Structure-Specific, Quantitative Methods for Analysis of Sphingolipids by Liquid Chromatography–Tandem Mass Spectrometry: “Inside-Out” Sphingolipidomics

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MATERIALS AND METHODS

Many methods have been developed for analysis of sphingolipids from the first intermediates of the *de novo* biosynthetic pathway (i.e., 3-ketosphinganine, sphinganine and sphinganine 1-phosphate, and dihydroceramides) through the metabolites of ceramide (ceramide phosphate, sphingomyelin, GlcCer, GalCer, and sphingosine and sphingosine 1-phosphate).

Biological samples

The biological sample can range from cells in culture (typically 1×10^6 cells, but depending on the analyte of interest, several orders of magnitude fewer cells might be sufficient). The same protocol is also effective with many other biological samples, such as 1 to 10 mg of tissue homogenates at 10% wet weight per volume in phosphate-buffered saline (PBS), small volumes (1 to 10 μl) of blood, urine, and the like.

However, the recovery and optimization of the extraction volume, time, and other aspects should be tested for each new sample type. Tissue culture medium (typically 0.1 to 0.5 ml) can also be analysed but should be lyophilized first (in the test tubes described in the next paragraph) before extraction to reduce the aqueous volume. When the samples are prepared, aliquots or duplicate samples should be assayed for the normalizing parameter (μg DNA, mg protein, cell count, etc.) to be used. In our experience, it is best to do these assays first because, if they fail, the effort invested in the lipid analysis will be wasted.

The samples should be placed in Pyrex 13 × 100–mm borosilicate, screw-capped glass test tubes with Teflon caps (the Corning number for these tubes with Teflon-lined caps is 9826–13). It is vital that these specific tubes be used because transfer of samples from one container to another is often a source of variability and frustration. The samples should be stored frozen at −80° until extracted. If it is necessary to ship the samples, this should be done in a shipping container that keeps the vials frozen, separated, and “right-side-up,” such as Exakt-Pak containers (catalog number for containers holding up to 20 vials, MD8000V20; for 40 vials, MD8010V40) (http://www.exaktpak.com/).

Extraction

The steps of this extraction scheme have been summarized in **Fig. 1**

1. Start with the samples in 13 × 100–mm screw-capped glass test tubes with Teflon caps. Most samples (washed cells, tissue homogenates, etc.) will already have an approximate aqueous volume of ∼0.1 ml; if not (e.g., if the sample has been lyophilized), bring to this volume with water.
2. Add 0.5 ml of methanol, then 0.25 ml of chloroform and the internal standard cocktail (which contains 0.5 nmol each of the C12:0 fatty acid homologs of SM, Cer, GlcCer, LacCer, and ceramide 1-phosphate; C25-ceramide; and d17:1 sphingosine, d17:0 sphinganine, d17:1 sphingosine 1-phosphate, and d17:0 sphinganine-1-phosphate) (Avanti Polar Lipids, Alabaster, AL)
3. Sonicate as needed to disperse the sample, then incubate overnight at 48° in a heating block.
4. Cool and add 75 μl of 1 *M* KOH in methanol, sonicate, and incubate 2 hr at 37°. (This step reduces clogging of the LC columns, but can be eliminated for some samples, if desired.)
5. Transfer half of the extract (which will be used for analysis of the more polar sphingolipids, such as sphingoid bases and sphingoid base 1-phosphates, by reverse phase LC-ESI-MS/MS) (0.4 ml) to a new test tube (this will be used in the next section).

6. To the half of the extract that remains in the original test tube(s), add 3 µl of glacial acetic acid to bring the pH to ~ neutral, then add 1 ml of chloroform and 2 ml of distilled, deionized water, and mix with a rocking motion to avoid formation of an emulsion.
7. Centrifuge in a table top centrifuge to separate the layers, then carefully remove the upper layer with a Pasteur pipette, leaving the interface (with some water).
8. Evaporate the lower phase to dryness under reduced pressure (using a stream of N_2 or a speed vac) and save this fraction for normal phase LC-ESI-MS/MS of the complex sphingolipids

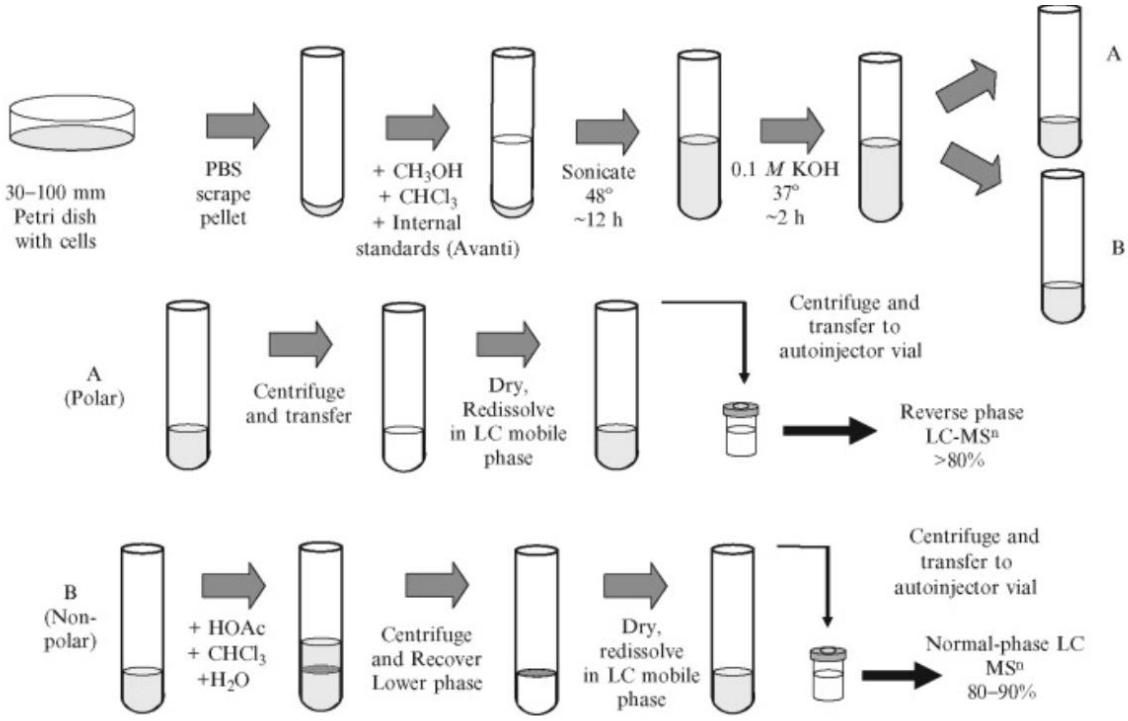

Figure 1. Summary of the extraction scheme used for these methods.

Preparation of samples for LC-MS/MS

For the sphingoid base fraction (Step 5 in "Extraction" section), use a table top centrifuge to remove insoluble material, then transfer to a new glass test tube and evaporate the solvent under N_2 or reduced pressure (speed vac). Add 300 µl of the appropriate mobile phase (as shown later) for reverse-phase LC-ESI-MS/MS, sonicate, then transfer to 1.5-ml microfuge tubes (organic solvent resistant) and centrifuge for several minutes or until clear. Transfer 100 µl of the clear supernatant into a 200-µl glass autoinjector sample vial for LC-ESI-MS/MS analysis. Prepare the extract for normal-phase LC-ESI-MS/MS (Step 8 in "Extraction" section) in the same manner but using the appropriate mobile phase.

Note: For some samples, slightly higher recoveries and/or more uniform recoveries across different sphingolipid subspecies are obtained if the residue from these redissolving steps are treated again and the extracts pooled.

Identification of the Molecular Species by Tandem Mass Spectrometry

The first step of the analysis is to identify the molecular subspecies of each category of sphingolipid that is present by analysis of their unique fragmentation products using precursor ion or neutral loss scans. Once these subspecies have been identified, the investigator can build an MRM protocol for quantitative analysis of those analytes for which both the ionization and dissociation (collision energy) conditions have been optimized for individual molecular species.

A general scheme for the instrumentation is given in **Fig. 2**.

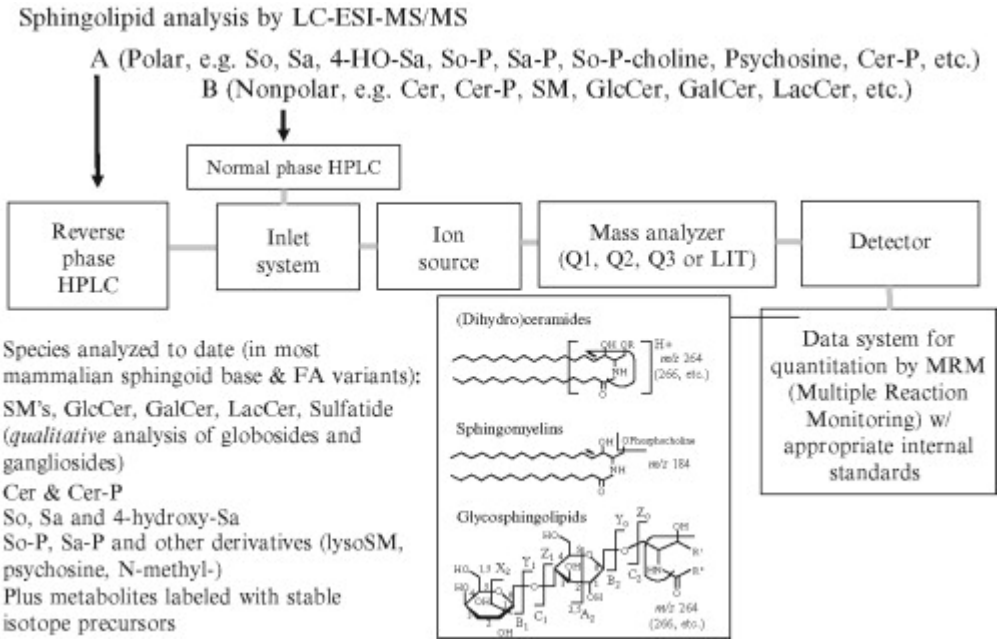

Figure 2. Summary of the work flow and metabolites analysed by LC-ESI-MS/MS of sphingolipids by the methods described in the text.

Materials for infusion and LC-ESI-MS/MS

Positive-ion infusion spray solution: CH_3OH/HCOOH (99:1) (v:v) containing 5-m*M* ammonium formate.
Reverse-phase LC:

1. The recommended reverse-phase LC column is a Supelco 2.1 mm i.d. × 5 cm Discovery C18 column (Supelco, Bellefont, PA).
2. The following are solvents for reverse phase LC:
   a) Reverse-phase LC solution A: CH_3OH/H_2O/HCOOH (74:25:1) (v:v:v) with 5-m*M* ammonium formate
   b) Reverse-phase LC solution B: CH_3OH/HCOOH (99:1) (v:v) with 5-m*M* ammonium formate
   Normal-phase LC:
1. The recommended normal-phase column is a Supelco 2.1 mm i.d. × 5 cm LC-NH_2 column, with the exception for the LC method for separation of GlcCer and GalCer, which uses a Supelco 2.1 mm i.d. × 25 cm LC-Si column.
2. The following are solvents for normal-phase LC:
   Normal-phase solvent A: $CH_3CN/CH_3OH/CH_3COOH$ (97:2:1) (v:v:v) with 5-m*M* ammonium acetate
   Normal-phase solvent B: CH_3OH:CH_3COOH (99:1) (v:v) with 5-m*M*ammonium acetate

Sphingolipid subspecies characterization prior to quantitative LC-MS/MS analysis

1. Dilute a 50-µl aliquot (one-sixth of complex fraction) of the reconstituted sample for complex sphingolipid analysis (as described previously the section of preparation of samples for LC-MS/MS) to a final volume of 1 ml with positive ion infusion spray solution.
2. Infuse this solution into the ion source with a 1-ml syringe at 0.6 ml/hr.
3. Perform precursor ion scan(s) for predetermined unique molecular decomposition products (e.g., fragment ions of *m/z* 264 and 266 for the d18:1 and d18:0 sphingoid base backbones, respectively, and *m/z* 184 for SM, etc.).
4. Identify the sphingolipid precursor and product ion pairs for species present, noting potential isobaric combinations of sphingoid base and N-acyl fatty acids.
   Note: At this point, it is usually most time-efficient to re-analyse the sample by LC-ESI-MS/MS using the LC method described below since the final optimization of the ionization parameters will need to be done with the same solvent composition as the compounds of interest elute from LC.
5. Using the "parts list" of species (**Fig. 4**) for each observed subspecies, determine the optimal ionization conditions and collision energy for each species. Typically, collision energy will need to be increased within each sphingolipid class as the N-acyl chain or sphingoid base increases in length.

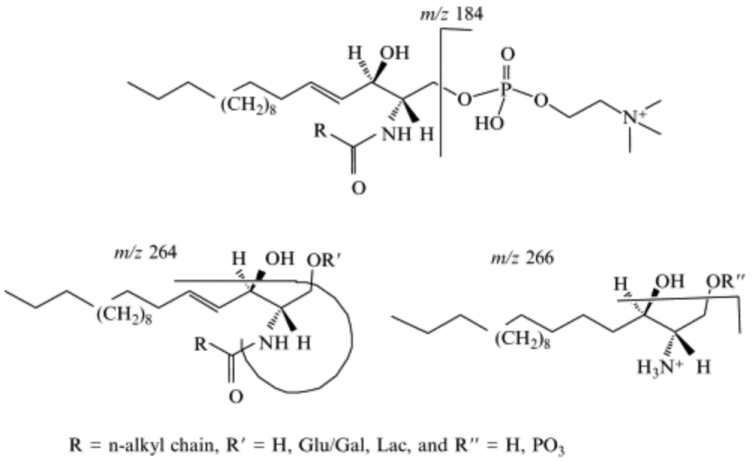

Figure 4. Fragmentation of sphingolipids observed in the positive ion mode. Fragmentation of long-chain bases, long-chain base phosphates, ceramides, and monohexosylceramides involves dehydration at the 3-position, dehydration at the 1-position, or cleavage of the 1-position moiety with charge retention on the sphingoid base. Sphingomyelin similarly cleaves at the 1-position; however, the charge is retained on the phosphoryl choline headgroup yielding the m/z 184 ion.

Quantitation by LC-ESI-MS/MS Using Multi Reaction Monitoring (MRM)

The optimized ionization and fragmentation conditions for each analyte of interest, combined with the LC elution position, are used to construct the MRM analysis protocols. In MRM, the mass spectrometer is programmed to monitor specific, individually optimized precursor and product ion pairs (with respect to instrument parameters for highest yield of the precursor and product ions of interest) in specific LC timeframes. The signal generated by each ion transition in most cases uniquely identifies a particular molecular species by retention time, mass, and structure, although there are still rare occasions where another compound adds to the signal; hence, whenever possible, samples should be examined for this possibility by another technique, such as further analysis of the ions of interest by tandem MS or by an orthogonal technique, such as comparing the results of other types of chromatographic separation, ionization (e.g., positive versus negative ion mode), and fragmentation. To determine the relationships between the signal response for the spiked internal standard (which can be very close to 1:1 for some instruments, such as the ABI 4000 QTrap when the MRM parameters have been optimized) and a correction factor (if needed), one typically compares the internal standard with the analyte of interest (e.g., the 17 carbon homolog of sphingosine [d17:1] versus naturally occurring sphingosine [d18:1]; in the case of more complex sphingolipids, C12-chain–length internal standard vs at least two chain length variants, such as the C16- and C24-chain–length versions of the analyte, which are usually available commercially [from Avanti Polar Lipids, Alabaser, AL, Matreya, Pleasant Gap, PA, among others]). This comparison has been performed for all of the species covered by the standard cocktail mix, and a very high equivalence exists between the ion yields of most of the naturally occurring analytes and the selected standard when analysed using the ABI 4000 QTrap; however, some categories show more deviation using the API 3000 triple quadrupole instrument, which illustrates the need to determine these relationships for the particular instrument being used for the analysis.

Analysis of sphingoid bases and sphingoid base 1-phosphates in positive ion mode

1. Portions of the reconstituted samples (prepared above) are diluted with reverse-phase LC solutions A and B for a final A:B ratio of 80:20 (v:v). (If it is desirable to concentrate the sample, the solvent can be removed by evaporation and then the residue re-dissolved in this solvent mixture.) The samples are centrifuged to remove any precipitate, and then placed in autosampler vials and loaded into the autosampler.
2. The LC column (reverse-phase C18) is equilibrated with reverse-phase LC solution A and B (80:20) for 0.5 min at flow rate of 1.0 ml/min.
3. The sample (typically 10 to 50 µl) is injected (then the needle is programmed to wash with at least 5 ml of reverse-phase solution A to prevent potential sample carryover) and the following elution protocol is followed (at a flow rate of 1.0 ml per minute):
    a) Wash the column for 0.6 min with reverse-phase solutions A and B (80:20, v:v).
    b) Apply a linear gradient to 100% of reverse-phase solution B over 0.6 min.

c) Wash with 100% reverse-phase solution B for 0.3 min.
d) Apply a linear gradient to reverse-phase solution A:B (80:20) over 0.3 min.
e) Re-equilibrate the column with a 80:20 mix of reverse-phase solutions A and B for 0.5 min.

4. Determine the areas under the peaks for internal standards and analytes of interest using extracted ion chromatograms.
5. Quantify analytes relative to internal standard spike.

During the LC run, the ions of interest with the salient points summarized here with comparisons between two types of mass analysers, a triple quadrupole MS/MS (the API 3000), and a hybrid quadrupole-linear ion trap (ABI 4000 QTrap, Applied Biosystems, Foster City, CA). Positive mode analysis of the $(M + H)^+$ ions of long-chain bases such as sphingosine (d18:1), sphinganine (d18:0), 4-D-hydroxysphinganine (t18:0), the 17-carbon homologs (d17:1 and d17:0), and the 20-carbon homologs (d20:1 and d20:0) by MS/MS reveals that they fragment via single and double dehydration to product ions of *m/z* 282/264, 284/266 (sphinganine also fragments to a headgroup ion with *m/z* 60 that can be followed to avoid overlap with sphingosine isotopes), 300/282/264, 268/250, 270/252, 310/292, and 312/294, respectively.

The ratios between single- and double-dehydration products varies by collision energy and type of mass spectrometer. Signal response for the single-dehydration products is greater than double-dehydration products in the ABI 4000 QTrap (even given higher collision energies), whereas in the API 3000 triple quadrupole, the ratio is reversed, with a much stronger double-dehydration signal than for single dehydration. Sphingoid base 1-phosphates derivatives undergo a similar dehydration and cleavage of the headgroup to yield the same *m/z* product ions as the double-dehydrated product ions described above; however, this does not interfere with their analysis in the same run because they are distinguished both by their precursor ion mass and retention times on LC.

Ionization parameters for sphingoid bases and sphingoid base 1-phosphates are similar for sphingoid bases containing 17 to 20 carbons. (In the API 3000 triple quadrupole ionization, settings are identical; however small eV differences are required in the ABI 4000 QTrap to achieve optimal signal response.) The main variable in these analyses is collision energy, which increases with sphingoid base chain length. Modifications to sphingoid bases such as N-methylation, additional sites of unsaturation, and hydroxyl addition will also require increasing collision energy and can be detected by shifts in precursor and product ion *m/z* as well as alteration of reverse-phase LC retention. Although one must check if there are changes in ion yield due to differences in the elution solvent, possible ion suppressing compounds in crude extracts, or other changes when compounds in a subspecies series elute slightly differently from the LC, small shifts in retention provide a useful verification of the identities of the species when product ions are otherwise identical.

Notes on 3-ketosphingoid base analysis: 3-Ketosphinganine (3kSa) can be analysed by reverse-phase LC with the other sphingoid bases. It has the same precursor *m/z* as sphingosine, and, although there are differences in the degree of dehydration in the fragments (sphingosine fragments primarily to *m/z* 264 whereas 3-ketosphinganine fragments to *m/z* 282), the most reliable way to differentiate these compounds is by examining the differences in elution on reverse-phase LC (3-ketosphinganine elutes after sphingosine). Interestingly, one also finds 3-keto-sphingoid bases as part of 3-ketodihydroceramides, which are identifiable by earlier elution on normal-phase LC as peak pairs with normal ceramide (with 3-ketodihydroceramide eluting first).

Analysis of (Dihydro) Ceramides, (Dihydro) Sphingomyelins, and (Dihydro) Monohexosyl-Ceramides in Positive Ion Mode

1. Portions of the reconstituted samples (as prepared in a previous section) are diluted with normal-phase solution A. (If it is desirable to concentrate the sample, the solvent can be removed by evaporation and the residue re-dissolved in this solvent mixture.) The samples are centrifuged to remove any precipitate and then placed in autosampler vials and loaded into the autosampler.
2. The normal-phase LC-NH_2 column (Supelco 2.1 mm i.d. × 5 cm LC-NH_2) is equilibrated for 0.5 min with normal-phase solution A (98:2, v:v) at 1.5 ml/min.
3. The sample (typically 10 to 50 µl) is injected (then the needle is programmed to wash with at least 5 ml of normal-phase solution A to prevent potential sample carryover), and the following elution protocol is followed (at a flow rate of 1.5 ml/min):
   a) Wash the column 0.5 min with normal-phase solution A.
   b) Apply a linear gradient to 10% normal-phase solution B over 0.2 min.
   c) Hold at A:B (90:10, v:v) for 0.5 min.
   d) Gradient over 0.4 min to normal-phase solutions A and B (82:18, v:v).

e) Hold at A:B (82:18, v:v) for 0.6 min.
f) Apply a linear gradient to 100% normal-phase solution B over 0.4 min.
g) Re-equilibrate the column with normal-phase solution A for 0.5 min.

Note: By this protocol, one monitors the specific precursor and product ion transitions for ceramides for the first 0.8 min, followed by monohexosylceramides for 1.15 min and then sphingomyelins for 1.05 min; however, due to age or brand of column, the elution of monohexosylceramides may shift, requiring time adjustment as needed.

Note regarding chromatography in general: It is useful to bear in mind that, when converting methods between two different size columns, it is advisable to convert flow rates, hold times, and gradients based on column volume. Pre-gradient holds should be at least five to six column volumes worth of solvent; for maximum resolution during a gradient, solvent change per column volume should be 2 to 4%. For the columns described in this review, the lengths described correspond to the column volumes in parentheses: 2.1 × 50 mm (0.11 ml), 2.l × 150 mm (0.33 ml), and 2.1 × 250 mm (0.55 ml). Also, as columns are changed, check the recommended flow rates for that column length.

4. Determine the areas under the peaks for internal standards and analytes of interest using extracted ion chromatograms.
5. Quantify analytes relative to internal standard spike.

Product ion analysis of the $(M + H)^+$ ions of ceramides reveals cleavage of the amide bond and dehydration of the sphingoid base to form highly abundant, structurally specific fragment ions. These product ions yield information regarding the number of carbon atoms in the chain, degree of hydroxylation, unsaturation, or other structural modifications of the long-chain base (e.g., sphingosine, *m/z* 264; sphinganine, *m/z* 266; and 4-hydroxysphinganine, *m/z*264—which is the same as for sphingosine-based ceramides, but these can be distinguished by differences in LC elution times). With this knowledge about the sphingoid base composition and the original precursor *m/z*, the identity of the fatty acids can be deduced.

Ionization parameters for complex sphingolipids are linked to their headgroup classes with size and charge of headgroup playing key roles. Accordingly, differences in N-acyl chain length somewhat shift the required collision energy (in our experience, ~2.5 eV every two to four carbons).

Product ion scans of the $(M + H)^+$ ions of GlcCer, LacCer, and more complex glycolipids reveal that these ions undergo dissociation by two pathways: cleavage at the glycosidic linkage(s) at low collision energies with loss of the carbohydrate headgroup as a neutral species with charge remaining on the ceramide moiety and cleavage of both the sugar headgroup and the fatty acid acyl chain at higher energies with charge retention on the dehydrated sphingoid base.

Low energy dissociation to remove the headgroup can be useful for analysing glycosidic linkages in neutral glycolipids and gangliosides; however, for quantitation of individual sphingoid base and N-acyl fatty acid combinations of monohexosylceramides, high energy dissociations to the sphingoid base-specific product ion are preferable. Choosing this fragmentation pathway allows distinction of isobaric d18:1/C18:0 versus d20:1/C16:0 glucosylceramides, which would have the same low energy deglycosylated fragment *m/z* 566 but high-energy conjugated carbocation fragments that differ (*m/z* 264 versus *m/z* 292, respectively).
Sphingolipids containing phosphodiester-linked headgroups, such as in sphingomyelin (SM), fragment very differently: the $(M + H)^+$ species fragments at the phosphate-ceramide bond, with charge retention on the phospho-headgroup to yield highly abundant ions of *m/z* 184. Ceramide phosphoethanolamines (CPE) also fragment at the phosphate-ceramide bond, but the headgroup is lost as a neutral species of mass 141 u.

Modifications for analysis of glucosylceramides and galactosylceramides

Because GlcCer and GalCer elute in the same fractions by the above method, biological samples that contain both of these monohexosylceramides must be analysed by a separate method.

1. Reconstitute the samples in normal-phase solution A and load into the autosampler.

2. Pre-equilibrate a normal phase LC-Si column (Supelco 2.1 mm i.d. × 25 cm LC-Si) for 1.0 min with a normal-phase solution A at 1.5 ml per min.
3. Inject 50 µl of each reconstituted extract and continue to elute with normal-phase solution A at 1.5 ml/min for 8 min. GlcCer elutes at 2.56 min and GalCer at 3.12 min using this isocratic normal-phase system.
   Note: GlcCer and GalCer are isobaric species, so only one MRM transition is needed per shared sphingoid base and fatty acid combination.
4. Determine the areas under the peaks for internal standards and analytes of interest using extracted ion chromatograms and quantify analytes relative to internal standard spike.

Analysis of ceramide 1-phosphates by positive and negative ion modes

Ceramide 1-phosphates may be examined by the reverse-phase LC method described above for sphingoid bases and sphingoid base 1-phosphates by extending the final wash with 100% reverse-phase LC solution B until the ceramide 1-phosphates elute (C12-ceramide phosphate elutes at the beginning of the change to this solvent, followed by longer chain-length species).

An orthogonal approach (normal-phase LC negative mode following phosphate as the product ion in the MRM) can also be employed using either silica (LC-Si) or diol (LC-Diol) columns (Supelco) in conjunction with elution solvents that contain triethylammonium acetate (TEAA) and begin with acetonitrile and gradient to methanol and water (formic acid and ammonium formate buffer systems can also be used because they allow CerP to ionize well in negative mode).

In positive mode, the major fragments from ceramide 1-phosphate arise from cleavage of the amide bond and dehydration of the sphingoid base to form *m/z*264 (for sphingosines), 266 (for sphinganines), and so on. As with the other complex sphingolipids, to maintain ionization efficiency, collision energy should be increased slightly with chain length. In negative mode, the major product ion from the precursor $(M - H)^-$ ions is phosphate. This can be used for quantitation; however, the fidelity of the MRM pair for ceramide 1-phosphate should be confirmed since the product is no longer structure-specific for the sphingoid base and N-acyl chain combinations.

Analytical methods for additional analytes

Methods for more complex glycosphingolipids are still under development. Sulfatides are readily analysed in negative ion mode and yield primarily $(M - H)^-$ ions that fragment to the sulfate group (*m/z* 96.9) (lower-abundance fragment ions representing the lipid backbone can also be observed). For LC, either reverse- (C18) or normal- (LC-Si or LC-Diol) phase columns can be used, with methanol/water gradients to methanol (with 5 m*M* ammonium acetate and 0.01% ammonium hydroxide to promote ionization) for reverse phase. For normal phase, acetonitrile, methanol, and water combinations (with the same mobile phase modifiers or triethylammonium acetate) achieve satisfactory LC resolution.

Gangliosides also ionize readily in the negative ion mode in methanol. For gangliosides analysed by the triple quadrupole MS/MS, the $(M - 2H)^{2-}$ ions fragment to yield highly abundant C1β-H_2O ions of *m/z* 290, which reflect the N-acetyl neuraminic acid moiety (**Figure 5 and 6**). The enhanced product ion scan feature that is available using the ion trapping function of the 4000 QTrap provides more structural information because it yields better sensitivity and more abundant high mass product ions (**Figure 6**). Additionally, cleavage at the glycosidic bonds produces characteristic Y_n-type ions and through ring cleavages, such as X2α2,4 and X3α2,4 (*m/z* 1351.9 and 1512.8, respectively), which are useful for determination of glycosidic bond linkage.

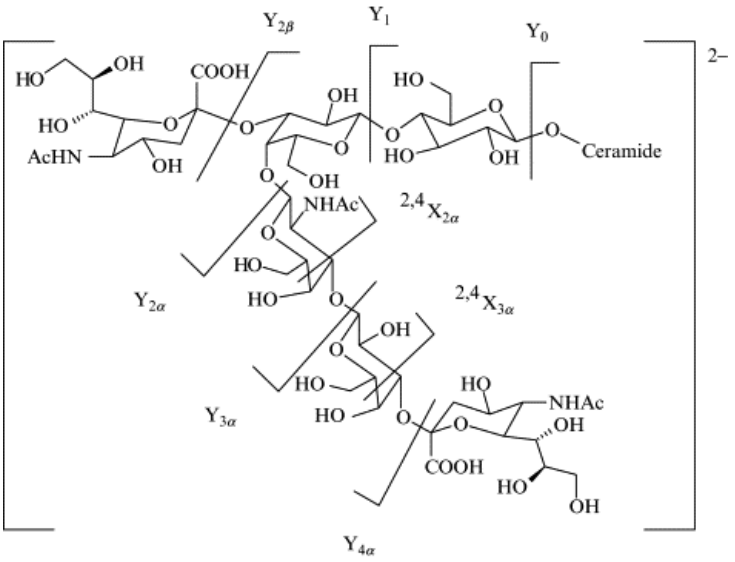

Figure 6. Structure of ganglioside GD1a and major cleavage sites and nomenclature.

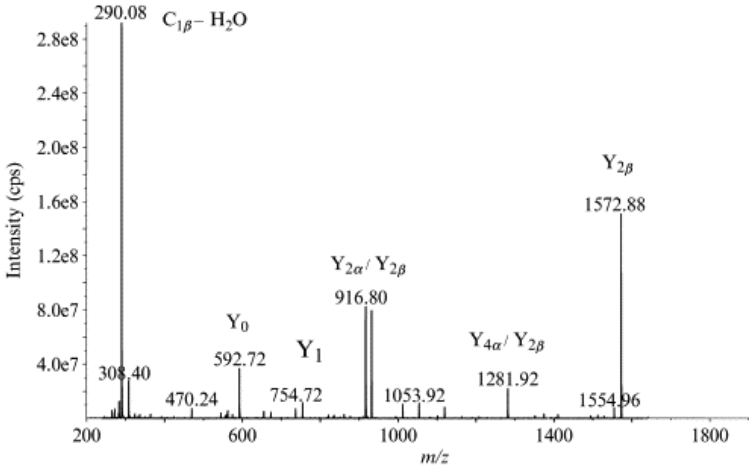

Figure 7. Tandem mass spectrometry analysis of ganglioside GD1a using the 4000 QTrap in enhanced product-ion scanning (EPI) mode (~55 fmol consumed). See **Figure 6** or the cleavages represented by these labels.

An MS/MS/MS (MS^3) analysis is performed much the same manner as a product ion scan. In this case the first mass analyser (Q1) is set to pass the precursor ion of interest, which is transmitted to Q2, where it collides with a neutral gas (N_2 or Ar) and dissociates to various fragment ions. Rather than mass analysing the resulting product ions, the linear ion trap (LIT) is set to trap and hold a 2-*m/z*-unit–wide window centered on the product ion of interest. The selected *m/z* is irradiated with a single wavelength amplitude frequency to induce further fragmentation to secondary product ions, which are then scanned out of the LIT to the detector. The resulting MS^3 spectrum shows the fragmentation pattern of the selected product ion, and yields additional structural details regarding the primary product ion.

MS^3 analysis provides critical structural information about higher order sphingolipids (such as gangliosides) that is not provided in the MS/MS spectrum. Typically, MS/MS data of these ions do not reveal any information about the components of the ceramide backbone. MS^3 analyses of the Y_0product ions (*m/z* 592.6), which comprise the core lipid part of the molecule, will determine the composition of the ceramide. In the example shown in **Fig. 8** the highly abundant S, T, U, and V + 16 ions (*m/z* 324, 308, 282, and 283, respectively) reveal that the fatty acid is C18:0, and the complementary P and Q ions (*m/z* 265 and 291, respectively) are characteristic of a d20:1 sphingoid base. Thus, MS^3 scans provide an additional level of structural analysis yielding critical information regarding sphingoid base, fatty acid, and headgroups in glycosphingolipids.

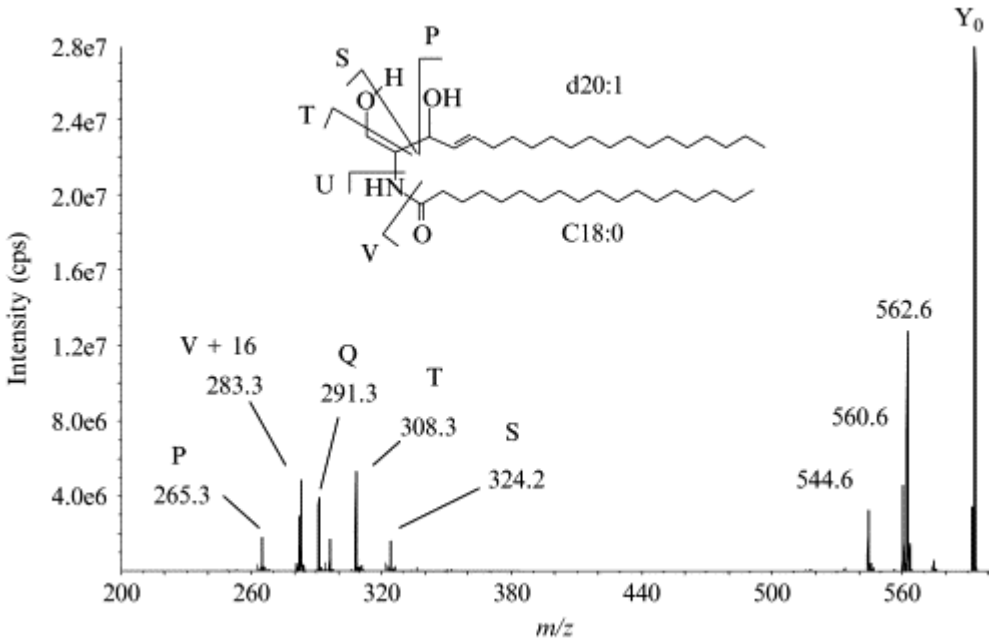

Figure 8. MS/MS/MS (MS^3) analysis of the Y_0 product ions (*m/z* 592.7) from Fig. 7 to reveal the highly abundant S, T, U, and V + 16 ions (*m/z* 324, 308, 282, and 283, respectively) that establish that the fatty acid is C18:0, and the complementary P and Q ions (*m/z* 265 and 291, respectively) that are characteristic of a d20:1 sphingoid base.