Acquisition of the depth profiles and reproducible mass spectra in matrix-assisted laser desorption/ionization of inhomogeneous samples

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RATIONALITY: In our previous analysis of the matrix-assisted laser desorption/ionization (MALDI) spectra of peptides, we treated their depth profiles in solid samples as homogeneous. Here, we wanted to determine if the reproducible MALDI spectra and linear calibration curves reported previously would be obtained even when the depth profiles were inhomogeneous.

METHODS: We derived a formula relating shot-number-dependent ion abundance data in temperature-controlled MALDI with the analyte depth profile in a solid sample. We prepared samples containing peptides, amino acids, and serotonin in α-cyano-4-hydroxycinnamic acid matrix by vacuum-drying and micro-spotting methods, recorded their MALDI spectra, and analyzed them with the aforementioned formula.

RESULTS: For the samples prepared by vacuum-drying, the analyte depth profiles were inhomogeneous and maximized at the sample surface. Although the MALDI spectra changed as the shot continued, their sum over the entire set was reproducible. Similarly, a high-quality calibration curve could be obtained with the spectral data summed over the entire set. Depth profiles were homogeneous for samples prepared by micro-spotting.

CONCLUSIONS: A method has been developed to obtain a reproducible MALDI spectrum and a linear calibration curve for an analyte with an inhomogeneous depth profile in a solid sample. Copyright © 2015 John Wiley & Sons, Ltd.

Matrix-assisted laser desorption/ionization (MALDI) is used to produce gas-phase ions from a solid-phase sample. It is thought that the abundance of each ion in a MALDI spectrum is irreproducible, which is an obstacle preventing its quantitative use. It is known that the inhomogeneity of a solid sample, which is an analyte-matrix mixture, is one of the culprits responsible for the irreproducibility of the spectrum.

In our recent study, the MALDI of peptides using α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix, we prepared samples by means of the vacuum-drying of solutions containing CHCA and peptides. Although the samples thus prepared appeared to be rather homogeneous, the MALDI spectra acquired by repetitive laser irradiation on a spot displayed shot-to-shot variation. This suggested that there was a factor(s) other than sample inhomogeneity that caused spectral irreproducibility. For these rather homogeneous samples, we observed that the spectral variation occurred systematically, not randomly. In particular, the variation became insignificant when the spectra displaying similar early plume temperatures (Tearly) were compared. Hence, we concluded that sample inhomogeneity and variation in the MALDI temperatures were two factors responsible for the spectral irreproducibility. We observed that the total ion count (TIC), i.e., the sum of the abundances of the peptide- and matrix-derived ions, increased as Tearly was raised. This was utilized to generate reproducible spectra.

With Tearly fixed, the TIC was constant regardless of the peptide concentration in a solid sample. That is, an increase in the total abundance of peptide-derived ions was matched by a decrease in that of matrix-derived ions. This was taken as evidence that a peptide ion ([A + H]+) was produced at the cost of a matrix ion ([M + H]+), most likely via a proton transfer.

\[
[M + H]^+ + A \rightarrow M + [A + H]^+ \tag{1}
\]

Its reaction quotient (Q) is as follows:

\[
Q = \frac{I([A + H]^+)}{I([M + H]^+)} / \frac{I(A)}{I(M)} \tag{2}
\]

I(X) denotes the amount of X in the plume. To estimate Q, we need I(A)/I(M) in the plume, a quantity that cannot be measured easily. A related quantity is the overall peptide-to-matrix ratio in a solid sample, which is known. This is termed the peptide concentration. We used it in the estimation of Q. For reaction (1) occurring at a fixed Tearly, Q was nearly constant regardless of the peptide concentration. We proposed the use of the resulting direct proportionality between \([I([A + H]^+)/I([M + H]^+)]\) and the peptide concentration as the calibration curve for peptide quantification.
Using the overall concentration in the solid sample as $I(\text{A})/I(M)$ in the plume produced by each laser shot is equivalent to assuming a homogeneous distribution, or a homogeneous depth profile, of the peptide along the sample depth – this is the $x$ direction with the sample surface constituting the $yz$-plane. In a typical experiment on a vacuum-dried spot of peptides in CHCA, we acquired a set of spectra through the repetitive irradiation of a spot, averaged them, and evaluated $(I(\text{A}+\text{H})^+)/I(M+\text{H})^+$, and used this result to draw a calibration curve. It was linear over a wide dynamic range.

In a subsequent study of the utility of our method in MALDI imaging, we noted that the thickness of a matrix on a tissue coated by a commercial sprayer was irregular. To estimate the analyte amount at each spot, we proposed the acquisition of the spectra from the spot until it becomes depleted, the evaluation of $(I(\text{A}+\text{H})^+)/I(M+\text{H})^+$ for each spectrum, and then summing them. We were also curious about the inhomogeneous depth profile of an analyte and its influence on the spectral reproducibility and quantification.

In this work, an algorithm to handle data from a sample with variable thickness and an inhomogeneous depth profile is derived. Cases displaying noticeably inhomogeneous depth profiles are presented. We show that reproducible MALDI spectra and linear calibration curves can be obtained even when a sample is inhomogeneous along the $x$-axis. Furthermore, we show that samples prepared with a micro-spotter are free from problems arising from the inhomogeneity along any direction.

**MODEL**

Recently, we observed that the extent of the fragmentation of the molecular ions of an analyte and a matrix produced by MALDI decreased steadily when a spot on a sample was repetitively irradiated by a pulsed laser at a fixed pulse energy. This indicated that the temperature ($T_{\text{early}}$) of the early plume where the in-source decay occurred decreased as the shot continued. When we fixed $T_{\text{early}}$ rather than the pulse energy, the abundances of all ions appearing in the MALDI spectrum became fixed. This was taken as an indication that the effective temperature of the matrix molecules undergoing ablation, $T_n$, was fixed when $T_{\text{early}}$ was fixed. This is plausible because $T_n$ and $T_{\text{early}}$ are related through the expansion cooling occurring in the very early plume. When the laser pulse energy, rather than $T_{\text{early}}$ is fixed, ion signals decrease as the shot continues and eventually disappear although some matrix molecules remain at the spot. The fact that the ion emission resumes when the pulse energy is increased is evidence that the depletion of the spot is incomplete at this stage. We explained the above observation as due to the lowering of $T_n$ as the shot at a spot continued; this may occur because conduction cooling becomes more efficient as a spot becomes thinner.

Our thermal explanation of MALDI suggests that the amounts of various chemical species generated by MALDI are fixed when $T_{\text{early}}$ and hence $T_n$ as well, is fixed. For example, the amount of the matrix ablated by a laser shot will be fixed when $T_n$ is fixed. This is one of the assumptions made in this work.

We will treat MALDI at a spot as the ablation of materials inside an elliptic cylinder with a constant cross-section. Hence, experimental data acquired using a laser with a flat-top cross-section may be more suitable for the analysis. The amounts of the matrix and the analyte in this cylinder will be denoted as $M$ and $A$, respectively. We will assume that a thin slice of this cylinder, an elliptic disc, is ablated by each laser shot. The amounts of the matrix and the analyte in the disc ablated by the $i$th laser shot will be denoted as $M_i$ and $A_i$, respectively. They are equivalent to the amounts of the matrix and the analyte in the $i$th plume, i.e., $I(M)$, and $I(A)_i$, respectively. With $T_n$ fixed, $M_i$ is constant throughout the measurement process. With $N$ as the number of shots that depletes a spot, $M_i$ becomes $M/N$. We will define the ion ($R_i^\text{A}$) and neutral ($R_i^\text{N}$) abundance ratios in the plume generated by the $i$th shot as follows:

$$R_i^\text{A} = I((\text{A}+\text{H})^+)/I((M+\text{H})^+)$$

$$R_i^\text{N} = I(A)^n/I(M)^n = A_i/M_i = (N/M)A_i$$

For the $i$th plume, $Q_i$ for the proton transfer defined in Eqn. (2) is as follows:

$$Q_i = R_i^\text{A}/R_i^\text{N}$$

or,

$$R_i^\text{A} = Q_i R_i^\text{N}$$

Previously, we noted that $Q_i$ was nearly constant ($= Q$) when $T_n$ was fixed. Thus, by inserting Eqn. (4) into Eqn. (6) and rearranging the resulting equation, the following expression is obtained:

$$A_i = \left[Q^{-1}(M/N)\right] I((\text{A}+\text{H})^+)/I((M+\text{H})^+)$$

Here, $A_i$ is the amount of the analyte in the $i$th disc. The set of $A_i$ ($A_i$) is the distribution of an analyte along $x$, or its depth profile. By summing $A_i$ over all shots needed to deplete a spot, the total amount of the analyte at the spot is obtained:

$$A = \Sigma A_i = \left[Q^{-1}(M/N)\right] \Sigma I((\text{A}+\text{H})^+)/I((M+\text{H})^+)$$

Accepting that $N$ is proportional to $M$ when $T_{\text{early}}$ is constant, Eqn. (8) simply states that the total amount of the analyte at a spot is proportional to the sum of the analyte-to-matrix ion abundance ratio, $R_i^\text{A}$, in the complete set of spectra acquired at the spot. This is in good agreement with the intuitive proposition presented in our previous study of samples with variable thicknesses. In the present paper, we will regard the set of the analyte-to-matrix ion abundance ratios measured at a spot, ($R_i^\text{A}$), as the depth profile.

**EXPERIMENTAL**

The home-built matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) instrument used in this work consists of an ion source with delayed extraction, an ion gate, a reflectron, and a microchannel plate detector. 337 nm output from a nitrogen laser (MNL100, Lasertechnik Berlin, Germany)
is focused on a sample. The number of ions in each peak of a MALDI spectrum was calculated by taking into account the detector gain.

A description of the TIC-control method[^9] used to acquire the MALDI spectra at a fixed T_{early} is given below. We first determine the minimum, or threshold, energy of a laser pulse needed for ion emission from the matrix.[^22] The pulse energy is then increased to two times the threshold and the TIC is measured, which is taken as the pre-set value of the TIC. Spectral measurement at a spot starts with a pulse energy corresponding to two times the threshold. After averaging the spectra over 10 consecutive shots, the TIC is calculated. When this is smaller than the pre-set value, the pulse energy is increased to compensate for the difference, and vice versa, and the spectral measurement is resumed (see data in Supporting Information). According to the model presented above, we must repeat this process until the materials in the spot are completely depleted. In the TIC-control mode, the pulse energy is increased when the TIC decreases, thereby restoring the TIC back to its pre-set level. A problem here is that the effective area of the spot contributing to the ion emission becomes larger (see data in Supporting Information) as the pulse energy increases.[^23] Hence, judging the time to stop the spectral acquisition is difficult. In practice, we stop the spectral acquisition when the pulse energy reaches three times the threshold. Above this pulse energy, fixing T_{early} via TIC-control becomes somewhat problematic. Also, an irradiated spot begins to appear dark due to the nearly complete depletion of the matrix. When 1 μL of a solution (25% acetonitrile and 0.1% trifluoroacetic acid (TFA) in water) containing 25 nmol of CHCA was vacuum-dried on a stainless steel target, a circular film with a diameter of ~2 mm was produced. When the density of CHCA was approximated as that of cinnamic acid (1.25 g cm^-3), the film thickness was ~1.2 μm. The pre-set TIC for such a sample was ~3000 and the number of shots (N) needed to deplete a spot was 160–180.

We also prepared samples with a micro-spotter (μMatrix Spotter, ASTA, Suwon, Korea).[^24] Both CHCA and 2,5-dihydroxybenzoic acid (DHB) were used as the matrix. To prepare the DHB samples, an amount of 3 μL per drops of a solution (20% methanol and 0.1% TFA in water) containing 25 nmol of CHCA was vacuum-dried on a stainless steel target, a circular film with a diameter of ~2 mm was produced. When the density of CHCA was approximated as that of cinnamic acid (1.25 g cm^-3), the film thickness was ~1.2 μm. The pre-set TIC for such a sample was ~3000 and the number of shots (N) needed to deplete a spot was 160–180.

For example, 1.0 pmol of an analyte in a solution of 70 nmol of DHB corresponds to ~10 fmol of the analyte in a sample with a diameter of 200 μm. Using the micro-spotter, we also prepared CHCA samples with a diameter of 200 μm and a thickness 1.2 μm. These were comparable to samples prepared by the vacuum-drying of 1.0 μL of a sample solution containing 25 nmol of CHCA and an analyte. We will quote the amount of the analyte in 25 nmol of CHCA as its concentration. A mixture of 80% ethanol and 0.1% TFA in water was used as the solvent. The pre-set TIC for the CHCA sample was ~3000 and the number of shots (N) needed to deplete a spot was 160–180.

From each spectrum obtained by spectral summing over ten consecutive shots, we calculated $R^2_i$. We also calculated the matrix suppression ($S$),[^25] as defined below:

$$
S = 1 - \frac{I_0([M+H]^+)}{I_0([M+H]^+)}
$$

Here, $I_0([M+H]^+)$ is the matrix ion abundance in MALDI of the pure matrix.

**Samples**

Peptide Y5R and Substance P were purchased from Peptron (Daejeon, Korea). Amino acids arginine and phenylalanine were also purchased from Peptron. Serotonin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Matrices CHCA and DHB and solvents were also purchased from Sigma-Aldrich.

**Figure 1.** Depth profiles, ($R^2_i$), of Y5R in vacuum-dried samples of (a) 0.10, (b)1.0, and (c) 10 pmol of the peptide in 25 nmol of CHCA. Error bars represent one standard deviation.
RESULTS AND DISCUSSION

For MALDI using CHCA as the matrix, or CHCA-MALDI, the usual sample preparation method of loading 1 µL of a solution containing CHCA and analytes on a stainless steel target and air-drying it produces a solid sample whose surface (yz-plane) appears to be highly inhomogeneous. In contrast, vacuum-drying of the same solution produces a solid sample that appears rather homogeneous. For DHB, which is another popular matrix, neither air- nor vacuum-drying produces samples that appear satisfactorily homogeneous. On the other hand, DHB samples prepared by micro-spotting appeared rather homogeneous. In this work, we will focus on CHCA-MALDI of samples prepared by vacuum-drying. Some data acquired for CHCA and DHB samples prepared by micro-spotting will also be presented.

Peptides

Peptides were the first analytes for which we observed the spectral reproducibility in MALDI when Tearly was fixed. In early works, we processed the spectral data as if the depth profile of a peptide in a vacuum-dried CHCA solid was homogeneous. In the present work, we calculated R² for each...
ten shot-averaged spectrum. The depth profiles, i.e., the $(R_i^1)$
values, of the peptide $Y_5R$ obtained at three concentrations –
0.1, 1.0, and 10 pmol in 25 nmol of CHCA – are shown in
Fig. 1. The depth profiles of $Y_5R$ in CHCA are rather flat,
except very close to the sample surface, which is ablated in
early shots. It seems that a fraction of $Y_5R$ follows the solution
phase until the last drop of the solvent evaporates during the
vacuum-drying process. This would result in a slight
difference between the spectra taken from the sample very
close to the surface and those from the bulk. To see this
difference, we took the sums of the spectra acquired from a
spot in a sample of 1.0 pmol $Y_5R$ in 25 nmol CHCA over
the shot number ranges of 1–10 and 141–150. These results
are shown in the Supporting Information. The abundance of
$[Y_5R + H]^+$ in the 1–10 shot number range was larger than that
in the 141–150 range only by 20%. We would like to mention
that the fragmentation pattern of the peptide ion, as well as
that of the matrix ion, remained the same regardless of the
shot number range used to construct a spectrum as long as $T_{early}$ was kept constant via the TIC-control method.

To check for spectral reproducibility, we acquired an entire
set of spectra that could be measured from a spot, took their
sum, and evaluated the abundance of $[Y_5R + H]^+$. This was
done at 10 spots. Relative standard deviations for the data
obtained for the samples with 0.10, 1.0, and 10 pmol of $Y_5R$
were 13, 14, and 11%, respectively. The results suggest that a
spectra with fair reproducibility can be obtained by
summing over the entire set of spectra even when the depth
profile is inhomogeneous.

In our previous work\cite{11} on the MALDI of peptides, we
acquired spectra at many spots on a sample, averaged them,
calculated the peptide-to-matrix ion abundance ratio in the
averaged spectrum, and used this ratio to construct a
calibration curve. No special effort was made to collect an
entire set of spectra from a spot. In the present work, we used
the analyte-to-matrix ion abundance ratios evaluated in
several different ways to construct calibration curves, e.g.,
sum of those in the shot number range of 1–50, 1–150, etc.
As shown in the Supporting Information, the calibration
curves thus obtained were indistinguishable. The same was
found for other peptides studied in this work. The results
for Substance P are listed in the Supporting Information. We
speculated that the calibration curves drawn by different
methods were similar because the depth profile of $Y_5R$ in CHCA
was rather flat. To see the influence of the inhomogeneity in
the depth profile on the spectral reproducibility, we studied
systems for which the analyte depth profiles were far more
inhomogeneous than those for peptides.

**Amino acids**

The depth profiles measured for 0.10, 1.0, and 10 pmol of
arginine in 25 nmol of CHCA are shown in Fig. 2. This figure
shows that the depth profile of arginine in CHCA is far more
inhomogeneous than that of $Y_5R$. The MALDI spectra
averaged over the shot number ranges of 1–10 and 141–150
for 5 pmol of arginine (R) in 25 nmol of CHCA are shown
in Figs. 3(a) and 3(b), respectively. It is clear that the CHCA-
MALDI spectrum of arginine will vary as the laser shot
continues, even when the temperature is fixed in the TIC-
control mode. Then, very careful control of the shot number
range for spectral acquisition will be needed to obtain
reproducible spectra. This will be difficult to implement. We
also acquired a spectrum by summing over the entire set of
spectra measured at a spot. The summed spectrum measured
for a sample with 5 pmol of arginine in 25 nmol of CHCA is
shown in Fig. 3(c). It is important to emphasize that the summed
spectrum displayed good spot-to-spot reproducibility.

At a given arginine concentration, we acquired an entire set
of spectra from a spot, evaluated $(R_i^1)$, summed the spectra,
and used the sum to construct the calibration curve. A log-
log plot of the curve is shown in Fig. 4(a). It is linear with a
slope close to 1. The same data plotted on an ordinary scale
are shown in the Supporting Information. That is, although
the depth profile of arginine in CHCA prepared by vacuum-
drying appeared to be highly inhomogeneous, a linear
calibration curve was obtained.

Instead of measuring the analyte-to-matrix ion abundance
ratio in each spectrum and taking their sum over the entire set,
we initially averaged the spectra over the entire set,

![Figure 4](image-url) **Figure 4.** Log-log plots of the calibration curves – the analyte-to-matrix ion abundance ratio vs. the analyte concentration in the solid sample – for vacuum-dried samples of arginine in CHCA. The ion abundance ratio was calculated by (a) summing $R_i^1$ over the entire set acquired at a spot and (b) summing the spectra over the set, measuring the ratio in the averaged spectrum, and multiplying the result by N. Error bars represent one standard deviation.
measured the ratio in the spectrum thus obtained, and multiplied the result by N. The calibration curve thus obtained is shown in Fig. 4(b). It is nearly identical to that in Fig. 4(a). We observed similar trends for the other analytes studied in this work. That is, spectral summing followed by ratio measurement represents a quick alternative to the more rigorous method of ratio measurement followed by summation. The former method is the method of choice here for the acquisition of reproducible spectra and the analyte quantification by MALDI.

Because the depth profiles of arginine measured at three different concentrations were similar, we speculated that we might be able to obtain a linear calibration curve by using the spectra acquired in a narrow shot number range. As examples, we constructed two calibration curves (not shown) by using the spectra acquired in the 1–10 and 141–150 shot number ranges. They were similar to those shown in Fig. 4.

Previously,[25] we reported that a log \( \frac{I([A+H]^+)/I([M+H]^+)}{\log (A/M)} \) plot deviates from linearity at high matrix suppression, e.g., at 70% or higher in CHCA-MALDI. In the CHCA-MALDI of arginine which is concentrated near the sample surface, matrix suppression will be higher in the spectra acquired in early shots than it is in late shots. In the MALDI of 10 pmol arginine in 25 nmol CHCA, matrix suppressions in the spectra acquired in the shot number ranges of 1–10 and 101–110 were 80 and 62%, respectively. According to the guideline mentioned above, the ion abundance ratio in an early shot would not be a correct representation of the arginine concentration near the surface. This results in the deviation of the calibration curve from linearity at a high arginine concentration, as shown in Fig. 4.

We also studied a few other amino acids and obtained similar results as for arginine. Data for phenylalanine are presented in the Supporting Information.

**Serotonin**

The structure of this neurotransmitter is shown in the Supporting Information. In the CHCA-MALDI spectrum of serotonin,[27] its molecular ion peak is weak. Although the abundance of its in-source decay product [serotonin + H– NH₃]^+ was higher, it was lower than those of typical peptide ions by one order of magnitude. Here, [serotonin + H– NH₃]^+ was used to acquire the depth profile and calibration curve.

The depth profiles of vacuum-dried samples with 1.0 and 10 pmol of serotonin in 25 nmol of CHCA are shown in Figs. 5(a) and 5(b), respectively. They were more inhomogeneous than those of arginine. Regardless, MALDI spectra summed over the entire set at each spot displayed good spot-to-spot reproducibility (spectrum not shown).

The calibration curve obtained by spectral summing followed by ratio measurement is shown in Fig. 6(a). The matrix suppressions in the shot number range of 1–10 for the samples containing 10 and 30 pmol of serotonin in 25 nmol of CHCA were 53 and 99%, respectively. Hence, quantification of serotonin by CHCA-MALDI will be erroneous when its concentration is well above 10 pmol, as also indicated by the deviation of the calibration curves from linearity near 30 pmol.

**Figure 5.** Depth profiles of serotonin in vacuum-dried samples of (a) 1.0 and (b) 10 pmol of the analyte in 25 nmol of CHCA and in a micro-spotted sample of 10 pmol of serotonin in (c) 25 nmol of CHCA and (d) 70 nmol of DHB. Error bars represent one standard deviation.
**Serotonin in samples prepared by micro-spotting**

A microscopic view\[^{[26]}\] of a vacuum-dried sample of DHB shows that it consists of a ring in the outskirts and a thin film inside. Although linear calibration curves for peptides were acquired from the film,\[^{[11]}\] the method was tricky because MALDI signals decayed rapidly. Later,\[^{[26]}\] we attempted to overcome this difficulty by preparing samples with a micro-spotter. We found that MALDI of peptides in DHB with a diameter of ~200 μm, comparable to the laser spot diameter, produced good calibration curves. In the present work, we checked whether the same method would give good results for the DHB-MALDI of serotonin.

As in CHCA-MALDI, [serotonin + H–NH\(_3\)]\(^+\) was a prominent serotonin-derived ion in DHB-MALDI. Although [serotonin + H]\(^+\) might have been produced as well, this was difficult to confirm because it overlapped with [DHB + Na]\(^+\). We used the abundance of [serotonin + H–NH\(_3\)]\(^+\) in the study of serotonin.

The depth profile of serotonin measured for a sample with a diameter of ~200 μm with 10 pmol of serotonin in 70 nmol of DHB prepared by micro-spotting is shown in Fig. 5(d). The actual amount of serotonin in this sample is 100 fmol. Unlike the depth profile of the vacuum-dried sample of serotonin in CHCA, that in the micro-spotted DHB sample appears to be homogeneous. Using the micro-spotter, we also prepared DHB samples containing peptides, amino acids, and some drugs. In all of the cases studied, homogeneous depth profiles were obtained. As expected for an analyte with a homogeneous depth profile, high-quality calibration curves could be constructed regardless of the method used to calculate the analyte-to-matrix ion abundance ratio. Figure 6(c) shows a calibration curve for serotonin in DHB prepared by micro-spotting.

We also prepared serotonin samples in CHCA with diameters of 200 μm by micro-spotting. The depth profiles of the serotonin in these samples were homogeneous as well (Fig. 5(c)). For a sample containing 30 pmol of serotonin in 25 nmol of CHCA prepared by micro-spotting, matrix suppression was 49%, whereas it was 99% for the spectra acquired in early shots on a comparable sample prepared by vacuum-drying. The calibration curve for the samples of serotonin-in-CHCA prepared by micro-spotting is shown in Fig. 6(b). This figure shows that the upper limit for the linearity of the curve is extended for samples with better homogeneity prepared by micro-spotting. However, the samples prepared with the micro-spotter used in this work are not useful for analyte quantification because a large volume of a sample solution, 1–2 mL, must be injected into the ink cartridge of the spotter. Finally, it is important to note that the linear parts of the two calibration curves drawn for CHCA-MALDI, one drawn with the vacuum-dried samples and the other drawn with the micro-spotted ones, are virtually identical.

**CONCLUSIONS**

We derived a general formula relating the concentration of an analyte in a solid sample with the analyte-to-matrix ion abundance ratio in the mass spectrum produced by MALDI. The formula, which facilitated the treatment of data from samples with variable thicknesses and/or with an inhomogeneous depth profile, necessitated the repetitive acquisition of MALDI spectra from a spot until it was completely depleted.

An analysis of spectral data with the formula showed that the depth profile of an analyte in a solid sample was inhomogeneous in general. In our initial studies of the reproducibility of CHCA-MALDI spectra, we adopted peptides as analytes. In retrospect, we were lucky to study peptides initially because their homogeneous depth profiles allowed a simple treatment of the spectral data.

Inhomogeneity along the sample surface direction (yz-plane) is also an obstacle to acquiring reproducible MALDI spectra. Thus far, we have failed to devise a simple method to overcome this problem. Preparation of a sample by micro-spotting can be a solution to the problem, although such a method is more complicated than dried-droplet methods.

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REFERENCES


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