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APPLICATION NOTE

Matrix Recrystallization for MALDI-MS Imaging of Maize Lipids at High-Spatial Resolution

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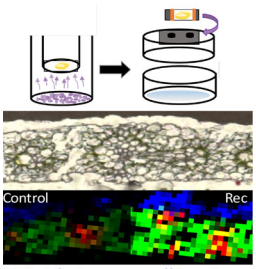

Lipid Recrystallization

Abstract. Matrix recrystallization is optimized and applied to improve lipid ion signals in maize embryos and leaves. A systematic study was performed varying solvent and incubation time. During this study, unexpected side reactions were found when methanol was used as a recrystallization solvent, resulting in the formation of a methyl ester of phosphatidic acid. Using an optimum recrystallization condition with isopropanol, there is no apparent delocalization demonstrated with a transmission electron microscopy (TEM) pattern and maize leaf images obtained at 10 μm spatial resolution.

Keywords: Mass spectrometry imaging, MALDI, Matrix, Recrystallization, Maize, Lipids, High spatial resolution

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Introduction

The spatial resolution of matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) has become routinely available at the size of 20 μm and has been demonstrated with a pixel size as small as 2.5 μm [1]. A critical challenge in high-spatial resolution MSI is the limited number of analytes available in a small sampling size. Hence, sample preparation should be carefully selected to enhance ion signals but not to induce delocalization. The vapor phase sublimation of organic matrix produces very small crystal sizes of around 1 μm or smaller and is most commonly used for high spatial resolution MALDI-MSI [2]. As a dry method, it does not induce analyte migration, but often shows poor sensitivity due to a lack of analyte incorporation into the matrix crystal [3].

Yang and Caprioli demonstrated that a recrystallization step added after sublimation can dramatically increase the sensitivity of proteins while keeping high spatial resolution at 10 μm [4]. Since then, the recrystallization method has been adopted for many applications [5–10]. Incubation with solvent vapor is most commonly used, but manual or automatic solvent spray is also used [7, 8]. Many of these applications, however, have been performed without testing potential delocalization during recrystallization. In addition, there has been no report for the application to plant tissues, which are fragile and quite different from animal tissues, mostly due to the cell walls and different lipid composition.

In the current study, we adopted and optimized the recrystallization method by Yang and Caprioli [4] to increase lipid signals in maize embryos and leaves while ensuring no apparent delocalization at high spatial resolution. Side reactions that may occur during recrystallization is a major concern, but has not been reported. Here, we also report unexpected side reactions between phosphatidic acid and methanol vapor.

Experimental

Materials

Isopropyl alcohol (IPA), methanol, water, chloroform, and acetone were purchased from Sigma-Aldrich (St. Louis, MO, USA) in CHROMASOLV LC-MS or Plus grade. Acetic acid was purchased from Fisher Scientific (Waltham, MA, USA) in glacial certified ACS Plus grade. 1,5-Diaminonaphthalene (DAN, 97%) was purchased from Sigma-Aldrich. Gelatin from porcine skin (300 bloom) was purchased from Electron Microscopy Sciences (Hatfield, PA, USA). Seeds (Zea mays, L., inbreed B73) were obtained from Dr. Marna Yandeau-Nelson at Iowa State University.

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Plant Growth and Harvest

Procedures for corn seed germination and plant growth are described in detail elsewhere [11, 12], and briefly described below. A maize seed was imbibed for 10 min and placed with the embryo side facing down on top of moist filter paper in a Petri dish that was placed in a climate-controlled greenhouse. After 28 h of germination, the seed was cut longitudinally, flash-frozen, and then placed in a cryo-mold with gelatin (10% w/v solution). For maize leaf imaging, maize seeds were planted in soil and grown in a greenhouse. Plant seedlings were harvested 11 d after planting and sections of leaves were collected at the midpoint. The fresh maize leaf section was embedded in gelatin before cryosectioning.

Sample Preparation

The molds were transferred to a cryostat (CM1850; Leica Microsystems, Buffalo Grove, IL, USA) pre-chilled to −20 °C, allowed to thermally equilibrate for 30 min, and sectioned at 10 µm thickness. The cryosectioned tissues were collected with Cryo-Jane tape (Leica Microsystems, Buffalo Grove, IL, USA), and attached to a cold microscope glass slide. The glass slides with sections were vacuum dried and DAN matrix was deposited via sublimation [2] at 140 °C for 4 min.

Recrystallization

A recrystallization chamber was made of a filter paper, a glass Petri dish, a metal plate, and a magnet [4]. The sublimated slide was adhered to a stainless steel plate using copper tape, and attached to the underside of the top part of the Petri dish by placing magnets on the exterior of the Petri dish. A piece of filter paper was placed in the bottom part of the Petri dish with 1 mL of a solution. The Petri dish was assembled to form a hydration chamber, sealed using tape, and placed in an incubator for various lengths of time.

Mass Spectrometry Analysis

MS imaging data was collected using a MALDI-linear ion trap (LIT)-Orbitrap mass spectrometer (MALDI-LTQ-Orbitrap Discovery; Thermo Scientific, San Jose, CA, USA). The instrument was modified to use an external 355 nm Nd:YAG laser (UVFQ; Elforlight Ltd., Daventry, UK) and a custom optical set-up [12]. TunePlus and Xcalibur software (Thermo Scientific) were used to define imaging acquisition parameters and for data acquisition. The laser energy used was 83%–85% at 60 Hz repetition rate. Maize seed and leaf images were acquired using 150 and 10 µm raster step size with a laser spot size of ~25 and ~9 µm, respectively. The laser spot size was adjusted using a beam expander. Mass spectra were acquired in negative mode using Orbitrap for *m/z* scan range of 350–1000 with a resolving power of 30,000 at *m/z* 400.

MS/MS spectra were acquired with Multiplex MSI [11] where each pixel (raster step) was divided into four spiral steps with a raster step size of 100 µm and a spiral step size of 50 µm. The first spiral step was collected with a full Orbitrap mass spectrum for *m/z* 350–1000. Data-dependent ion trap MS/MS spectra were collected in the other three steps for the three most abundant ions from the preloaded precursor mass list. An isolation window of ±1.0 Da and collision energy of 35% was used. Dynamic exclusion was used with a repeat count of 2, a repeat duration of 30 s, and an exclusion duration of 180 s.

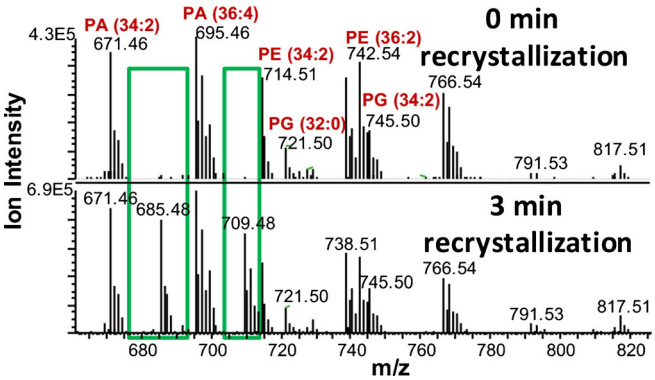

Figure 1. Mass spectra of maize embryo section after 0 and 3 min of recrystallization with 5% methanol. Green boxes indicate the newly appeared peaks after recrystallization

Observed and imaged peaks are all deprotonated, $[M-H]^-$. Peak assignments were made based on accurate mass values and MS/MS. MS images were generated using ImageQuest (Thermo) with a mass window of ±0.003 Da and without normalization to total ion count.

Results and Discussions

Matrix Recrystallization Optimization

Cross sections of ~28 h germinated maize embryos were used to optimize recrystallization, which was then applied to maize leaf

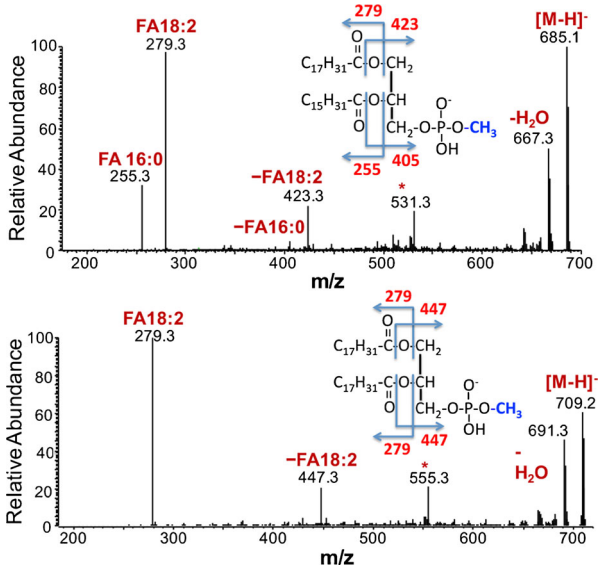

Figure 2. MS/MS spectra of *m/z* 685.481 (top) and *m/z* 709.481 (bottom) shown in Figure 1. * represents fragment from precursor contaminant

cross sections at 10 μm resolution. This study was confined to negative ion mode with DAN as a matrix because many small metabolites are readily ionized in negative ion mode, as well as several lipids of our interest as previously published [13].

First, recrystallization solvents were varied at a fixed temperature and time (39 °C and 1.5 min). Yang and Caprioli obtained optimal recrystallization for MSI of proteins using a small amount of organic solvent in water. In addition to 5% methanol and 5% acetic acid they used, a few more organic solvents in water were tested (5% IPA, 5% acetone, 5% chloroform). As shown in Supplementary Figure S1, four solvents in water (5% acetone, 5% IPA, 5% methanol, and 5% acetic acid) increased phospholipid ion signals of phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphadylinositols (PI), and phosphatidylglycerols (PG), as well as a sulfolipid, sulfoquinovosyl diacylglycerol (SQDG).

Further optimization was made for the four solvents by varying incubation time at a temperature slightly lower than the boiling point (i.e., 39 °C, 55 °C, 39 °C, and 80 °C for 5% acetone, 5% IPA, 5% methanol, and 5% acetic acid, respectively). As shown in Supplementary Figure S2, optimum incubation time was determined as 1.5 min, 2 min, 3–4 min, and 2–3 min, respectively. Ion images were also improved as shown in Supplementary Figure S3. Overall performance was similar at the optimum conditions, but 5% IPA gave a slightly better result in terms of signal improvement.

In a close look at the mass spectra obtained with 5% methanol, two new peaks (*m/z* 685.481 and 709.481) appeared after recrystallization (Figure 1). No such peaks were found with other solvents. The two compounds were initially assigned as odd number fatty acids, PA 35:2 and PA 37:4, respectively, based on accurate mass search against Metlin database (metlin.scripps.edu). Odd number fatty acids are rare in plants and it is very unlikely recrystallization would induce dramatic signal enhancement only for PAs with odd number fatty acids. MS/MS experiments were performed (Figure 2), which identified these peaks as a methyl phosphoester of PA produced during recrystallization. In addition, the isotope patterns

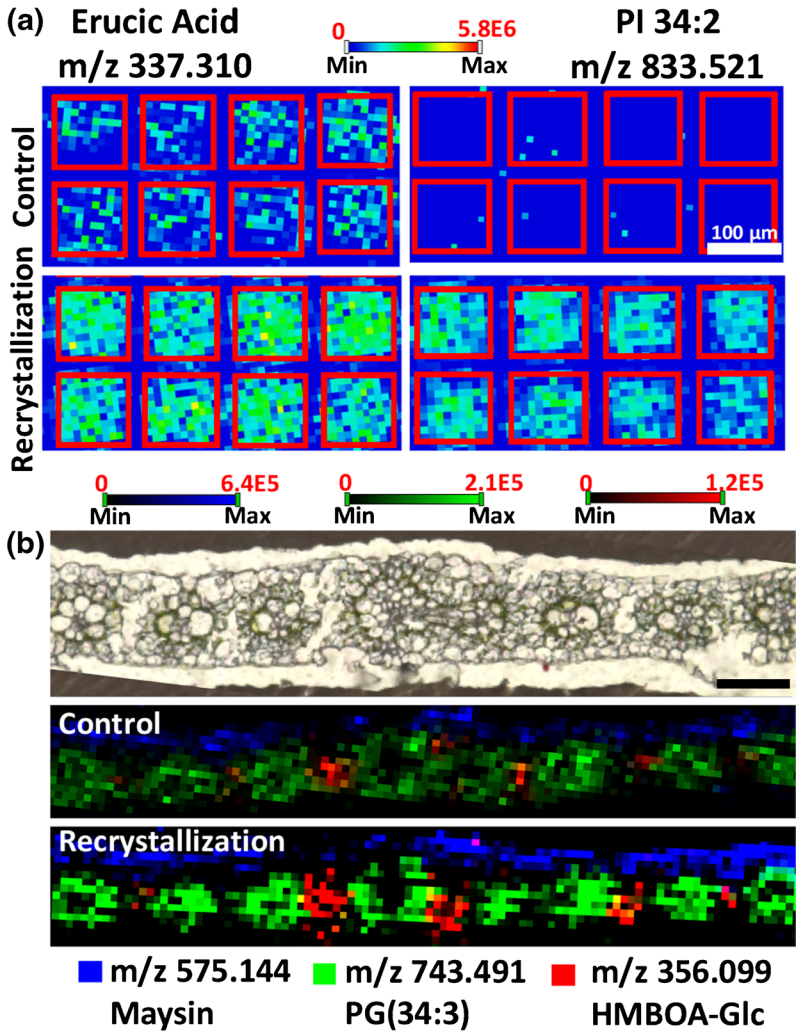

Figure 3. **(a)** MS images of erucic acid and PI 34:2 on a TEM grid pattern without (top) and with (bottom) recrystallization. **(b)** Optical image (top) and MS images for selected lipids (middle and bottom) of a cross section of maize leaf without and with recrystallization. All MS images were obtained at 10 μm spatial resolution. HMBOA-Glc: 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside

of *m/z* 685.48 and *m/z* 709.48 exactly match those of *m/z* 671.46 and *m/z* 695.46 further supporting their assignment as methylated PA.

Recrystallization with 5% IPA at 55 °C for 2 min was selected as the most optimal condition and used for high-resolution imaging below. Unlike methanol, phospho-esterification of PA did not occur with IPA. The use of 5% acetone or 5% acetic acid was not further considered because of potential delocalization or side reactions for small metabolite molecules.

High-Resolution MALDI-MS Imaging

A copper transmission electron microscopy (TEM) grid was used to make fine analyte patterns to test analyte delocalization during recrystallization. Two TEM grids were secured on a glass slide and a solution containing soy lipid extracts and erucic acid (22:1 fatty acid) were sprayed on top of the grids (see Supplementary Figure S4 for the workflow). After the removal of the grids, the slides were sublimated with DAN, recrystallized with 5% IPA at the optimum condition, and MS imaging data were acquired with 10 μm spatial resolution. Figure 3a shows MS images of erucic acid and PI 34:2. The ion signals are very low before recrystallization, but after recrystallization there are significant signal improvements with almost no signal outside the grid pattern.

Finally, MS imaging was performed for cross sections of maize leaf with 10 μm spatial resolution. Representative ion images are shown in Figure 3b for maysin, PG 34:3, and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one glucoside (HMBOA-Glc) that have distinct localizations. After recrystallization, the ion signals were improved by ~three times, thus improving the image quality, but there were no apparent changes in their localizations. It should be noted that ion signals are actually decreased for some molecules, especially caffeic and ferulic acids, as shown in Supplementary Figure S5.

Conclusions

Spatial resolution in MALDI-MSI is limited by four major factors: (1) delocalization during sample preparation, (2) matrix homogeneity, (3) laser spot size, and (4) sensitivity. Sensitivity has become a critical factor recently, thanks to many advances in sample preparation and instrumentation. This work demonstrates recrystallization can be performed to improve lipid ion signals on plant tissues while producing minimal or no analyte delocalization appropriate for high spatial resolution MALDI-MSI. The effect of matrix recrystallization on very small molecules (<500 Da) shows mixed results, suggesting caution needs to be taken for these molecules. Most importantly, recrystallization data should be carefully inspected for potential side reactions, as we have demonstrated the occurrence of phosphoester formation between methanol vapor and PA.

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