A Simple Method for Quantification of Peptides and Proteins by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry

Kyung Man Park,† Yong Jin Bae,‡ Sung Hee Ahn,‡ and Myung Soo Kim †,*

†Department of Chemistry, Seoul National University, Seoul 151-742, Korea

Supporting Information

ABSTRACT: Even though matrix-assisted laser desorption ionization (MALDI) is a powerful technique for mass spectrometry of peptides and proteins, it is not quite useful for their quantification that is one of the outstanding problems in quantitative proteomics. The main difficulty lies in the poor reproducibility of MALDI spectra. In this work, a simple method to circumvent this problem has been developed. The method is based on a previous observation that the reaction quotient for the matrix-to-peptide proton transfer evaluated in temperature-selected MALDI was nearly constant regardless of the peptide concentration in the solid sample. This implied a direct proportionality between the relative abundance of an analyte ion in a temperature-selected MALDI spectrum and the concentration of the corresponding neutral in the solid sample. This relation has been confirmed by calibration curves obtained for some peptides. Another characteristic of the relation is that it holds even when other analytes are present. This has been demonstrated for mixtures containing peptides and proteins. This and the fact that the method does not require the addition of internal standards allow rapid and inexpensive quantification of any analyte amenable to MALDI.

Matrix-assisted laser desorption ionization (MALDI) and electrospray ionization are the two most popular ionization techniques in mass spectrometry of biological molecules.1−3 Sample matrix effect, or suppression of analyte ion signal,2,4 is one of the difficulties in these techniques, especially in their use for quantitative analysis—quantification of peptides and proteins is one of the outstanding problems in quantitative proteomics.5−9 Sample cleanup and coupling with a chromatograph are used to deal with this problem.

The fact that an analyte ion signal can be observed even when contaminants are present in a sample and that spectral acquisition can be made rapidly are distinct advantages of MALDI mass spectrometry.10−12 However, MALDI is not regarded as the method of choice for quantitation because analyte ion signals generated by this technique display poor sample-to-sample, spot-to-spot, and shot-to-shot reproducibilities.13 The best way to cope with the signal irreproducibility has been to add an internal standard whose physicochemical properties are similar to those of the analyte. The analyte molecules that are chemically or metabolically labeled with stable isotopes such as 13C and 15N can be useful for this purpose.14−19 One of the problems in this approach is that the complexity of biological samples sometimes makes it difficult to find an internal standard with an m/z different from all the substances in the sample. Another problem is that you need an isotopically labeled internal standard for each analyte to be quantified. The latter problem can be avoided by tagging the analyte with an isotopically labeled reagent.20−22 For example, in the method called ICAT (isotope-coded affinity tag)20 developed for the quantification of proteins, a part of the tag called linker is labeled with stable isotopes. Sample loss and long analysis time are the potential problems common to the analytical schemes utilizing on-site chemical labeling.

Recently, we investigated MALDI of some peptides by collecting mass spectra from different samples, from different spots on the same sample, and from different laser shots on the same spot.23 We estimated the effective temperature in the early matrix plume, Tearly, associated with each spectrum by kinetic analysis of the fraction of peptide ions that did not dissociate until they passed the ion source exit, or the survival probability.24 Then, we observed that the overall patterns of MALDI spectra obtained under different experimental conditions were similar, or reproducible, when those associated with the same Tearly were compared. In particular, the reaction quotient for the matrix-to-peptide proton transfer was constant regardless of the peptide concentration in the solid sample.23 Kinsel et al. 25 observed near equilibrium in MALDI of amino acids even without temperature selection. There, the observed near equilibrium arose possibly because only the spectral data collected in a narrow range of shots were compared and hence the temperature did not change much. From the near constancy of the reaction quotient for the matrix-to-peptide proton transfer at a specified temperature, we realized that this would allow us to quantify analytes by MALDI without adding any

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internal standard. Such a capability will be demonstrated in this work for some peptides and proteins.

## EXPERIMENTAL SECTION

Two home-built MALDI-TOF instruments were used, instruments I\textsuperscript{26} and II\textsuperscript{27}. Overall layouts for the two instruments are similar, even though they differ in details such as in the total length. In each instrument, 337 nm output from a nitrogen laser (MNL100, Lasertechnik Berlin, Berlin, Germany) was used for MALDI. The focal lengths of the lenses were 100 and 250 mm, respectively, for the instruments I and II. Instrument I was used mostly for MALDI with α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix, while instrument II was for MALDI with 2,5-dihydroxybenzoic acid (DHB). The threshold laser pulse energies were 0.30 and 1.65 μJ/pulse, respectively, for CHCA- and DHB-MALDI. Spectral measurements were made using the laser pulse energy corresponding to two times the threshold.\textsuperscript{28} Quantification results for the same peptides were the same within error limits regardless of the instrument used. To improve the signal-to-noise ratio, spectral data from every ten laser shots were summed. Then, the results from twenty different spots on a sample were summed. Detector gain vs m/z of ion was calculated by measuring the charge for each single ion pulse. The number of ions in each peak was calculated by integrating its current vs TOF data and dividing the result by the detector gain.\textsuperscript{29,30}

**Sample Preparation.** Angiotensin I (DRVYIHPFH), angiotensin II (DRVYIHPF), substance P (RPKPQ2FPGM-NH\textsubscript{2}), YGGFL, insulin, β-amyloid 1-42, CHCA, and DHB were purchased from Sigma (St. Louis, MO, USA). Remaining peptides were purchased from Peptron (Daejeon, Korea). Nonpeptide samples, i.e. histamine, glucosamine, fumonisin B1, and creatinine were also purchased from Sigma. Aqueous stock solution of each analyte except insulin was diluted to a desired concentration and mixed with 1:1 water/ acetonitrile solution of CHCA or DHB. In the case of insulin, acetonitrile was used to prepare stock solution. One μL of a mixture was loaded on the target and vacuum-dried. With CHCA, spectral data were taken from any location on a solid sample because samples prepared by vacuum-drying were rather homogeneous. In the case of DHB, crystallites at the rim of a sample were much larger than those near the center. For ease of experiment, spectral data were taken from the center.

## RESULTS AND DISCUSSION

Since a sample used in MALDI is a mixture of a matrix (M) and an analyte (A), the overall pattern of a MALDI spectrum would be determined by three factors. First are the fragment ion-to-precursor ion (AH\textsuperscript{+}) abundance ratios for the analyte. Second are the fragment ion-to-precursor ion (MH\textsuperscript{+}) abundance ratios for the matrix. Third is the analyte ion-to-matrix ion abundance ratio, [AH\textsuperscript{+}]/[MH\textsuperscript{+}].\textsuperscript{25} Then, the similar overall pattern at the same T\textsubscript{early} mentioned in a previous section meant that not only the relative abundances of the fragment ions from AH\textsuperscript{+} and MH\textsuperscript{+} but also the [AH\textsuperscript{+}]/[MH\textsuperscript{+}] ratio was thermally determined. This, in turn, suggested that the reaction quotient for the matrix-to-peptide proton transfer, MH\textsuperscript{+} + A → M + AH\textsuperscript{+}, was nearly constant at the temperature of T\textsubscript{early}.\textsuperscript{23} To check this, we performed experiments for samples with various matrix-to-analyte ratios, selected spectra associated with the same T\textsubscript{early}, and estimated the reaction quotient Q = ([M]/[A])([AH\textsuperscript{+}]/[MH\textsuperscript{+}]).\textsuperscript{23} Assuming that the proton transfer preceded the fragmentation of AH\textsuperscript{+} and MH\textsuperscript{+}, we estimated [AH\textsuperscript{+}] and [MH\textsuperscript{+}] as the sums of the analyte- and matrix-derived ions, respectively. However, as far as checking the constancy of Q was concerned, the abundance of any analyte-derived ion, whether it was AH\textsuperscript{+} ion, one of its fragments, or their combinations, might as well be used because the abundance of each fragment ion relative to that of AH\textsuperscript{+} was fixed when T\textsubscript{early} was specified. The same applied to [MH\textsuperscript{+}]. For the matrix-to-analyte neutral ratio in the plume, [M]/[A], we used the ratio in the solid sample. Then, Q turned out to be independent of the matrix-to-analyte ratio. Even though the constancy of Q is generally taken as manifestation of equilibrium, a kinetic quasi-steady state may be a better description for the situation in the early matrix plume because it is undergoing a rapid time evolution.\textsuperscript{34} The above relation can be written in the following form

\[
\]

With Q constant, the analyte-to-matrix ion abundance ratio measured from a spectrum is directly proportional to the analyte-to-matrix ratio in the solid sample. Such a direct proportionality holds only when the ion abundance data are taken from the spectra associated with the same T\textsubscript{early}. Equation

\[10333\]

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suggests that the concentration, or the amount, of an analyte in a solid sample can be determined from the abundances of analyte- and matrix-derived ions. From the description of the method used to calculate Q, it is obvious that the abundance of any ion derived from the analyte, and from the matrix also, can be used for the analysis. In the case of the analyte, its molecular ion (AH+) would be the natural choice.

When there are two analytes, A and A′, in a sample, a relation similar to eq 1 will hold for the second component also.

\[
\frac{[AH^+]}{[MH^+]} = Q'(\frac{[A']}{[M]})
\] (2)

Regardless of the participation of the second proton transfer reaction, however, eq 1 may hold. That is, analyte quantification based on eq 1 may be made even when the analyte is a component of a mixture. Another important aspect of the present quantification scheme is that one does not have to add an internal standard, or one may say that the matrix is serving as the internal standard.

Spectral Selection. It is well-known that some of the matrix and peptide ions undergo dissociation in the hot early matrix plume—in-source decay (ISD).24,32 Intuitively, ISD will become more efficient as the early plume gets hotter. In a previous study, we treated the internal energy of a peptide ion undergoing ISD as displaying thermal distribution at an effective temperature, T_early. Then, we devised a method to determine T_early by kinetic analysis of the probability for a peptide ion to avoid ISD until it passed the source exit, that could be calculated from a MALDI spectrum.24,28 The method requires knowledge on the rate-energy relation, k(E), for the dissociation of this ion that must be determined by a separate study. An easier way to estimate T_early is to utilize the fact that the extent of fragmentation for a matrix ion is thermally determined also.23 In this work, we took CHCA-MALDI spectra of Y₅K—dissociation kinetics of [Y₅K+H]⁺ was determined previously33 and calculated T_early associated with each spectra. From the abundance vs T_early data for several matrix-derived ions, we decided to use the [M + H – H₂O]⁺-to-[M + H]⁺ ion abundance ratio as a measure of T_early. This ion abundance ratio vs T_early data are shown in Figure 1(a). For example, this ratio of 3.0—4.5 corresponds to T_early of 870—900 K. We did the same for DHB-MALDI utilizing the dissociation kinetics of [Y₆+H]⁺ (Figure 1(b)).33 In this case, the [M + H – H₂O]⁺-to-[M + H]⁺ ratio of 4.0—11.5 in DHB-MALDI corresponds to T_early of 780—800 K. We would like to emphasize that the accuracy of the present method is not critical and that the magnitude of the number evaluated is meaningless because we use it only to select spectra associated with the same T_early.

In the previous study on the reproducibility of temperature-selected MALDI spectra,23 we did not deal with DHB-MALDI because the spot-to-spot spectral variation was more serious than in CHCA-MALDI. In this work, we collected spectral data

![Figure 2. Calibration curves in CHCA-MALDI of (a) Y₅R, (b) Y₅K, (c) YLYELAR, and (d) angiotensin II. [AH⁺]/[MH⁺] vs [A]/[M] is drawn in log–log scale. The amount of each peptide, 0.01—250 pmol, in 2.5 nmol of CHCA in a solid sample was used to calculate [A]/[M]. [AH⁺]/[MH⁺] was calculated from MALDI spectra with the [CHCA + H – H₂O]⁺-to-[CHCA + H]⁺ ion abundance ratio of 3.0—4.5 (870—900 K in T_early). The abundance of the protonated peptide was taken as [AH⁺], while the sum of the abundances of [CHCA + H]⁺, [CHCA + H – H₂O]⁺, and [CHCA + H – CO₂]⁺ was taken as [MH⁺]. Error bars indicate one standard deviation from triplicate measurements.]
from the central part, rather than the rim, of a dried DHB sample to reduce such variation.

**Calibration Curve.** To check the utility of eq 1 in analyte quantification, we carried out MALDI studies for solid samples with \(0.01 - 250\) pmol of Y5R, Y5K, YLYEIAR, or angiotensin II in 25 nmol of CHCA. For each sample, we obtained a set of spectra as a function of the laser shot number, selected those with \([\text{CHCA} + \text{H} - \text{H}_2\text{O}]^+\)-to-\([\text{CHCA} + \text{H}]^+\) ion abundance ratio lying in the range of 3.0 - 4.5 (870 - 900 K). Each spectrum was normalized to the abundance of \([\text{CHCA} + \text{H} - \text{H}_2\text{O}]^+\).

The log-log plots of \([\text{AH}^+] / [\text{MH}^+]\) vs \([\text{A}] / [\text{M}]\) obtained for the four peptides are shown in Figure 2. In the four plots, it is obvious that \([\text{AH}^+] / [\text{MH}^+]\) is directly proportional to \([\text{A}] / [\text{M}]\) up to around 30 pmol of a peptide in 25 nmol of CHCA—the reason for the deviation from linearity at higher peptide concentration is under investigation. We also observed the direct proportionality at other \(T_{\text{early}}\) and at other laser pulse energies.

**Table 1. Quantification Results**

<table>
<thead>
<tr>
<th>analyte</th>
<th>amount loaded</th>
<th>calibration curve</th>
<th>one-point calibration</th>
<th>amount determined</th>
<th>calibration curve</th>
<th>one-point calibration</th>
<th>amount loaded</th>
<th>calibration curve</th>
<th>one-point calibration</th>
<th>amount determined</th>
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<tr>
<td>YLYEIAR</td>
<td>0.30</td>
<td>0.29 ± 0.04</td>
<td>0.30 ± 0.04</td>
<td>0.30</td>
<td>0.13 ± 0.02</td>
<td>0.13 ± 0.02</td>
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<td>0.30</td>
<td>0.23 ± 0.03</td>
<td>0.21 ± 0.03</td>
<td>1.0</td>
<td>0.78 ± 0.08</td>
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<td>0.25 ± 0.04</td>
<td>0.30</td>
<td>0.34 ± 0.04</td>
<td>0.37 ± 0.05</td>
<td>3.0</td>
<td>0.35 ± 0.08</td>
<td>0.34 ± 0.08</td>
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<tr>
<td>DRVYHIPF</td>
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<td>0.29 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.30</td>
<td>0.36 ± 0.04</td>
<td>0.36 ± 0.04</td>
<td>3.0</td>
<td>0.34 ± 0.07</td>
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<td>FKDLGEEHK</td>
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<td>0.38 ± 0.11</td>
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<td>0.36 ± 0.03</td>
<td>0.38 ± 0.11</td>
<td>10</td>
<td>11 ± 3</td>
<td>11 ± 3</td>
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<tr>
<td>DRVYHIPFHL</td>
<td>0.30</td>
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<td>0.36 ± 0.03</td>
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<td>0.31 ± 0.01</td>
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<td>3.0</td>
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<td>HLVDPEQNLK</td>
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<td>0.36 ± 0.03</td>
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<td>0.30</td>
<td>0.36 ± 0.04</td>
<td>0.36 ± 0.04</td>
<td>10</td>
<td>11 ± 3</td>
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<tr>
<td>RPKFQFGLM-NH2</td>
<td>0.30</td>
<td>0.36 ± 0.03</td>
<td>0.36 ± 0.03</td>
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</table>

Errors denote one standard deviation from triplicate measurements. Amount in number of picomoles of an analyte in 25 nmol of CHCA. Amount in number of picomoles of an analyte in 50 nmol of DHB.

**Figure 3.** (a) MALDI spectrum for an equimolar mixture of nine peptides (YLYEIAR, Y5K, DLGEEHK, Y5R, DRVYHIPF, FKDLGEEHK, DRVYHIPFHL, HLVDPEQNLK, and RPKFQFGLM-NH2), 0.30 pmol each in 25 nmol of CHCA. (b)-(e) are MALDI spectra of 0.30 pmol of one peptide in 25 nmol of CHCA for (b) YLYEIAR, (c) Y5K, (d) Y5R, and (e) RPKFQFGLM-NH2. Temperature selection was made by selecting spectra with \([\text{CHCA} + \text{H} - \text{H}_2\text{O}]^+\)-to-\([\text{CHCA} + \text{H}]^+\) ratio of 3.0 - 4.5 (870 - 900 K).

Figure 4. MALDI spectrum for a mixture of nine analytes, i.e. YLYEIAR (0.10), Y5K (1.0), DLGEEHK (3.0), Y5R (0.30), YGGFL (10), histamine (1.0), glucosamine (30), fumonisin B1 (1.0), and creatinine (0.30). Here the number in each parenthesis denotes the number of picomole of each analyte in 25 nmol of CHCA. Temperature selection was made by selecting spectra with \([\text{CHCA} + \text{H} - \text{H}_2\text{O}]^+\)-to-\([\text{CHCA} + \text{H}]^+\) ratio of 3.0 - 4.5 (870 - 900 K).
The relative abundance of a peptide ion, represented by $[AH^+]/[MH^+]$, is a component of the mixture. According to eq 1, the ion is similar whether the peptide is the only analyte in the sample or is a component of the mixture. According to eq 1, the ion is similar whether the peptide is the only analyte in the sample. The presence of other analytes in the sample reduces the abundance of a selected analyte ion. Weakly in the MALDI spectrum of a mixture, an analyte can be quantified as a highly systematic version of an internal standard. In this sense, the present method might be as a highly systematic version of an external standard method. Alternatively, it might be classified as an absolute quantification method using the solid sample as the internal standard.

**CONCLUSION**

Even though MALDI is a sensitive, rapid, and relatively inexpensive method for the identification and structure determination of biological molecules, its utility in their quantification has been limited due to the poor spectral reproducibility. In this work, our previous observation that temperature-selected MALDI spectra were reproducible has been translated into the direct proportionality between the analyte-to-matrix ion abundance ratio and the analyte-to-matrix ion ratio in the solid sample, allowing easy quantification of the analyte. The relation has been found to hold even when the analyte is a component of a mixture. A salient feature of the method is that one can quantify an analyte without adding an internal standard. In this sense, the present method might be classified as a highly systematic version of an external standard method. Alternatively, it might be classified as an absolute quantification method using matrix as the internal standard. We expect that the method will become an inexpensive technique suitable for quick quantitative screening of any analyte amenable to MALDI such as peptides and proteins. The fact that the analyte concentration is proportional to the analyte-to-matrix ion abundance ratio can be used for very quick comparison of the relative amounts of a particular analyte in two or more biological samples.

**Figure 5.** MALDI spectrum for a mixture containing 3.0 pmol of β-amyloid 1-42, 3.0 pmol of insulin, and 0.3 pmol each of YLYEIAR, Y,K, DLGEEHFK, Y,R, DRVYIHPF, FKDLGEEHFK, HLVDQPQLNLIK, and RPKPQFFGLM-NH$_2$ in 50 nmol of DHB. Temperature selection was made by collecting spectra with the $[DHB + H^-]/[DHB + H^+]$ ratio of 4.0−11.5 (780−800 K in T$_{early}$).
**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.

**AUTHOR INFORMATION**

**Corresponding Author**
*E-mail: myungsoo@snu.ac.kr.*

**Notes**
The authors declare no competing financial interest.

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