Efficient Hydrogen-Deuterium Exchange in Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry Imaging for Confident Metabolite Identification

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Abstract

Highly efficient hydrogen deuterium exchange (HDX) is developed for mass spectrometry imaging (MSI) with low vacuum matrix-assisted laser desorption ionization (MALDI). HDX efficiency of 73-85% is achieved by introducing D_2O vapor into a heated MALDI source in combination with deuterium-labeled matrix, which allows correct determination of the number of possible H/D exchanges for up to 17 labile hydrogens. This provides valuable orthogonal information to supplement *m/z*, allowing for increased confidence in metabolite identification while retaining the spatial information MSI supplies. When combined with high-throughput METASPACE annotation, this approach can systematically improve untargeted metabolite annotations in MALDI-MS imaging. The developed method was applied to MALDI-MS imaging of the top surface, bottom surface and the middle section of *Lemna minor* fronds. Out of a total of 56 on-sample annotations made with the BraChem database using a 10% false discovery rate, 31 of these annotations (55%) matched our HDX data, providing additional confidence. For the remaining 45%, our data allowed us to narrow down structural possibilities and eliminate incorrect structures, greatly increasing confidence in metabolite identifications.

Introduction

Matrix-assisted laser desorption ionization mass spectrometry imaging (MALDI-MSI) is a well-established analysis technique that provides spatial localization information within tissues[1,2]. When combined with high-accuracy *m/z* data it is a powerful tool for detection and analysis of metabolites in biological samples with up to single cell resolution. However, MSI is incompatible with chromatography, making it difficult to identify compounds among structural isomers. MS/MS is commonly used to determine the correct structure, but it has limited throughput and the databases used are often incomplete. Ion mobility combined with MALDI-MSI is capable of differentiating some structural isomers using collision cross-sections,[3,4] but the development of additional tools to provide other orthogonal information would be highly beneficial.

Hydrogen-deuterium exchange (HDX)-MS has long been used for structural analysis of proteins.[5] Recently, its utility has also been demonstrated for small molecule analysis to determine the number of labile hydrogen atoms (referred to below simply as labile hydrogens)

and differentiate structural isomers[6] in various applications including laser ablation electrospray ionization (LAESI)-MSI.[7] Gas phase HDX was previously applied to MALDI-MSI by the Nikolaev group by introducing D_2O vapor into a 10 Torr low vacuum MALDI source[8]. This approach assisted lipid identification in MALDI-MSI; however, its application to compounds with a large number of labile hydrogens was limited due to limited HDX efficiency. For example, they tentatively assigned GalCer 22:0(OH):d18:1 after detecting 7 exchanges, with a labeling efficiency of ~45%. However, the measured protonated adduct has 9 labile hydrogens, so they do not detect significant amounts of the two highest mass isotopologues. While incomplete HDX is still useful to determine a minimum number of labile hydrogens and can be applied to most lipids, it cannot be widely applied to other metabolite classes, especially those with low abundance or many labile hydrogens, such as flavonoids.

Here we adopt a similar approach but present a significant improvement in HDX efficiency by combining introducing D_2O vapor with the use of a deuterated matrix and heated MALDI source. We rely on the high temperatures and rapid reaction kinetics occurring in the MALDI laser plume to achieve a rapid and efficient H/D exchange. The new method allows us to correctly determine the total number of labile hydrogens for each molecule in MALDI-MS imaging even for the metabolites with many labile hydrogens. Furthermore, a systematic approach is presented, combining HDX data with METASPACE analysis, a web-based automatic platform for metabolite annotation of mass spectrometry imaging data[9], to determine or narrow down the correct structural isomers. This approach is demonstrated in MALDI-MSI of *Lemna minor* fronds to explore the metabolites on the surfaces and the middle layer of this plant system.

Methods

Some non-essential experimental details are described in the supporting materials. *Lemna minor* was grown at ambient temperature in sterile 0.5x Schenk and Hildebrandt media under cool white LED lights with 16 hours of light per day and 60 $\mu mol/m^2 \cdot s$ photon flux. To prepare surface samples, fresh fronds were placed with the top or bottom side up on double sided tape that was attached to a glass slide. The fronds were then dried in a vacuum chamber for 1 hour at approximately 60 mTorr. For fractured sample preparation[10], the fronds were placed on single sided packing tape and dried at ~60 mTorr for 4 hours. The tape was then folded over itself and

the folded tape was rolled through a mill and pulled apart, exposing the center of the fractured fronds. Potassium acetate was applied to *L. minor* tissues samples using a TM Sprayer and then a gold sputter was applied to provide conductivity on the surface. Deuterated or undeuterated 2,5-dihydroxyacetophenone matrix was then sublimated onto the tissue samples.

An Orbitrap mass spectrometer (Q Exactive HF; Thermo Scientific, San Jose, CA) connected with a low vacuum MALDI source (Spectroglyph, Kennewick, WA) was used in this study, with the source pressure maintained between 8 and 10 Torr. A 349 nm laser (Explorer One; Spectra Physics, Milpitas, CA) with a 500 Hz repetition rate and an energy of ~4 μJ per shot was used for data collection. To accomplish HDX, a blank flange on the base of the MALDI source was replaced with 1/4" Swagelok adapter that is connected to a leak valve. Nitrogen gas was bubbled through D_2O and then introduced through this leak valve. This introduces D_2O vapor directly to the ion funnel, allowing for gas phase H/D exchange supplemented by labile deuterium atoms in the exchanged MALDI matrix. The outside of the MALDI source housing was heated to ~140°C using a heating tape (BriskHeat, Columbus, OH) to remove surface-adsorbed H_2O. In this condition, the temperature of the sample stage reached ~50°C. Data was collected at 240,000 resolving power at *m/z* 200 with a *m/z* range of 100-1200 and a fixed ion injection time of 492 msec, using a spatial resolution of 30 μm for positive ion mode samples and 50 μm for negative ion mode. METASPACE[9] and MSiReader[11] were used for the metabolite annotation and visualization. The control datasets analyzed with METASPACE are publicly available with the project title of "ISU_2022_duckweed_HDX_controls" at METASPACE (https://metaspace2020.eu) or at the following URL: (https://metaspace2020.eu/project/isu_hdx).

A direct infusion ESI-MS experiment was conducted using a duckweed extract dried, reconstituted in 50:50 CH_3OD:D_2O, and equilibrated for two hours.

Results

Similar to Kostyukevich et al.,[8] HDX efficiency was insufficient in our initial experiments to determine the number of labile hydrogens for some metabolites with low abundance or with many labile hydrogens because the completely labeled isotopologue was often too low abundance to be detected. We initially hypothesized that this low efficiency was due to the equilibration time required for D_2O vapor to permeate the source and replace ambient gas. The deuteration efficiency is improved over time after exposing to D_2O vapor for a long period;

however, even after several hours of exposure to D_2O the measured deuteration efficiency did not reach a steady state. We then hypothesized that the slow improvement of HDX over time is due to the slow desorption of H_2O from the walls of MALDI chamber. Water vapor in ambient air is readily adsorbed on the inside metal surface when the vacuum system is vented, then desorbed from the surface very slowly under vacuum unless it is "baked" for rapid removal. Our MALDI chamber is regularly exposed to ambient air, especially when changing the sample plate, making it vulnerable to ambient water vapor adsorption. To accelerate the removal of surface-adsorbed water molecules, the outside MALDI source is heated for 1 hour with a heating tape while D_2O is introduced. This served a dual purpose of removing adsorbed H_2O while also allowing time for the D_2O to diffuse into the source for the subsequent gas phase HDX. To confirm that this heating process does not damage the tissue samples, additional replicate tissues were imaged 1) before and after heating, and 2) with exposure to H_2O vapor or dry nitrogen; however, no significant differences were seen due to heating or introducing vapor. Additionally, while the outside MALDI source is heated to ~140°C, the sample plate only reaches ~50°C. Furthermore, considering the tissue samples already went through a drying step as a part of sample preparation, it is very unlikely the heating would cause any negative effect.

To further improve HDX MALDI-MS, we also adopted the use of deuterated MALDI matrix, which is applied to the sample through sublimation in order to prevent back exchange during application. Combining the exposure to D_2O vapor with the source heating and deuterated MALDI matrix, we could achieve very high MALDI-HDX efficiency. To determine how many labile hydrogens can be effectively exchanged in a complex sample medium during the short MALDI process, a series of oligosaccharide standards were applied to tissue samples and analyzed by HDX MALDI-MS. As can be seen in **Figure 1**, the maximum possible deuteration peaks, 11, 14, and 17 for maltotriose, maltotetraose, and maltopentaose, respectively, were readily detected. We also collected the HDX MALDI-MS spectra of various standards during heating with the deuterated matrix and D_2O vapor. The HDX efficiency reached a steady state after about one hour of heating (**Figure S1**): ~85% for arginine and citric acid, and ~73% for maltotriose, maltotetraose, and maltopentaose.

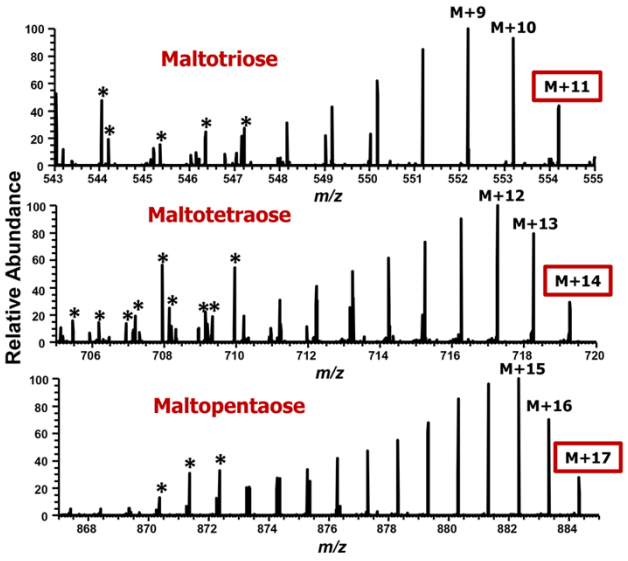

Figure 1. Deuterium labeling of saccharide standards sprayed on duckweed fronds showing detection of up to 17 H/D exchanges.

Determining the maximum HDX is complicated by the contribution from natural ^{13}C, especially for high mass metabolites where there are tens of carbons and the mass resolution is not sufficient to resolve the difference between ^{13}C and ^{2}H peaks. The mass spectrometer used in this study has a high mass resolution of 240,000 at *m/z* 200, or 120,000 at *m/z* 800. However, a theoretical resolution of 270,000 is required at *m/z* 800 to resolve ^{2}H labeled peaks from ^{13}C peaks (a difference of 2.92 mDa). We developed a simple strategy to determine the maximum HDX using a combination of accurate mass and relative intensity as explained in detail in the Supporting Information. In short, we first identify the molecular formula using accurate mass or METASPACE analysis of the control dataset, then calculate the relative abundance of the ^{13}C peak from the number of carbons. Alternatively, one may simply take the relative abundance of the ^{13}C peak from a control dataset. We then compare each pair of M+X and M+X-1 peaks for their relative abundance and mass difference to determine the potential X number of deuterium labels. If there is significant deuterium labeling, the relative abundance would be much greater than that of ^{13}C natural abundance. Similarly, the mass difference would be closer to the mass difference of ^{2}H and ^{1}H (1.0063 Da) than ^{13}C and ^{12}C (1.0033 Da). An example of DGDG 36:6 shown in **Figure S2**. It is determined to have the maximum of 7 HDX because M+7 peak is much greater than 55% of M+6 peak while the mass difference, 1.0059 Da, is much closer to

1.0063 Da than 1.0033 Da. For a large-scale dataset, this process can be automated using a home-made Python code.

Once we developed a method for effective HDX in MALDI-MSI, this approach was systematically applied to spatial metabolomics using METASPACE. MSI datasets from control samples were uploaded to METASPACE and searched using the BraChem database for potential metabolite annotations. BraChem is a rapeseed database and the main plant metabolite database available in METASPACE. Though this is not ideal for *L. minor*, it is a useful starting point as there is no comprehensive duckweed metabolite database available. A total of 56 metabolite annotations were made with a 10% false discovery rate (FDR) providing well-defined ion images from the top surface, bottom surface, and middle section of fractured samples of duckweed.

To confirm that the maximum exchange detected in HDX MALDI-MS corresponded to the actual number of labile hydrogens, duckweed extract in deuterated solvent was analyzed with direct infusion ESI after in-solution HDX. Out of 56 metabolites that we had identified using METASPACE analysis of MALDI-MSI data, 36 were also seen in ESI-MS. Every one of these 36 metabolites showed the same maximum number of deuterium labeling from in-solution HDX as in the gas phase HDX approach in MALDI. It should be noted that ESI-MS does not show 100% HDX efficiency, but is comparable to that of HDX MALDI-MS, due to back exchange with ambient H_2O vapor during the ESI process. However, the high ESI ion signals allowed us to be confident in the maximum labeling detected in this experiment. An example of the comparison is shown in **Figure 2** for $[C_{42}H_{46}O_{23}+K]^+$, where a maximum of 15 HDX was detected for both HDX MALDI-MS and direct infusion ESI-MS.

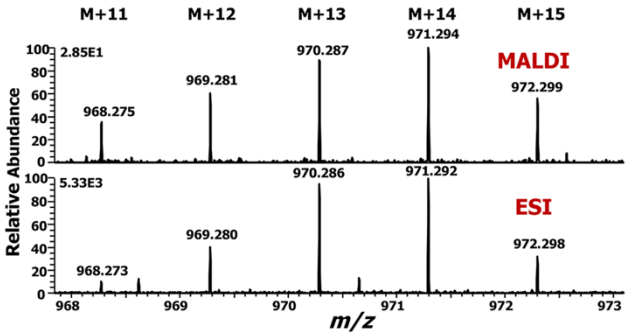

Figure 2. Comparison of deuterium labeling between MALDI-MS with gas-phase HDX and ESI-MS with in-solution HDX for $[C_{42}H_6O_{23}+K]^+$ in duckweed MS imaging and extract, respectively.

As an additional confirmation that we can detect the maximum labeling, HDX MALDI-MS data was also collected in negative ion mode. The annotations from METASPACE in positive ion mode were used to calculate the theoretical deprotonated masses in negative ion mode. Out of the 56 annotations from positive ion mode, 26 corresponding *m/z* were seen in negative ion mode. Similarly to the ESI-MS data, 25 detected negative mode ions showed the same deuterium labeling as the corresponding positive mode ions with similar localization. The one exception, *m/z* 455.098 in negative ion mode, which showed different maximum labeling, was a contamination detected in the off-tissue region. Most of the annotations that were not seen in negative ion mode were lipid species that we did not expect to ionize, including 4 phosphatidylcholine species and 9 triacylglycerol species. Negative mode labeling confirmation in also indicated in **Table S1.**

As we have confirmed that our HDX efficiency is high enough to confidently determine the number of labile hydrogens in metabolites, at least up to 17 HDX, we compared our labeling data to the structures provided by the database. **Figure 3a** shows a Venn diagram comparing the metabolites from top, middle, and bottom portions of duckweed. There are 19 metabolites common to all three, but there are also many unique annotations. **Table S1** shows a full list of the annotated metabolites comparing the labile hydrogens present in structures provided by the database and the number of detected exchanges. As summarized in **Figure 3b**, most of the annotations are consistent with our HDX data (blue), giving additional confidence in these annotations. Some of them have multiple annotations and our labeling data only matches part of those annotations (orange). In these cases, HDX labeling allows us to eliminate incorrect structures, narrowing the possibilities for the correct annotation. There were also some metabolites for which our labeling data did not match any of the database annotations (gray). In these situations our data indicates that the annotations are incorrect, primarily because the database is incomplete or incompatible. This is not surprising, because many metabolites in *L. minor* may not be present in the BraChem database. However, it shows situations where further investigation is needed and HDX data can be used to propose and support other possibilities.

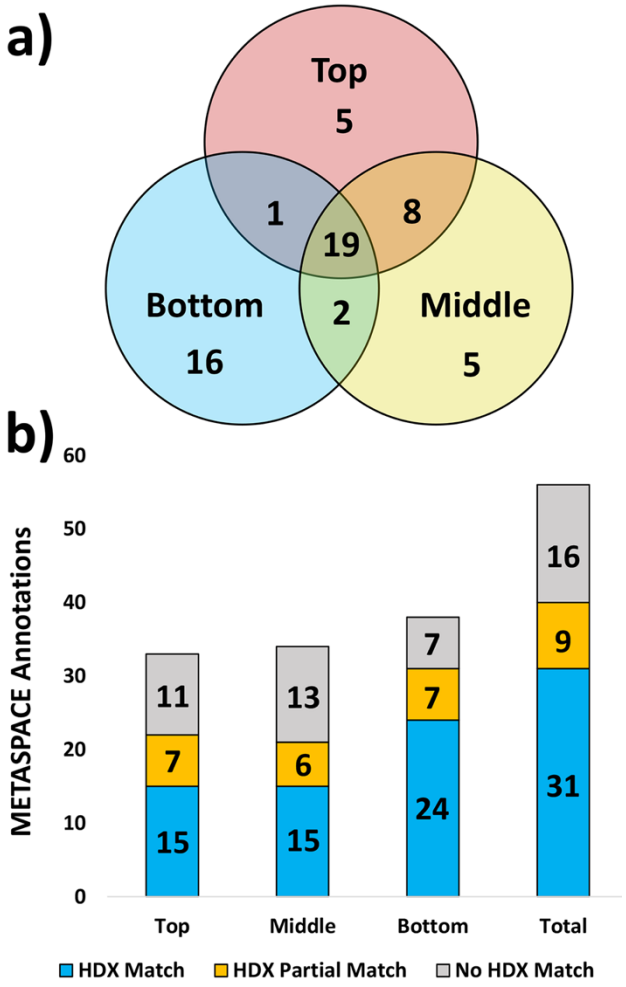

Figure 3. (a) Summary of annotated metabolites in control MALDI-MSI dataset of *L. minor* using METASPACE at 10% FDR comparing top surface, bottom surface, and middle layer. **(b)** Comparison of METASPACE annotations with HDX labeling data for agreement with the number of labile hydrogens.

It is notable that the database annotations are more compatible with HDX labeling data for the metabolites in the bottom surface of *L. minor* than the top surface and middle (**Figure 3b)**. The detectable metabolites in the bottom surface of duckweed are mostly lipids, primarily triacylglycerols, most of which are relatively well conserved between different plant species and have well defined structures. In contrast, the top surface has numerous flavonoids, many of which are also detected in the middle layer and may be unique to duckweed and not present in rapeseed. This is especially relevant to the HDX method developed here because most flavonoids have one or two sugar units and high numbers of labile hydrogens, requiring high-efficiency

HDX to properly analyze. HDX was critical to filter out incorrect metabolites for more than half of top surface and middle section annotations. In most of these cases, more exchanges are observed than predicted from database structures and one could misassign the compounds if they had insufficient HDX efficiency.

Figure 4 shows MS images of some select metabolites comparing the monoisotopic peak (M) in a control and the maximum HDX labeled isotopologue in a labeled sample. The intensity is lower for the maximally labeled image because the signals in the labeled samples are divided into multiple isotopologues with varying numbers of exchanges; however, sufficient ion signals are detected to show clear MS images of the highest possible exchange. The localization is consistent between the control and HDX datasets and there is no evidence of artificial image distortion due to HDX. Lower mass isotopologues with fewer exchanges showed the same localization patterns (not shown). It should be noted that there is a minor difference between each pair of images because they are from different biological replicates. Some compounds are detected outside the tissue area as a result of the fracturing process and mounting fronds onto tape, but these slight issues are consistent in the control and labeled data and not a result of the HDX reaction.

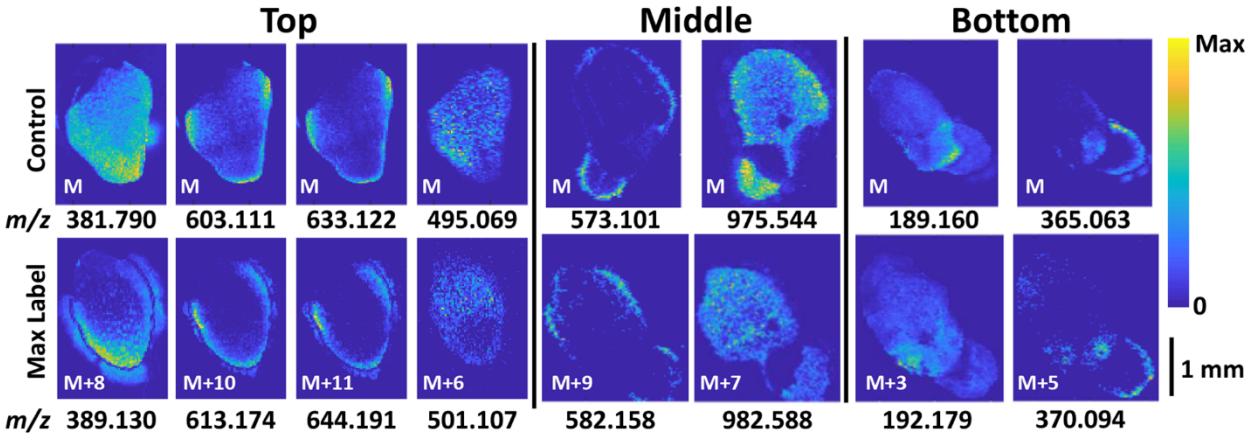

Figure 4. Selected MS images of control and completely labeled isotopologues from the top surface, fractured center, and bottom surface of *L. minor* fronds.

Conclusions

Introducing D_2O vapor in a heated low vacuum MALDI source in combination with a deuterated organic matrix allowed high-efficiency gas phase hydrogen-deuterium exchange of metabolites in MALDI-MS imaging. While HDX MALDI-MSI is previously reported[8], this is

the first-time a heated source and deuterated matrix are utilized in combination with introduced D_2O vapor to dramatically improve the HDX efficiency and correctly determine the maximum number of labile hydrogens for low abundance metabolites and metabolites with many labile hydrogens. Its usefulness is further demonstrated in systematic metabolite annotation using an automatic annotation tool, METASPACE. When applied to *Lemna minor*, HDX data could filter out many incorrect metabolite annotations from METASPACE analysis of control tissue. The practical and broadly applicable MALDI imaging HDX developed in this work can dramatically improve metabolite annotations in large-scale metabolomics by supplementing the accurate mass only annotations in typical MALDI-MS imaging.

Supporting Information

Supplemental methods, deuterated efficiency of various standards over time, supplementary discussion how to determine the maximum number of deuterium labeling (PDF). HDX labeling of METASPACE annotated metabolites (XLSX).

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