K, Y5R, YLYEIAR, YGGFL, creatinine and histamine in 25 nmol CHCA at 900 $\pm$ 5 K (spectra are shown in Supporting information). The total numbers of ions measured from these spectra also listed in Table 1 are virtually the same as those for Y5K. The aforementioned independence, in turn, suggests that the total numbers of ions generated from peptide mixtures will be the same as that from pure CHCA. To check this, we obtained a set of MALDI spectra by repetitive irradiation of a sample with 1.0 pmol each of Y5K, Y5R, YLYEIAR, YGGFL, creatinine and histamine in 25 nmol CHCA. Then, we selected the spectrum with $T_{\text{early}}$ of 900 $\pm$ 5 K (spectrum is shown in Supporting information) and calculated the total number of ions. The total number of ions listed in Table 1, around 4100 ions per pulse, is close to those obtained for the other samples. For all the samples studied so far, we also counted the numbers of ions in the spectra at $T_{\text{early}}$ of 875 $\pm$ 5 K and listed the results in Table 1. It is evident that the total number of gas-phase ions generated by MALDI is virtually the same regardless of the identity, concentration and the number of analytes in a sample when $T_{\text{early}}$ is the same. This number increases with the temperature, consistent with our postulation that the role of laser is in ablation of a solid sample, not in ionization of neutrals. We carried out similar, even though less extensive, measurements for DHB-MALDI and obtained similar results as summarized in Table 2.

### Table 1. Total number of gas-phase ions versus analyte concentration in CHCA-MALDI

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration$^a$</th>
<th>Total number of ions per pulse$^b$, $T_{\text{early}}$ = 875 $\pm$ 5 K</th>
<th>$T_{\text{early}}$ = 900 $\pm$ 5 K</th>
</tr>
</thead>
<tbody>
<tr>
<td>–$^c$</td>
<td>0</td>
<td>1700 $\pm$ 170</td>
<td>4000 $\pm$ 510</td>
</tr>
<tr>
<td>Y5K</td>
<td>0.10</td>
<td>1600 $\pm$ 350</td>
<td>4100 $\pm$ 510</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1300 $\pm$ 230</td>
<td>3400 $\pm$ 350</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1700 $\pm$ 290</td>
<td>3800 $\pm$ 170</td>
</tr>
<tr>
<td>Y5R</td>
<td>0.10</td>
<td>1600 $\pm$ 170</td>
<td>3900 $\pm$ 170</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>1500 $\pm$ 400</td>
<td>3900 $\pm$ 400</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1800 $\pm$ 230</td>
<td>3800 $\pm$ 170</td>
</tr>
<tr>
<td>Mixture$^d$</td>
<td>1.0/analyte</td>
<td>1800 $\pm$ 170</td>
<td>4100 $\pm$ 170</td>
</tr>
</tbody>
</table>

CHCA, x-cyano-4-hydroxycinnamic acid; MALDI, matrix-assisted laser desorption ionization.

$^a$Number of picomoles of analyte in 25 nmol of CHCA in the solid sample.

$^b$Averages over three or more measurements with one standard deviation.

$^c$Pure CHCA.

$^d$1.0 pmol each of Y5K, Y5R, YLYEIAR, YGGFL, creatinine and histamine in 25 nmol of CHCA.

### Table 2. Total number of gas-phase ions versus analyte concentration in DHB-MALDI

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration$^a$</th>
<th>Total number of ions per pulse$^b$, $T_{\text{early}}$ = 780 $\pm$ 5 K</th>
<th>$T_{\text{early}}$ = 800 $\pm$ 5 K</th>
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<tbody>
<tr>
<td>–$^c$</td>
<td>0</td>
<td>1200 $\pm$ 90</td>
<td>3600 $\pm$ 420</td>
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<td>Y6</td>
<td>2.0</td>
<td>1100 $\pm$ 90</td>
<td>3400 $\pm$ 250</td>
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<td></td>
<td>20</td>
<td>1200 $\pm$ 280</td>
<td>3700 $\pm$ 320</td>
</tr>
<tr>
<td>Mixture$^d$</td>
<td>2.0/analyte</td>
<td>1200 $\pm$ 250</td>
<td>3700 $\pm$ 510</td>
</tr>
</tbody>
</table>

DHB, 2,5-dihydroxybenzoic acid; MALDI, matrix-assisted laser desorption ionization.

$^a$Number of picomoles of analyte in 100 nmol of DHB in the solid sample.

$^b$Averages over three or more measurements with one standard deviation.

$^c$Pure DHB.

$^d$2.0 pmol each of Y6, Y5R, YLYEIAR, YGGFL, creatinine and histamine in 100 nmol of DHB.

### Conclusion

In our previous study on the efficiency of gas-phase ion formation in CHCA-MALDI, we observed two empirical rules concerning the total number of ions generated from an entire sample: (1) the total number of ions is independent of the analyte concentration or it is the same as that from pure matrix and (2) the total number is also independent of laser fluence. In the subsequent study of shot-number-dependent variation of CHCA-MALDI spectral pattern, two more empirical rules were observed: (3) mass spectral pattern strongly correlates with or is virtually determined by the temperature in the early plume and (4) the matrix-to-analyte proton transfer is in quasi-equilibrium. In the present CHCA-MALDI and DHB-MALDI studies, the empirical rules analogous...
to (1) and (2), but pertinent to temperature-selected single-shot spectra, have been found: (5) the spectral pattern and the absolute ion abundances are independent of the laser fluence, (6) the total number of ions is independent of the analyte and its concentration, (7) rule (6) is valid even when more than one analyte is present. Rules (1), (6) and (7) indicate that the matrix ion, \([\text{M} + \text{H}]^+\), is the main primary ion generated by MALDI, whereas rules (3) and (4) indicate that the analyte ion, \([\text{A} + \text{H}]^+\), is formed by the matrix-to-analyte proton transfer and that the pressure in the early plume is high enough to assure quasi-equilibrium for the reaction. Rules (2) and (5) indicate that laser-induced ionization of a matrix molecule is not the mechanism for the formation of \([\text{M} + \text{H}]^+\). On the other hand, postulation of laser-induced ablation as the role of laser in MALDI is compatible with all the empirical rules observed. Accordingly, we suggest that the gas-phase ions are generated via two thermal reactions. The first step generates the matrix ion, presumably via the autoprotolysis of the matrix. Then, the analyte ion is generated via the matrix-to-analyte proton transfer. More importantly, both of these reactions are in quasi-equilibrium in the early plume.

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Supporting information

Supporting information may be found in the online version of this article.

References