Reproducibility of Temperature-Selected Mass Spectra in Matrix-Assisted Laser Desorption Ionization of Peptides

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ABSTRACT: Matrix-assisted laser desorption ionization (MALDI)1-4 combined with time-of-flight (TOF) mass spectrometry is one of the most useful techniques for peptide analysis. Here, gas-phase peptide ions are generated by laser irradiation on a solid matrix containing a tiny amount of a peptide. Since the MALDI-TOF spectrum for a peptide is the mass spectrum of a peptide-matrix mixture, its overall pattern is affected by the peptide-to-matrix ion abundance ratio and the fragmentation patterns of the protonated peptide (PH+, peptide ion) and matrix (MH+, matrix ion).

In a typical MALDI-TOF instrument, delayed extraction, i.e., application of the ion extraction voltage at some time after the laser irradiation, is used together with a reflectron to achieve a good mass resolution.5 Fragment ions of PH+ and MH+ generated inside the ion source (in-source decay, ISD)6 appear as narrow peaks in a spectrum recorded with such an instrument. Recently, ISD of peptide ions occurring via bimolecular hydrogen atom transfer is attracting attention in relation to peptide sequencing.6-8 However, we are interested in the unimolecular ISD that is dominant for small peptide ions studied in this work. This generates b and y type ions vs “c” and “z” types in ISD after hydrogen atom transfer, see refs 9 and 10 for the fragment ion notation. Post-source decay (PSD)11 of peptide ions generates b and y type ions as in the unimolecular ISD. When a MALDI-TOF equipped with a reflectron with a linear-plus-quadratic potential inside is used,12-14 both ISD and PSD product ions form narrow peaks. This allows the measurement of the abundances of a precursor ion and its ISD and PSD products from the MALDI-TOF spectrum, all under the same experimental conditions.

In this laboratory, MALDI was used to generate peptide ions for their kinetic study.15 The dissociation rate constant $k(E)$, this is specified by the critical energy ($E_0$) and entropy ($\Delta S^\text{f}$) in Rice–Ramsperger–Kassel–Marcus (RRKM) modeling.16,17 for a peptide ion was determined by simultaneous analysis of its PSD and time-resolved photodissociation spectra.15,18,19 Also determined in the study was the effective temperature of the peptide ion coming out of the source or the temperature ($T_{\text{late}}$) in the “late” matrix plume. Subsequently, we estimated the effective temperature ($T_{\text{early}}$) of the peptide ion undergoing unimolecular ISD under the assumption of thermal equilibrium in the “early” plume.13,14,20 $T_{\text{early}}$ thus estimated was near 900 K when $\alpha$-cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix. Through a combined UV- and IR-MALDI investigation,14 we concluded that $T_{\text{late}}$ was substantially lower than $T_{\text{early}}$ due to the expansion cooling14,21,22 occurring in the plume.

It is well-known to practitioners of MALDI that it is difficult to generate steady ion signals and reproducible overall spectral patterns with this technique.23 Part of the irreproducibility originates from the inhomogeneity of samples prepared for MALDI.23,24 Signal reproducibility improves when liquid matrices are used such as glycerol in IR-MALDI and room temperature ionic liquids in UV-MALDI.24-26 In our recent study of the ionization efficiency in MALDI of peptides,27 we noted that vacuum-drying, rather than usual air-drying, of CHCA in water/acetonitrile solution produced solid samples that were fairly homogeneous in terms of the ionization efficiency.27,28 Regardless, significant variations were observed...
for the spectra recorded by repetitive irradiation of a spot on such a sample. Then, we noted that the spectral variations were not occurring randomly but systematically. It is to be recalled that we assumed a thermal equilibrium in our previous kinetic analysis of peptide ISD.\textsuperscript{13,20} If the assumption is adequate for the description of ISD, it may be also adequate to describe other features in MALDI spectra. In this work, we will show that the overall mass spectral pattern in MALDI is thermally determined and hence that mass spectral pattern becomes reproducible when the thermal condition is specified.

### EXPERIMENTAL SECTION

The home-built MALDI-TOF instrument and its operation were reported previously.\textsuperscript{27,29,30} One of the key features of the instrument is the installation of a reflectron with a linear-plus-quadratic potential inside.\textsuperscript{15,29} This allows the simultaneous detection of prompt ions and their ISD and PSD products.\textsuperscript{13,14} Unless otherwise specified, 337 nm output from a nitrogen laser (MNL100, Lasertechnik Berlin, Berlin, Germany) focused by an \( f = 100 \text{ mm} \) lens was used for MALDI. Also used was the 355 nm output from an Nd:YAG laser (SL III-10, Continuum, Santa Clara, CA) focused by the same lens.

To improve the signal-to-noise ratio, spectral data from every 20 laser shots were summed. Then, the results at the same shot number interval collected from 20 different spots on a sample were summed. Hence, each point in such spectra corresponds to the summation of over 400 shots. The method to evaluate the number of ions in each peak was reported previously.\textsuperscript{27,28}

The threshold laser pulse energy,\textsuperscript{15} or threshold, for CHCA-MALDI of peptides at 337 nm was 0.50 \( \mu \text{J/pulse} \). This was a little smaller than 0.75 \( \mu \text{J/pulse} \) reported previously\textsuperscript{27,28} due to a better beam shaping. The threshold at 355 nm was around 0.40 \( \mu \text{J/pulse} \).

**Sample Preparation.** Peptides \( Y_5X \) (X = K and R) were purchased from Peptron (Daejeon, Korea). Angiotensin II (DRVYIHPF) and CHCA were purchased from Sigma (St. Louis, MO). Aqueous stock solution of each peptide was diluted to a desired concentration and mixed with a water/acetonitrile solution of CHCA. A volume of 1 \( \mu \text{L} \) of each mixture was loaded on the target and vacuum-dried. Typically, a sample contained 1 or 3 pmol of a peptide in 25 nmol of CHCA.

**Method to Estimate the Early Plume Temperature.** Details of the kinetic method to estimate the early plume temperature were reported previously.\textsuperscript{14,20} Experimentally, the product ion abundances for ISD, PSD, and PSD of ISD products were measured from a MALDI-TOF spectrum. From these data, the survival probabilities of the peptide ion at the source exit (\( S_{\text{in}} \)) and at the detector (\( S_{\text{out}} \)) were evaluated. Let us explain the method using the dissociation of \([Y_5+H]^+\) as an example. Its total dissociation rate constant, \( k(E) \), was known from the previous time-resolved photodissociation study.\textsuperscript{31} In the kinetic analysis, 50 ns was taken as the threshold lifetime for ISD, which corresponded to 1.4 \( \times 10^7 \text{ s}^{-1} \) in rate constant or 13.157 eV in internal energy as read from \( k(E) \). Then, the effective temperature in the early plume was determined such that the area below 13.157 eV in the internal energy distribution became \( S_{\text{in}} \). The late plume temperature was determined similarly, using 5.4 \( \times 10^7 \text{ s}^{-1} \) as the threshold rate constant. \( T_{\text{early}} \) determined in this work is somewhat higher than 881 K reported in a previous study\textsuperscript{13} because the laser fluence was larger.

In the method explained above, \( k(E) \) of a peptide ion is needed to estimate its early plume temperature by the kinetic method. In a previous study,\textsuperscript{13} we showed that \( k(E) \) itself could be estimated by using the above method in reverse and reported the dissociation kinetic parameters of \( E_0 = 0.660 \text{ eV} \) and \( \Delta S^f = -27.2 \text{ eu} \ (1 \text{ eu} = 4.184 \text{ J mol}^{-1} \text{ K}^{-1}) \) for \([Y_5+H]^+\) and \( E_0 = 0.630 \text{ eV} \) and \( \Delta S^f = -27.6 \text{ eu} \) for \([Y_5K+H]^+\). For each peptide ion, the RRKM rate-energy relation (\( k(E) \)) calculated with the above \( E_0 \) and \( \Delta S^f \) was used to determine the early plume temperature under various experimental conditions.

### RESULTS AND DISCUSSION

**Shot Number Dependence of the Overall Spectral Pattern.** By repetitively irradiating a spot on a sample with a 337 nm nitrogen laser and collecting data, a set of MALDI spectra was obtained. Some of those from the first 200 shots in MALDI of 3 pmol of \( Y_5R \) in 25 nmol of CHCA obtained with six times the threshold are shown in Figure 1. In addition to the peptide \([Y_5R+H]^+\) and matrix \([\text{CHCA}+H]^+\) ions, their ISD product ions appear in the spectra such as the immonium ion \( Y \) from the peptide ion and \([\text{CHCA}+\text{H}2\text{O}]^+\) and \([\text{CHCA}+\text{H}+\text{CO}_2]^+\) are those of \([\text{CHCA}+H]^+\). PSD peaks are marked with asterisks (*).

![Figure 1](image)

**Figure 1.** Spot on a sample with 3 pmol of \( Y_5R \) in 25 nmol of CHCA was repetitively irradiated at 337 nm with 6 times the threshold pulse energy. MALDI spectra were integrated in the shot number ranges of (a) 1–20, (b) 41–60, (c) 81–100, (d) 141–160, and (e) 181–200. The immonium ion \( Y \) is the major ISD product of \([Y_5R+H]^+\) while \([\text{CHCA}+\text{H}+\text{H}_2\text{O}]^+\) and \([\text{CHCA}+\text{H}+\text{CO}_2]^+\) are those of \([\text{CHCA}+H]^+\). PSD peaks are marked with asterisks (*).
Earlier, we mentioned that the overall pattern of a MALDI spectrum was characterized by the peptide-to-matrix ion abundance ratio and the fragmentation patterns of peptide and matrix ions. All of these changed as the shot continued, as can be seen in Figure 1. First, the abundance of the immonium ion Y relative to that of the peptide ion decreased steadily, the same also occurred for other ISD product ions. Second, the mass spectral pattern for the matrix changed steadily, with the relative abundance of \([\text{CHCA} + H − \text{CO}_2]^{+}\) getting weaker. Third, the peptide-derived ions became relatively more abundant than the CHCA-derived ions. We observed similar trends for the other peptides, Y₅K and angiotensin II (see the Supporting Information).

**Shot Number Dependence of the Effective Temperature.** Decrease in the relative abundances of the ISD products means that the average internal energy of the peptide ion decreased steadily as the shot continued. Or, under the assumption of thermal equilibrium in the early plume, \(T_{\text{early}}\) was getting lower. One thing that happens as the irradiation continues is the gradual decrease in the sample thickness at the irradiated spot. Then, our observation indicates that \(T_{\text{early}}\) gets lower as the sample gets thinner. In the study of the temperature of matrix surfaces exposed to a laser pulse by detecting the blackbody radiation, Zenobi et al. observed a higher peak temperature for a thicker sample than for a thinner one. This led them to conclude that thermal conduction played a role in surface cooling in MALDI. To see if more effective thermal conduction in a thinner sample was responsible for the steady decrease in \(T_{\text{early}}\), we prepared samples with the same composition, viz., \(\text{Y}_5\text{R:CHCA} = 1:25\,000\) but with different thicknesses (0.9–2.1 μm); homogeneous samples thicker than 2.1 μm were difficult to prepare. We also prepared similar samples on the hydrophobic part of an anchor chip plate coated with a 50 nm fluorocarbon layer. The laser pulse energy was kept at 6 times the threshold, and the spectra were averaged over the first 20 shots. For each spectrum, \(T_{\text{early}}\) was estimated from \(S_P\). \(T_{\text{early}}\) vs the initial sample thickness plot shown in Figure 2 is consistent with the hypothesis that thermal conduction is more efficient in a thinner sample. \(T_{\text{early}}\) for samples loaded on the fluorocarbon layer was higher than that on the bare metal plate, suggesting that the fluorocarbon layer played an insulator to the heat flow. It is to be emphasized that the fact that \(T_{\text{early}}\) can be determined from the peptide ion dissociation yield and that it goes down as the shot continues provide a rare opportunity to investigate whether and how the temperature change will affect the spectral pattern.

**Shot Number Dependence of the Fragmentation Pattern for [CHCA + H]⁺.** Since the time span for PSD (around 10 μs) is significantly longer than that of ISD (several tens of nanoseconds) or the rate constant for PSD is smaller, a lower energy process is relatively more favored in PSD than in ISD. In the PSD spectrum of \([\text{CHCA} + H]^+\) (see the Supporting Information), \([\text{CHCA} + H − \text{H}_2\text{O}]^+\) was the most abundant product while \([\text{CHCA} + H − \text{CO}_2]^{+}\) was only 10% of \([\text{CHCA} + H − \text{H}_2\text{O}]^+\) in abundance, indicating that the loss of \(\text{H}_2\text{O}\) is a lower energy process than that of \(\text{CO}_2\). In the MALDI spectra shown in Figure 1, the abundance of \([\text{CHCA} + H − \text{CO}_2]^{+}\) generated by ISD relative to that of \([\text{CHCA} + H − \text{H}_2\text{O}]^+\) decreased steadily as the shot continued. This was consistent with the fact that \(T_{\text{early}}\) got lower as the shot continued. That is, the mass spectral pattern of CHCA seems to be thermally determined, as was assumed to be the case for the peptide.

**Peptide-to-Matrix Ion Abundance Ratio.** Probably the most interesting spectral change was that the peptide-derived ions got relatively more abundant than the matrix-derived ions as the shot continued. Even though this might happen if the peptide concentration was higher in the deeper part of the sample, such a possibility could be eliminated experimentally (see the Supporting Information). Then, it is likely that the peptide-to-matrix ion abundance ratio changed due to the change in \(T_{\text{early}}\) or the ratio is thermally determined. Suppose that we record sets of MALDI spectra for samples with the same peptide-to-matrix ratio but under different experimental conditions. Also suppose that we select one spectrum with the same \(T_{\text{early}}\) from each set. Then, the thermal determination of the peptide-to-matrix ion abundance ratio and the thermal dissociation of the peptide and matrix ions suggest that the overall patterns of the spectra selected above will be the same. As a test, we collected sets of spectra for samples with the peptide-to-matrix ratio of 1:8300 under four different experimental conditions.

**Proton Transfer Equilibrium.** It is widely accepted that the matrix-to-peptide proton transfer occurs in MALDI, i.e., \(\text{M}^+ + \text{P} \rightarrow \text{M}^+ + \text{PH}^+\). Here \(\text{M}^+\) is the proton donor that might be \([\text{CHCA} + H]^+\), \([\text{CHCA} + H − \text{H}_2\text{O}]^+\), or \([\text{CHCA} + H − \text{CO}_2]^{+}\) in the present case. The fact that the peptide-to-matrix ion abundance ratio is thermally determined suggests that the proton transfer is almost in thermal equilibrium. One way of checking such a possibility is to measure the reaction quotient, \(Q = ([\text{M}^+]/[\text{P}])([\text{PH}^+]/[\text{M}^+]\)) for samples with various peptide concentrations at

![Figure 2. The temperature in the early plume (T_early) vs sample thickness. Various amounts of a sample with Y₅R:CHCA = 1:25000 were loaded either on a bare stainless surface (●) or on a 50 nm fluorocarbon layer of an anchor chip plate (○). First 20 shot spectra from each fresh sample were integrated. 6 times the threshold pulse energy at 337 nm was used.](image-url)
a specified $T_{\text{early}}$ and see if it is independent of the concentration. Accordingly, we recorded a set of MALDI spectra by repetitively irradiating a sample containing 0.3–20 pmol of Y$_5$R or Y$_5$K in 25 nmol of CHCA and determined $T_{\text{early}}$ for each spectrum. Then, we selected one spectrum from each set with a specified value of $T_{\text{early}}$ thereby generating a new set with the same $T_{\text{early}}$ but different composition in the solid sample. The abundances of the matrix- and analyte-derived ions were measured for the spectra in the new set. At this stage, one needs to know the identity of $M^+$ to calculate $Q$. However, as far as checking the constancy of $Q$ is concerned, one can use the abundance of any of the potential proton donors mentioned above, or their combinations, because the relative abundances of all the matrix-derived ions were fixed when $T_{\text{early}}$ was fixed. The concentration independence of the fragmentation pattern of matrix ions further suggests that a fragment ion such as [CHCA + H – H$_2$O]$^+$ is not the main proton donor because if it were, its abundance would decrease more rapidly than that of [CHCA + H]$^+$ as the amount of peptide increases. That is, it is likely that [CHCA + H]$^+$ is the main proton donor. Assuming that some of the matrix ions that survive deprotonation undergo fragmentation, we took the total abundance of the matrix-derived ions, $\sum$[matrix-derived ion], as [M$'$$H^+$] in the calculation of $Q$. Similarly, $\sum$[peptide-derived ion] was used as [PH$^+$]. For the concentration ratio of the neutrals in the gas phase, i.e., ([M$'$/[P])/(PH$^+$/[M$'$H$^+$])), the matrix-to-peptide ratio in the solid sample was used. $Q$ values determined at $T_{\text{early}}$ of 950 K vs the peptide amount are plotted in Figure 4. It is evident that $Q$ is essentially independent of the peptide amount, indicating that the proton transfer reaction is almost in thermal equilibrium. That is, $Q$ shown in the figure is essentially the equilibrium constant, $K$. $K$ for the matrix-to-peptide proton transfer is larger for Y$_5$R than for Y$_5$K, in agreement with the fact that arginine (R) is a stronger base than lysine (K).35

As a further test, van’t Hoff plots were drawn for the $K$ vs $T_{\text{early}}$ data obtained from a set of spectra collected over 1–200 shots on a spot on the sample containing 3 pmol of Y$_5$R or Y$_5$K in 25 nmol of CHCA. As can be seen in Figure 5, ln $K$ vs 1/$T_{\text{early}}$ plots for Y$_5$R and Y$_5$K are linear, supporting that the matrix-to-peptide proton transfer is almost in equilibrium. $\Delta H^o$ estimated from the slope was $-86$ and $-56$ kJ mol$^{-1}$ for Y$_5$R and Y$_5$K, respectively. The exothermic nature of the proton transfer reactions is consistent with the equilibrium constants much larger than 1. Also, larger exothermicity for Y$_5$R than for Y$_5$K is in agreement with the fact that arginine is the stronger base.35 The linear pattern of the van’t Hoff plots suggests that the present experimental technique, i.e., repetitive irradiation of a spot on a sample, can become a way of collecting

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**Figure 3.** Spectrum with $T_{\text{early}}$ near 968 K was selected from each MALDI spectral set for samples with Y$_5$R:CHCA = 1:8300 obtained under four different experimental conditions denoted as (no. of pmol of Y$_5$R, no. of nmol of CHCA, laser pulse energy in unit of the threshold, laser wavelength in nm): (a) 71–90 shot number range of (3, 25, x6, 337), (b) 51–70 shot number range of (3, 25, x4, 337), (c) 101–120 shot number range of (4, 2, 35, 337), and (d) 31–50 shot number range of (3, 25, x6, 355).

**Figure 4.** Reaction quotient ($Q$) for the matrix-to-peptide proton transfer at $T_{\text{early}}$ of 950 K vs the peptide amount in the solid sample. Details of the calculation are explained in the text. ● and ○ denote the data for Y$_5$R and Y$_5$K, respectively. Horizontal lines are the averages. Error bars represent 1 standard deviation.

**Figure 5.** ln $K$ vs 1/$T_{\text{early}}$ plot. Data were taken from the MALDI spectra of 3 pmol of peptide in 25 nmol of CHCA collected over 1–200 shots at 6 times the threshold. $K$ was approximated as ([M$'$/[P])/(PH$^+$/[M$'$H$^+$])). Data for Y$_5$R and Y$_5$K are shown as ● and ○, respectively. $\Delta H^o$ estimated from the slope was $-86$ and $-56$ kJ mol$^{-1}$ for Y$_5$R and Y$_5$K, respectively.

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thermodynamic data for gas-phase peptide ions. Further study will be needed to develop this technique into such a thermodynamic tool.21

■ CONCLUSIONS

To summarize, the early plume temperature ($T_{early}$) associated with each single shot MALDI spectrum obtained by repetitive irradiation of a spot was estimated by kinetic analysis of peptide ion dissociation. With each spectrum tagged with $T_{early}$, its accuracy does not really matter, it became very clear that our perception of the erratic mass spectral pattern in MALDI arose partly because we were comparing MALDI spectra associated with different $T_{early}$. Controlling various experimental factors such as sample thickness may be a way of improving spectral reproducibility. There may be easier ways, such as selecting spectra based on the fragmentation pattern of the matrix ion. With each spectrum tagged with $T_{early}$, interpreting the time-evolution of MALDI spectra became quite straightforward also. It was obvious that the dissociations of peptide and matrix ions were determined by thermal kinetics while the peptide-to-matrix ion abundance ratio was determined by quasi-thermal equilibrium in the proton transfer. Even though the reactions occurring inside the ion source have been found to be governed by $T_{early}$, the same does not apply to the reactions occurring outside the source, i.e., PSD. Previously, we showed that the effective temperature in the late plume ($T_{late}$), which governs PSD, was substantially lower than $T_{early}$ due to expansion cooling of the matrix plume.

■ ASSOCIATED CONTENT

Supporting Information
Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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