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Considerations for quantification of lipids in nerve tissue using MALDI mass spectrometric imaging

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Abstract

MALDI mass spectrometric imaging is a technique that provides the ability to identify and characterize endogenous and exogenous compounds spatially within tissue with relatively little sample preparation. While it is a proven methodology for qualitative analysis, little has been reported for its utility in quantitative measurements. In the current work, inherent challenges in MALDI quantification are addressed. Signal response is monitored over successive analyses of a single tissue section to minimize error due to variability in the laser, matrix application, and sample inhomogeneity. Methods for the application of an internal standard to tissue sections are evaluated and used to quantify endogenous lipids in nerve tissue. A precision of 5% or less standard error was achieved, illustrating that MALDI imaging offers a reliable means of *in situ* quantification for microgram-sized samples and requires minimal sample preparation.

Keywords

lipids; quantification; mass spectrometric imaging; biomedical

INTRODUCTION

MALDI mass spectrometric imaging has recently gained popularity for the *in situ* analysis of thin tissue sections. The robustness and relatively simple sample handling procedures have aided in promoting the widespread use of this methodology. The technique has successfully demonstrated the ability to identify and characterize, in two dimensions, a host of compounds including both endogenous and exogenous species.¹ Creating ion-specific images has become an invaluable tool for correlating histological features to the absence or presence of diseased tissue^{2,3} localization of pharmaceutical compounds and their metabolites^{4,5} and monitoring the anatomical distribution of both large^{6–8} and small^{9–11} biomolecules.

The growth of the technique has currently generated interest in moving MALDI imaging beyond a qualitative method into a quantitative one. Recent quantitative efforts employing MALDI (without imaging) have shown success in quantifying small molecule drugs, amino acids, peptides, proteins, oligosaccharides, bile acids, and oligonucleotides.¹² A quantitative

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evaluation of sphingomyelins and glucosylceramides was performed by Fujiwaki and colleagues¹³ on tissue from patients with Niemann Pick and Gaucher disease; however, the tissue was homogenized and interrogated as spots on a MALDI plate. Each of these studies demonstrates the capabilities of making quantitative measurements with MALDI, but in each case, the spatial information obtained from the imaging process was not retained.

Due to inherent variability in signal, direct microprobe analysis of tissue has generally been considered a qualitative technique. This variability is mostly caused by an inhomogeneous sample surface and inhomogeneous crystallization of the MALDI matrix. The impact of laser shot-to-shot variability is minimized by averaging successive time-of-flight spectra or by using multiple laser shots (typically 10) to fill an ion trap for each mass spectrum. In addition, careful choices of matrixes, optimization of solvent systems, improved matrix deposition techniques, and the use of suitable internal standards have the potential to minimize MALDI signal variability and provide a rapid method of quantification.

In this study, signal variability in sequential tissue analyses and differing approaches to the application of an internal standard to nerve tissue are evaluated. A non-endogenous phosphatidylcholine, PCho(10:0/10:0) was chosen to provide similar ionization and extraction efficiency to the phospholipids of interest. Potential migration and uniformity of application of the internal standard are monitored for each approach to internal standard application. MALDI-MS imaging experiments are carried out to study the effect of applying an internal standard on the distribution of endogenous compounds. Finally, the internal standard is used to calculate the relative concentration of endogenous phospholipids in both brain and spinal cord sections.

EXPERIMENTAL

Animal Treatment and Tissue Preparation

All animal studies were approved by the local IACUC at UCSD and were performed in an AAALAC-approved vivarium. Adult female Sprague-Dawley rats (Harlan, San Diego, CA) were housed 3 per cage under a 12 hr light:dark cycle with free access to food (Harlan Teklad 7001) and water. The animals were sacrificed after they achieved a weight of 250 g. Excised tissue was immediately flash frozen in liquid nitrogen and stored at -80°C in plastic tubes until shipped on dry ice to the University of Florida, where it was again stored at -80°C until processed. Frozen tissue was sectioned to a thickness of $10\text{ }\mu\text{m}$ at a temperature of -25°C using a Leica CM1850 cryotome (Houston, TX). The tissue was attached to the cryotome sample stage using water to avoid the mass spectral interferences caused by traditional techniques that use polymers to affix the tissue to the sample stage.

Internal Standard and Matrix Application

Sectioned tissue was thaw-mounted either on uncoated glass microscope slides or on slides that were first coated with a 100-ppm solution of the internal standard, PCho(10:0/10:0) in 70:30 methanol:water. Slides prepared for mounting of spinal cord were coated completely with the internal standard (under wet tissue: UWT), while those prepared for mounting of brain sections were coated with one, 3x3 mm square of the internal standard. The tissue mounted on uncoated slides was then separated into two sets. One set was immediately coated with a 10 ppm solution (70:30 methanol:water) of the internal standard while still wet (on top of wet tissue: TWT). A lower concentration was applied on top of tissue to avoid masking the signal from endogenous lipids. The other set of tissue was dried in a vacuum desiccator for 30 minutes and then coated with a 10 ppm solution of the internal standard (on top of dry tissue: TDT). Tissue used to study signal variability was placed on uncoated glass slides. All tissue was then vacuum dried for 30 minutes and coated with matrix, 40 mg/ml 2,5-dihydroxybenzoic acid (DHB) with 10 mM sodium acetate in 70:30 methanol:water.

These procedures for application of the internal standard are illustrated in Figure 1. Both the internal standard and the matrix solutions were applied using an Epson R260 inkjet printer.¹⁴ The microscope slides were placed in a modified CD tray holder and passed through the printer 20 times for internal standard application and 15 times for matrix application. By weighing the slide after deposition, a 10 ppm solution of the internal standard was calculated to deposit 2.5 pg/mm² per pass. Therefore, 20 passes of the 10 ppm solution deposited 50 pg/mm², and 20 passes of the 100 ppm solution deposited 500 pg/mm². Parameters within the Epson CD printing software were set for photo quality printing on glossy paper. Evenness was monitored visually using a dissecting microscope with transmitted illumination through a blue filter.

Instrumentation

Studies were carried out on a linear ion trap fitted with a MALDI ion source that operates at intermediate pressure (LTQ XL with vMALDI option, Thermo Fisher Corporation, San Jose, CA), as described previously.¹⁵ Briefly, a N₂ laser with a wavelength of 337 nm is directed to the source by means of a fiber optic cable. Optics external to the vacuum chamber allow laser spot diameters in the range of 80 to 120 μ m that are focused at an incident angle of 32° onto the sample plate. The source operates at a pressure of 0.17 Torr, which is $\sim 10^5$ times higher pressure than a traditional high-vacuum MALDI source, but $\sim 10^4$ times below that of an atmospheric pressure (AP) MALDI source. Two vacuum-rated stepper motors control the two-dimensional movement of the sample plate. A set of modified quadrupole rods, q00, at the front of the multipole assembly permits entry of the laser beam and access by the camera to view the sample plate. The rest of the system is identical to that of a typical LTQ XL. Preliminary high-resolution studies were performed on a hybrid linear ion trap/Orbitrap mass spectrometer with an intermediate pressure MALDI ion source (Thermo Fisher Scientific, Bremen, Germany).

Tissue Analysis

Microscope slides affixed with coated tissue were adhered to a modified MALDI sample plate using double-sided tape (3M, St. Paul, MN). The laser raster size was set to 100 μ m, which approximately equals the laser spot diameter. All experiments were performed with automatic gain control turned off, and the signal was initially optimized by manually adjusting the laser power and the number of laser shots fired. Optimum mass spectra were based on a normalized intensity signal of at least 10^3 for the lipid base peak (m/z 782.5) with little to no mass shifting, which indicated no effects of space-charging. Full-scan MS experiments were typically run at a relative laser power of 30 (arbitrary units) with 10 laser shots per step. MS² and MS³ experiments usually required a slightly higher relative laser power with the same number of laser shots per step as in full-scan experiments. To monitor lateral migration of PCho(10:0/10:0) within tissue, three serial sections of brain tissue were treated as previously described. Two sections were placed on uncoated slides and a 3×3 mm square of internal standard was applied to one wet section and to one dry section. The third section was placed on top of a 3×3 mm square printed onto a glass slide. Quantitative data were acquired from spinal cord taken from the cervical portion of the spine and from a sagittal section of the brain. Tissue images were generated by interrogating serial sections of the spinal cord from the lumbar region of the spine and 3 mm² area of serial coronal brain sections. Quantitative data were acquired by interrogating on-tissue only and signal was averaged over an entire tissue section using Qualbrowser (Thermo Fisher Scientific). Qualitative data were obtained using Qualbrowser and ImageQuest (Thermo Fisher Scientific) and Surfer imaging software (Golden Software, Golden, CO), and quantitative results were processed using Microsoft Excel.

RESULTS AND DISCUSSION

Signal Variability

To generate reliable quantitative data, signal stability is of great importance. To counteract signal variability inherent to MALDI, experiments on a time-of-flight (TOF) mass spectrometer typically average from 100 to 1000 laser shots per spot.^{16–19} With an ion trap, the number of laser pulses is optimized to fill the trap without creating a space-charge effect; software sets a maximum of 20 laser shots per scan. At relatively high laser attenuation, 10 laser shots produce desirable spectra without space-charging. As in conventional MALDI/TOF methods, multiple scans can be averaged to reduce this variability. Figure 2 is a graph that plots the signal intensity at one tissue position of four ions over the course of 20 analyses (each with 10 laser shots) of a spinal cord section at a relative laser power of 30. This represents a total of 200 laser shots after 20 analyses at one spot. The first analysis (from the first 10 laser shots) is dominated by the DHB matrix at m/z 273; while it remains a relatively intense ion, increased signal is observed for the lipid ions in subsequent analyses. Signal for all the ions begins to tail off around the 11th examination (110 laser shots) and appears to be exhausted by the 20th (200 laser shots). The signal increase of DHB observed in the 15th analysis is most likely due to inhomogeneous crystallization. The ratios of the intensities of the three lipid ions in Figure 2 from analysis to analysis are fairly reproducible (SM/PCho 1.0 ± 0.04 ; SM:PCho/Chol 2.4 ± 1.06). Nevertheless, by taking the average signal of the first ten or eleven analyses, a more representative ion signal can be used for quantification purposes.

Internal Standard Evaluation

Initial studies were designed to evaluate the utility of a non-endogenous phospholipid as an internal standard in nerve tissue. A phospholipid was chosen as an internal standard to provide similar ionization and extraction efficiency between the analytes and the internal standard. The phospholipid PCho(10:0/10:0) is not known to occur naturally within the tissue of the nervous system, therefore allowing the interference-free analysis of endogenous lipids. Fig. 3(a) is a full-scan averaged spectrum of the lipid region of a coronal brain section in the central gray substance region without the application of the internal standard; Fig. 3(b) is the mass spectrum of the same area of an adjacent serial brain section with 50 pg/mm² of internal standard applied on top of dry tissue (TDT). The characteristic molecular ions of lipids are observed in the m/z region 700–900. The $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions of the internal standard are seen at nominal masses 566, 588, and 604, respectively. This m/z region is relatively free from background interference; however, signal from ions at m/z 566 and 604 shows a slight interference for the $[M+H]^+$ and $[M+K]^+$ ions. MS/MS was performed on these ions from a blank tissue sample to confirm that they were not a contribution from PCho(10:0/10:0). Therefore, the $[M+Na]^+$ ion at m/z 588 was chosen for further analysis.

When sodium acetate is added to the matrix solution to force formation of sodium adducts, a noticeable difference in the two spectra is a change in relative ion intensity among the lipids, most notably m/z 798 $[PCho(16:0/18:0) + K]^+$ and 810 $[PCho(18:0/18:1) + Na]^+$. These two ions are adducted with different cations and have been shown to be distributed differently throughout tissue. The sodiated lipid at m/z 810 is predominantly found in white matter whereas the potassiated lipid at m/z 798 is primarily found in gray matter, which may suggest a difference of ionization efficiency between white and gray matter once an internal standard is applied. However, further examination shows that other potassiated species exhibit a decrease in ion intensity as well. It has been reported that the sodium and potassium levels in gray matter of the spinal cord are slightly higher than in the white matter, but the ratio of the cations is the same in each.²⁰ Intensities of the sodiated

PCho(10:0/10:0) ion at m/z 588 extracted from white and gray matter separately show no significant difference, which implies similar extraction and ionization efficiencies. Therefore, the change seen between the spectra may be accounted for as additional sodium ions being applied with the internal standard.

A primary concern in using a phospholipid as an internal standard is the potential lateral migration of the lipid within the tissue. To monitor possible migration, an inkjet printer was used to apply a 3×3 mm square of internal standard under wet tissue (UWT) or on top of wet (TWT) and dry tissue (TDT), as described in the Methods section. The mass spectral images of m/z 588 extracted from tissue with internal standard applied TWT, TDT, and UWT can be seen in Figure 4(a-c), respectively. Previous research in our lab has generally shown that migration does not exceed the diameter of the laser spot size (100 μm); here, no evidence of migration greater than the error in inkjet application is seen. The most intense and even signal is produced from applying internal standard on TDT. This indicates very little horizontal migration of the internal standard into the tissue which could lead to suppression of ion signal from endogenous compounds. A slightly more intense signal is observed in the image where internal standard was applied UWT when compared to application on TWT. This demonstrates that the internal standard is efficiently extracted from under the tissue and suggests possible extraction in the opposite direction when applied on TWT. An anomaly in signal is noticed in the bottom left of each image in Fig. 4(a) and 4(b), where internal standard was applied on top of tissue. Because these samples had internal standard applied at the same time and the abnormal data are seen in the same area, it is likely that a printer error occurred and caused an uneven coat of internal standard to be applied. A slight unevenness is also observed from the top to the bottom of the image in Fig. 4(c).

The effect of applying an internal standard on the distribution of endogenous lipids was also investigated by imaging mass spectrometry. The entire area of a tissue section including a small margin around the tissue was interrogated to create an image representative of actual tissue shape. Figure 5 shows the images created by extracting the intensity of m/z 756 [PCho(16:0/16:0) + Na]⁺ divided by the intensity of m/z 588 for each strategy of internal standard application. The distinct H-pattern of gray matter is clearly observed in each image, and this signal appears to be consistent between tissue sections, which implies an even signal distribution of both the analyte and the internal standard. However, the most well-defined tissue shape is seen in Fig. 5(c), application of internal standard UWT. The edges of the tissue as well as the gray matter pattern are less clearly defined in the other two images, Fig. 5(a) and 5(b), which may suggest some migration of the analyte. Areas of increased intensity, known as hot spots, are seen in all three images and are likely due to inhomogeneous crystallization of the MALDI matrix rather than an increased concentration of lipids in those areas. For reasons discussed above, further experiments of endogenous lipids were performed with internal standard applied UWT.

Quantification

Table 1 compares relative concentrations of PCs of varying fatty acid composition from published²¹ results of extracted phospholipids in rat brain tissue with MS results acquired in this study from one section of rat brain and three sections of spinal cord. In the published study²³, phospholipids were extracted from the homogenized forebrain region and separated by TLC then hydrolyzed. The hydrolyzed fatty acids were analyzed by reverse phase HPLC for each lipid class and the fractions were collected, converted to their methyl esters, and identified by GC. Mol% was calculated based on the addition of a single internal standard, 1,2-distearoyl-*sn*-glycerol. For this study, the PCho(10:0/10:0) internal standard was applied on top of dry tissue and mol% was calculated from brain or spinal cord sections by averaging the spectra across the entire section and recording the intensity of each ion. One

sagittal brain section was analyzed once, and three spinal cord sections were each analyzed three times. It was assumed that the MALDI response factor for each PC (including the internal standard) is the same, not an unreasonable assumption within a single compound class, as here. Thus, the mol% can be calculated simply based on intensity% (just for PCs). The number of moles was calculated using the following equation

$$\text{moles} = (\text{Int}_{\text{AN}} / \text{Int}_{\text{IS}}) (W_{\text{IS}}) (MW_{\text{AN}})^{-1} (A)$$

where Int_{AN} is the intensity of the analyte peak, Int_{IS} is the intensity of the internal standard peak, W_{IS} is the amount of internal standard applied (50 pg/mm²), MW_{AN} is the molecular weight of the analyte, and A is the area of the tissue. Area can be measured from the image of the tissue. The sum of the total mol% stated in the literature for lipids chosen for comparison is 84.2%. Generation of mol% was achieved by summing the number of moles for each ion and dividing individual mole counts by a sum to give a mol% total of 84.2%.

As shown in Table 1, the mol% for the PCs from the MS data taken from the brain section shows a remarkable correlation to the literature values. A previous comparison based on relative abundance of lipid ions generated by MALDI has also shown similar results.²² A good correlation between data from the spinal cord and the brain is observed for a majority of the ions, but a larger deviation from the literature values is seen when compared to those data from the brain section. For example, PCho(18:0/18:1) and PCho(18:0/20:4) are notably higher, while PCho(14:0/16:0), PCho(16:0/16:0), and PCho(16:0/20:4) are lower. To the authors' knowledge, no literature exists that reports the individual lipid content found in spinal cord. More insight into these differences could be gained by comparing the mass spectra collected from the brain section and the spinal cord sections.²³

An attempt to quantify other lipid classes found in nerve tissue presents a unique challenge. The preformed positive charge of the quaternary amine of PCs and SMs enhances the detectability in MALDI of these lipids over that of other lipid species.²⁴ Identification of lipids through the use of MS/MS shows that the majority of ions detected in positive ion mode is PCs, and rarely is a PE or PS detected as the dominant species.¹⁸ Therefore, a more reliable method for quantifying low abundance ions would be through MS/MS analyses. The MS/MS spectrum of m/z 856 can be seen in Figure 6. The spectrum is dominated by the ion at m/z 797, which arises from a neutral loss of 59 corresponding to the trimethylamine of a PC. The ions at m/z 813 and 769 correspond to the neutral loss of 43 and 87 indicating the presence of a PE and a PS, respectively. The ions at m/z 673, 572, and 524 coincide to the neutral losses of the PC headgroup and fatty acid tails of the lipids present. Due to the presence of isobars, the quantification of low abundance species could be accomplished through MS/MS experiments; however, a separate MS/MS scan would have to be performed for the internal standard, which would introduce greater variability in the quantification process.

CONCLUSIONS

Strategies for quantifying endogenous lipids in nerve tissue have been demonstrated here. First, signal intensity was monitored in successive analyses of a single tissue section to provide insight into the variation of signal response with an increasing number of laser shots. Such an experiment is necessary to minimize errors due to signal variability and for direct comparisons to other MS and quantitative techniques.

A non-endogenous phospholipid, PCho(10:0/10:0), was explored for use as an internal standard. It was chosen because it does not interfere with ions typically found in the lipid

mass range (m/z 700 – 900) as well as having similar ionization and extraction efficiency to the endogenous lipids of interest. Three different application methods of the internal standard were evaluated, on top of dry tissue (TDT), on top of wet tissue (TWT), and under wet tissue (UWT). For quantitative purposes, it was determined that application of the internal standard UWT provided ionization and extraction efficiencies most similar to endogenous lipids as well as generating tissue images with the greatest spatial resolution and most accurate anatomical structure.

Finally, endogenous lipids were quantified in rat brain and spinal cord tissue sections. Results showed that quantification performed by MALDI was similar to published results. Sample preparation for MALDI is significantly less tedious than for traditional quantitative methods such as LC/MS, and requires only micrograms of sample. The quantitative results presented here illustrate the potential of this technique to provide a rapid and reliable means of quantification.

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Figure 1.

Three approaches to applying internal standard to tissue. These techniques depict the internal standard being applied to discrete areas of the tissue and slide; however, some experiments were performed with internal standard applied everywhere on the tissue and slide.

Figure 2.

Graph plotting the signal intensity of four ions over the course of 20 analyses from one section of spinal cord at one tissue position. A relative laser power of 30 and 10 laser shots were used for each analysis. (DHB: 2,5-dihydroxybenzoic acid m/z 273 $[2M + H - 2H_2O]^+$; Cholesterol: m/z 369 $[M + H - H_2O]^+$; SM: sphingomyelin m/z 753 $[M + Na]^+$; PC: phosphatidylcholine m/z 756 $[M + Na]^+$)

Figure 3.

Mass spectra of the phospholipid region of serial rat brain sections (a) with no internal standard applied and (b) with internal standard. The $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$ ions of the internal standard are labeled in (b).

Figure 4.

Mass spectral images of the $[M+Na]^+$ ion of the internal standard, m/z 588, applied in a 3-mm square to serial sections of rat brain (a) on top of dry tissue, (b) on top of wet tissue, and (c) under wet tissue.

Figure 5.

Mass spectral images of the $[M+Na]^+$ ion of PCho(16:0/16:0), m/z 756, divided by the intensity of the internal standard, m/z 588, applied (a) on top of dry tissue, (b) on top of wet tissue, and (c) under wet tissue.

Figure 6.

MS/MS spectrum of m/z 856 showing the neutral losses (NL) of 43, 59, and 87, which indicates the presence of isobaric lipids. (PE: phosphatidylethanolamine (42:7) + K; PC: phosphatidylcholine (18:0,22:6) + Na; PS: phosphatidylserine (18:0,20:4) + 2Na)

Table 1

Literature and MS data comparison of relative concentration (mol%) of phosphatidylcholines of varying fatty acid tail composition

Phosphatidylcholine	Literature (Brain)	MS (Brain)	MS (Cord) ($\pm S_E$)
14:0,16:0	3.1	1.1	0.1 (± 0.004)
16:0,16:0	19.2	14.3	4.6 (± 0.3)
16:0,18:1	36.2	33.3	37.5 (± 0.5)
16:0,20:4	4.4	5.4	0.7 (± 0.05)
18:0,18:1	14.1	15.6	20.5 (± 0.5)
18:0,20:4	3.8	8.7	2.5 (± 0.07)
18:1,18:1	3.4	5.9	18.3 (± 0.5)

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