Video Article

Preparation of Homogeneous MALDI Samples for Quantitative Applications

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URL: http://www.jove.com/video/54409
DOI: doi:10.3791/54409

Keywords: Biochemistry, Issue 116, MALDI, Imaging Mass Spectrometry, Spatial Homogeneity, Quantitative Analysis, Hydrodynamic Flows, Dried-droplet Method, Substrate Temperature

Date Published: 10/28/2016

Citation: Ou, Y.M., Tsao, C.W., Lai, Y.H., Lee, H., Chang, H.T., Wang, Y.S. Preparation of Homogeneous MALDI Samples for Quantitative Applications. *J. Vis. Exp.* (116), e54409, doi:10.3791/54409 (2016).

Abstract

This protocol demonstrates a simple sample preparation to reduce spatial heterogeneity in ion signals during matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. The heterogeneity of ion signals is a severe problem in MALDI, which results in poor data reproducibility and makes MALDI unsuitable for quantitative analysis. By regulating sample plate temperature during sample preparation, thermal-induced hydrodynamic flows inside droplets of sample solution are able to reduce the heterogeneity problem. A room-temperature sample preparation chamber equipped with a temperature-regulated copper base block that holds MALDI sample plates facilitates precise control of the sample drying condition. After drying of sample droplets, the temperature of sample plates is returned to room temperature and removed from the chamber for subsequent mass spectrometric analysis. The areas of samples are examined with MALDI-imaging mass spectrometry to obtain the spatial distribution of all components in the sample. In comparison with the conventional dried-droplet method that prepares samples under ambient conditions without temperature control, the samples prepared with the method demonstrated herein show significantly better spatial distribution and signal intensity. According to observations using carbohydrate and peptide samples, decreasing substrate temperature while maintaining the surroundings at ambient temperature during the drying process can effectively reduce the heterogeneity of ion signals. This method is generally applicable to various combinations of samples and matrices.

Video Link

The video component of this article can be found at http://www.jove.com/video/54409/

Introduction

Mass spectrometry (MS) is one of the most important analytical techniques for analyzing the molecular compositions of complex samples. Among all the ionization methods used in MS, matrix-assisted laser desorption/ionization (MALDI) is the most sensitive and widely used method in bioanalytical applications.[1] In comparison to other ionization techniques, MALDI has the highest sensitivity and high tolerance to salt contaminants. Such analytical properties make MALDI the first choice for carbohydrate analysis and many proteomics applications. However, sample preparation is a crucial step for obtaining high quality data in MALDI-MS.

The most commonly used sample preparation method for MALDI-MS is the dried-droplet method, in which sample droplets are deposited on a surface and dried under ambient conditions. This drying method is simple and generally effective.[2-5] However, a common problem in the dried-droplet method is that the resultant analyte/matrix crystals normally distribute irregularly. In many cases, the crystals aggregate at the periphery of sample areas, resulting in the so-called ring-stain formation.[6-8] The heterogeneous crystal morphologies affect the spatial distribution of analyte molecules, which results in severe fluctuation in ion signal over sample areas. Such severe signal fluctuations and poor data reproducibility are known as the "sweet spot" problem in MALDI-MS.[9] Thus, there is a great need for reducing spatial heterogeneities in MALDI-MS dried droplet applications.

Hydrodynamic flows in the sample droplet play an important role in determining the spatial distribution of samples prepared with the dried-droplet method.[10-12] It was found that the evaporation of solvent induces outward capillary flows within droplets, which are responsible for the ring-stain formation.[7,10] In contrast, recirculation flows induced by tangential surface-tension gradients may counterbalance the outward capillary flows.[13] If the recirculation flow speeds are higher than that of the outward capillary flows, the samples can be efficiently redistributed to reduce the heterogeneity problem.[14]

In this work, we demonstrate a detailed protocol for preparing samples with a simple drying chamber to induce efficient recirculation flows during droplet drying processes. Droplet drying conditions are precisely controlled, including the temperatures of the sample plate and its surroundings, and the relative humidity within the chamber. The model analytes include maltotriose and bradykinin chain (1-7). The matrix used for the demonstration is 2,4,6-trihydroxyacetophenone (THAP). The samples are examined with time-of-flight (TOF) MS, and the data are analyzed quantitatively to show the reduction of heterogeneity.

Protocol

NOTE: This protocol is developed for reducing the spatial heterogeneity of maltotriose and bradykinin fragment (1-7) prepared with the dried-droplet method. The protocol consists of three main steps, including preparation and preconditioning, sample deposition and drying, and mass spectrometry data analysis. The procedures are outlined and described in more detail below:

1. Preparation and Preconditioning

1. Cleaning the Sample Plate
    1. Wear nitrile gloves and hand-wash the sample plate gently with detergent and distilled-deionized water (DDW).
    2. Rinse the sample plate with methanol (MeOH) and DDW.
    3. Insert the sample plate in a 600 ml beaker and fill with DDW.
    4. Sonicate the sample plate in DDW for 15 min in an ultrasonic bath (200 W, 40 kHz).
    5. Remove DDW from the beaker and fill the beaker with MeOH.
    6. Sonicate the sample plate in MeOH for 15 min in the ultrasonic bath (200 W, 40 kHz).
    7. Blow off the solvent drops on the plate with nitrogen gas and keep the sample plate dry before sample deposition.

2. Regulating Drying Chamber Temperature
    NOTE: The drying chamber is a 35 x 20 x 45 cm^3 (W x D x H) acrylic chamber. **Figure 1** shows the picture of this drying system. The chamber is purged with room temperature nitrogen gas through a gas flowmeter at a constant flow rate to maintain a low relative humidity condition monitored by a calibrated hygrometer installed inside the drying chamber. A copper base block in the drying chamber equipped with a programmed constant temperature water circulator is used to accommodate stainless steel sample plates. The copper base block is able to regulate the sample plate temperature from 5 to 25 °C. The temperatures of air, copper base block, and the sample plate are monitored by K-type thermocouples.
    1. Open the door and rapidly put the sample plate on the copper base block then close the door.
    2. Manually adjust the gas flowmeter to set the nitrogen flow rate to 10 standard cubic feet per hour (SCFH).
    3. Monitor the relative humidity in the drying chamber by the hygrometer and fine tune the gas flowmeter to ensure the relative humidity is always below 25%.
    4. Monitor the temperature of the sample plate by K-type thermocouples and adjust the water circulator temperature manually until the sample plate reaches 5 °C for experiment or room temperature (25 °C) for control.
        NOTE: In order to stabilize the sample plate at a designed temperature, the water circulator temperature is typically set 0 to 5 °C lower than the designed sample. For example, to maintain 5 °C at the sample plate, the temperature setting of the water circulator is in the range of 0 to 2 °C; to maintain the sample plate at 25 °C, the temperature setting of the water circulator is in the range of 23 to 25 °C.
    5. Ensure the required temperatures and the relative humidity are reached (**Table 1**) before sample deposition.
        NOTE: All parameters as well as their setting values for the drying processes with different sample plate temperatures are shown in **Table 1**.
        NOTE: At a low sample plate temperature, water condensation on the sample plate may occur if the chamber door is open for a long time. If water condensation occurs, close the door and **DO NOT** deposit any sample on it until water condensation is dried out.

3. Preparation of Matrix and Analyte Solutions
    1. Preparation of matrix solutions
        1. Prepare 0.1 M THAP solution with 50% acetonitrile (ACN): 50% DDW aqueous solution.
    2. Preparation of analytes
        1. Prepare 10^{-4} M maltotriose solution with DDW.
        2. Prepare 10^{-5} M bradykinin fragment (1-7) solution in 50% acetonitrile (ACN) : 50% DDW aqueous solution.

2. Sample Deposition and Drying

1. Premix 0.25 µl of 0.1 M THAP solution and 0.25 µl of 10^{-4} M maltotriose or 10^{-5} M bradykinin fragment (1-7) solutions in a microcentrifuge tube.
2. Vortex the mixed solution for 3 sec.
3. Centrifuge the mixed solution for 2 sec (2,000 x g) to collect the solution at the bottom of the centrifuge tube.
4. Open the door of the drying chamber, carefully deposit 0.1 µl of the solution on the sample plate with pipette and close the door immediately.
5. Wait for the sample droplet to dry out.
    NOTE: The typically observed drying times with different sample plate temperatures are listed in **Table 1**. For sample plate temperature of 5 °C, the average drying time is 800 to 1,000 sec; for sample plate temperature of 25 °C, the average drying time is 100 to 150 sec.
6. After drying, open the door of the drying chamber.
7. Set the water circulator temperature to room temperature (25 °C).
    NOTE: Skip this step if the sample plate is kept constantly at room temperature (25 °C) during the drying process.
8. After the sample plate temperature returns to room temperature (25 °C), remove the sample plate from the drying chamber.
9. Examine the sample morphology under a 5X stereomicroscope and take a snapshot bright-field image.
    NOTE: If the crystal morphologies are not as expected, it is necessary to prepare a new sample with the same procedure. Typical crystal morphologies are shown in the upper panels of **Figure 2**.

NOTE: In the cases with low sample plate temperatures, such as 5 °C, it is important to warm up the sample plate to room temperature before taking it out of the drying chamber. When depositing the samples, **DO NOT** keep the premixed solution in the tip of the pipette over 10 sec. **DO NOT** use the premixed solution again after depositing the samples. The upper panels of **Figure 2** show bright-field images of samples prepared with different sample plate temperatures.

3. Mass Spectrometry Data Analysis

1. Mass Spectrometry Data Acquisition
   NOTE: After preparation, the sample can be analyzed using imaging mass spectrometry. In the current study, the imaging MS experiments are conducted using a laboratory-built synchronized dual-polarity TOF (DP-TOF) imaging mass spectrometer.[15] Commercial MALDI-TOF mass spectrometers with imaging capability are also suitable for such experiments. The mass spectrometer is operated in linear extraction and positive ion modes with optimized extraction delays. The kinetic energy of ions is 20 kV. The laser beam size is 35 μm in diameter on the sample surface, and the spectrum of every spot is the average of 5 laser shots.
   1. Insert the sample plate into the MALDI mass spectrometer.
   2. Perform imaging mass spectrometry analysis to the sample prepared in steps 2.1-2.9.
   3. Select a characteristic mass peak from the mass list shown in the result window and click "2D" to plot a two-dimensional ion image.
      NOTE: For maltotriose mixed with THAP, the characteristic peaks are sodiated maltotriose, protonated THAP, and sodiated THAP. For bradykinin fragment (1-7) mixed with THAP, the characteristic peaks include protonated bradykinin fragment (1-7), protonated THAP, and sodiated THAP.
   4. Click the adjustment buttons in the popup window to determine the upper and lower limits of the signal intensity and click "save a picture". This setting defines the contrast of ion images.
      NOTE: In every individual set of data, the cracked regions and the null spots showing low brightness are eliminated.
   5. Observe and compare the ion image with the bright-field image that was taken at step 2.9.
      NOTE: Imaging mass spectrometry and construction of images of particular ions can be achieved with commercial instruments. Due to the variety of data acquisition and analysis software, the users should follow software instructions provided by the instrument vendor to get high quality images.

2. Data Analysis
   NOTE: The heterogeneity of samples is analyzed quantitatively. In this demonstration, every sample is divided into multiple concentric areas by software developed in-house to analyze the spatial distribution of the ions. The analysis can also be performed using stand-alone data analysis software.
   1. Click the null spots and the cracked regions in the ion image shown in the result window to remove unimportant areas.
      NOTE: This procedure defines the essential area of ion image.
   2. Click "find edge" button to find the outmost layer of the ion image.
   3. Click "deduct" to save the ion abundance information of the outmost layer in a database and remove this layer from the ion image simultaneously. A check box representing this outmost layer will appear in the "output data" list of the result window.
   4. Repeat steps 3.2.2 and 3.2.3 until the center of the ion image is defined.
   5. Click and select all the check boxes in the "output data" list and click "export" to export the data.
   6. Open the exported data using spreadsheet software to calculate the average ion abundance of every layer to obtain the spatial distribution information of ions.

Representative Results

The bright-field images as well as the MS images of maltotriose and bradykinin fragment (1-7) prepared with sample plate temperature of 5 and 25 °C are shown in **Figure 1**. In the case of sodiated maltotriose, the ion signal mainly populates at the periphery of the sample area when it is prepared with a sample plate temperature of 25 °C. By decreasing the sample plate temperature to 5 °C, the signal populates homogeneously over the entire sample area. The only noticeable downside when preparing samples under 5 °C is that there are more cracks than the samples prepared under 25 °C. The ion image of protonated bradykinin fragment (1-7) shows a similar trend as those of sodiated maltotriose. The results of imaging MS suggest that preparing samples under a lower sample plate temperature can significantly redistribute the molecules and reduce heterogeneity.

Based on the experimental results, statistical analyses show that the heterogeneity of ion signals obtained under a sample plate temperature of 5 °C reduces by 60-80% with respect to that under 25 °C. **Figure 3** shows the results of statistical analyses for maltotriose and bradykinin fragment (1-7) prepared under sample plate temperatures of 5 and 25 °C. For each sample, the average intensity is normalized. In the case of sodiated maltotriose with a sample plate temperature of 25 °C, the signal intensities at the centers are much lower than those with the sample plate temperature of 5 °C. The result of protonated bradykinin fragment (1-7) also shows less variation when decreasing the sample plate temperature from 25 to 5 °C.

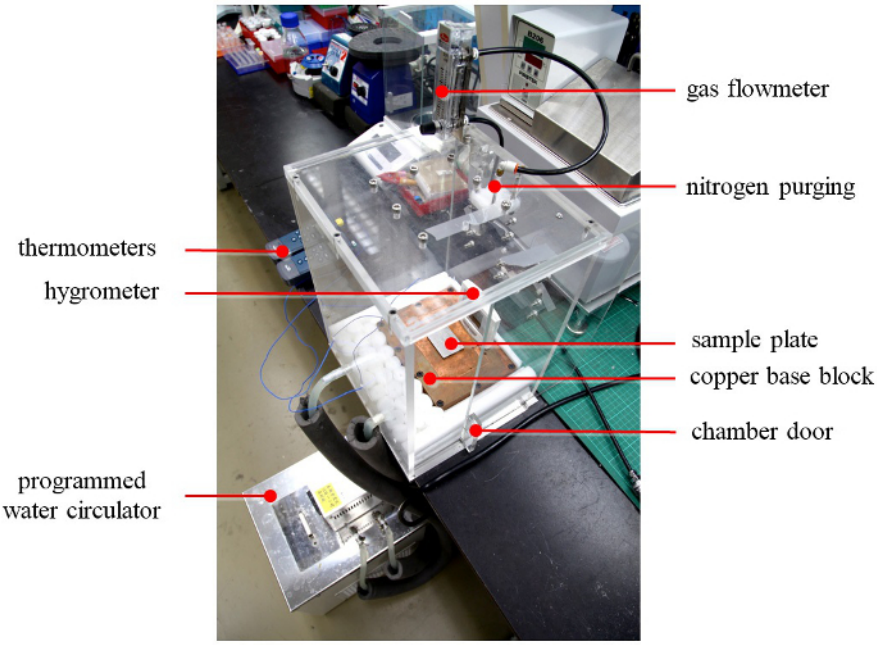

Figure 1: Picture of the sample drying system. The drying chamber is made of acrylic. The chamber is purged with room temperature nitrogen gas to maintain a low relative humidity condition. A copper base block equipped with a programmed constant temperature water circulator is used to regulate the temperature of stainless steel sample plates. The thermometers monitor air, copper base block, and the sample plate. Please click here to view a larger version of this figure.

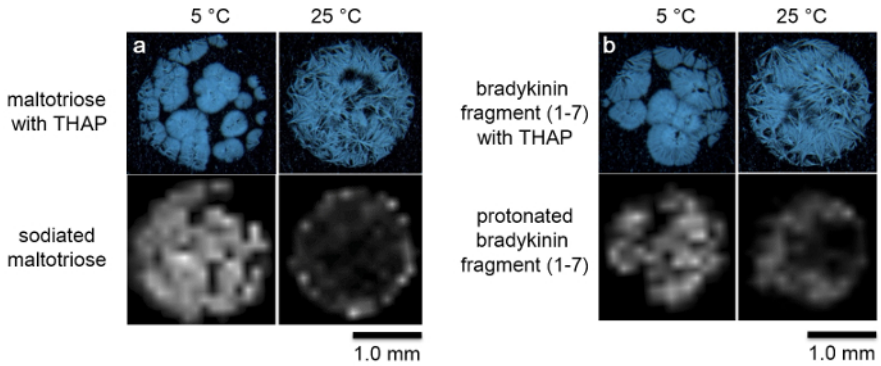

Figure 2: Lowering sample plate temperature results in better signal homogeneity. The bright field images (upper images) as well as the MALDI images (lower images) of maltotriose (**a**) and bradykinin fragment (1-7) (**b**) prepared with THAP under different sample plate temperatures. The MALDI images were obtained by extracting the sodiated maltotriose (m/z: 527) and protonated bradykinin fragment (1-7) (m/z: 757) from the total spectrum, respectively. The pixel size of the ion images is 35 µm. Please click here to view a larger version of this figure.

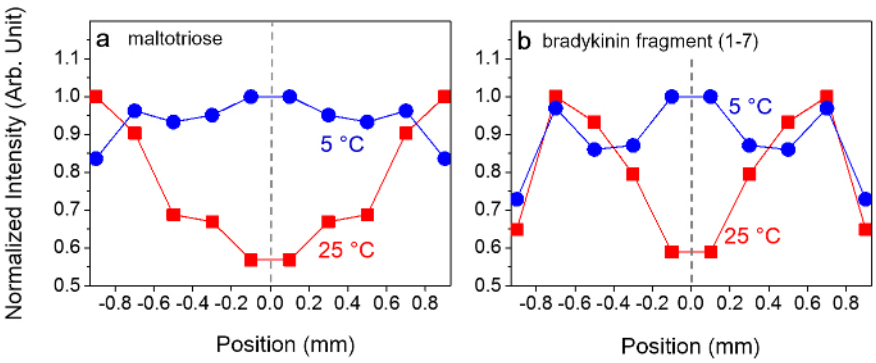

Figure 3: Signal variation reduces as sample plate temperature decreases during drying process. The MALDI images are obtained with maltotriose (**a**) and bradykinin fragment (1-7) (**b**) prepared with THAP under different sample plate temperatures. Red and blue data indicate the sample prepared at the sample plate temperatures of 25 and 5 °C, respectively. Please click here to view a larger version of this figure.

Sample Plate Temperature (°C)	Sample	Air Temperature (°C)	Relative Humidity (RH %)	Drying Time (sec)
5	maltotriose with THAP	20 ± 3	<25	800 - 1,000
	bradykinin fragment (1-7) with THAP			
25	maltotriose with THAP	25 ± 3		100 - 150
	bradykinin fragment (1-7) with THAP			

Table 1: Experimental parameters and drying conditions under different sample plate temperatures.

Discussion

Based on previous theoretical predictions, temperature-induced hydrodynamic flows within droplets can overcome outward capillary flows induced by solvent evaporation. The efficiency of such internal recirculation of molecules is enhanced when the temperature gradients within a droplet increase. According to the predicted results, when keeping the sample plate temperature under 5 °C while maintaining its surroundings at ambient temperature, the average velocity of recirculation flows within the droplet is about 4 times faster than that of the outward capillary flows. If the sample plate temperature is the same as the surroundings, the average velocity of recirculation flows is 1,800 times slower than the outward capillary flow. The results of this calculation indicate that decreasing sample plate temperature during sample preparation is advantageous. The experimental observations agree with this prediction.

The sample plate temperature should be precisely controlled throughout the sample preparation process. **Table 1** shows the typical droplet drying time with 0.1 µl of sample under different sample plate temperatures. Before depositing sample solution on the plate, it is important to ensure that the sample plate surface is dry. If water condensation occurs when preparing samples under low temperatures, deposition of the sample solution is not recommended because condensed water enlarges sample areas and dilutes solutions. Thus, it is important to keep the relative humidity of the drying chamber below 25%. In addition, when preparing samples under low temperatures, the sample plate should be warm up to room temperature before taking it out of the drying chamber. Although minor water condensation after completion of sample crystallization does not alter the sample population, significant condensation should be avoided.

The use of freshly premixed solutions is recommended. Once the premixed solutions are exposed to air, pre-crystallizations of the sample solutions occur and the final crystal size and morphology may change. Therefore, the pipetting procedure should be performed with reasonable efficiency, typically within 10 seconds, to prevent the sample droplet from pre-crystallization within the pipette tip. It is recommended to observe sample morphologies under a microscope to ensure suitable crystal morphologies are produced before mass spectrometry analysis. If the crystal morphologies are not as good as expected, repeating the deposition process as necessary.

According to our theoretical and experimental studies, preparing samples with a low temperature sample plate installed under ambient conditions greatly improves the data reproducibility and quality in MALDI-MS. Subsequent experiments also show significant enhancement of signal intensity with this sample preparation method. The experimental data obtained by this method considerably improve the reliability of MALDI mass spectra for quantitative analyses. In comparison with other methods involving solution composition or surface property changes,[8,16-18] changing drying condition is simpler and more generally applicable for conventional samples. Thus, most mass spectrometry users can benefit from it in regular applications.

Improving MALDI signal homogeneity with decreasing sample plate temperature is also effective for some other popular matrices. For example, improved α-cyclodextrin (α-CD) signal homogeneity with THAP and α-cyano-4-hydroxycinnamic acid (CHCA) as the matrix under low-temperature sample drying conditions has been reported recently.[14] The disadvantage with changing sample plate temperature is that the method is currently unsuitable for high-throughput analysis due to the long sample drying time in low-temperature conditions.

Disclosures

The authors declare no competing financial interest.

Acknowledgements

This work is supported by the Genomics Research Center, Academia Sinica and the Ministry of Science and Technology of Taiwan, the Republic of China (Contract No. 104-2119-M-001-014).

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