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Sphingolipidomics: High-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry

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Abstract

Sphingolipids are a highly diverse category of compounds that serve not only as components of biologic structures but also as regulators of numerous cell functions. Because so many of the sphingolipids in a biological system are bioactive and are often closely related structurally and metabolically (for example, complex sphingolipids ↔ ceramide ↔ sphingosine ↔ sphingosine 1-phosphate), to understand the role(s) of sphingolipids in a given context one must conduct a "sphingolipidomic" analysis—i.e., a structure-specific and quantitative measurement of all of these compounds, or at least all members of a critical subset. Liquid chromatography tandem mass spectrometry (LC MS/MS) is currently the only technology with the requisite structural specificity, sensitivity, quantitative precision, and relatively high-throughput capabilities for such analyses in small samples ($\sim 10^6$ cells). This review describes a series of protocols that have been developed for the relatively rapid analysis of all of the molecular species from 3-ketosphinganines through sphingomyelins and some glycosphingolipids (including all the compounds that are presently regarded as sphingolipid "second messengers") using normal- and reverse-phase LC to separate isometric and isobaric species (such as glucosylceramides and galactosylceramides) in combination with triple quadrupole (for MS/MS) and hybrid quadrupole–ion trap (for MS^3) mass spectrometry. Also discussed are some of the issues remaining to be resolved in the analysis of the full sphingolipidome.

Keywords: Sphingolipid analysis; Lipid mass spectrometry; Bioactive sphingolipids; Sphingolipid metabolism; Lipidomics

1. Introduction

The family of compounds termed sphingolipids is one of the most complex and structurally diverse due to the large number of possible combinations of hydrophobic backbones and headgroups (each of which number in the hundreds, hence, the possible combinations are in the tens of thousands) [1]. Sphingolipids are highly bioactive compounds that serve not only as components of biologic structures such as membranes and lipoproteins, but also as regulators of cell proliferation, differentiation, cell–cell and cell–matrix interactions, cell migration, intracellular (and extracellular) signaling, membrane trafficking, autophagy, and cell death [1–3]. For most of these functions, there is structural specificity with respect to the major sphingolipid subclass (for example, in many cases ceramides are growth inhibitory whereas sphingoid base 1-phosphates usually stimulate growth) [4]. And within subclass, changes as minor as the presence or absence of a double bond can have a major impact on function [5].

It should be evident from this brief summary that studies of the roles of sphingolipids in biologic systems must analyze all the possible subspecies within each class

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with respect to the sphingoid base type, the fatty acid sidechain, and headgroup. Liquid chromatography, electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS)[1] is the only method that has been shown to date to be able to provide an in-depth profile of all of the "signaling" sphingolipids (i.e., all the backbones including ceramides, sphingoid bases, and sphingoid base 1-phosphates as well as species such as ceramide 1-phosphate, sphingosylphosphocholine, *N*-methyl-sphingoid bases, and others) in small samples (e.g., $\sim 10^6$ cells) [6–9]. This methodology, including sample preparation and data analysis, will be described in this review. In addition, some newer developments using a hybrid quadrupole-trap instrument (the ABI 4000 Q Trap) (Applied Biosystems, Foster City, CA) will be described because it allows extension of the analyses to more complex glycosphingolipids to help provide a complete picture of all of the species in a biologic system.

2. Structures of sphingolipids

Sphingolipids are characterized by their "sphingoid" base backbones, which are long-chain alkanes or alkenes (sometimes with additional unsaturation) of approximately 14–20 carbons in length, with an amino group at position 2 and hydroxyl-substituents at positions 1 and 3, as shown in Fig. 1 for sphingosine (which is also called sphing-4-enine, 2-aminooctadec-4-ene-1,3-diol, and *trans*-D-erytho-2-amino-octadec-4-ene-1,3-diol). The alkyl chain length is usually defined fairly narrowly for a given species, for example, in humans the sphingoid bases are almost entirely 18 carbons in length with the exception of brain gangliosides, where substantial amounts of 20-carbon species appear with advancing age.

Other common variations are for the alkyl chain to be fully saturated (which have the name "sphinganines") or have an additional hydroxyl at position 4 (which is named 4-D-hydroxysphinganine, (2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecanetriol, or phytosphingosine—the latter being a misnomer since this compound is not unsaturated). Mammals have small amounts of sphingoid bases with hydroxyl groups at other positions, such as carbon 6 (a species found in skin) and plants and fungi have sphingoid bases with double bonds at other positions (for example, sphing-8-enine and 4-hydroxy-8-sphingenine) and methyl-branched species. An abbreviated nomenclature for sphingoid bases is for the number of

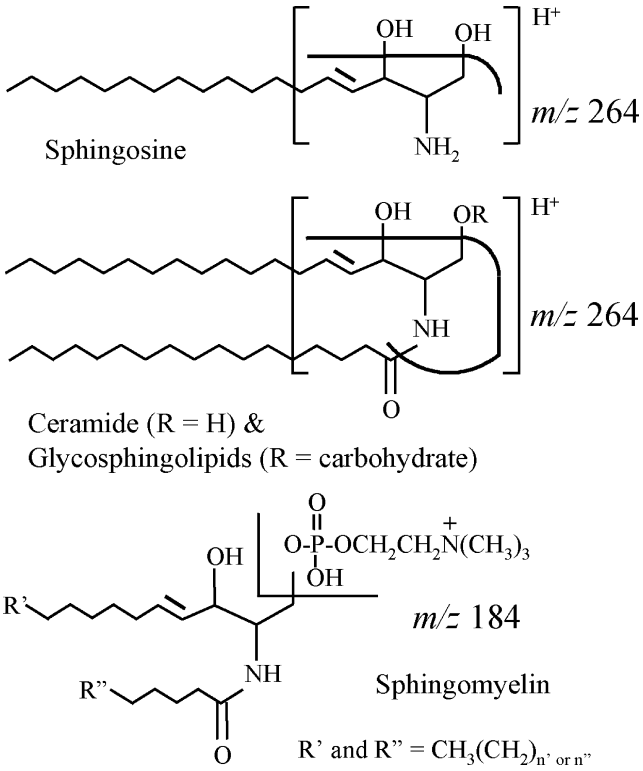


Fig. 1. General structures of sphingolipids and sites of cleavage.

hydroxyls to be designated by "d" (di-) or "t" (tri-) followed by the number of carbons, then the number and position of the double bonds; for example: d18:1$^{\Delta 4}$ for sphingosine, d18:2$^{\Delta 4,8}$ for 4,8-sphingediene (or sphing-4,8-diene), t18:0 for 4-D-hydroxysphinganine, and t18:1$^{\Delta 8}$ for 4-D-hydroxysphing-8-enine.

Free sphingoid bases are usually present in cells in very small amounts, with most being derivatized on the 2-amino group with a long-chain fatty acid and conjugated at the 1-hydroxyl with a polar headgroup to produce "complex" sphingolipids. The *N*-acyl-derivatives are generically called "ceramides" although this term is more often applied specifically to *N*-acylsphingosines to distinguish them from dihydroceramides (*N*-acylsphinganines). The fatty acids vary in chain length (14–32 carbon atoms), degree of unsaturation (but are mostly saturated), and presence or absence of a hydroxyl group on the α- (or in skin, the ω-) carbon atom. The headgroups are mainly linked by phosphodiester bonds (e.g., sphingomyelins, ceramide phosphoethanolamines, and ceramide phosphoinositols) and glycosidic bonds (glycosphingolipids). Glycosphingolipids are classified into broad types on the basis of carbohydrate composition [1,10]. The neutral glycosphingolipids of mammals contain uncharged sugars such as glucose (Glc), galactose (Gal), *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), and fucose (Fuc) (and in other organisms, mannose and other sugars). Acidic glycosphingolipids contain ionized functional groups such as phosphate, sulfate (sulfatoglyco-

[1] *Abbreviations used:* Cer, ceramide (*N*-acylsphingosine); DHCer, dihydroceramide (*N*-acylsphinganine); ESI, electrospray ionization; GalCer, galactosylceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; LC, liquid chromatography; LysoSM, sphingosylphosphocholine; MALDI, matrix-assisted laser desorption ionization; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; Q, quadrupole; SM, sphingomyelin.

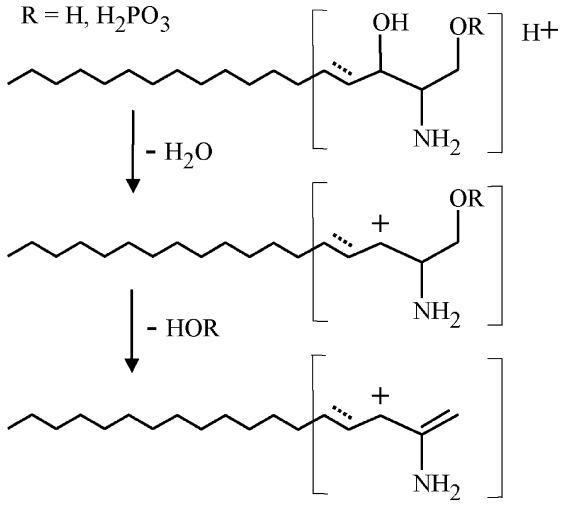
sphingolipids), or charged carbohydrate residues such as sialic acid (*N*-acetylneuraminic acid) in gangliosides or glucuronic acid in some plant glycosphingolipids. Gangliosides are often denoted by the "Svennerholm" nomenclature that is based on the number of sialic acid residues (e.g., GM1 refers to a monosialo-ganglioside) and a number reflecting, in many instances, the relative position of the ganglioside upon thin-layer chromatography (for example, the R_f of GM3 > GM2 > GM1). A few sphingolipids are referred to by their historic names as antigens and blood group structures, such as Forssman antigen (a pentosylceramide that is found in many mammals) and the Lewis blood group antigens (a family of α1-3-fucosylated glycan structures, which include Lewis x and sialyl Lewis x, etc.) [10].

In recent years, considerable attention has been given to other types of sphingoid base derivatives, namely the 1-phosphates and other "lyso" types of compounds (sphingosyl-phosphocholine, galactosylsphingosine, etc.) because these are highly bioactive. *N*-Methyl-derivatives (*N*-methylsphingosine, *N*,*N*-dimethylsphingosine, and *N*,*N*,*N*-trimethylsphingosine) have also been found in biological systems, but little is known about their origin or function(s).

3. Why select mass spectrometry for analysis of sphingolipids?

Sphingolipids have been studied using mass spectrometry for at least four decades [11,12] using many ionization methods, including MALDI [13–16] and electrospray [6–9,17–25], and mass analyzers, such as sectors [26], quadrupoles (Q) [6,7,20], time-of flight (TOF) [13–15,17–19], ion traps [9,25], and Fourier-transform ion cyclotron resonance (FT-ICR) [16]. These studies have established that mass spectrometry is the method of choice for a sphingolipidomic analysis because it provides: (a) a high level of specificity with regard to identification of complex compounds via molecular mass, especially when analyzed by tandem mass spectrometry (MS/MS); (b) levels of sensitivity that are orders of magnitude lower than classical techniques, so compounds can be detected even if they are present in fmol amounts per $\sim 10^6$ cells (or sometimes less); (c) a signal response can be correlated to analyte concentration provided there are appropriately matched internal standards to normalize for differences in ionization and fragmentation of individual molecular species; and (d) a dynamic range of several orders of magnitude for some forms of MS/MS, which allows analysis of compounds that vary in abundance over this same range in biological samples (for example, sphingomyelins versus backbone sphingolipid signaling metabolites). Nonetheless, to achieve these goals, a number of decisions regarding the handling of the samples (both extraction methods and separation of isomeric and isobaric species prior to mass spectrometry), internal standards, ionization technique, instrument, and mass spectrometry scanning method must be made judiciously.

The success of mass spectrometric analyses fundamentally depends on the gas-phase chemistry of the compounds of interest and whether or not they produce ions that are useful as unique and sensitive identifiers for the individual species. In this regard, sphingolipids have proven to be particularly amenable to mass spectrometric analyses because they are relatively easily ionized, and many fragment to products that are characteristic for the headgroup and backbone subclasses. For example, both long-chain bases and complex sphingolipids readily ionize via positive-ion ESI to form primarily $(M+H)^+$ ions, and sphingoid base-1-phosphates, SM, sulfatides, and gangliosides may also form $(M-H)^-$ ions via negative-ion ESI. Furthermore, the fragmentation profiles for both sphingoid bases (Figs. 1 and 2) and many complex sphingolipids (Figs. 1 and 3) provide information about both the headgroups and types of sphingoid bases and fatty acids in the backbones. However, when fragmentation leaves the ion on the headgroup rather than the ceramide backbone (as occurs with SM, for example), only the overall composition of the backbone is determined, not the nature of the individual components, unless additional techniques (MS/MS/MS, as will be described later) are used. To deal with the complexity of the compounds that are being



Sphingosines	*m/z* 264
Sphinganines	*m/z* 266
4-Hydroxysphinganines	
(double dehydration)	*m/z* 282
(triple dehydration)	*m/z* 264

Fig. 2. Major route of dissociation of free sphingoid bases (shown for sphingosine, where the dashed line is a 4,5-double bond, and sphinganine) via single and double dehydration. The free sphingoid base and the 1-phosphates fragment similarly.

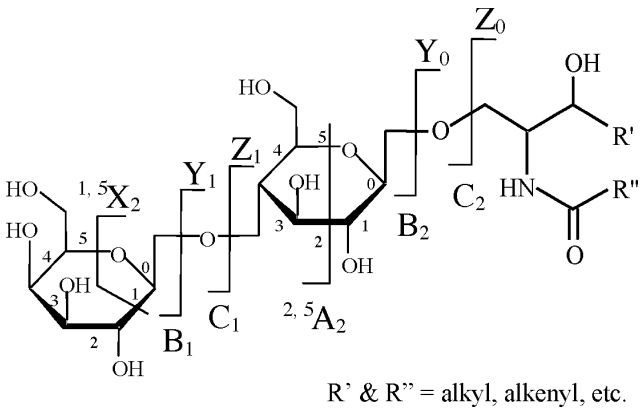


Fig. 3. Examples of headgroup cleavage sites in complex glycosphingolipids and nomenclature. Capital letters are used to label cleavages where the charge is retained on the fragment starting from the carbohydrate end of the compound (A, B, C…) versus cleavages where the charge is retained on the fragment with the ceramide backbone (…X, Y, Z).

analyzed (especially for the higher order glycosphingolipids) and, hence, the potential number of fragments, a systematic nomenclature has been developed which describes these fingerprint fragmentations [26–29].

4. Sample preparation

One of the challenges in working with sphingolipids is that some of the species are highly water soluble (e.g., sphingosine 1-phosphate and the more complex glycosphingolipids, especially gangliosides with multiple sialic acids) whereas others are highly hydrophobic (e.g., ceramides); therefore, there is usually loss of some species using the standard two-phase extraction methods (e.g., Bligh-Dyer and Folch) that are applied to other lipids. While this has sometimes been utilized to fractionate subcategories of sphingolipids (for example, by using the organic phase for analysis of SM, ceramides, etc. and saving the aqueous phase for analysis of gangliosides), this approach can be less effective when profiling all the species in a biological sample because some compounds may be lost in the solvent interface. One approach is to prepare an initial azeotrophic solvent mixture and use this for analysis of the most polar species, and carry a portion through the usual two-phase extraction for analysis of the less polar compounds in the organic phase, as described in Protocol 1.

4.1. Protocol 1: an extraction procedure for the preparation of sphingolipids for analysis by LC MS/MS

This protocol is designed for analysis of a cell pellet containing approximately $1–10 \times 10^6$ cells, which are usually obtained by scraping the cells from culture dishes in a small volume of phosphate-buffered saline (PBS) then centrifuging the suspended cells to allow removal of excess PBS for a final volume of 0.05–0.1 ml. It is also applicable to tissue homogenates or subcellular fractions that contain ~1–10 mg of protein in 0.05–0.1 ml, or for approximately 10–20 μl of whole blood, serum or plasma. Cell culture medium can also be analyzed, but the amount of protein should fall within the range described above (for studies of sphingolipids in culture medium, we often reduce the serum to 1% and extract 1–2 ml of medium after the water has been removed or reduced by lyophilization). When lyophilized samples (including cells or other materials) are analyzed, the equivalent volume of water is added to the extraction solvents in step 2.

1. Place the samples in 13×100 mm screw-capped, borosilicate glass test tubes with Teflon caps. It is usually better to place the samples in these tubes as soon as possible (if possible, before freezing and thawing) to minimize clumping and other losses (if samples are lyophilized, they should be lyophilized in the tubes that will be used for the extraction).
2. Add 0.5 ml of methanol then 0.25 ml of chloroform and the internal standards. The internal standards are prepared as a working stock in methanol and chloroform (2:1 v/v) that can be added in a small volume to each test tube, or they can be mixed with the 0.25 ml of chloroform that is added above. The composition of the internal standard mixture will depend on the analyses that are being conducted, but for a general survey, it is prepared to deliver 0.5 nmol of each of the following (per sample): ceramide (d18:1/12:0-Cer), sphingomyelin (d18:1/12:0-SM), glucosylceramide (d18:1/12:0-GlcCer), and lactosylceramide (d18:1/12:0-LacCer) (note that all have a 12-carbon fatty acid sidechain), and C17-sphingosine, C17-sphinganine, C17-sphingosine 1-phosphate, and C17-sphinganine 1-phosphate (the C17-chain length is not found in most samples, but this should be verified for each new type of sample that is analyzed). All of these are available from Avanti Polar Lipids (Alabaster, AL). If the samples already contain water, proceed to step 3; if not, add 0.05–0.1 ml of water now.
3. Sonicate the test tubes in a bath-type sonicator until they appear evenly dispersed, then incubate overnight at 48 °C in a heating block. This heating step can often be shortened, but it is conventional in extraction of sphingolipids [30] because they have high phase transition temperatures.
4. Cool the tubes and add 75 μl of 1 M KOH in methanol, sonicate, and incubate for 2 h at 37 °C. This step removes most of the interfering glycerolipids, in particular phosphatidylcholines that can mask sphingomyelins in a simple MS scan (see Section 6).
5. Cool the samples to room temperature and transfer half (0.4 ml) to a new test tube. This portion (named Extract A) will be used for separation of the more

polar sphingolipids by reverse-phase LC: remove the solvent using a Speed Vac-type concentrator (ThermoSavant), and redissolve in the reverse-phase LC solvent as described in Section 5.

6. To the half of the extract that remains in the original test tube, add 3 µl of glacial acetic acid to bring the pH near neutral, add 1 ml of chloroform and 2 ml of water, mix (vortex), and centrifuge to separate the phases.
7. Carefully remove the upper layer with a pasteur pipette (discard), leaving the interface (w/some water). Evaporate the solvent from the lower layer (named Extract B) using a Speed Vac-type concentrator (ThermoSavant), and redissolve in the normal-phase LC solvent as described in Section 5.

The extracts are kept refrigerated and should be analyzed as soon as possible to minimize possible changes in composition. Based on disappearance of the internal standards, the sphingoid bases appear to be most unstable, but this does not become noticeable for a few weeks. Some samples have been reanalyzed for Cer, SM, and GlcCer after several years of storage and produced results that were not discernibly different from the original. Note that Extract A should contain all the sphingolipids that are recovered in Extract B (and Extract B will have most, but not all, of those in Extract A); therefore, it is possible to use the extracts for analyses other than the ones for which they were originally intended if circumstances require.

5. Liquid chromatography

Because more than one compound can have the same elemental composition but different structures, liquid chromatography (LC) has been used fairly widely to prepare samples for analysis by mass spectrometry [6–9,21,25,31–34]. The goal of the LC separation is somewhat different in LC–MS/MS than for conventional liquid chromatography because the mass spectrometer is able to discriminate between many of the components of a mixture (for example, all of the fatty acid chain length variants of Cer), therefore, the purpose of the chromatography is 2-fold: (a) to separate isomeric and isobaric species that cannot be distinguished by mass spectrometry alone, and (b) to reduce the number of compounds that are being analyzed in a given volume of eluate to improve accuracy and sensitivity. The latter can also be improved by decreasing the flow rate of the mobile phase, however, this increases the run time, so the goal is to achieve an acceptable balance between these factors.

Two types of chromatography have proven to be very useful with sphingolipids: reverse-phase LC for separations based on differences in hydrophobicity (for example, to separate sphingosine and sphinganine) and normal-phase LC to separate compounds based mainly on differences in their polar components (for example, Cer from SM, etc.). It may not be obvious why some separations are needed, for example, that of sphingosine and sphinganine since their precursor and product ions have easily resolved *m*/*z*. However, when a biologic sample contains much higher amounts of sphingosine, the ^{13}C isotopic component of sphingosine will interfere with the quantitation of sphinganine unless the compounds have been separated by LC. There have been reports of methods to analyze sphingolipid by "shotgun" techniques where the samples are infused directly into the mass spectrometer, which has the advantage of being simpler and potentially more sensitive [35]. Unfortunately, these are only valid under the special circumstance where isometric and isotopic species do not co-exist, which is rare,[2] or if the samples have already undergone chromatography.

5.1. Protocol 2: reverse-phase LC

Free long-chain sphingoid bases, sphingoid base 1-phosphates, lyso-sphingolipids, *N*-methyl-derivatives of sphingoid bases, and ceramide 1-phosphates are separated by reverse-phase LC before analysis by MS/MS [6]. The selection of the LC column depends somewhat on the number of compounds to be analyzed because short columns have the advantage of very rapid run times, but a longer column is sometimes needed for greater separation. The following protocol is for a typical short-column LC separation would use a 2.1 × 50 mm Discovery C_{18} column (Supelco, Bellefonte, PA) packed with 5 µm particles, with the eluent directly connected to the mass spectrometer.

1. Dissolve the sample (Eluant A from Section 4.1) in 0.2 ml of mobile phase RA and RB at 80:20, v/v, with RA = methanol–H_2O–formic acid (74:25:1, v/v/v), and RB = methanol–formic acid (99:1,v/v); both RA and RB also contain 5 mM ammonium formate. Sonicate to disperse fully, then centrifuge to clarify before transferring most of the sample to an autosampler vial.
2. Pre-equilibrate the LC column with RA–RB (80:20, v/v) for 0.5 min then inject the sample (typically 10–50 µl).
3. Continue the mobile-phase RA–RB at 80:20 for 0.6 min (at a flow rate of 1 ml/min) then begin a 1.8-min linear gradient to 100% RB, and hold at 100% RB for 0.6 min.

[2] Unfortunately, even if there is a literature precedent for a system containing only one species (for example, GlcCer but not GalCer), it is risky to assume this does not change. We have noted, for example, that the appearance of GalCer in Hek293 cells depends on the cell culture conditions (J. Kollmeyer, J. Allegood, unpublished observations).

4. After each sample, equilibrate the column with RA–RB at 80:20, v/v, for 0.3 min before injecting the next sample.

An example chromatogram is shown in Fig. 4 where the eluates have been analyzed using the ABI 4000 Q Trap with identification of each species by MRM (see Section 6.4). Note that it is possible to complete each run in approximately 3 min. Only one transition appeared as two peaks (the 20-carbon homolog of sphinganine, d20:0) and thus appears to generate a false signal (i.e., one that appears at the elution time for another sphingoid base, C20:1). The false signal is generated because this transition differs from d20:1 in both the precursor and product ion transition by only 2 *m*/*z* units, so the portion of the d20:1 that contains two ^{13}C will appear in the d20:0 transition. The natural isotopic contribution of ^{13}C in the d20:1 ion is ~2.7%, which closely approximates magnitude of this peak. Quantitation can be performed using the data shown in Fig. 4 because the two peaks observed for the d20:0 transition (the true d20:0 eluate and the d20:1 isotope) are baseline resolved. Alternatively, the isotopic contribution can be eliminated by choosing a product ion transition of *m*/*z* 60, which does not display this behavior (not shown).

When analyzing multiple samples, load each series into the autoinjector with the first vial containing only the mobile phase (to check the cleanliness of the column, injector needle, etc. of the LC system), the next vial containing the internal standard mixture (added directly to the vial to verify the elution times and areas for the standards), then only mobile phase in the next vial to detect sample-to-sample carryover. If unacceptably high amounts of carryover are found (and this cannot be eliminated by adjusting the autoinjector needle or changing the LC tubing or the guard or main LC column), insert a vial containing only mobile phase between each sample vial. After running the samples (or more frequently, if necessary), reanalyze a vial containing the internal standard mixture to ensure that neither the LC elution times nor the performance of the MS have changed during the run.

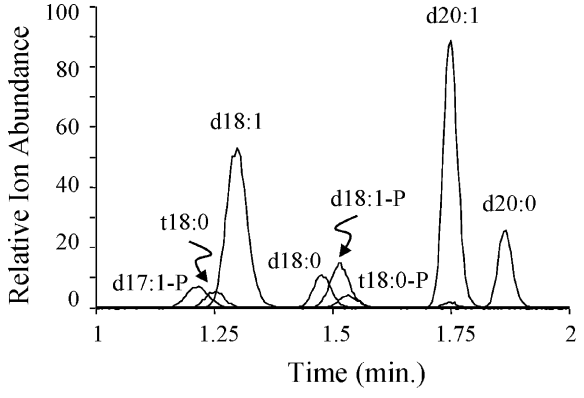


Fig. 4. Reverse-phase LC–MS/MS with the extracted ion chromatograms for the free long-chain base and 1-phosphate standards analyzed using the Q-Trap 4000 using the transitions shown in Table 1B. See *Protocol 2* in the text for more information about the chromatography conditions.

In general, we have found reverse-phase LC to be most useful for sphingoid bases (including 3-ketosphinganine), sphingoid base 1-phosphates, *N*-methyl-(mono-, di-, and tri-) sphingoid bases, lysosphingolipids (sphingosylphosphocholine and psychosine), and ceramide 1-phosphates [6–9].

5.2. Protocol 3: normal-phase LC

Analysis of complex sphingolipids is usually performed using normal-phase LC–MS/MS, which provides several advantages versus reverse-phase. Most importantly, the sphingolipids are separated largely by headgroup, which means that all chain lengths within a given class of sphingolipids elute simultaneously (including the shorter chain, C12-internal standard); therefore, all will be ionized under identical solvent composition conditions, which avoids most ionization efficiency issues. The main exceptions are sphingolipids that have ceramide backbones with extra hydroxyl groups in the sphingoid base (e.g., 4-D-hydroxysphinganine) or fatty acid (e.g., α-hydroxy fatty acid), which generally elute after their counterparts without the extra hydroxy groups. In addition, because the samples have been extracted into chloroform prior to analysis by LC–MS/MS, there is less interference from salts and other components that might suppress ionization as well as from alkali metals (Na^+, Li^+, etc.) that can form adducts. Because all of the subspecies of each sphingolipid type elute together, it is absolutely necessary to analyze the eluate by tandem mass spectrometric methods (such as multiple reaction monitoring, which will be described in Section 6.4), to distinguish the subspecies as their individual precursor/product ion *m*/*z* pairs.

The following protocol is for a typical short-column, LC separation using a 2.1 × 50 mm LC-NH_2 amino column (Supelco, Bellefonte, PA) with the eluent directly connected to the mass spectrometer (see Fig. 5).

1. Dissolve the sample (Eluant B from Section 4.1) in 0.2 ml of mobile-phase NA where NA = acetonitrile–methanol–acetic acid (97:2:1, v/v/v) containing 5 mM ammonium acetate. Sonicate to disperse fully, then centrifuge to clarify before transferring most of the sample to an autosampler vial.
2. Pre-equilibrate the LC column with 100% NA for 0.5 min at a flow rate of 1.5 ml/min then inject the sample (typically 10 μl).
3. Continue the mobile-phase NA for 0.5 min followed by a linear gradient to NA–NB (90:10, v/v) in 0.2 min (where NB = methanol–acetic acid (99:1, v/v) containing 5 mM ammonium acetate) and hold in this mobile phase for 0.5 min, then increase NB to an NA–NB

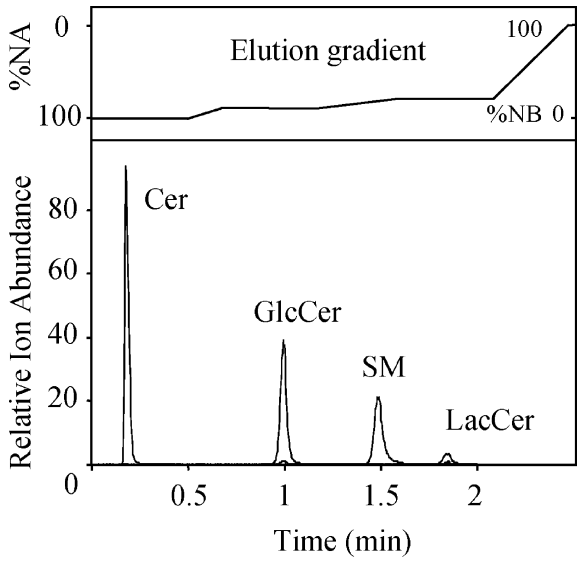


Fig. 5. Normal-phase LC–MS/MS extracted ion chromatograms of the complex sphingolipid standards (i.e., w/C12 fatty acids) for ceramide (Cer), glucosylceramide (GlcCer), sphingomyelin (SM), and lactosylceramide (LacCer) using the 4000 Q Trap using the transitions shown in the tables. The upper panel shows the % of mobile phase A and B at each timepoint. See *Protocol 3* in the text for more information about the chromatography conditions..

ratio of 82:18 (v/v) over 0.4 min and hold at this composition for 0.6 min, ending with a linear gradient to 100% NB over 0.4 min.

4. After each sample, equilibrate the column with 100% NA for 0.3 min before injecting the next sample.

Load each series of samples into the autoinjector in the order: (1) a vial containing only the mobile phase, (2) a vial containing the internal standard mixture, (3) a vial containing only mobile phase, (4) sample 1, (5) a vial containing only mobile phase, (6) sample 2, etc. until the end, when the last vial should contain the internal standard mixture to ensure that neither the LC elution times nor the performance of the MS have changed during the analyses.

5.3. Protocol 4: normal-phase LC to separate GlcCer and GalCer

When samples contain both GlcCer and GalCer with similar ceramide backbones, these isomers are not resolved using the amino column in Protocol 3, but can be separated using a Supelco LC-Si column (2.1×250 mm and 5 μm particles). The separation is achieved by isocratic elution using 100% mobile-phase NA at a flow rate of 1.5 ml/min (total elution time, ca. 7 min).

6. Mass spectrometry

The mass spectrometric methods described in this article use electrospray ionization and either triple quadrupole or hybrid quadrupole–ion trap mass spectrometers as the mass analyzers. Descriptions of these are presented in the following subsections.

6.1. Electrospray ionization

Analysis of the eluates from the LC columns by mass spectrometry requires removal of the solvent and ionization of the sphingolipids by some means. This can be conducted by collection of LC fractions and analysis by a method such as MALDI or by flowing the LC eluate directly into the mass spectrometer and generating intact molecular ions by electrospray ionization (ESI) [6–9]. In ESI, the LC eluate is infused into an ion source through a needle or capillary held at a high potential (1–6 kV) to form charged droplets. As the droplets enter the mass spectrometer they undergo rapid desolvation due to the high vacuum inside the mass spectrometer and the neutral solvent molecules are pumped away. The positively or negatively charged analytes (depending on which mode of ionization and ion selection is being used) are then separated by various means that are described in Section 6.2. Modern mass spectrometers are capable of handling a wide range of flow rates, from μl per min for capillary LC to ml per min for larger columns, with the latter requiring a very high vacuum, heating of the ion source and/or the assistance of a bath gas to aid desolvation.[3]

ESI is a soft ionization technique (i.e., ions are formed with little fragmentation in the ion source) and the signal response is often proportional to analyte concentration over several orders of magnitude, so it is nearly ideal for quantitation. A limitation of ESI is that solvent ions and other co-eluting species may interfere with detection of the compounds of interest, particularly at lower *m*/*z* ratios where solvent ions predominate. However, as long as the interfering ions and co-eluants do not suppress the formation of ions of interest (as happens, for example, in samples that contain high concentrations of salts), the ions of interest can often be differentiated from solvent ions by use of MS/MS (as described below). Tandem mass spectrometry is generally the method of choice for profiling sphingolipids because many of the ions of interest (e.g., sphingoid bases, sialic acid, etc.) are in the *m*/*z* range where solvent ions and clusters interfere.

[3] A recently developed alternative to injecting the LC eluates directly into the mass spectrometer is to collect the eluates and either mix them with MALDI matrix material for analysis as spots on a MALDI plate (which have been applied automatically with a device such as a Probot) (LC Packings/Dionex, Sunnyvale, CA) or to load them into capillaries that are automatically infused using a device such as a NanoMate automated nanoelectrospray device (Advion BioSciences, Ithaca, NY).

6.2. Tandem mass spectrometry

Tandem mass spectrometry (MS/MS) may be generally defined as the arrangement of two mass spectrometers in series so that the ions formed in the ion source can be selected by the first mass spectrometer, then fragmented, and the fragments analyzed using the second mass spectrometer. The fragment ions provide important clues about the structure and reactivity of the intact molecular ion, but are also useful practically as a signature that identifies an ion of interest against a background of ions that might have the same or similar *m*/*z* in the first mass spectrometer. The fragment ions may be formed by metastable dissociation (M*), meaning decomposition of molecular ions from internal energy inherent to them when they are formed (these fragment ions are generally low energy and reveal cleavage of the weakest chemical bonds), or by collision-induced dissociation (CID), which arises from collision(s) between analyte ions in motion and neutral gas molecules (in CID, the energy deposited, and hence the degree of fragmentation, can be controlled by changing the velocity of the precursor ions or the mass of the target gas).

The results with sphingolipids that are described in this review have been obtained using two types of tandem mass spectrometers: a triple quadrupole mass spectrometer (the API 3000) and a hybrid quadrupole–linear ion trap tandem mass spectrometer (the ABI 4000 Q-Trap). The latter instrument will be used to demonstrate several mass spectral scanning techniques with regard to their role in identification, and structure elucidation of complex sphingolipids prior to analysis via LC–MS/MS. Both instruments perform tandem mass spectrometric analyses such as precursor, product, and neutral loss scans which can be combined with multiple reaction monitoring (MRM) for the analysis of both simple and complex sphingolipids in a high-throughput mode. Additionally, the ABI 4000 Q-Trap provides enhanced resolution of higher order glycosphingolipids, enhanced product ion analysis, and more complete structure elucidation using MS/MS/MS (MS^3).

Selection of the type of mass spectrometer depends on a number of factors. Triple quadrupole instruments such as the API 3000 and 4000 (Applied Biosystems, Foster City, CA) tend to be robust and sensitive for all of the backbone sphingolipids (sphingoid bases, sphingoid base 1-phosphates, and ceramides), SM, and glycosphingolipids with one or two carbohydrates (GlcCer, GalCer, and LacCer) (detecting all of these in pmol amounts); however, in our experience, the API 3000 has not been as useful for higher order glycosphingolipids or for analysis of anionic sphingolipids in the negative-ion mode. The 4000 Q Trap was strikingly more sensitive for these compounds (for example, gangliosides could be analyzed in fmol amounts); furthermore, the MS^3 function allows identification of both the carbohydrate headgroups and the ceramide backbones of complex glycosphingolipids [9]. A recent report states that the 4000 Q Trap is also able to characterize the backbone composition of SM [25], and we have found it also to be useful for sulfatide analysis by following the product ion for the sulfate, *m*/*z* 96.9, in negative-ion mode with backbone determination using MS^3 (although the charge retention by the ceramide is 2 orders of magnitude lower than the sulfate upon fragmentation) (J. Allegood et al., unpublished observations).

For both types of MS/MS, a pre-MS separation procedure such as LC is required to distinguish isotopic, isomeric, and isobaric species (as illustrated above for the double ^{13}C-isotope of C20-sphingosine (d20:1) appearing at the same transition, but different elution time, as C20-sphinganine (d20:0). This contribution could be corrected arithmetically, however, if the species making the isotopic contribution is present in much higher amounts, this can seriously compromise the accuracy of the analysis of the more minor species). Other examples are the separation of D-*erythro*-sphinganine and L-*threo*-sphinganine, which is of interest because the latter compound, which is also called "safingol," is undergoing clinical trials as a possible anti-cancer drug, and the closely related monohexosylceramides, GlcCer and GalCer. It is prudent to keep in mind that for most compounds there are alternative structures (such as enantiomers) that are also consistent with the mass spectrometric data, but are not considered because previous investigations using complementary methods such as NMR have not found them in biological systems. Nonetheless, considering the high level of sensitivity of modern instruments, it is likely that some unexpected variants will be detected where the biosynthetic enzymes are highly selective but not absolutely specific.

6.3. Tandem mass spectrometric scanning techniques: product ion analysis, MS^3, precursor ion analysis, and neutral ion loss

Due to the complexity and diversity of the structures of sphingolipids, a number of MS/MS techniques are useful for their analysis:

Product ion analysis. In a product ion scan, the first mass analyzer (referred to Q1 for the triple quadrupole MS/MS or the Q-Trap) is set to pass a single ion of interest (*m*/*z*). This precursor ion is transmitted to a chamber (Q2) that is filled with a neutral gas such as N_2 or Ar, and operated in a mode to pass all ions. In Q2, the precursor ions undergo multiple collisions with the target gas and are induced to decompose. The product ions are transmitted to the second mass analyzer (Q3), which is scanned across a range of *m*/*z* values to allow the fragment ions to reach the detector sequentially. The resulting tandem mass spectrum shows the fragmentation pattern of the selected precursor ions, and yields structural information in the

form of both product ions detected and neutral species lost. The relative ion abundances of the product ions detected are reflective of the kinetics of the various dissociation pathways and vary with collision energy.

Analysis of long-chain bases such as sphingosine (d18:1), sphinganine (d18:0), 4-D-hydroxysphinganine (t18:0), 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, 300/282, 310/292, and 312/294, respectively, as were shown in Fig. 2. The single dehydration products are much more abundant than the double dehydration products over a range of collision energies. Long-chain base-1-phosphate derivatives undergo a similar dehydration and cleavage of the headgroup to yield the same *m/z* product ions described above, however, this does not interfere with their being analyzed in the same run as the other sphingolipids because they are distinguished by their precursor ion mass (and retention times on LC).

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 O″ fragment ions (Fig. 1) [7]. 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.

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 bond with loss of the carbohydrate headgroup as a neutral species with charge retention remaining on the ceramide moiety (forming the Y_n/Z_n, where $n=0,1$, type ions) (Fig. 3); and cleavage of both the sugar headgroup and the fatty acid acyl chain at higher energies with charge retention on the dehydrated sphingoid base (yielding predominantly N″ ions, which are structurally identical to the O″ ions from ceramides) (the difference in nomenclature is attributed to the GlcCer and LacCer having a headgroup other than a hydrogen atom) (Figs. 1 and 3).

Sphingolipids containing phosphodiester-linked headgroups, such as in 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 C ions of *m/z* 184 [6–9]. Ceramide phosphoethanolamines (CPE) also fragment at the phosphate–ceramide bond, but the headgroup is lost as a neutral species of mass 141 u [9]. This difference in charge retention for SM and CPE probably results from differences in the gas-phase basicity of the quaternary and primary nitrogens of phosphocholine and phosphoethanolamine.

For gangliosides, the $(M-2H)^{2-}$ ions fragment to yield highly abundant $C_{1\beta}$–H_2O ions of *m/z* 290 which reflect the sialic acid moiety when analyzed by the triple quadrupole MS/MS. The enhanced product ion scan feature that is available using the ion trapping function on the 4000 Q Trap yields much more structural information because it provides better sensitivity and more abundant high mass product ions (Fig. 6, lower panel). Furthermore, cleavage at glycosidic bonds also produce characteristic Y_n-type ions and through ring cleavages such as $^{2,4}X_{2\alpha}$ and $^{2,4}X_{3\alpha}$ (*m/z* 1323.8 and 1484.8, respectively), which are useful for determination of glycosidic bond linkage.

MS/MS/MS (MS³) analysis. An MS^3 analysis is performed in much the same way as a product ion scan: the first mass analyzer (in this case, Q1) is set to pass the selected precursor ion of interest, which is transmitted to Q2 to collide with a neutral gas (N_2 or Ar) and decompose to product ions that enter the linear ion trap (LIT) of the 4000 Q Trap. Instead of proceeding to the detector (as occurs in triple quadrupole MS/MS), the LIT is set to trap and hold a 2 *m/z* unit wide window centered on the

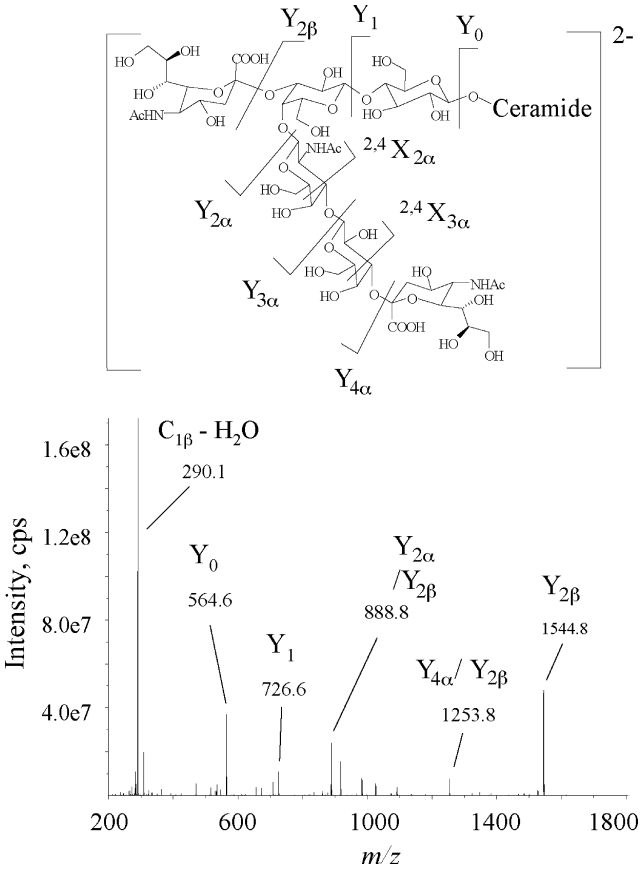


Fig. 6. MS analysis of ganglioside GD1a using the 4000 Q Trap. Shown in the upper half are the total cleavages seen; the lower portion shows a typical enhanced product ion scan (~0.055 pmol consumed).

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 then are 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 by an MS/MS spectrum. Whereas MS/MS spectra do not reveal any information about the components of the ceramide backbone, MS^3 analyses of the Y_0 product ions (*m/z* 564.6), which comprise the core lipid part of the molecule, will determine the composition of the ceramide. In the example shown in Fig. 7, 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 complimentary P and Q ions (*m/z* 237 and 263, respectively) are characteristic of the d18:1 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 [9] as well as SM (the latter was followed by selection of $[M - CH_3]$ specifically obtained from SM) [25].

Precursor ion analysis. In a precursor ion analysis, the second mass analyzer (in this case, Q3) is set to pass the *m/z* of a structure-specific product ion, such as *m/z* 264 if one were interested in scanning for all species that contain a sphingosine backbone (mindful of the already-mentioned caveats that SM and higher order glycosphingolipids are not cleaved to this product in an MS/MS analysis and require MS^3). By scanning a range of precursor ions in Q1, the species that enter the collision chamber (Q2) and reach the detector only if they yield the product ions that have been prescribed by Q3. This type of analysis not only allows identification of specific molecular species in a complex mixture, but also greatly reduces background chemical noise.

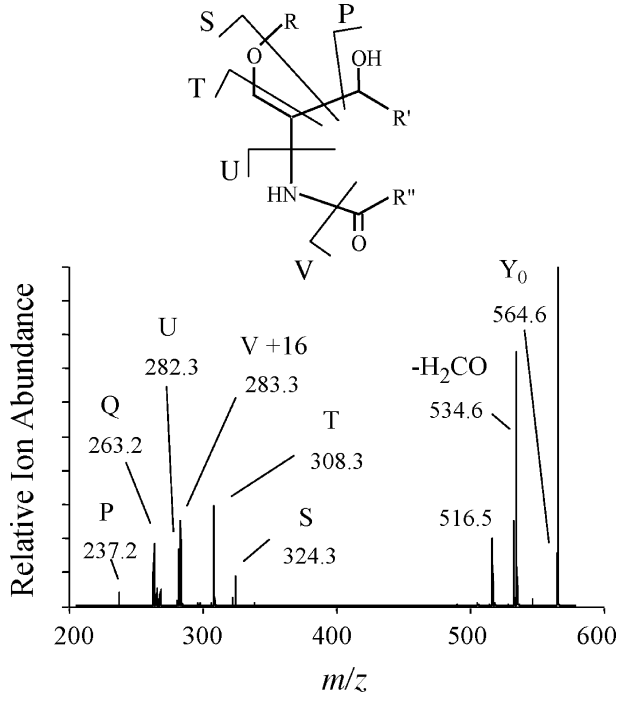


Fig. 7. MS^3 spectrum of the core lipid Y_0 ions (*m/z* 564.6) arising from fragmentation of ganglioside GD1a $(M - 2H)^{2-}$ ions (*m/z* 917.5). The cleavages resulting in these ions are shown above the spectrum.

Precursor ion scans are also extremely useful for analysis of sphingoid bases and sphingoid base 1-phosphates which typically are present at low analyte concentrations. One important feature to keep in mind is that species containing a Δ4 double bond yield more abundant dehydration products than do saturated species of similar concentration (by approximately 8-fold), presumably due to the formation of a stable conjugated carbocation upon dehydration allylic to the double bond (Fig. 2). This necessitates the inclusion of internal standards for both the saturated and unsaturated species for accurate quantitation. The saturated species (i.e., sphinganines) also yield a prominent fragment ion of *m/z* 60 at higher collision energies, which was not as sensitive, but more specific, than the single dehydration product. Optimization of ionization and dissociation conditions for the free sphingoid bases has revealed other interesting points regarding their accurate quantitation [6–9]. Comparison of the analysis of sphingoid bases using the 4000 Q Trap versus the API 3000 has demonstrated that the Q-Trap 4000 has much higher sensitivity [9].

Quantitation of sphingolipids with different headgroups and backbones using precursor ion scanning is complicated by a number of factors. First, the process of ionization during charged droplet formation and desolvation is complex and subject to suppression by the wide variety and quantity of molecules that are present in crude extracts. This underscores the importance of having the appropriate internal standards for the species to be quantitated and for them to be in the same LC eluate as the unknowns so all will be subject to the same factors that influence ionization (see Table 1).

Attention must also be given to the influence of the effective size of the species of interest (i.e., alkyl chain length and unsaturation) on ionization and fragmentation. Ions that have fewer atoms, and thus fewer degrees of freedom, will have more collision energy per degree of freedom and may undergo additional fragmentation. This is contrasted with larger ions, which have more atoms and more degrees of freedom, and will have less collision energy per degree of freedom. These ions may, therefore, not fragment as efficiently, yielding lower abundance product ions, and thus, reduced signal response in the precursor ion scan. This effect is clearly seen when a compound such as *N*-acetylsphingosine (C2-ceramide) is compared with naturally occurring, long-chain ceramides [6], but can also be seen to a lesser extent as the chain lengths vary among naturally

Table 1
Summary of precursor/product ion *m*/*z*'s, and associated collision energy, declustering potential, and dwell time used for MRM detection of individual molecular species of free sphingoid bases[a] and 1-phosphates (-P) by (A) API 3000 triple quadrupole and (B) 4000 Q TRAP mass spectrometers

Free sphingoid bases	Precursor/product ion *m*/*z*	Collision energy (eV)	Declustering potential (eV)	Focusing potential (eV)	Dwell time (ms)
(A) API 3000 triple quadrupole mass spectrometer					
d17:1	286.3/250.4	30.0	30	180	20
d17:0	288.3/252.4	30.0	30	180	20
d18:1	300.3/264.4	30.0	30	180	20
d18:0	302.3/266.4	30.0	30	180	20
t18:0	318.3/282.3	32.5	30	180	20
d20:1	328.4/292.4	35.0	30	180	20
d20:0	330.3/294.4	35.0	30	180	20
d17:1-P	366.4/250.4	35.0	30	180	20
d17:0-P	368.4/252.4	35.0	30	180	20
d18:1-P	380.4/264.4	35.0	30	180	20
d18:0-P	382.4/266.4	35.0	30	180	20
(B) 4000 Q TRAP mass spectrometer					
d18:1	300.3/282.3	18	40		25
d18:0	302.3/60.00	45	50		25
t18:0	318.3/300.3	22	50		25
d20:1	328.4/310.3	20	40		25
d20:0	330.3/312.3	23	50		25
d17:1-P	366.4/250.4	23	50		25
d18:1-P	380.3/264.4	25	50		25
t18:0-P	398.4/300.4	22	50		25

[a] In addition, 3-keto-sphingoid bases can be followed by loss of CH_2O from rearrangement and loss of the position 1 carbon, which for 3-ketosphinganine gives a transition from *m*/*z* 300.7 to 270.2. The 3-keto-sphingoid bases are also separated from the corresponding sphinganines and sphingosines by the LC conditions described in Protocol 2.

occurring species (e.g., from 16 to 26 carbon atoms). Fortunately, across this range the differences are small enough that they can be compensated for by adjusting the collision energy, as shown in Tables 2–5.

Another factor to bear in mind for precursor ion scans is that they are very inefficient with regard to duty cycle, or the amount of instrument time spent detecting ions of interest. For example, if a sample contains approximately 10 distinct individual sphingolipid species in a given 200 u mass range, a precursor ion scan over this mass range will utilize only 5% of the scan time detecting the ions of interest—the remaining 95% of the scan time is essentially wasted. This reduces sensitivity (and hence produces poorer ion statistics), and may compromise the ability to obtain reliable quantitation. A more efficient method is to focus on the species of interest, which is the basis for a technique that is discussed in Section 6.4.

Constant neutral loss scans. In a constant neutral scan, the first and second mass analyzers (Q1 and Q3) are scanned together but the *m*/*z* that Q3 will pass is offset from Q1 by a fixed value that corresponds to the mass of neutral molecule(s) of interest. Thus, only those precursor ions that lose the correct mass fragment(s) as a neutral species will be passed to the detector by Q3. This is effective for identification of specific molecular species in complex mixtures, and greatly reduces background noise; however, constant neutral loss scans share the same types of limitations as precursor ion scanning with respect to quantitation (i.e., ionization suppression, kinetics of dissociation, and sensitivity).

6.4. Multiple reaction monitoring

Multiple reaction monitoring (MRM) is a powerful tool for increasing the efficiency and accuracy of quantitative MS/MS analyses. In MRM, the first mass analyzer is set to pass a specific precursor ion *m*/*z*, and the second mass analyzer is set to pass a specific product ion *m*/*z*, therefore, only ions that meet both precursor and product ion *m*/*z* conditions simultaneously will be transmitted to the detector. The analysis is not limited to a single precursor–product pair, but can continue on to other transitions and/or cycle repeatedly. Thus, it is possible to monitor transitions corresponding to numerous analytes very rapidly on an LC time frame, and because essentially all of the instrument time is spent detecting ions of interest, the detected signal intensities are greatly enhanced. Sensitivity can be further enhanced by optimization of ionization and dissociation conditions for each individual molecular species *m*/*z* transition pair. Thus, used in conjunction with LC and the appropriate internal standards, MRM provides more accurate quantitative data as a result of addressing critical issues with regard to ionization suppression, kinetics of ion dissociation, and sensitivity.

7. Internal standards

Accurate and precise quantitation of sphingolipids requires the use of internal standards to control for sample losses in extraction, differences in chromatographic

Table 2
Summary of precursor/product ion *m/z*'s and associated parameters for MRM detection of individual molecular species of ceramides[a] by (A) an API 3000 triple quadrupole and (B) 4000 Q TRAP mass spectrometers

Species	Precursor/product ion *m/z*	Declustering potential (eV)	Focusing potential (eV)	Dwell time (ms)	Collision energy (eV)
(A) API 3000 triple quadrupole mass spectrometer					
Cer[a]					
d18:1/12:0	482.6/264.4	40	220	25	35.0
d18:1/16:0	538.7/264.4	40	220	25	40.0
d18:0/16:0	540.7/266.4	40	220	25	40.0
d18:1/18:0	566.7/264.4	40	220	25	42.5
d18:0/18:0	568.7/266.4	40	220	25	42.5
d18:1/20:0	594.7/264.4	40	220	25	45.0
d18:0/20:0	596.7/266.4	40	220	25	45.0
d18:1/22:0	622.8/264.4	40	220	25	47.5
d18:0/22:0	624.8/266.4	40	220	25	47.5
d18:1/24:1	648.9/264.4	40	220	25	50.0
d18:0/24:1	650.9/266.4	40	220	25	50.0
d18:1/24:0	650.9/264.4	40	220	25	50.0
d18:0/24:0	652.9/266.4	40	220	25	50.0
d18:1/26:1	676.9/264.4	40	220	25	52.5
d18:0/26:1	678.9/266.4	40	220	25	52.5
d18:1/26:0	678.9/264.4	40	220	25	52.5
d18:0/26:0	680.9/266.4	40	220	25	52.5
(B) 4000 Q TRAP mass spectrometer					
Cer					
d14:1/20:0	538.6/208.3	30		20	40
d14:1/22:0	566.6/208.3	30		20	44

[a] 4-Hydroxysphinganine- (Phyto-) based ceramides (t18:0) can also be monitored by as 16 u higher precursor ions that fragment to product ions of *m/z* 282.4 and 264.4 (triple dehydration). Sphingolipids with α-hydroxy fatty acids in the ceramide backbone also appear as 16 u higher precursor ions and fragment to *m/z* 264.4 product ions, but are distinguished from non-hydroxy and t18:0 based ceramides by mobility on normal-phase LC. The 3-keto-ceramides fragment to *m/z* 270.2 (for the d18:0 homolog) which, with their shift in LC mobility, allows them to be distinguished, as well.

retention, ionization efficiency, and fragmentation. The ideal internal standard would be a stable isotope labeled version of each species to be analyzed so it would have identical physical and chemical properties as the sphingolipid of interest, and for there to be some way to uniformly mix the internal standard with the endogenous sphingolipid so the recovery would be truly representative. Unfortunately, there are too many individual species for addition of a matched internal standard for every analyte, not to mention difficulties in the synthesis of these compounds or the expense of commissioning custom syntheses since few are commercially available. Instead, one can use a panel of internal standards that represent each subclass of sphingolipids to be analyzed, selecting compounds that closely approximate the analytes' behavior during extraction and LC elution in addition to their MS/MS ionization and dissociation chemistry.

A major challenge in the quantitative analysis of sphingolipids is efficient extraction of compounds with such a wide range of properties since some are highly hydrophobic (e.g., ceramides) and others are as, or more, soluble in the aqueous phase than the chloroform phase of a traditional lipid extract. As noted in *Protocol 1*, this is solved by using the initial one-phase (azeotropic) solvent mixture for analysis of some categories of sphingolipids. Perhaps, the most difficult factor to know with complete confidence is that the internal standard and the analyte of interest have been uniformly mixed because many proteins and other biomolecules aggregate upon addition of the lipid extraction solvents and may trap some of the endogenous sphingolipids. When this occurs, the estimation of the recovery from the internal standards (which are already dissolved) overestimates the recovery of the unknowns. Penetration of the solvents into such aggregates is often achieved by use of a combination of solvents that have both polar and nonpolar properties in proportions that maintain an azeotrophic mixture (i.e., as characterized in the classic Bligh and Dyer extraction protocol) combined with vortexing and sonication to disperse aggregates into small particles. This is generally effective with small samples such as cell culture extracts, and is relatively easy to perform with bath-type sonicators. Because most sphingolipids are relatively chemically stable, sonication of the extract can be done without addition of ice to keep the bath from heating somewhat; in fact, it is customary to incubate sphingolipid extracts at ~50 °C for at least several hours because ceramides and complex sphingolipids typically have phase transition temperatures in this vicinity due to the mostly saturated alkyl chains. If visual examination of the extracts reveals large clumps that are not dispersed during sonication, it may be necessary to use a small, close-fitting glass homogenizer (this is more often the case with tissue extracts than cells in culture).

Table 3
Summary of precursor/product ion *m/z*'s and associated parameters for MRM detection of individual molecular species of monohexosylceramides by (A) an API 3000 triple quadrupole and (B) 4000 Q TRAP mass spectrometers

Species	Precursor/product ion *m/z*	Declustering potential (eV)	Focusing potential (eV)	Dwell time (ms)	Collision energy (eV)
(A) API 3000 triple quadrupole mass spectrometer					
MonohexCer[a]					
d18:1/12:0	644.6/264.4	50	300	25	45.0
d18:1/16:0	700.7/264.4	50	300	25	40.0
d18:0/16:0	702.7/266.4	50	300	25	40.0
d18:1/18:0	728.7/264.4	50	300	25	52.5
d18:0/18:0	730.7/266.4	50	300	25	52.5
d18:1/20:0	756.7/264.4	50	300	25	55.0
d18:0/20:0	758.7/266.4	50	300	25	55.0
d18:1/22:0	784.8/264.4	50	300	25	57.5
d18:0/22:0	786.8/266.4	50	300	25	57.5
d18:1/24:1	810.9/264.4	50	300	25	60.0
d18:0/24:1	812.9/266.4	50	300	25	60.0
d18:1/24:0	812.9/264.4	50	300	25	60.0
d18:0/24:0	814.9/266.4	50	300	25	60.0
d18:1/26:1	838.9/264.4	50	300	25	62.5
d18:0/26:1	840.9/266.4	50	300	25	62.5
d18:1/26:0	840.9/264.4	50	300	25	62.5
d18:0/26:0	842.9/266.4	50	300	25	62.5
(B) API 4000 QTRAP mass spectrometer					
MonohexCer					
d14:1/20:0	700.6/208.3	50		20	45
d14:1/22:0	728.8/208.3	50		20	45

[a] 4-Hdroxysphinganine- (Phyto-) based monohexosylceramides (t18:0) can also be monitored by as 16 u higher precursor ions that fragment to product ions of *m/z* 282.4 and 264.4 (triple dehydration). Sphingolipids with α-hydroxy fatty acids in the ceramide backbone also appear as 16 u higher precursor ions and fragment to *m/z* 264.4 product ions, but are distinguished from non-hydroxy and t18:0 based ceramides by mobility on normal-phase LC.

Table 4
Summary of precursor/product ion *m/z*'s and associated parameters for MRM detection of individual molecular species of dihexosylceramides by an API 3000 triple quadrupole mass spectrometer

Species	Precursor/product ion *m/z*	Declustering potential (eV)	Focusing potential (eV)	Dwell time (ms)	Collision energy (eV)
DihexCer[a]					
d18:1/12:0	806.6/264.4	50	300	25	55.0
d18:1/16:0	862.7/264.4	50	300	25	50.0
d18:0/16:0	864.7/266.4	50	300	25	50.0
d18:1/18:0	890.7/264.4	50	300	25	62.5
d18:0/18:0	892.7/266.4	50	300	25	62.5
d18:1/20:0	918.7/264.4	50	300	25	65.0
d18:0/20:0	920.7/266.4	50	300	25	65.0
d18:1/22:0	946.8/264.4	50	300	25	67.5
d18:0/22:0	948.8/266.4	50	300	25	67.5
d18:1/24:1	972.9/264.4	50	300	25	70.0
d18:0/24:1	974.9/266.4	50	300	25	70.0
d18:1/24:0	974.9/264.4	50	300	25	70.0
d18:0/24:0	976.9/266.4	50	300	25	70.0
d18:1/26:1	1000.9/264.4	50	300	25	72.5
d18:0/26:1	1002.9/266.4	50	300	25	72.5
d18:1/26:0	1002.9/264.4	50	300	25	72.5
d18:0/26:0	1004.9/266.4	50	300	25	72.5

[a] 4-Hydroxysphinganine- (Phyto-) based dihexosylceramides (t18:0) can also be monitored as described in the footnote of Table 3.

Sample size is an important factor since it is relatively easy to exceed the capacity of the solvent mixture to dissolve all the compounds of interest; hence, if larger samples are analyzed, the amounts of the other extraction components should be increased. To determine if these procedures are effective, one usually extracts the samples once by a standard protocol, then reextracts the remaining residue to determine the amount and composition of the sphingolipids in the second extract. If the percentage in the second extract is low (typically ca. 10%) without

Table 5
Summary of precursor/product ion *m/z*'s and associated parameters for MRM detection of individual molecular species of sphingomyelins with d18:1 and d18:0 sphingoid bases using an API 3000 triple quadrupole mass spectrometer

Species	Precursor/product ion *m/z*	Declustering potential (eV)	Focusing potential (eV)	Dwell time (ms)	Collision energy (eV)
SM[a]					
d18:1/12:0	647.7/184.4	40	220	25	45.0
d18:1/16:0	703.8/184.4	40	220	25	40.0
d18:0/16:0	705.8/184.4	40	220	25	40.0
d18:1/18:0	731.8/184.4	40	220	25	52.5
d18:0/18:0	733.8/184.4	40	220	25	52.5
d18:1/20:0	759.8/184.4	40	220	25	55.0
d18:0/20:0	761.8/184.4	40	220	25	55.0
d18:1/22:0	787.9/184.4	40	220	25	57.5
d18:0/22:0	789.9/184.4	40	220	25	57.5
d18:1/24:1	813.9/184.4	40	220	25	60.0
d18:0/24:1	815.9/184.4	40	220	25	60.0
d18:1/24:0	815.9/184.4	40	220	25	60.0
d18:0/24:0	817.9/184.4	40	220	25	60.0
d18:1/26:1	841.9/184.4	40	220	25	62.5
d18:0/26:1	843.9/184.4	40	220	25	62.5
d18:1/26:0	843.9/184.4	40	220	25	62.5
d18:0/26:0	845.9/184.4	40	220	25	62.5

[a] 4-Hydroxysphinganine- (Phyto-) and α-hydroxy fatty acid containing sphingomyelins (t18:0) can also be monitored by the 16 u shift of the precursor ion. They are distinguished from each other by LC mobility.

an enrichment of any particular subspecies, the extraction protocol is judged adequate for most applications.

The internal standards and the compounds of interest should ideally co-elute from the LC columns because this ensures that any factor that alters the ionization efficiency will be shared by the standard and analyte. And because the internal standard and analyte must both ionize and fragment the same for MS/MS, the least perturbation of the compound's properties is to substitute stable isotopes, with preference for ^{13}C or ^{15}N over ^{2}H since the latter sometimes alters the chemical properties of the compound (and if all or most of the ^{12}C can be replaced by ^{13}C, that is most ideal because it will shift the *m/z* away from the natural abundance ^{13}C). If stable isotopes are not available, an alternative for sphingolipids is to prepare unnatural homologs that are nearly identical in chemical and physical properties to the compounds of interest, such as sphingosine homolog with a 17-carbon chain length. In our experience, this homolog is not present in most cell lines and tissues, however, this should be tested with new samples because odd-chain-length sphingoid bases are found in bovine tissues, including the serum used in cell culture media (17-carbon homologs can often be used nonetheless if the background amounts are trace compared to the internal standard). The C17-homologs of sphingosine, sphinganine, and the 1-phosphates are available commercially (Avanti Polar Lipids, Alabaster, AL), and both are needed because the presence or absence of the Δ4 double bond yields much different signal responses for these species.

For more complex sphingolipids (Cer, SM, and glycosphingolipids), internal standards with uncommon fatty acids in the ceramide backbone can be used. Several classes of sphingolipids with a C12:0 fatty acid, which is rarely seen in sphingolipids, are also commercially available (Avanti) and can be used as internal standards. As the alkyl chain length increases, it will usually be increasingly difficult to fragment the compound (i.e., higher eV must be applied, see Tables 2–5), hence, the signal from a very short-chain species (such as *N*-acetylsphingosine) will differ substantially from that of a long-chain fatty acid containing sphingolipid unless the collision energy is optimized for both species. This is accommodated by conducting the MS/MS using MRM and varying the eV as larger sphingolipids are being analyzed (for this example, the collision energies that produce the same signal for C2-ceramide and C24:1-ceramide are 25 and 45 eV, respectively) [6,7]. As for the free long-chain bases, it is desirable to have separate internal standards for complex sphingolipids with and without a Δ4 double bond in the sphingoid base backbone (or with the 4-D-hydroxysphinganine backbone if that category of sphingolipid is being analyzed).

Other considerations in the use of internal standards are: (1) If an internal standard is not available, it is often possible to quantify a compound of interest by spiking the extract with a known amount of the same compound available commercially (or prepared by the investigator) and comparing the MS/MS signal with and without the spike. However, if this method is used, careful controls must be conducted to ascertain that the % recovery of the spike is proportional to the amount added at concentrations close to the unknown; otherwise, the recovery of the unknown may change due to the spike and compromise the quality of the data. (2) The amounts of the internal standard should be sufficiently close to that of the

unknowns for their behavior during extraction, etc. to be comparable. (3) The internal standard should be sufficiently stable in solution so the amounts added to the samples will be reproducible. Free sphingoid bases tend to decompose in some types of test tubes, and sphingoid base 1-phosphates are often difficult to dissolve.

8. Strategies for analysis of sphingolipids by mass spectrometry

There are many approaches that one could take in setting up the LC–MS/MS method for analysis of a new biological material. The following protocol outlines one approach.

8.1. Protocol 4: order of steps to follow in setting up an LC–MS/MS for a new biological sample

1. Extract the sphingolipids using the basic protocol described in Section 4.1 but prepare two extracts, one with and the other without the internal standards. All of the analyses below will be conducted with the sample that has been extracted without the internal standard; however, the other will be checked to ensure that the recoveries have been reasonable. After the new material has been surveyed, it may be necessary to modify the extraction further to obtain higher yields (Protocol 1 is generally effective with mammalian cells, tissues, and blood; however, much different extraction conditions are needed for other organisms such as plants and fungi).
2. Using the extract, conduct Q1 scans in negative- and positive-ion modes using constant infusion of the sample to identify possible sphingolipids. Compare detected precursor ions with prepared tables of sphingolipid *m*/*z*'s. Experienced users may conduct precursor ion or neutral loss scans in place of Q1 scans if some sample information is known (i.e., screening for *m*/*z* 264.4 in mammalian samples which have large amounts of d18:1 sphingoid bases). This technique is useful for detecting methylated or hydroxylated species that may be in low abundance and hence not initially noticed.
3. Optimize conditions for generation of precursor ion signal for each species of interest by performing Q1 scans varying ionization conditions one parameter at a time (starting at a low value and increasing until an optimum is achieved). Optimization of each analyte individually is important because lipid structure can significantly impact ionization. Input the optimum values for each parameter.
4. Perform product ion scans on each analyte to identify abundant, structure-specific fragmentations to be used as signature product ions for each species. To determine a signature product ion for a given species, perform several product ion scans at increasing collision energies. Choice of signature product ions is paramount in achieving accurate results. Typically, it is best to choose an ion that categorizes the compounds (such as the conjugated carbocation of 264.4 *m*/*z* for sphingosine, Fig. 2). A functionality that is shared by a large number of other compounds, such as phosphate, can be chosen as a signature ion, but this may produce an unacceptably high background. Once the signature ion is identified, vary collision conditions (collision energy, collision gas, exit potential, and detector settings) to optimize formation and transmission of the desired product ion.
5. From the information gained from the precursor and product ion scans, generate a "parts list" of compounds expected to be in the samples (similar to the lists in the tables).
6. Build these precursor–product ion pairs (i.e., the "parts list") into a MRM method for a given system.
7. Inject the sample into LC and examine the elution profiles compared to standards for that family of compounds. Typically, sphingolipids are separated by headgroup in normal phase and by alkyl chain length in reverse-phase LC.

Thus, only those molecular species with the appropriate retention time, molecular mass, and fragment ion of interest will be annotated. Any anomalously behaving species are usually studied further because they may also be compounds of interest. However, the additional stage of selectivity provided by MS/MS greatly reduces the amount of background chemical noise in the LC profiles for each precursor–product pair.

9. Issues remaining to be resolved in the analysis of the full sphingolipidome

The methods described here allow quantitative analysis of all the compounds in the sphingolipidome from the initial condensation product of serine palmitoyltransferase (3-ketosphinganine) through SM, GlcCer, GalCer (with essentially any type of 'ceramide' backbone), as well as several more complex sphingolipids (thus far) in addition to the turnover (and signaling) metabolites sphingosine, sphingosine 1-phosphate, lysosphingolipids, etc. (Fig. 8). This summary includes several interesting metabolites that are not usually regarded to be part of the sphingolipid metabolic pathway, such as (a) sphinganine 1-phosphate, which is usually present in very small amounts, but can be increased by 1–2 orders of magnitude when cells are exposed to an inhibitor of ceramide synthase, such as fumonisin B_1 [6]; (b) *N*-acetyl-sphingoid bases, which are also seen mostly when cells are exposed the fumonisin B_1 [36]; (c) *N*-methyl-sphingoid bases, which are found in highest amounts in

cells that have been treated with exogenous sphingoid bases or sphingoid base analogs (S. Pruett, Y. Liu, et al., unpublished observations); (d) 3-ketodihydroceramides, which are found in some cell types (e.g., HeLa) and are more prevalent than 3-keto-sphingoid bases (i.e., it appears that if the 3-keto-intermediate is not reduced quickly, it is acylated) (A. Momin, J. Allegood, et al., unpublished observations); and (e) complex sphingolipids with dihydroceramide backbones [8]. The high sensitivity of these methods will undoubtedly provide other surprises.

These methods have been applied to several dozen different mammalian cell types and the each typically contains 50–80 of the sphingolipid subspecies (i.e., headgroup and backbone variants) shown in Fig. 8. Because SM is usually present in high amounts (several nmol/10^6 cells), half or more of the total sphingolipid mass has probably been accounted for in most cases, with most of the compounds that remain to be analyzed being more complex glycosphingolipids. The success in analyzing ganglioside GD2a by MS^3 using the Q-Trap 4000 lends some optimism that such new instruments will allow the analysis of a substantial number of the remaining compounds. Nonetheless, a major factor in the ultimate success of this endeavor will be the ability to separate closely related classes of compounds by new types of LC, such as the use of enantiomeric columns or perhaps affinity chromatography using lectins or

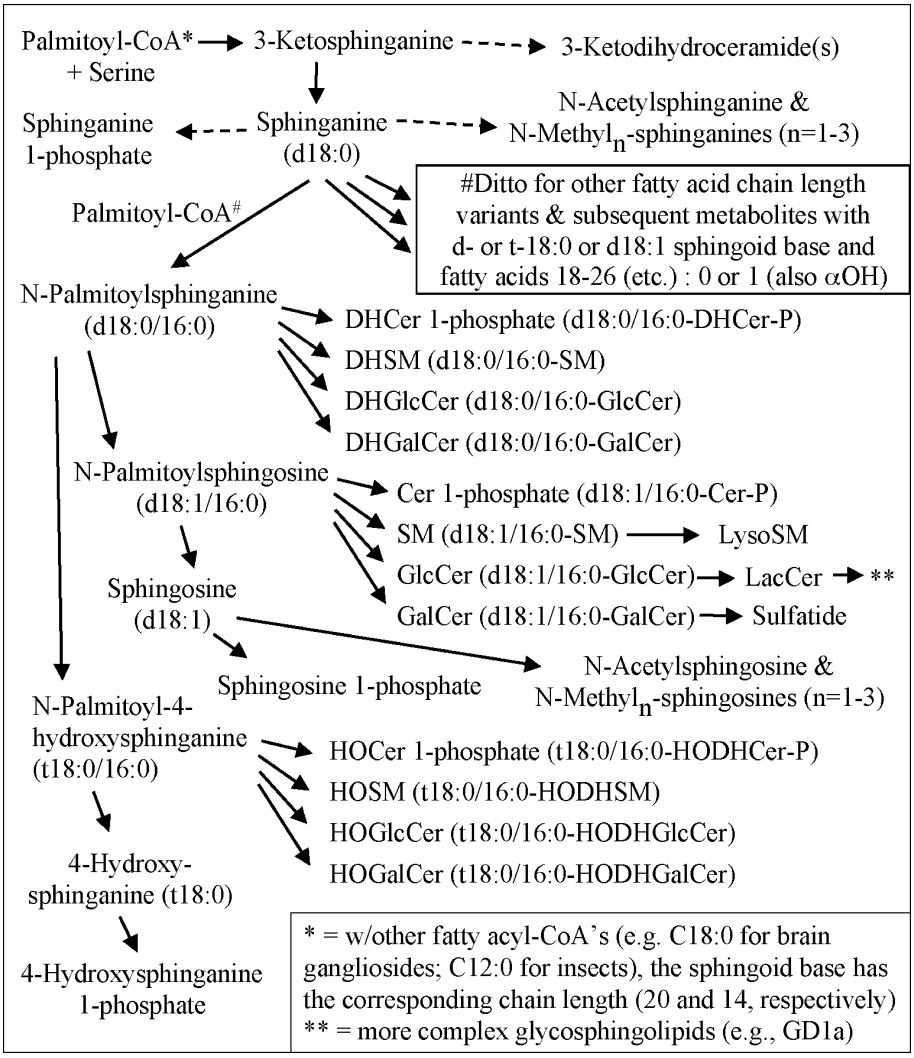


Fig. 8. Summary of sphingolipids that have been analyzed by the methods presented in this article. The abbreviations are: Cer, ceramide (*N*-acylsphingosine); DHCer, dihydroceramide (*N*-acylsphinganine); HODHCer, 4-D-hydroxydihydroceramide or phytoceramide (*N*-acyl-4-D-sphinganine); GalCer, galactosylceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide; LysoSM, sphingosylphosphocholine; *N*-methyl$_n$-sphingoid base, *N*-monomethyl-, *N*,*N*-dimethyl-, and *N*,*N*,*N*-trimethyl-sphingoid bases; and SM, sphingomyelin. The backbone abbreviations refer to the sphingoid base (d- and t- for di- and tri-hydroxy, followed by the number of carbons and number of double bonds) and fatty acid (number of carbons followed by number of double bonds); for example, *N*-palmitoylsphingosine is abbreviated d18:1/16:0 (αOH refers to fatty acids with an α hydroxyl group). The asterisks indicate structural variations that have also been analyzed by these methods. Note that although most of the compounds are arranged according to their position in sphingolipid biosynthesis, they are also formed by sphingolipid turnover.

antibodies for compounds that are impossible to resolve by other procedures. New accessories for mass spectrometry, such as automated nanoelectrospray, as well as tools and ideas from related disciplines such as proteomics will probably play important roles in development of the methodologies for analysis of the full sphingolipidome.

In addition to these technical aspects, there are complementary issues that need to be dealt with, some of which may be as or more difficult than quantitation of all of the sphingolipids. One is the ability to identify and quantify the origin of the compounds of interest; for example, if a highly bioactive species such as ceramide changes, is this the result of an increase in de novo biosynthesis or turnover of more complex sphingolipids? Even more troubling is the prospect that the turnover of a compound in one compartment of the cell may mask the formation of that species elsewhere. Mass spectrometry will be able to help answer these questions in some cases, for example, if one is able to selectively label newly made sphingolipids using a stable isotope precursor such as (U-[^{13}C]) palmitic acid. Identification of the subcellular localization is also possible using classical fraction methods, but these are currently relatively slow and laborious.

A second need is for computational and visualization tools to facilitate analysis and interpretation of the large amounts of data from sphingolipidomic analyses. In even a relatively simple experiment, there can be over 1000 data points, hence, the data sets will be analogous to those from gene microarrays. A new field of sphingolipid bioinformatics will be needed to deal with this information and to integrate it with the other "omic" disciplines. Such efforts have begun with initiatives such as LipidMaps (www.lipidmaps.org) and the affiliated SphinGOMAP (www.sphingomap.org), and lipid ontologies will be constructed in analogy to (and connected to) the "GO" (i.e., Gene Ontology) tools that have been developed by the Gene Ontology Consortium (www.geneontology.org/), the Kyoto Encyclopedia of Genes and Genomes (KEGG, www.genome.jp/kegg/) and others. One begins to wonder if in naming "sphingosin," J.L.W. Thudichum had a premonition that sphingo would someday become sphinGO.

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