Discovering a solvent effect preventing quantitative profiling by matrix-assisted laser desorption/ionization and its treatment

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RATIONALITY: In analyte profiling by matrix-assisted laser desorption/ionization (MALDI), drawing a quantitative profile map is an outstanding problem. Recently, we developed a method to quantify an analyte by MALDI, which is needed to solve the problem. Another requirement for quantitative profiling is the quantitative sample-to-matrix analyte transfer, which is investigated in this work.

METHODS: MALDI-time-of-flight (TOF) spectra were acquired for samples produced by two methods. In one, a sample solution containing a matrix and an analyte was loaded with a pipet and dried. In the other, a sample was prepared by a consecutive process, i.e., loading-drying of an analyte solution followed by that of a matrix solution. Two different micro-spotters were used in the second method. Various mixtures of organic solvents with water were used to prepare matrix solutions.

RESULTS: The organic solvent, matrix, and analyte used in the study did not affect the analyte transfer efficiency, whereas it improved as the water content in the solvent increased. It also improved as the liquid droplet emitted by a micro-spotter got larger. Use of a more polar solvent or a larger droplet increases the contact time between a solution and a matrix solution droplet is one of the requirements for quantitative profiling.

CONCLUSIONS: Sample-to-matrix analyte transfer occurred efficiently when polar solvents and/or large liquid droplets were used to produce solid samples for MALDI profiling with a micro-spotter. A long contact time between the sample surface and a matrix solution droplet is one of the requirements for quantitative profiling. Copyright © 2015 John Wiley & Sons, Ltd.
matrix layer.\textsuperscript{[19]} For accurate results, we prepared a solution containing both a matrix and an analyte, loaded it onto a clean tissue, and dried the tissue. We refer to this as the premixed sample preparation process. We quantified the analytes in premixed samples using this method and observed excellent agreement with the prepared concentrations.

However, premixed sample preparation is not how a sample is pretreated for a profiling experiment. Here, the analyte is present in the sample from the beginning and a solution containing the matrix is put onto the sample surface and dried.\textsuperscript{[20,21]} During this process, there is no guarantee that all of the analytes in the original sample will be transferred to the matrix layer.\textsuperscript{[21]} To check this, we devised a stepwise sample preparation method in which the analyte and matrix solutions were loaded and dried one after another. Using this method, we observed that as the water contents of the mixed solvents used to prepare matrix solutions got lower, the quantification results for analytes became smaller than the prepared amounts. The origin of the discrepancy, which turned out to be a kind of solvent effect, is presented together with remedies.

**EXPERIMENTAL**

The apparatus used in this work is a home-built MALDI-TOF (time-of-flight).\textsuperscript{[22]} It consists of an ion source with delayed extraction, an ion gate, a reflectron, and a microchannel plate detector. Pulsed output of a nitrogen laser (MNL100, Lasertechnik Berlin, Berlin, Germany) at 337 nm is used to induce MALDI.

All spectra were acquired at a fixed $T_{\text{early}}$, by controlling the total ion count (TIC).\textsuperscript{[15]} The TIC represents the total number of the analyte- and matrix-derived ions in a spectrum. For each measurement at a spot, we averaged the spectra over ten consecutive laser shots, measured the TIC, changed the pulse energy to keep the TIC constant, and resumed the spectral acquisition process. Spectral acquisition at a spot continued until the spot was nearly depleted. In practice, we began spectral acquisition at two times the threshold pulse energy and ended when it reached three times the threshold.

We took the sum of the spectra acquired at each spot, evaluated the ion ratio in the summed spectrum, and multiplied it by the number of spectra taken from the spot. The result is called the total ion ratio. According to our previous study, the total ion ratio is proportional to the total amount of the analyte at the spot.\textsuperscript{[19,23]}

In the stepwise method devised to mimic the sample pretreatment for profiling, first we produced an analyte spot with a circular cross section (200 $\mu$m in diameter) on a metallic sample plate using an inkjet printer.\textsuperscript{[24]} A circular matrix layer was then prepared at the same location using the same printer. Initially, we used an inkjet printer ($\mu$Matrix Spotter; ASTA, Suwon, Korea) that emitted a liquid jet at a fixed volume (3 pL per droplet). In the subsequent study conducted to explain the discrepancies in the quantification results, a different inkjet printer was used (CHIP-1000; Shimadzu, Kyoto, Japan). This second printer was more versatile than the first in various aspects. Concerning the present study, the most important aspect was that the volume of a liquid jet droplet was controllable, with a minimum of ~100 pL. The stepwise methods using the two inkjet printers will be differentiated by the corresponding notations stepwise-$\mu$Spotter and stepwise-CHIP1000. The amounts of the matrix and the analyte loaded onto a sample plate were calibrated by UV/VIS spectrophotometry. The volumes of the liquid jets were calibrated similarly.

We also prepared solid samples using premixed solutions, i.e., those containing both a matrix and an analyte. They were used to acquire reference data, calibration curves in particular. Use of the two inkjet printers here will be differentiated by the notations premixed-$\mu$Spotter and premixed-CHIP1000. $a$-Cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were used as matrices. For solid samples using CHCA as the matrix, the conventional method of loading a premixed solution with a pipet and then vacuum-drying it was also used, referred to here as premixed-pipet. The notation CHCA-premixed-pipet represents the preparation of a sample containing CHCA as the matrix.

Mixtures of ethanol (EtOH) and water were used as solvents in the stepwise preparation of solutions containing CHCA as the matrix. We also used mixed solvents containing acetonitrile (ACN) or methanol (MeOH) instead of EtOH. For example, 25% ACN in water was used for CHCA-premixed-pipet. We will denote this experimental condition as CHCA-premixed-pipet-ACN/H$_2$O(25/75). The use of solvents with a water content level greater than 80% or less than 70% produced inhomogeneous samples. During the preparation of samples with CHCA using either of the inkjet printers, EtOH/H$_2$O(80/20) was used. In this case, the use of a solvent with a water content level greater than 20% clogged the inkjet printer. In the case of DHB, premixed-pipet did not produce a homogeneous sample. When DHB samples were prepared using either of the inkjet printers, the ethanol content in the water could be changed in the range of 20–80%, as clogging did not occur. Water was the solvent during the preparation of the analyte (Y$_5$R and imipramine) solutions. The structure of imipramine is illustrated in the Supporting Information. All of the solutions contained 0.1% trifluoroacetic acid.

Peptide Y$_5$R was purchased from Peptron (Daejeon, Korea). All of the other chemicals were purchased from Sigma (St. Louis, MO, USA).

**RESULTS AND DISCUSSION**

During the pretreatment of a sample for profiling, a solution containing a matrix is loaded onto the sample surface to extract an analyte(s) from the sample. In this work, we attempted to investigate the analyte transfer process during this step by studying samples prepared by the stepwise loading-drying of solutions containing an analyte and a matrix. We will first present the calibration curves used to quantify the analytes. The efficiency of the analyte transfer will be presented thereafter.

**Calibration curves**

Previously, we acquired the calibration curve of the peptide Y$_5$R in CHCA using samples prepared with premixed-pipet-ACN/H$_2$O(25/75).\textsuperscript{[16]} 1.0 $\mu$L of each premixed solution contained various amounts of Y$_5$R and 25 nmol of CHCA. In the present work, we acquired the calibration curve under the same condition. The result drawn on the log-log scale is
shown in Fig. 1(a). The slope of 1.04, which is close to 1.0, indicates that the total ion ratio is proportional to the analyte concentration over a wide range of analyte concentrations. The calibration curve in Fig. 1(a) drawn in linear scales is shown in the Supporting Information to demonstrate the direct proportionality of the curve. The curve shown in Fig. 1 (a) is essentially identical to that in the previous report. We would like to reiterate our claim in the previous report\cite{25} that the calibration curve was insensitive to changes in various experimental conditions such as the instrument used and its tuning and the solvent in the sample solution. Also, as was observed for Y5K in CHCA,\cite{25} we expect that the calibration curve constructed using spectral data for samples prepared by premixed-inkjet is identical to that acquired by premixed-pipet. To verify this, we constructed the calibration curves for Y5R in CHCA prepared by premixed-\(\mu\)Spotter-EtOH/H\(_2\)O(80/20) and by premixed-CHIP1000-EtOH/H\(_2\)O (80/20). These curves are shown in Figs. 1(b) and 1(c), respectively. The three calibration curves are nearly identical, indicating that an analyte in a matrix can be quantified using a calibration curve constructed for premixed samples prepared in any way one chooses. We will use the calibration curve acquired by premixed-\(\mu\)Spotter-EtOH/H\(_2\)O(80/20) for the quantification of Y5R in CHCA.

For Y5R in DHB, we acquired the calibration curve using the samples prepared by premixed-\(\mu\)Spotter-EtOH/H\(_2\)O(20/80). We did not use EtOH/H\(_2\)O(80/20) as the solvent in this case because the samples thus prepared were often inhomogeneous. The calibration curve for Y5R in DHB is shown in the Supporting Information. We also constructed the calibration curve for imipramine in DHB using the samples prepared by premixed-\(\mu\)Spotter-EtOH/H\(_2\)O(20/80). This is also shown in the Supporting Information.

**Solvent dependence in the quantification of analytes in samples prepared by stepwise-\(\mu\)Spotter**

In the initial stage of our study of the sample-to-matrix analyte transfer process, we prepared a circular spot of Y5R (200 \(\mu\)m in o.d.) on a metal target using the \(\mu\)Matrix Spotter. Then, a layer of CHCA was prepared on the same spot by \(\mu\)Spotter-EtOH/H\(_2\)O(80/20). Figure 2 shows the MALDI spectrum of the stepwise sample containing 30 fmol of Y5R and 250 pmol of CHCA together with that of the premixed sample with the same overall analyte concentration. It is remarkable to note that the abundance of [Y5R+H]\(^+\) in the spectrum of the stepwise sample is much lower than that of the premixed case. Quantification of Y5R in the stepwise sample using the calibration curve in Fig. 1 resulted in 2 ± 1 fmol of Y5R, which is 7% of the correct overall concentration. When we changed the solvent to 100% EtOH, a similarly erroneous result of 0.9 ± 0.2 fmol of Y5R was obtained. The data indicated that Y5R was not efficiently transferred to the matrix layer when using samples prepared by stepwise-\(\mu\)Spotter-EtOH/H\(_2\)O(80/20), presumably due to a solvent effect (see below) which arises during the transfer of the analyte. We could not repeat the CHCA-MALDI experiment with different solvents because use of a solvent with a water content level higher than 20% clogged the \(\mu\)Matrix Spotter. To avoid this difficulty, we decided to study the phenomenon using DHB as the matrix, which dissolves well in various solvents.

The DHB-MALDI spectra of Y5R samples prepared by various methods are shown in Fig. 3. In all cases, the overall amounts of Y5R and DHB were 30 fmol and 700 pmol,
respectively. Because the abundances of \([\text{DHB} + \text{H}]^+\) in the four spectra are similar, the concentration of Y5R in the matrix layer of each sample will be nearly proportional to the abundance of \([\text{Y5R} + \text{H}]^+\) in each spectrum. Figures 3(a) and 3(b) show the spectra of samples prepared by premixed-\(\mu\)-Spotter-EtOH/H\(_2\)O(80/20), and stepwise-\(\mu\)-Spotter-EtOH/H\(_2\)O(80/20), respectively. Compared to the abundance of \([\text{Y5R} + \text{H}]^+\) in Fig. 3(a), that of the same ion in Fig. 3(b) is much lower. This is the same trend as observed for CHCA. The quantification results for Y5R in the two samples were 33 and 1.5 fmol. Compared to the prepared amount of 30 fmol, the quantified amount of 33 fmol is a reasonable value whereas 1.5 fmol is too low. The results for various amounts of Y5R in DHB prepared by stepwise-\(\mu\)-Spotter-EtOH/H\(_2\)O(80/20) and stepwise-\(\mu\)-Spotter-EtOH/H\(_2\)O(20/80) are listed in Table 1.

Figs. 3(c) and 3(d) show the spectra of samples prepared with relatively more water in the solvent, i.e., premixed-\(\mu\)-Spotter-EtOH/H\(_2\)O(20/80) and stepwise-\(\mu\)-Spotter-EtOH/H\(_2\)O(20/80), respectively. It is to be noted that the matrix ion abundances are similar in the two spectra. In addition, the abundances of \([\text{Y5R} + \text{H}]^+\) in the two spectra are also similar. These indicate similar concentrations of Y5R in the two samples. In fact, the quantification results were 31 and 32 fmol of Y5R, respectively (more comprehensive data is given in the Supporting Information). We made similar measurements for stepwise samples of Y5R prepared with MeOH/H\(_2\)O(80/20), MeOH/H\(_2\)O(20/80), ACN/H\(_2\)O(80/20), and ACN/H\(_2\)O(20/80). These results are listed in the Supporting Information, where they are shown to be similar to those obtained with EtOH/H\(_2\)O as the solvent. We also made similar measurements for the DHB-MALDI of imipramine using the same six combinations of solvents. These results are also listed in the Supporting Information; they are nearly identical to those for Y5R.

To summarize, the sample-to-matrix analyte transfer efficiency was poor when a sample was pretreated by stepwise-\(\mu\)-Spotter-X/H\(_2\)O(80/20) (X = ACN, EtOH, or MeOH), whereas a nearly complete transfer occurred upon a pretreatment by stepwise-\(\mu\)-Spotter-X/H\(_2\)O(20/80). The same held true for CHCA- and DHB-MALDI and for the two analytes we thoroughly tested, i.e., Y5R and imipramine. In short, the water content in the solvent of the matrix solution was responsible for the discrepancies in the quantification results observed in this work.

One may attribute the solvent effect summarized above to various possibilities. First, this solvent effect may manifest if the solubility of an analyte in a solvent decreases significantly when the water content decreases. This explanation is unlikely, however, because the analytes used here dissolve well even in pure EtOH. Second, the analytes may somehow not be easily incorporated into the matrix crystal as the water content of the solvent decreases. This is not likely either, as the DHB-MALDI of the analytes is efficient regardless of the composition of the solvent used in the premixed preparation of the samples.

In addition to the conventional manifestations of the solvent effect mentioned above, another factor which can arise during the sample pretreatment process can affect the efficiency of the analyte transfer. This is the contact time between the sample surface and a droplet of the matrix solution that has been deposited onto the sample surface. As the solvent becomes more polar, the droplet will evaporate much more slowly. Alternatively, as the water content of a solvent increases, the surface tension of a solvent drop increases, which may result in a longer evaporation time for the drop. Then, a longer evaporation time would allow a longer contact time between the sample and the matrix solution. This may result in more efficient analyte extraction. As a rough check of the feasibility of this idea, we loaded 1.0 \(\mu\)L of EtOH/H\(_2\)O(80/20) and EtOH/H\(_2\)O(20/80) onto a metal plate and measured the decrease of their masses as a function of time. Their half-lives under the room condition were ~60 and ~200 s, respectively. The same measurement carried out with 10 \(\mu\)L of the solvents showed half-lives of ~100 and ~1000 s, respectively. Hence, it is clear that it takes noticeably more time for a droplet of EtOH/H\(_2\)O(20/80) to evaporate as compared to that required for a droplet of EtOH/H\(_2\)O(80/20). This provides a partial explanation of the solvent effect encountered here. Another way to increase the sample-solvent contact time is to use a larger liquid jet droplet. Such a test could not be done using the \(\mu\)Matrix Spotter because the volume of the liquid droplets emitted by the device was fixed at 3 \(\mu\)L.

![Figure 3](image_url)

**Figure 3.** Raw MALDI spectra of samples with circular cross section (200 \(\mu\)m o.d.) containing 30 fmol of Y5R in 700 pmol of DHB prepared by (a) premixed-\(\mu\)-Spotter-EtOH/H\(_2\)O(80/20), (b) stepwise-\(\mu\)-Spotter-EtOH/H\(_2\)O(80/20), (c) premixed-\(\mu\)-Spotter-EtOH/H\(_2\)O(20/80), and (d) stepwise-\(\mu\)-Spotter-EtOH/H\(_2\)O(20/80). The TIC of each spectrum was controlled at 1200.

**Table 1.** Quantified amounts of Y5R in 700 pmol of DHB prepared by stepwise-\(\mu\)-Spotter-EtOH/H\(_2\)O

<table>
<thead>
<tr>
<th>Correct amount (fmol)</th>
<th>Quantified amount (fmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EtOH/H(_2)O(80/20)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>3.0</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>10</td>
<td>1.8 ± 0.9</td>
</tr>
<tr>
<td>30</td>
<td>1.3 ± 0.5</td>
</tr>
</tbody>
</table>
Analyte transfer efficiency versus volume of a matrix solution droplet

When using the other inkjet printer, CHIP-1000, we can control the volume of an emitted liquid droplet. The adjustment of the volume of a droplet deposited at a spot was done as follows. We first calibrated the volume of an emitted droplet by UV/VIS spectrophotometry of a solution containing a known amount of CHCA. This value was ~150 pL for EtOH/H₂O(80/20). We then utilized the capability of CHIP-1000 to consecutively emit many droplets at a time. These consecutive droplets arrive at a spot on a sample virtually at the same time. Hence, they can be regarded collectively as one liquid drop with a larger volume, V_d. Because V_d was not proportional to the number of droplets deposited per shot, calibration by UV/VIS spectrophotometry was necessary.

In all of the MALDI experiments carried out to observe the drop-size effect on the transfer efficiency, we first deposited 30 fmol of Y5R onto a circular spot (200 μm o.d.). Then, we deposited a fixed total volume of a matrix solution with a fixed concentration onto the same circular spot. In this step, we varied V_d by varying the number of consecutive liquid droplets. When viewed with a microscope in the apparatus, liquid droplets with V_d ~150 pL deposited on the target disappeared rapidly, whereas those with larger V_d values stayed longer, in agreement with our explanation.

The amounts of Y5R quantified versus V_d in CHCA- and DHB-MALDI are shown in Fig. 4. It should be noted that the quantified amounts increase steadily as V_d increases. Eventually at a V_d value of 600 pL, the quantitation results approach the correct amounts of 30 fmol. The transfer efficiencies observed in CHCA- and DHB-MALDIs tend to change similarly to the change of V_d, providing strong evidence that the observed discrepancies are unrelated to the matrix used.

### Table 2. Quantified amounts of Y5R in 700 pmol of DHB prepared by stepwise-CHIP1000-EtOH/H₂O

<table>
<thead>
<tr>
<th>Correct amount (fmol)</th>
<th>Droplet volume 150 pL</th>
<th>Droplet volume 900 pL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>3.0</td>
<td>3.4 ± 1.0</td>
<td>3.7 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>5.4 ± 1.6</td>
<td>9.0 ± 2.3</td>
</tr>
<tr>
<td>30</td>
<td>6.1 ± 1.0</td>
<td>32 ± 7.9</td>
</tr>
</tbody>
</table>

Profilng of Y5R by DHB-MALDI

We acquired quantification results for samples containing 1–30 fmol of Y5R prepared by various methods. Among these data, those acquired by stepwise-μSpotter-EtOH/H₂O (80/20) and by stepwise-μSpotter-EtOH/H₂O(20/80) are shown in Table 1. In addition, two sets of data acquired by stepwise-CHIP1000-EtOH/H₂O(80/20), one produced with a V_d value of 150 pL and the other with that of 900 pL, are shown in Table 2. Similar data acquired for samples prepared using water/methanol and water/acetonitrile as solvents are listed in the Supporting Information.

In Fig. 5, these data are presented in the form of color-coded profile maps. Also shown in the figure is a profile plot of the correct amounts, i.e., the results for samples prepared by premixed-μSpotter-EtOH/H₂O(20/80). The figure clearly demonstrates that the water content of the solvent used to extract an analyte(s) from a sample can critically affect the quantitativeness of the analyte.

![Figure 4](image-url)

**Figure 4.** The quantified amounts of Y5R vs. the droplet volume (V_d) in CHCA- and DHB-MALDI of samples prepared by stepwise-CHIP1000-EtOH/H₂O(80/20). CHCA- and DHB-MALDI data are drawn with filled (●) and open (○) circles, respectively. The prepared amount of Y5R was 30 fmol either in 250 pmol of CHCA or in 700 pmol of DHB, which is drawn as a horizontal line.

![Figure 5](image-url)

**Figure 5.** Columns (a), (b), (c), and (d) show the color-coded profile maps for the samples of Y5R in DHB prepared by stepwise-μSpotter-EtOH/H₂O(80/20), stepwise-μSpotter-EtOH/H₂O(20/80), stepwise-CHIP1000-EtOH/H₂O(80/20) with V_d of 150 pL, and stepwise-CHIP1000-EtOH/H₂O(80/20) with V_d of 900 pL, respectively. The prepared (‘correct’) profile is shown in column (e). The scale for the amount of Y5R, in number of fmol in 700 pmol of DHB, is drawn in column (f).
profile. It also shows that the above problem can sometimes be avoided by increasing the volume of the matrix solution droplet.

CONCLUSIONS

Two of the requirements for the quantitative profiling of an analyte in a sample by MALDI are a quantitative sample-to-matrix analyte transfer and the capability of quantifying an analyte incorporated inside a solid matrix. Recently, we developed a method which facilitates the second requirement.\cite{9}

Concerning the first requirement, we found in this work that the use of a matrix solution with a low water content level resulted in a poor sample-to-matrix analyte transfer. We could avoid this negative solvent effect with a more polar solvent or by increasing the size of the matrix solution droplet deposited by a printer. Based on these observations, we suggest that the aforementioned solvent effect arises because the evaporation time of a liquid droplet decreases as the solvent becomes less polar.

Even when the two requirements for quantitative profiling are met, a profiling result still may not be quantitative if the analyte transfer from the bulk of a sample to its surface, and eventually to the matrix layer, does not occur efficiently. This can be particularly troublesome during the profiling of analytes in biological samples. A thorough investigation of this process is therefore needed.

Acknowledgements

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REFERENCES


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