Analysis of mammalian sphingolipids by liquid chromatography tandem mass spectrometry (LC-MS/MS) and tissue imaging mass spectrometry (TIMS)

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LC-MS/MS materials and methods

The following methods have been developed for the analysis of SL from the first step of the de novo biosynthetic pathway (i.e. all long chain bases) through the metabolic products of Cer (and their dihydro counterparts) which include: GlcCer, GalCer, Cer1P, LacCer, SM, and sulfatides (ST).

Internal standards and solvents

The LIPID MAPS™ internal standard cocktail (catalogue number LM-6002) by Avanti Polar Lipids (Alabaster, AL) is provided in sealed ampoules and is certified to be > 95% pure and within 10% of the specified amount (25 μM). It contains four different 17-carbon chain length sphingoid base analogues: C17-sphingosine, (2S,3R,4E)-2-aminoheptadec-4-ene-1,3-diol (d17:1-So); C17-sphinganine, (2S,3R)-2-aminoheptadecane-1,3-diol (d17:0-Sa); C17-sphingosine 1-phosphate, heptadecasphing-4-enine-1-phosphate (d17:1-So1P); and C17-sphinganine 1-phosphate, heptadecasphinganine-1-phosphate (d17:0-Sa1P); and five C12-fatty acid analogues of the more complex SL: C12-Cer, N-(dodecanoyl)-sphing-4-enine (d18:1/12:0); C12-Cer 1-phosphate, N-(dodecanoyl)-sphing-4-enine-1-phosphate (d18:1/12:0-Cer1P); C12-SM, N-(dodecanoyl)-sphing-4-enine-1-phosphocholine (d18:1/12:0-SM); C12-GlcCer, N-(dodecanoyl)-1-β-glucosyl-sphing-4-enine (d18:1/12:0-GlcCer); and C12-LacCer, N-(dodecanoyl)1-β-lactosyl-sphing-4-enine (d18:1/12:0-LacCer) as described in Section 6.2.2. Other chain length subspecies of these SL were compared to the internal standards to validate their suitability for accurate quantitation. Similarly, internal standards for sulfatides (d18:1/12:0-sulfatide, ST) and GalCer (d18:1/12:0-GalCer) are obtained from Avanti and Matreya (Pleasant Gap, PA), respectively for this as well. When the dihydro- (i.e., sphinganine backbone) versions of the standards were not commercially available, they were synthesized by reduction of the backbone double bond using hydrogen gas and 10% Pd on charcoal (Aldrich-Sigma, St. Louis, MO) and were verified by LC-MS/MS analysis that the conversion was complete.

HPLC grade solvents were used at all times (acetonitrile, # EM-AX0145; chloroform, # EM-CX1050; hexane, # JT9304-33; and methanol, # EM-MX0475, as well as formic acid (ACS grade, # EM-FX0440-7) was obtained from VWR, West Chester, PA), and acetic acid (ACS grade, # A38C-212) was obtained from Fisher (Pittsburg, PA).

Biological samples

These include cultured cells, tissue homogenates, blood, plasma, urine, etc. For cells, typically 106 cells are sufficient, however, an order of magnitude more or less may be required depending on the abundance of the analyte of interest in that particular cell line. Generally, 1–10 mg of homogenized tissue at 10% wet weight per volume of phosphate buffered saline (PBS) is sufficient for SL analyses. Small volumes of biological fluids such as blood, plasma, or urine, typically on the order of 1–10 μL can be effectively extracted as well. Similarly, tissue culture medium may be extracted for SL, but larger volumes should be used (100–500 μL), and the medium should be lyophilized prior to extraction to reduce the aqueous volume.

Initially, samples should be prepared in aliquots or as duplicates so that one may be assayed to provide a normalizing parameter between different samples such as μg DNA, mg protein, or cell count. As a word of caution, we have noted that one should perform the analyses for the normalizing factor prior to the SL extraction because any errors and variation in analysis of protein, DNA, etc. are often more likely than for failure in the lipid analysis. Therefore, by doing them first, the time and effort for the extraction and subsequent MS analysis are not wasted if the normalizing parameter cannot be measured accurately enough.

Samples should be placed into Pyrex 13 × 100 mm borosilicate tubes with a Teflon-lined cap (catalogue #60827-453, VWR, West Chester, PA). It is critical that these specific tubes be used because SL will stick to some types of glass. Therefore, when samples are transferred from one container to another, some lipids may be left behind,

resulting in variability (and frustration). Unextracted samples should be stored at − 80 °C. Finally, if samples require shipment, this should be done in a container that can keep them separate, in their proper upright orientation, and can maintain a low temperature. An effective shipping container can be obtained from Exakt-Pak (cat. No. MD8000V20 and MD8000V40 for 20 and 40 vial capacity, respectively; http://www.exaktpak.com/).

Sphingolipid extraction protocol

Samples are homogenized using sonication or another type of homogenizer as needed to get uniform dispersion of the samples. Following this an aliquot is removed for normalization purposes via cell number, protein, DNA, etc. The remaining sample is divided, in a measured volume, into two 13 × 100 mm screw-capped glass test tubes with Teflon caps. One will be extracted for high recovery of free sphingoid bases (test tube A) and the other will be extracted differently for high recovery of the complex SL (test tube B).

First, add 1 mL of CH_3OH to both test tubes. Then, add 0.5 mL of methylene chloride (CH_2Cl_2) to test tube A and 0.5 mL of chloroform ($CHCl_3$) to test tube B. Next, add the internal standards to test tubes A and B. Typically, 20 µL of the SL cocktail is appropriate for the sample amounts mentioned previously. If additional analytes will be quantified (e.g., sphingosylphosphocholine, sulfatides, etc.), they can be added to the internal standard cocktail. It should be noted that the amount of the internal standard should be roughly estimated when performing the initial profiling in the presence of the internal standard, so that the concentration of the analytes and standards are within 1–2 orders of magnitude of each other.

Both test tubes A and B should be capped and sonicated for ~ 30 s. The test tubes then should be incubated overnight at 48 °C in a heating block or bath. Allow the test tubes to cool, then add 150 µL of 1 M KOH in CH_3OH to each. This is followed by sonication for 30 s and incubation for 2 h at 37 °C to hydrolyse unwanted species. After base hydrolysis, allow the tubes to cool to room temperature and adjust pH to neutral by adding an appropriate volume of glacial acetic acid (typically ~ 6 µL).

Test tube A is now assigned for further work-up as the “single phase extract,” test tube B will be designated as the “organic phase extract”, which will be treated as follows:
The single phase extract from test tube A should be centrifuged to pellet any insoluble material and the remaining solvent should then be transferred to a new glass test tube. Next, re-extract the pellet by adding another 1.0 mL CH_3OH and 0.5 mL CH_2Cl_2 to the original test tube followed by sonication, vortexing, and then centrifugation. Remove the solvent and combine with the first extraction. The pellet may now be discarded and the combined extracts may be reduced to dryness via speed vac, being careful not to overheat.

The organic phase extraction from test tube B is processed by adding 1 mL of $CHCl_3$ and 2 mL of H_2O, followed by vortexing then centrifugation. Carefully remove the lower layer with a Pasteur pipette, leaving the interface, and put into a new test tube. Re-extract the pellet by addition 1 mL of $CHCl_3$ to the upper layer followed by vortexing and centrifugation. As before, remove the lower layer, leaving the interface, and add this to the first. The recovered $CHCl_3$ may be reduced to dryness by speed vac, being careful to not overheat.

The dried single phase extraction residue may be reconstituted in 300 µL of the initial condition mobile phase used for reverse-phase LC-MS/MS. The dried organic phase extraction residue is similarly reconstituted in 300 µL of the initial condition mobile phase used for normal-phase LC-MS/MS. Both are sonicated for ~ 15 s, then each is transferred to separate 1.5-mL microfuge tubes (organic solvent resistant) and centrifuged for several minutes or until clear. Approximately 70 µL of the clear supernatant is transferred from each into separate 200 µL tapered glass autoinjector sample vials for LC-MS/MS analysis. The remainder may be saved for profiling and further future analyses. It has been demonstrated that the recovery of SL using these methods is very high and will be discussed in detail below. A summary of the extraction protocol is provided (**Fig. 1**).

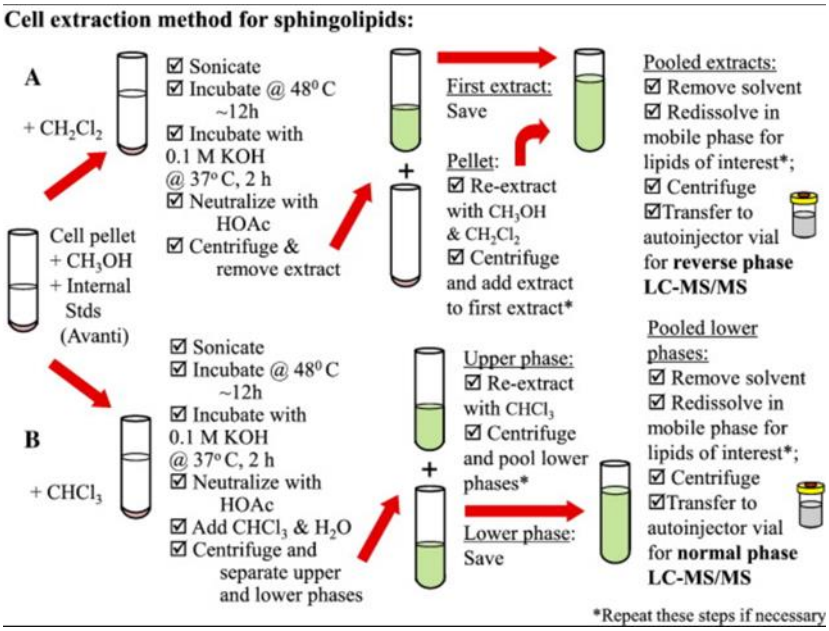

Fig. 1. Protocol for sphingolipidomic analyses via LC-MS/MS using (A) the single phase extraction for high recovery of LCB, their phosphates, Cer1P, and ST; and (B) the organic phase extraction for high recovery of complex sphingolipids Cer, Glc/GalCer, LacCer, and SM.

Sphingolipid profiling

A 50-µL aliquot of the reconstituted extract should be diluted to 500 µL with 99:1 CH_3OH/HCOOH (v/v). This solution may then be infused into the mass spectrometer at a flow rate of 5–10 µL/min (or lower if using nanospray). Sphingolipids such as So, Sa, S1P, Sa1P, Cer, GlcCer, LacCer, and SM ionize readily in the positive ion mode and give strong $(M + H)^+$ ions. Alternatively, anionic SL such as Cer1P, ST, and gangliosides ionize more readily in the negative ion mode and give strong $(M–H)^-$ and $(M–nH)^{n-}$ ions. MS/MS on the positive ion species reveals that most SL fragment to give a common product ion of m/z 264 indicative of a doubly dehydrated sphingosine base, and SL having a sphinganine backbone give rise to fragment ions of m/z 266 (**Fig. 2**). SM is the exception to this, as it fragments primarily to yield an ion of m/z 184, which is indicative of the phosphocholine head group (**Fig. 2**). In the negative ion mode Cer1P, ST, and gangliosides fragment to yield highly abundant ions of m/z 79, 97, and 290, respectively. These fragmentations are indicative of PO_3^-, HSO_4^-, and a dehydrated N-acetyl neuraminic acid (NANA) residue, respectively.

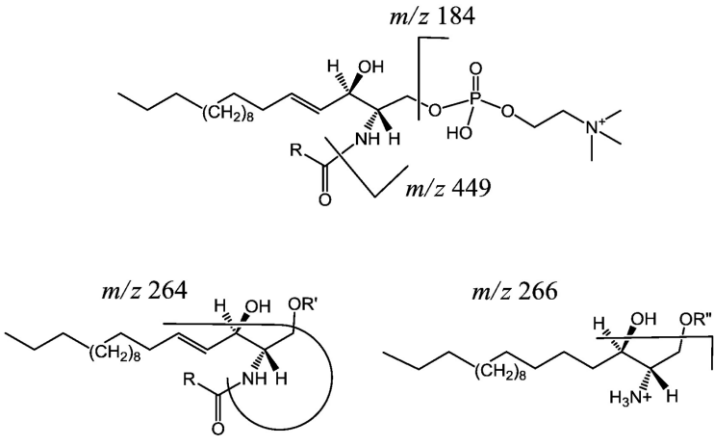

Fig. 2. Common fragmentations of sphingomyelin (top center) showing cleave of the phosphocholine headgroup in the positive mode and neutral loss of the fatty acid in the negative mode. Complex sphingolipids fragment via

neutral loss of the fatty acid and dehydration of the sphingoid base (lower left), as do the long chain sphingoid bases (lower right). These unique species are used for determination of sphingoid base, fatty acid, and head group combinations for building a "parts list" for LC-MS/MS.

These unique structural fragmentations of SL provide a way to identify specific sphingoid base, fatty acid, and headgroup species in complex mixtures (Fig. 3). Here, precursor ion scans of the m/z values mentioned above are performed. Only ions which fragment to give the product ion specified will pass to the detector and be observed. When used in this manner, other interfering species are reduced and primarily the SL of interest will be detected. For example, a scan of precursors of m/z 184.1 can be used to detect the subspecies of SM. A neutral loss of 141u or 162u can be performed to identify CPE or HexCer, respectively. Similarly, scans of the precursors for m/z 264.4 (d18:1 backbone) and 266.4 (d18:0 backbone) can be performed over a wide range of collision energies (35–75 eV) and masses (m/z 200–1000) to check for other subspecies of SL. This also detects subspecies with a 4-hydroxysphinganine (phytosphingosine, t18:0) backbone and/or an α-hydroxy-fatty acid because these have a 16 amu higher precursor m/z yet also produce a m/z 264.4 fragment (it is easily distinguished from the d18:1 species not only by mass but also by LC retention). To check for other sphingoid base backbones, the precursor m/z is varied by ± 14 amu for likely homologs (e.g., − 14 for 17:1 and + 28 for 20:1) and by increments of − 2 amu for additional double bonds or other unsaturation (e.g., d18:2).

The results from these survey scans are used to construct a "parts list" of the subspecies of each class of SL that are present the biological sample of interest. The list will contain all the precursor and product ion pair information necessary to populate those parameters for the multiple reaction monitoring (MRM) analyses. Other conditions regarding ionization and fragmentation will need to be optimized individually for each ion pair for maximum sensitivity and reproducibility. It has been observed that collision energy will need to increase with increasing N-acyl or sphingoid base chain length within each class of SL.

Multiple reaction monitoring (MRM) optimization

Optimal conditions for both ionization and fragmentation are determined for each analyte of interest as well as the internal standards. Standard curves should be generated for the internal standards to determine the limit of detection (LOD = 3:1 signal to noise, s/n), limit of quantitation (LOQ = 10:1 s/n), and the linear dynamic range of the mass spectrometer. Each SL standard can be dissolved at a concentration of 1–10 pmol/μL in the appropriate solvent mixture in which it elutes from the column. For example, Cer elutes early in the normal phase run so it is infused in the initial high acetonitrile solution, whereas SM elutes late and is infused in the methanol solution.

The declustering potential (DP) and focusing potential (FP) will then be varied to determine their optimal settings to give the greatest $(M + H)^+$ signal without generating any in-source fragmentation. After this, the exact center of the mass of the precursor is determined to pass the most ions to Q2 for fragmentation. The structurally specific product ions mentioned previously (i.e. m/z 264, 266, 184, etc.) is then identified and both their optimal collision energy (CE) and collision cell exit potential (CXP) is determined. Once these conditions are optimized, they are entered into the instrument software to establish a MRM detection channel for that specific molecular species. The dwell time for each channel is typically on the order of 20 ms with a 5 ms interchannel delay using the ABI 4000 QTrap. Thus, it takes 0.250 s to cycle through 10 MRM pairs and thus generates 4 data points/s of HPLC elution time. A 15 s wide LC peak would therefore have approximately 60 data points. The dwell time should be adjusted to give the optimal number of data points depending on the number of MRM pairs, the width of the chromatographic peak, and the speed at which the instrument can effectively cycle between MRM channels.

Liquid chromatography separation conditions

Summarized in **Fig. 3** are the types of LC columns and elution conditions that were found to be optimal for analysis of different subcategories of SL.

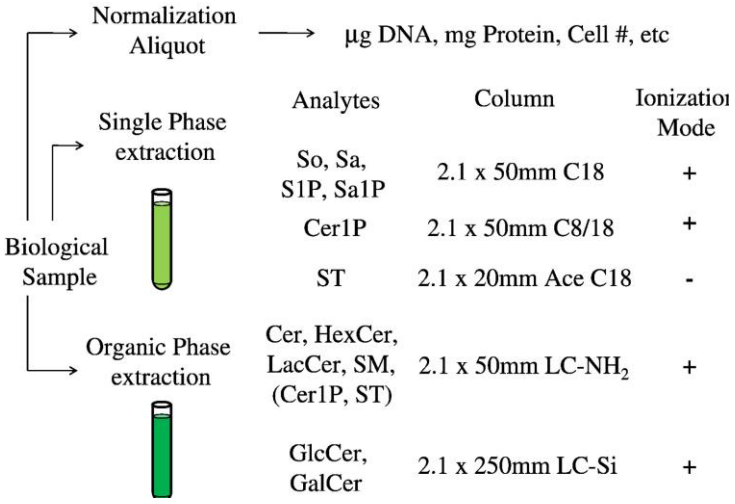

Fig. 3. The sphingolipid analysis pipeline detailing the sample normalization protocol and which classes of sphingolipids may be analysed using a specific extraction procedure, HPLC column, and ionization mode.

LCBs and Cer1P

These compounds are recovered in the single phase extract because we have noted that their recovery into the organic phase of a traditional lipid extraction was highly variable (especially for S1P). The LCBs are easily separated by reverse phase LC using a Supelco 2.1 (i.d.) × 50 mm Discovery C18 column (Sigma, St. Louis, MO) and a binary solvent system at a flow rate of 1 mL/min. Prior to injection of the sample, the column is pre-equilibrated for 0.4 min with a solvent mixture of 60% Mobile phase A ($CH_3OH/H_2O/HCOOH$, 58/41/1, v/v/v, with 5 mM ammonium formate) and 40% Mobile phase B ($CH_3OH/HCOOH$, 99/1, v/v, with 5 mM ammonium formate). After sample injection (typically 50 µL), the A/B ratio is maintained at 60/40 for 0.5 min, followed by a linear gradient to 100% B over 1.8 min, which is held at 100% B for 0.8 min, followed by a 0.5 min re-equilibration wash of the column with 60:40 A/B before the next run. The total run time for LCB analysis is ~ 4 min. Analysis of Cer1P species is performed simply by extending the hold at 100% B for an additional 4.5 min, yielding a total run time of ~ 9 min.

Cer1P has proven to be difficult to analyse via the method mentioned above. It has been observed that there is significant carryover of Cer1P on the LC column of > 1%. This occurs with reverse phase columns obtained from different vendors as well as with different lots of columns from the same vendor. As an alternative, Cer1P can be analysed instead using a Supelco 2.1 (i.d.) × 50 mm Ascentis C8 column (Sigma, St. Louis, MO), with the column heated to 60 °C and a binary solvent at a flow rate of 0.5 mL/min. Prior to the injection, the column is equilibrated for 2 min with a solvent mixture of 70% mobile phase A ($CH_3OH/H_2O/THF/HCOOH$, 68.5/28.5/2/1, v/v/v, with 5 mM ammonium formate) and 30% Mobile phase B ($CH_3OH/THF/HCOOH$, 97/2/1, v/v/v, with 5 mM ammonium formate). After sample injection (30 µL), the A/B ratio is maintained at 70/30 for 0.4 min, followed by a linear gradient to 100% B over 1.9 min. The flow is held at 100% B for 5.3 min, followed by a 0.5 min wash of the column with 70:30 A/B before the next run.

Cer, HexCer, LacCer, SM, ST, and Cer1P

These compounds are analysed using the "Organic phase extract" and normal phase LC using a Supelco 2.1 (i.d.) × 50 mm LC-NH_2 column at a flow rate of 1.0 mL/min and a binary solvent system. Prior to injection, the column is equilibrated for 1.0 min with 100% mobile phase A ($CH_3CN/CH_3OH/HCOOH$, 97/2/1, v/v/v, with 5 mM ammonium formate). After sample injection, 100% mobile phase A is continued for 3 min. Following this, a 1.0-min linear gradient to 100% Mobile phase B ($CH_3OH/H_2O/HCOOH$, 89/6/5, v/v/v, with 50 mM triethylammonium acetate) is performed. This is held for 3.0 min, then restored to 100% A by a 1.0-min linear gradient and maintained at 100% A for 1 min to re-equilibrate the column. In addition to these analytes, Cer1P and sulfatides (ST) can also be analysed in the organic phase extract (although recoveries can be low and errors can increase accordingly). It should be noted that prior to extraction, the samples should be spiked with 500 pmol of C12-sulfatide (d18:1/C12-GalSulfate from Avanti Polar Lipids) since this lipid is currently not part of the SL cocktail. The "Organic phase extract" can be used as qualitative screen of whether or not sulfatides are present. The "Single phase extract" should be used for quantitation because it has the higher recovery (> 50%) of ST.

Separation of GlcCer and GalCer

GlcCer and GalCer can be resolved from samples arising from the "Organic phase extract" using a silica based normal phase column (Supelco 2.1 (i.d.) × 250 mm LC-Si) and an isocratic elution protocol. The initial mobile phase A is $CH_3CN/CH_3OH/H_3CCOOH$, 97/2/1, v/v/v, with 5 mM ammonium acetate (note acetate buffering vs. formate buffering above) flowing at a rate of 1.5 mL/min. The column is pre-equilibrated for 1.0 min, then the sample (dissolved in mobile phase mentioned above) injected, and the column eluted isocratically for 8 min. The isomeric GlcCer and GalCer elute approximately 0.5–1 min apart and should be nearly baseline separated. This should be confirmed during the analysis by interspersing vials with these internal standards throughout large sample batches. Use of isocratic conditions enhances the likelihood that column performance can degrade as the silica surface builds up chromatographically retained undesirable debris (CRUD). When reduced separation of the standards is observed the column will need to be back flushed and regenerated via the manufacturer's protocols.

Generation of standard curves

Once the LC and MRM protocols have been determined, standard curves should be generated under these conditions. The concentration of the individual components of the Avanti internal standard cocktail is 25 μM. If additional SL subspecies intend to be quantitated they should be dissolved in methanol to produce a stock solution at 0.5 mg/mL concentration. These may subsequently be serially diluted into the appropriate LC solvent immediately before analysis to provide 0.5 to 1000 pmol of each standard per 50 μL injection. It should be noted the 4000 QTrap may go much lower in concentration range, and new instrumentation and techniques may also as well.] Each may then be analysed by the appropriate LC-MS/MS protocol to generate the standard curves such as those shown previously, then the linear regression lines and fit may be calculated.

Data analysis

The elution profiles for each MRM pair are examined to quantify the amounts of the SL analytes of interest in biological sample extracts. The areas under the peaks generated for both analytes and internal standards are integrated via the native mass spectrometer software (i.e. Analyst 1.5.1 or newer for Applied Biosystems instruments). Using identical integration settings (number of peak smooths = 2–3, bunching factor = 5–10, and noise threshold = 1 × 104) to integrate both internal standard and analyte allowed quantitation via measuring the area under the peak, then the pmol of the analyte is calculated using the following formula:

where

K*analyte* = correction factor for the analyte versus the internal standard,

Analyte = area of the analyte;

AIS = area of the added internal standard.

The K*analyte* factor adjusts for differences between the analyte and the internal standard with respect to ion yield per unit amount for the selected MRM pair. This calculation also includes any correction for differences in isotopic abundance (~ 1.1% per carbon), which are insignificant for analytes with alkyl chain lengths similar to the internal standard but becomes more substantial when the number of carbon atoms in the analyte is larger than the internal standards (i.e. very-long-chain species). To calculate this "lost signal" one first calculates the ratio of $(M + H)^+$, $(M + H + 1)^+$, and $(M + H + 2)^+$ for the number of carbons in the internal standards and analytes, which is then used to adjust for differences between these compounds.

No correction was necessary for differences in extraction recovery because the extraction conditions were chosen to achieve similar recoveries, as described below. Following the LIPID MAPS convention, the quantities of the SL are then expressed as pmol analyte/μg of DNA. For comparison, it has been our experience that 1 × 106 RAW 264.7 cells contain approximately 3 μg of DNA and approximately 0.25 mg of protein.

Quality control

Each batch of samples submitted for LC-MS/MS analysis should include analysis of the internal standards alone at the beginning, middle, and end of the run. In addition, blank samples (containing only the LC solvent) should also be analysed at varying intervals throughout the run to assess possible carryover. If carryover or shifts in the LC retention times for any of the analytes or standards is observed, the run should be halted and the column should be back flushed and cleaned before resuming the batch run. As noted above, some columns had an unacceptable level of carryover of Cer1P, and when that was the case, the column was cleaned or replaced as appropriate.

Tissue imaging mass spectrometry (TIMS) materials and methods

The following methods have been developed for direct profiling and mapping of SL from tissue surfaces using MALDI.

Chemicals

The chemicals used for imaging SL include 2,5 dihydroxybenzoic acid (DHB) (Aldrich Chemicals, Milwaukee, WI), trifluoroacetic acid (TFA) (Fisher Scientific, Pittsburgh, PA), sulfatides (porcine brain) and a total ganglioside extract (porcine brain) (Avanti Polar Lipids Inc., Alabaster, AL). Also needed are monosialogangliosides GM1, GM2, and GM3 (Matreya LLC, Pleasant Gap, PA), and hematoxylin–eosin (H&E) staining solution (VWR, West Chester, PA). All solvents must be HPLC grade (EMD Chemicals, Gibbstown, NJ), and nanopure water (18 MΩ) is required as well.

Tissue sectioning

Tissue to be dissected is typically frozen in liquid nitrogen and stored at − 80 °C. When ready to section the frozen tissue is placed in a sealed dry ice box and allowed to equilibrate for 1 h. Afterwards the tissue is transferred into the cryostat at − 20 °C for 1 h, then sectioned into 8–10 μm thick slices. These thin slices are then thaw-mounted onto chilled MALDI plates. The following section may be cut in an identical fashion and placed onto a glass slide for histological staining. This process may be continued alternating between MALDI plate and slide with some slices saved for extraction and analysis via LC-MS/MS as detailed above. The slices on the MALDI plate are then slowly brought to room temperature in a desiccator for matrix application for subsequent analysis.

Oscillating capillary nebulizer (OCN) matrix application

A diagram and description of the nebulizer has been previously published. Briefly, a matrix solution of DHB at a concentration of 30 mg/mL in 1:1 (v:v) acetonitrile/water with 0.1% TFA may be delivered to the OCN at a flow rate of 60 μL/min via syringe pump, which is delivered through the inner needle of the apparatus. Ultra high purity nitrogen is then passed at ~ 50 psi through the outer needle surrounding the inner needle. This gas flow generates a high frequency oscillation of the inner needle at the tip, which extends slightly outside the outer needle. This oscillation serves to nebulize the matrix solution into a fine and uniformly dispersed spray. The size of the spray droplet, which is subsequently deposited on the tissue, can then be controlled by 3 variables: matrix solution flow rate, gas flow rate, and distance from the spray tip to the tissue slice. For SL imaging the smallest droplets provide the most homogenous and uniform crystals and prevent lipid migration. The sample stage can then be used to uniformly apply the matrix to a thickness of 10–20 μm. Typical spraying time for a 4 cm^2 sample area is ~ 5 min.

MALDI-MS

Images have been acquired using either a Voyager DE STR MALDI TOF MS or a QStar XL (both Applied Biosystems). Both instruments use a 337 nm N_2 laser with the Voyager operating at 3 Hz having a laser spot size of ~ 100 μm. Best results on the Voyager are acquired using delayed extraction in reflector mode. Accelerating voltage, grid voltage, and delay time are typically set to 22 kV, 70%, and 400 ns, respectively. The mass range is calibrated using a sulfatide and total ganglioside mix. Typically, 9 laser shots are acquired per spot, and the sample stage is typically moved in 60 μm steps across the tissue. Imaging MALDI MS data sets are acquired using modified MALDI MS Imaging Tool (MMSIT) without the 32k data limitation. The ion images are generated using the freely available (http://www.maldi-msi.org/) BioMap software package (Novartis Pharma AG, Basel, Switzerland).

Extraction, quantitation, and localization of sphingolipids via LC-MS/MS and TIMS

Analysis of the extraction efficiencies

The use of an internal standard is based on the assumption that it has the same chemical and physical properties as the analyte of interest, not only for LC ESI-MS/MS but also during the extraction procedures leading up to the analysis. To this end, a series of 6 samples were extracted 4 different times using the single phase extract protocol. It was clearly demonstrated that all the long chain base species and their related internal standards have better than 80% recovery in the first extract, approximately 10% in the second, and virtually none in the third or fourth extract. A similar experiment using the “organic phase” extraction procedure was also performed with strikingly similar results. In this case it was unequivocally shown that all the chain length variants including the internal standard of the complex SL Cer, Cer1P, HexCer, LacCer, and SM were 80–90% recovered in the first two extractions. It was also shown that other SL could be recovered, including ST, which was found to be more highly enriched in the single phase extract.

Standard curves and linear dynamic range

A manuscript detailing the linear dynamic range, overall sensitivity, and the correlation of the internal standard to different chain lengths of SL has been published. There the instruments used were an older ABI 3000 triple quadrupole (QQQ) and a newer ABI 4000 quadrupole linear ion trap (QTrap). They were both operated as QQQ

using MRM protocols similar to the ones above. The QTrap used is at least two generations behind currently available instruments. It is therefore expected that state-of-the-art mass spectrometers would have even greater sensitivity for all classes of SL listed below, given the significant improvements generating, sampling, and transmitting ions as well as newer separation techniques such as ultra high pressure liquid chromatography (UHPLC).

Sphingoid bases and sphingoid base 1-phosphates

It has been shown that all of the LCBs, their internal standards, and their 1-phosphates have a linear signal response from 0.5 to 1000 pmol. The major difference in signal response was observed to occur between the saturated and unsaturated species (e.g., d18:1 vs. d18:0), which demonstrates the need for internal standards for each sphingoid base backbone. It was noted that the QTrap was approximately 3 to 4 orders of magnitude more sensitive for analysis of these compounds than the QQQ. Therefore, this instrument is capable of detecting these SL down in the single digit fmol range with a dynamic range in excess of 4 orders of magnitude.

(Dihydro) ceramide

Like the LCBs, all of the subspecies displayed a linear signal response from 0.5 to 1000 pmol on both instruments, with little difference in signal response due to the length of the fatty acyl-chain (Cer C12:0/C16:0/C18:0/C24:0/C24:1/C25:0 and DHCer C16:0/C18:0/C24:0/C24:1). The chain length dependence should be analysed on different instruments and even the same instrument as conditions change, including aging of the ion source heater, changes in instrument parameters after periodic maintenance, and other procedures. The biggest difference between these particular instruments was observed to be that the signal for Cer on the QQQ is 6 to 8 times higher than for the respective DHCer species. This was in contrast to the QTrap, where the signals for DHCer were much closer (~ 85%). Additionally, it was again observed that the Qtrap yielded 3 to 4 orders of magnitude greater signal response than the QQQ and has a corresponding 2 to 3 orders of magnitude lower detection limit than the range over which it was evaluated.

It was also shown that the C12:0 internal standard could be used for all Cer and DHCer species owing to the similarity in signal response across all chain lengths (C12:0 to C25:0) for both Cer and DHCer on the QTrap, while noting the minor correction above. It was also observed that the correction factor on the older QQQ system was much greater for DHCer using the C12:0 Cer standard and some correction was needed for the longer chain species. This was identified as a reason for the inclusion of Cer d18:1/25:0 in the initial LIPID MAPS™ SL internal standard cocktail. However, subsequent analyses revealed it in fact produced artifacts not observed in the QTrap, therefore, it has since been removed from the SL internal standard cocktail.

Ceramide 1-phosphate

It was shown that the highest recoveries of these SL were found in the “Single Phase Extract” (as discussed later). It was further demonstrated that Cer1P can be analysed by either reverse or normal phase (RP and NP, respectively) LC. Using HPLC for identification and quantitation of Cer1P was reported to have several advantages. First, it is necessary that these species be strongly retained, de-salted, and chromatographically focused because of the high salt content of the single phase extraction procedure and the low abundance of the analyte. Second, they can be analysed in the positive ionization mode where these molecules fragment to give the more structure-specific backbone fragment. This was found to preferable to the ubiquitous phosphate fragment ion of m/z 79 and 97 that is observed in negative mode, which may arise from a number of other phosphorylated species.

In this case it was shown that all the chain length variants of Cer1P analysed (C12:0/C16:0/C24:0) and DHCer1P (C16:0/C24:0) displayed a linear signal response from 0.5 to 1000 pmol on both instruments and little difference in signal response related to the length of the fatty acyl-chain was noted. It was, therefore, concluded that the C12:0 species would be an appropriate internal standard for quantitation of a wide variety of Cer1P chain lengths and backbone variants. It was also noted that the QTrap was approximately an order of magnitude more sensitive than the QQQ. Therefore, detection for these SL was estimated to be 1 to 2 orders of magnitude lower than that used in the experiment.

It should be pointed out that some lots of C18 packing material gave rise to significant carryover of Cer1P, in some cases as high as 10%. To address this, another LC method using a weaker retention solid phase (C8) was developed for those instances. Additionally, the normal phase LC method for complex SL may also be used to analyse Cer1P, however, the peaks are broad and diffuse and have reduced sensitivity. Care must also be taken to neutralize the lipid extracts to minimize hydrolysis of SM to Cer1P, as discussed previously.

Sphingomyelin

Sphingomyelin was likewise reported to have a linear signal response from 0.5 to 1000 pmol on both instruments, and there was little difference in signal response with regard to chain length for SM (C12:0/C18:0/C24:0) or DHSM (C12:0/C18:0/C24:0). Unlike the LCB, DHCer and Cer, the difference in signal response between the QQQ and the QTrap for all SM species was an order of magnitude. It was also noted that SM and DHSM are analysed via loss of the phosphocholine headgroup rather than backbone cleavage. Therefore, the presence or absence of the Δ4 double bond may not have as much an effect on the signal response for these the molecular species. Further, the magnitude of the signal response on the low end of the concentration range was such that both instruments were capable of detecting these species at least 2 orders of magnitude lower concentrations.

Monohexosylceramide

The monohexosylceramides like the previous SL species were observed to have a linear signal response from 0.5 to 1000 pmol for all of the chain length variants HexCer and DHHexCer analysed (C12:0/C16:0/C18:0/C24:1) on both instruments and little variation in signal response relative to chain length was reported. Much like the LCBs, DHCer, and Cer the signal response for GlcCer was reported to be ~ 2.5 orders of magnitude higher on the QTrap versus the QQQ, and little correction was needed for the DHGlcCer species. This was in contrast to the QQQ, in which an 8-fold correction factor was reportedly needed. As was also the case for the other SL the detection limit for the QTrap is on the order of single digit fmol for these species as well.

Several comparisons of standard GlcCer and GalCer with similar backbones did not reveal any differences in signal response between the two species. It was therefore concluded that the C12-GlcCer internal standard may be used for the quantitation of total HexCer if the species are not to be differentiated. It was noted that this might not be the case, however, for other backbones, such as ones with α-hydroxy-fatty acids. It is worthy of mentioning that many cells do not contain galacto-family SL, therefore, it is not necessary to distinguish GlcCer and GalCer in some cases. However, it has been observed that in some cases galacto-sphingolipids may not be present under initial conditions but may be greatly abundant after some stimulus.

Dihexosylceramide (lactosylceramide)

LacCer (C12:0/C16:0/C24:0) and DHLacCer (C16:0/C24:0) have displayed a linear signal response from 0.5 to 1000 pmol on both instruments and some differences in signal response were attributed to the length of the fatty acyl-chain. It was likewise concluded that the C12:0 LacCer species would be an appropriate internal standard for the quantitation of a wide variety of alkyl-chain lengths of these SL. It was observed that the signal response for LacCer is the lowest of the SL analysed in the positive ion mode. Furthermore, like the Cer1P and SM species the QTrap was seen to yield approximately an order of magnitude greater signal response than the QQQ. It was also shown that the difference in signal response for DHLacCer was only 20% lower than that of the LacCer when analysed using the QTrap, but they were ~ 5 fold lower when analysed via the QQQ. Given the magnitude of the signal response generated from the QTrap at the lowest concentration in the experiment, it is estimated that the lower limit of detection is approximately one order of magnitude lower than tested.

Analysis of other sphingolipids via protocol modification: sulfatide

The ability to analyse additional SL is a continual and growing process. It should be possible to expand the list of analytes extracted and detected by supplementation of the internal standard cocktail with the appropriate reference compound. It was reported that a commercially available C12-sulfatide (ST d18:1/12:0) was used to quantify sulfatides in RAW264.7 cells. The highest recoveries of these compounds were obtained in the “Single Phase Extract” procedure.

A comparison of the signal responses for sulfatides on the QQQ and QTrap revealed that the latter was approximately one order of magnitude more sensitive than the former. It was further demonstrated that the signal response was virtually identical for all chain length species investigated (C12:0/C16:0/C24:0), and linear from 0.5 to 1000 pmol for both instruments as well. Similar comparisons with DHST and ST containing α-hydroxy-fatty acid were not possible because of a lack of the necessary standards.

Estimation of the coefficient of variation (CV) for RAW264.7 cells

The coefficients of variation (CV) for extraction and quantitative analysis of Cer, GlcCer and SM using these methods on three separate cultures of cells (with 6 individual dishes per culturing) have previously been determined. It was shown that the average CV for the most abundant SL (SM) was 8 ± 4%, and the agreement among the three separate cells cultures was also found to be high. The CV determined for Cer and HexCer was found to be somewhat higher (12 ± 5%) when calculated for the subspecies that were more prevalent (i.e., > 1

pmol/μg DNA), and as much as twice this percentage for minor subspecies (below 1 pmol/μg DNA). Agreement among the three separate experiments was also high for Cer and HexCer.

The CV for the free sphingoid base were reported to be on the order of 15–25% depending on the amount present, with the greater variability observed at lower quantities. The CV for Cer1P was reported to be on as high as 50% in some cases. It was hypothesized that this variation could be the result of their low quantity, or dish to dish variability, as these molecules are mainly associated with cell signalling.

Tissue imaging mass spectrometry of sphingolipids

Multiple parameters such as matrix solvent flow, N_2 gas flow, and distance from the surface were evaluated for the application of MALDI matrix to a brain tissue slice. Optimal coating conditions produced very fine matrix crystals, which were observed by scanning electron microscopy. It was determined that discrete spraying and drying cycles yielded optimal results for TIMS.

TIMS analysis of a mutant Tay/Sachs-Sandhoff (hexb −/−, knockout) mouse brain showed several striking features. The knockout mouse brain slices when ionized in the negative ion mode showed two prominent ions of m/z 888.9 and 1383. The image generated from the former showed it to be localized mainly to the myelinated fiber region of the cerebellum and fairly evenly distributed in the brain stem. This was contrasted by the m/z 1383 ion, which was more localized to the granular cell region in the cerebellum and was not detected in the brain stem.

Analysis of the mutant mouse brain in the positive ion mode again revealed a number of ions between m/z 700–1400. Ions of m/z 772.6 and 1132 were seen to have significantly different distributions from each other. The ion of m/z 772.6 was observed primarily in the molecular layer region. This was contrasted by the m/z 1132 ion which was seen in the granular cell region, similar to that of the m/z 1383 ion in negative mode. These molecules were hypothesized to be a potassium phosphatidylcholine (PC) and GA2, respectively, based on their correlation to theoretical mass.

ESI MS/MS and MS/MS/MS were performed to elucidate the structures and determine the identity of the molecules observed in TIMS. The MS/MS spectrum of the ion of m/z 888.9 was determined to be a ST having a sphingoid base and fatty acid combination of d18:1/24:1. The MS/MS spectrum of the ion of m/z 1383 was found to be ganglioside GM2 via prominent glycosidic bond cleavages, however, no peaks corresponding to fragmentation of the Cer core were observed. Therefore, MS3 was subsequently performed on the Y0 fragment ion, which revealed that the Cer core had a d18:1/18:0 composition.

The normal mouse brain was observed to have some similarities and some differences when compared to the mutant. For example, both the normal and the mutant mouse brains displayed highly abundant m/z 888.9 ions in the negative mode, which were also localized mainly in the myelinated fiber region of the cerebellum. In contrast, neither m/z 1383 (GM2) in the negative mode nor m/z 1132 (GA2) in the positive mode were detected in the normal mouse brain.

Multiple other prominent ions were observed to be localized to distinct regions of the brain. Several other chain length variants of both sulfatide and GM2 were also seen to have the same localization as the ones detailed above. In addition, other phospholipids such as PC and PI could also be seen. This clearly demonstrates that specific individual molecular species may be localized in specific regions of the brain, and that these experiments provide spatial information that is complimentary to other types of lipidomic analyses.