Published in final edited form as:
Biochim Biophys Acta. 2011 November ; 1811(11): 838–853. doi:10.1016/j.bbalip.2011.06.027.

Analysis of Mammalian Sphingolipids by Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) and Tissue Imaging Mass Spectrometry (TIMS)

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Abstract

Sphingolipids are a highly diverse category of molecules that serve not only as components of biological structures but also as regulators of numerous cell functions. Because so many of the structural features of sphingolipids give rise to their biological activity, there is a need for comprehensive or “sphingolipidomic” methods for identification and quantitation of as many individual subspecies as possible. This review defines sphingolipids as a class, briefly discusses classical methods for their analysis, and focuses primarily on liquid chromatography tandem mass spectrometry (LC-MS/MS) and tissue imaging mass spectrometry (TIMS). Recently, a set of evolving and expanding methods have been developed and rigorously validated for the extraction, identification, separation, and quantitation of sphingolipids by LC-MS/MS. Quantitation of these biomolecules is made possible via the use of an internal standard cocktail. The compounds that can be readily analyzed are free long-chain (sphingoid) bases, sphingoid base 1-phosphates, and more complex species such as ceramides, ceramide 1-phosphates, sphingomyelins, mono- and di-hexosylceramides sulfatides, and novel compounds such as the 1-deoxy- and 1-(deoxymethyl)-sphingoid bases and their N-acyl-derivatives. These methods can be altered slightly to separate and quantitate isomeric species such as glucosyl/galactosylceramide. Because these techniques require the extraction of sphingolipids from their native environment, any information regarding their localization in histological slices is lost. Therefore, this review also describes methods for TIMS. This technique has been shown to be a powerful tool to determine the localization of individual molecular species of sphingolipids directly from tissue slices.

Keywords

Sphingolipids; LC-MS/MS; tissue imaging mass spectrometry; tandem mass spectrometry; quantitation; internal standards

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1. INTRODUCTION

Sphingolipids (SL) comprise a complex family of compounds that are found in a wide variety of prokaryotic and eukaryotic organisms as well as viruses, and are involved in many aspects of cell structure, metabolism, and regulation [1, 2]. Mammalian SL are synthesized *de novo* primarily via the condensation of serine with palmitoyl-CoA to yield 3-ketosphinganine, which is subsequently reduced to sphinganine (Sa) [2, 3]. This sphingoid base backbone is comprised of an18-carbon linear alkane having a 1, 3-dihydroxy-2-amino head group as shown in Figure 1, which illustrates the early steps in the biosynthesis of the major mammalian sub species. This core backbone may then be N-acylated resulting in the formation of the complex sphingolipid dihydroceramide (DHCer). The fatty acids used may vary in chain length from C14 to C26 (and longer) and may be saturated or mono-unsaturated and sometimes α-hydroxylated. These DHCer species can be further modified in mammals, the two major species being ceramide (Cer) and phytoceramide (phytoCer). The former is generated via introduction of a double bond at the 4, 5 position of the sphingoid base to yield N-acyl-sphingosines, which are the central locus of much greater sphingolipid diversity. The latter is formed via hydroxylation at C4 of the sphingoid base to yield “phytoceramides.” These lipid backbones may then be derivatized further via linkage at the 1-hydroxy position of either complex carbohydrates or other polar species. Counting just those headgroup species known for mammals, this results in an estimated over 450 categories of more complex glycosphingolipids and phosphosphingolipids (see http://www.sphingomap.org/) [2].

In addition to the biosynthetic pathways, other signaling sphingolipid molecular species such as sphingosine arise via SL degradation pathways (catabolism or turnover and some of these, such as sphingosine and sphingosine 1-phosphate, are highly bioactive and are also utilized by cells for signaling). Thus, the resulting ensemble of all of the SL in a given organism—its “sphingolipidome”--has the potential to encompass many tens of thousands of individual molecular species depending on how many combinations of sphingoid bases, fatty acids and headgroups are present. This biodiversity illustrates the need for structure-specific methods of identification and quantitation of all the subspecies of SL. This will not only reveal what species are present (presumably for functions important to the organism), but will also distinguish the pathways (biosynthetic or turnover) that give rise to specific molecular species.

Although many methods are available for analysis of SL [3], liquid chromatography (LC) in conjunction with tandem mass spectrometry (MS/MS) is currently the only technology that can provide the necessary sensitivity, structural specificity, separation, quantitative precision, and relatively high throughput for sphingolipidomic analyses in limited sample sizes. The most recent description of the methods developed by our laboratory as part of the LIPID MAPS Consortium (www.lipidmaps.org) is found in reference [4] and some comments about its application are made in this review. In optimizing these methods, we typically utilize the following approach: Initially, representative samples are profiled via precursor ion and neutral loss scans to identify the various individual molecular species of sphingolipids that are present. Following this, the analytes of interest are identified and quantified by LC-MS/MS using multiple reaction monitoring (MRM), a technique where the eluate is repetitively scanned for selected precursor-product ion pairs to enhance the sensitivity and specificity of the analysis. These techniques provide 3 orthogonal dimensions of separation and identification: retention time, mass, and structure. This serves to minimize interferences arising from isomeric, isobaric, and isotopic species, which limit 2-dimensional techniques.

A critical aspect which allows quantitative analysis of sphingolipids by LC-MS/MS has been the development of internal standards to control variability in recovery from the biological material as well as other factors that can negatively affect ionization. A stable isotope-labeled version of each analyte of interest would traditionally be selected as an ideal internal standard. However, it is physically, technically, and fiscally impractical for these to be synthesized for the large numbers of compounds measured in a sphingolipidomic study. A more practical approach has been introduced to identify internal standards that are similar in structure and physical properties, as well as ionization and fragmentation characteristics for the categories of compounds under investigation. To this end, an internal standard cocktail has been developed for the LIPID MAPS Consortium [5, 6] (which is commercially available from Avanti Polar Lipids) that contains uncommon chain-length sphingoid bases (C17) for sphingosine (So), sphinganine (Sa) and their 1-phosphates (S1P and Sa1P) and C12:0 fatty acid analogs of Cer, ceramide 1-phosphate (Cer1P), SM, and mono- and dihexosylCer (HexCer and diHexCer). The types of analytes can be expanded by supplementation with additional internal standards. We now typically include sulfatides, 1-deoxy-, and 1-(deoxymethyl)-sphingoids in our routine analysis, which has been continuously expanded and refined [4] since first published more than a decade ago [7, 8]

The LC-MS/MS methods described in this review have been developed specifically to address the mouse macrophage RAW 264.7 cell line using an ABI 3000 triple quadrupole (QQQ) and an ABI 4000 quadrupole / linear ion trap (QTrap) tandem mass spectrometry systems. These methods may be used on other instrument systems while noting that some instrumental parameters such as ion source and fragmentation conditions will usually have to be altered somewhat for optimal results. Similarly, these methods may be used to investigate the sphingolipidome of other organisms. However, it should be noted that initial profiling is critical to determine the appropriate MRM pairs used for LC-MS/MS (as well as other factors, such as extraction efficiency, etc.).

One of the more interesting biological questions regarding SL pertains to their distribution within tissues and ultimately compartmentally within cells. Recent advances in the application of MALDI matrix have enabled the ability to ionize intact SL directly from the surface of a tissue slice without lipid migration, which can result from the applied organic matrix solution [9, 10]. Moreover, MS and MS/MS analysis may be performed in an ordered array across the entire surface of the tissue. The subsequent spectra may then be queried and discrete *m/z* values plotted in x, y-space, which can yield images of the distribution of specific individual molecular species on the tissue surface. This tissue imaging mass spectrometry (TIMS) technology is emerging as a powerful tool for the identification and localization of sphingolipids, which may serve as biomarkers of disease or its progression as will be addressed in this review as well as included in others in this Special Edition.

2. SPHINGOLIPID NOMENCLATURE AND STRUCTURE

2.1 Sphingoid Long Chain Bases (LCBs)

A structurally precise way of describing the individual molecular species of SL which have been identified is an important foundation on which to build “-omic” research. To this end, a systemic nomenclature for all lipids has been proposed [11], and is presented below. In the case of SL, this means rigorously identifying the composition of the sphingoid base, fatty acid, and headgroup, and any other modifications. Mass spectrometry (MS) is clearly the most powerful method to obtain this information. However, it should be noted that in some cases it is not possible to prove without doubt some aspects of structure, such as stereochemistry, by only mass spectrometry alone. Therefore, other techniques may be required.

As mentioned previously SL are generally categorized as having the backbone moieties termed “long-chain sphingoid” bases (LCB), which are (usually) linear 18 carbon alkane or alkene having a 1, 3-dihydroxy-2-amino groups as defining parts of their molecular structure. It has been known for a long time that there are numerous variations on this theme [12, 13], and some of these were found in humans. This thinking has been solidified by the finding of considerable variation in the backbones of, for example, the sphingomyelins of human plasma [14], and the production *de novo* of even sphingoid bases without the 1-hydroxyl-group [15].

The most common sphingoid base found in mammalian cells is D-*erythro*-sphingosine, which has the formal chemical description of (2S, 3R, 4E)-2-aminooctadec-4-ene-1,3-diol. A more concise way to describe sphingoid bases is to first assign a letter that indicates the number of hydroxyl groups, such as “m”, “d”, or “t” for mono-, di-, or tri-hydroxylated, respectively. This is then followed by a number designation of the number of carbon atoms present in the alkyl chain (for example, typically 18 for most mammalian sphingolipids), which is then separated by a colon from the number of units of unsaturation (rings or double bonds) also present in the alkyl chain. Thus, for sphingosine (2-aminooctadec-4-ene-1,3-diol), this is typically “d18:1”. If, however, the position and stereochemistry of the double bond in sphingosine is known, this can be added to give the abbreviations 4E-d18:1, 4-*trans*-d18:1, or d18:1$^{trans\Delta 4}$. This system is readily applicable to many other sphingoid bases that occur in mammals which are known to expresses variation in alkyl chain length and/or number and position of double bonds. This includes the d20:1 sphingoid bases found in brain, and 4-*trans*,14-x-d18:2 long-chain bases in the sphingolipids of atherosclerotic human aorta [16]. Similarly, a 1-deoxy-sphingoid base, such as 1-deoxysphinganine [15] can be designated m18:0, and tri-hydroxy (“phyto”) sphingoid bases, which have an additional hydroxyl group can be described as t18:0 for 4-hydroxysphinganine, or more formally (2S, 3R, 4R)-amino-octadecane-1,3,4-triol.

Derivatives of sphingoid bases may have mono-methyl branched chains (at ω-1, ω-2, or other positions), additional hydroxylations (at 4- and 6-positions), 1-phosphorylations [17], and mono-, di-, and tri-N-methyl species. Other derivatives of “free” (i.e., not N-acylated) sphingoid bases, such as lysosphingomyelin (sphingosyl-phosphocholine), psychosine (galactosylsphingosine), and other sphingosyl-hexoses are also found, and are thought to arise from degradation of more complex species as described below. It should be noted that in mammals double bonds located at the Δ4, and other positions of *de novo* biosynthesized sphingoid bases are thought to arise from desaturation of dihydroceramide(s) not directly from the production of unsaturated free sphingoid bases and is the major source of sphingosine based sphingolipids [18]. This said, unsaturated sphingoid bases are common in organisms that are consumed by humans (as well as microorganisms that might be part of the GI microflora) [13], hence, might appear from recycling of these compounds when taken up by the body. Much, however, is degraded in the GI track [19] via the (currently) only known degradative exit of sphingolipids from their metabolic pathway, which occurs via cleavage of the 2, 3 carbon-carbon bond of sphingoid base phosphates by sphingosine phosphate lyase [20].

2.2 Dihydroceramide and Ceramide

Dihydroceramide (DHCer) and ceramide (Cer) refer to sphingoid bases which have undergone N-acylation with fatty acids of chain lengths C16 and longer (usually to C24, but longer are seen) (DHCer are amide linked to sphinganine (Sa) and Cer to sphingosine (So), respectively). Specification of the N-acyl chain length is the result of the fatty acyl-CoA substrate specificity of six known human (dihydro)ceramide synthase isoforms [21, 22], whose names have been proposed to be CerS1-6. DHCer can be converted to Cer via a DHCer desaturase, which introduces a 4,5-E double bond [23]. It may be similarly

converted to phytoceramide by introduction of a 4-hydroxy group, by Δ4-dihydroceramide desaturase 2 [24]. Hydrolysis of DHCer gives rise to the formation of Sa, and provides a different pathway for formation of this LCB. Similarly, hydrolysis of Cer and phytoCer gives rise to So, and 4-OH So, respectively. Thus, these free long chain bases arise from degradation pathways and may be further metabolized as is sphinganine.

The abbreviated notation for (DH)Cer describing the nature of the two lipid moieties begins with the LCB followed by a similar description of the fatty acyl group. Therefore, N-palmitoyl-sphinganine would be “d18:0/16:0”. Here the “d18:0” indicates a dihydroxy, 18 carbon chain with no double bonds for sphinganine, and the “16:0” describes a 16 carbon fatty acid with no double bonds. When double bonds are present, they are assigned to the appropriate chain as determined by MS/MS. In some cases the fatty acid contains an additional hydroxyl group, a lower case “h” is placed before the description of the fatty acid (d18:0/h16:0, for example).

2.3 Sphingomyelin, Ceramide, Ceramide Phosphoethanolamine, and Ceramide-1-Phosphate

Some of the most abundant complex SL of mammals, sphingomyelin (SM), are formed by polar head group addition via phosphodiester linkage at the 1-hydroxy position to either dihydroceramides or ceramides. In addition to SM, linkage of a phosphoethanolamine at the 1-hydroxy position results in the formation of ceramide phosphoethanolamine (CPE), a common SL of insects and other organisms and is reported in small amounts in mammals. Cer can also be phosphorylated or acylated to yield ceramide-1-phosphate (Cer1P) and 1-O-acyl ceramide, respectively. Hydrolysis of DHCer1P and Cer1P gives rise to the signaling molecules sphinganine-1-phosphate (Sa1P) and sphingosine-1-phosphate (S1P), respectively. Similarly, hydrolysis of SM yields sphingosyl-phosphocholine or lysoSM.

The abbreviated nomenclature for these SL follows that of the DHCer and Cer, where the headgroup is listed followed by the ceramide core. Therefore, N-palmitoyl sphingosine phosphorylcholine is written as, SM d18:1/16:0, and N-steroyl sphinganine-1-phosphate is Cer1P d18:0/18:0.

2.4 Glycosphingolipids

Glycosphingolipids (GSLs) comprise the most structurally diverse and complex category of SL. For mammalian GSL, a carbohydrate linked to the 1-hydroxy position to yield a monohexosylceramide. This is typically either glucose (for glucosylceramide, GlcCer) or a galactose (for galactosylceramide, GalCer). It is noteworthy that these isomers cannot be distinguished from one another either by mass spectrometry (MS) or tandem MS (MS/MS), and are referred to collectively as cerebrosides. These two species are the building blocks upon which hundreds of unique molecular structures may be synthesized by addition or removal of other additional carbohydrates and functional groups (such as sulfate, for the sulfatides). In the case of GSLs these typically include additional glucose or galactose units, their N-acetyl versions, as well as acidic glycans containing carboxylic acid or sulfate moieties, as well as others. More complex GSLs may be generally characterized as being either neutral or acidic. These are divided further into the ganglio-, globo-, isoglobo-, lacto-, neolacto-, arthro-, and mollo- series with each having a characteristic tetrasaccharide root structure [25].

One system of nomenclature for SL is that of Svennerholm [26] wherein the letter “G” is used to designate the presence of a sialic acid-containing GSL (ganglioside) family followed by a second letter which indicates the number of sialic acid residues (GM, GD, GT, or GQ for mono-, di-, tri-, or quad-, respectively). A number is added afterward to indicate other

structural differences, such as the order in which the species are resolved via thin-layer chromatography, (e.g., the GD3 > GD2 > GD1 series representing the subspecies with increasing numbers of additional carbohydrate moieties). Other systems of describing the carbohydrate moiety have been reviewed as well [3]. It should be clear given the number of different carbohydrates the many possible sequences in which they can be added, the multiple positions at which the carbohydrates can be linked to each other (and in either α or β-glycosidic bonds), and the backbone complexity, that the likely number of possible SL is in the tens of thousands. This results in a vast area for potential discovery and identification.

3. SPHINGOLIPID ANALYSIS VIA MASS SPECTROMETRY

3.1 Electron Ionization (EI)

Early investigations of sphingolipids used EI MS to determine the structures of both Cer [27, 28] and neutral GSL [29, 30]. These experiments revealed both the intact molecular ion, and fragment ions, which could be used to differentiate SL isomers [31, 32]. It is interesting to note that these species had to be converted into either trimethylsilyl [33] or permethyl ethers in order to decrease their polarity, thereby increasing their volatility for analysis in the gas phase. Given the high energy of the EI process, extensive fragmentation resulted and in some cases intact molecular masses of some larger species could not be observed. Analysis of complex mixtures of SL via these methods proved extremely difficult given that there were multiple species present that gave rise to even more fragment ions, which highlighted the need for chromatographic separations.

3.2 Fast Atom Bombardment (FAB) and Liquid Secondary Ionization Mass Spectrometry (LSIMS)

FAB and LSIMS are softer ionization techniques relative to EI. Here a sample either in solution or solid form is mixed with a non-volatile matrix (i.e. glycerol, thioglycerol, or 3-nitrobenzyl alcohol) on a probe tip. When the probe is inserted inside the instrument, the analyte is ionized off the probe tip via collisions with an energetic beam of either atoms or ions, respectively. In this case, SL may be ionized directly without the need for prior derivatization, and often yield intact molecular ions; however, in some cases fragmentation can be observed. These ionization techniques represented a great leap forward because they allowed the analysis of complex mixtures of LCBs, Cer, HexCer, and SM, whereby many individual molecular species could be differentiated on the basis of *m/z* value [34-35].

Given that both FAB and LSIMS generated primarily intact molecular ions of SL, tandem mass spectrometry (MS/MS) was required to elucidate structural information. Here a precursor ion of interest may be selected with the first mass analyzer, after which the ions enter a region filled with a neutral gas (i.e., He, N_2, Ar, etc.). The ions then collide with the gas and part of their translational energy is converted into internal energy and they may undergo fragmentation. Following the collision event, any surviving precursors as well as fragment ions are resolved by the second mass analyzer yielding a product ion spectrum for the selected precursor. Fortunately, in either the positive or negative ion mode, SL fragment at specific locations and yield product ions diagnostic for structural portions of the lipid, namely the sphingoid base, fatty acid or headgroup [36]. Interestingly, it was observed that these fragmentation pathways could be altered by addition of alkali metal ions, forming the $(M + Me^+)^+$ species (where $Me^+ = Li^+$, Na^+, K^+, Rb^+, and Cs^+) and the position of some double bonds may determined by charge remote fragmentations [37].

There are several limitations in the use of either FAB or LSIMS for the analysis of SL. The most prominent of these is the matrix used for ionization. It produces a great deal of background chemical noise in the lower mass region, which limits sensitivity in the detection of the LCBs. Another issue is the short time in which a sample could be analyzed

before the probe would have to be removed and the sample replenished. Further, one could not couple other analytical techniques such as liquid chromatography to mass spectrometry so that complex mixtures could be separated into individual molecular species for identification and structure elucidation. Development of Dynamic FAB helped to address some of these issues [38, 39]. Here, sample and matrix could be diluted ~ 100 fold with volatile organic solvents and allowed to flow through a silica capillary directly onto the probe tip. Under the high vacuum conditions in the source the solvent could be pumped away leaving a continuous deposition of the analyte and matrix on the probe tip for subsequent ionization. However, over time, matrix would build up and chemical noise would prevent detection of species at low analyte concentrations.

3.3 Electrospray Ionization (ESI)

Electrospray ionization revolutionized analysis of SL via mass spectrometry. With this technique, a solution containing the SL in an organic solvent can be directly infused into the ion source of a mass spectrometer via a hollow metal needle held at a high potential (either positive or negative). At the tip of the needle, the solution is dispersed into highly charged droplets, which are drawn into the orifice of the mass spectrometer by both potential and pressure difference. As the droplets transition between atmospheric pressure and high vacuum, the charged droplets undergo evaporation whereby the neutral solvent is pumped away. As the droplet grows smaller, the ratio of charge vs. surface area greatly increases leading to a coulomb explosion creating many new, smaller, charged droplets. This process rapidly repeats itself eventually leaving the analyte as an ion in the gas phase. When performed at low temperatures and potentials, this evaporative cooling effect generates primarily intact molecular ions and is a very soft ionization technique with little fragmentation. However, instruments that possess heated glass or metal capillaries can thermally decompose many SL and must be carefully controlled to obtain intact molecular ions.

Infusion analyses were typically limited to 1-10 μL/min. flow rates and multiple stages of pumping were required to remove the solvent. ESI proved to be a much more sensitive technique by orders of magnitude vs. FAB or LSIMS owing to greatly reduced background chemical noise. Improvements in pump and ion source technology have allowed extremely high or low flow rates to be accessed providing additional capabilities. The high flow rate conditions use heated gas flows to nebulize several mL/min of solvent flow and allow very high throughput LC-MS/MS analyses. This is contrasted by the very low flow rates of nanospray (typically on the order of 10-500 nL/min.), which allows very high sensitivity and greatly reduced sample consumption.

3.4 ESI-MS/MS (2-D) Techniques

Large families of lipids including SL have been reported to be identified and quantified using triple quadrupole tandem mass spectrometry techniques [40, 41]. Here, aqueous LiOH and/or LiCl may be added to a solution containing the lipids. The resulting solution may be sprayed via infusion in either positive or negative ion mode, and multiple MS and MS/MS (precursor ion and neutral loss) scans indicative of key parts of a lipid may then be acquired. The resulting data can be presented in a 2-dimensional plot to reveal the structural composition of numerous lipid species. The technique is relatively easy to perform and it is a rapid way to identify many lipid species including: phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidic acid (PA), diacyl- and triacylglycerol (DAG and TAG, respectively), cardiolipin, free fatty acids, SM, cerebroside, Cer, and others [40]. However, it should be noted that this technique has several limitations including ionization suppression,

interferences from isotopic and isobaric species, and the inability to distinguish many isomeric species.

This technique has been extended to other instrumental configurations via use of nanospray ionization and high resolution MS and MS/MS [42-44]. Here, very small volumes of sample are infused at nL/min flow rates via a chip containing a high density array of miniature nanospray nozzles into a Q-TOF or linear ion trap-orbitrap tandem mass spectrometer. The greatly reduced flow rates provides enhanced sensitivity through reduced background noise and samples being reconstituted in smaller volumes resulting in higher concentrations. The very low flow rates also result in the ability to extend acquisition time allowing numerous product ion scans to be performed across a user defined mass range. The resulting product ion data can then be searched for structure specific fragment ions or neutral losses that correspond to characteristic portions of a lipid molecule, which for SL would be sphingoid base, fatty acid, or headgroup. The high resolution of either the TOF or Orbitrap mass analyzer in MS/MS mode allows the fragment ion or neutral loss to be much more narrowly specified. This serves to reduce the misidentification of some lipids as would occur on triple quadrupole instruments operating at a lower resolution.

3.5 High Performance Liquid Chromatography Electrospray Ionization and Tandem Mass Spectrometry (HPLC-MS/MS)

Coupling of the eluates from liquid chromatography to ESI MS and MS/MS is a logical extension of the electrospray technique and provides many benefits otherwise unavailable. The primary of these is analytical ability to separate and resolve closely related SL. Use of chromatography also allows SL to be desalted, chromatographically focused, and selectively eluted all of which enhance sensitivity and specificity. Another benefit provided by the use of HPLC is added robustness whereby complex mixtures can be separated into individual components. This serves to reduce the complexity of the eluent at any given time which minimizes ionization suppression from other species, and reduces interferences that arise from isomers, isobars, and isotopes. The result of this is improved quantitative accuracy, sensitivity, and reproducibility versus MS only based techniques.

LC-MS/MS has been used to identify, quantify, and elucidate the structures of LCBs and their phosphates, Cer, HexCer (both GalCer and GlcCer), LacCer, SM, and other complex SL for over a decade [7, 8]. A baseline separation of all individual molecular species within a given class of SL is not necessary so long as all non-chromatographically resolved species have the same chemical and physical properties and a unique precursor/product ion *m/z* value. Here the similarity of the chemical and physical properties ensures that one species does not affect the ionization of the others. Additionally, the uniqueness of the precursor/product ion pair allows the mass spectrometer to differentiate between many components in a complex mixture (i.e. all N-acyl chain variants of Cer), even in the event that they co-elute from the HPLC. The addition of internal standards that co-elute with the analytes of interest is of critical importance. This allows the resulting signal to be normalized and takes into consideration any ionization suppression or enhancement that may occur during that elution window. One caveat to this is the occurrence of isomeric species, such as GlcCer and GalCer, which must be baseline resolved via HPLC to obtain accurate quantitative data.

Separations of SL by HPLC have classically been performed either by reversed or normal phase chromatography. In reversed phase chromatography separations are based on the length and (un)saturation of the sphingoid base and/or N-acyl fatty acid [45-47]. This is contrasted by the normal phase method in which separations are based on the polarity of the head group (i.e. separation of Cer, HexCer, LacCer, and SM from each other) [47-49]. The need for HPLC separations are driven by the previously mentioned interferences arising from isotopes, isobars, and isomers. So and Sa, for example, have easily resolved *m/z* values

of both precursor (*m/z* 300 vs. 302) and product (*m/z* 264 vs. 266) ions. However, in biological samples, So is of much greater abundance than Sa, therefore, the naturally occurring (M + 2) isotope of So (~2% abundance) will pass the MRM pair of Sa and give a falsely high signal unless these species are separated by LC, or an alternate fragmentation pathway is used. The isobaric situation occurs when two distinct SL have the same mass and fragment to the same product ion. This has been observed to happen for the Cer1P and HexCer species, which further demonstrates the need for their separation via LC. The problem presented by isomers such as GlcCer and GalCer is difficult to resolve via MS only based methods, however, they are easily separated by HPLC.

3.6 Matrix Assisted Laser Desorption Ionization (MALDI)

Matrix assisted laser desorption ionization has been successfully used to ionize multiple classes of SL such as Cer, HexCer, LacCer, ST, globosides, gangliosides, and SM [50-52]. Sample preparation for MALDI analysis usually occurs by mixing a solution of small organic acid (matrix) with a solution containing the analyte of interest. A small volume, typically on the order of 0.5-1 μL, of this mixture is then spotted on a metal target plate and allowed to dry. When dry, the analyte is imbedded throughout the matrix crystals which have formed. The plate can then be placed inside the high vacuum region of the ion source. Ionization of the sample occurs when the spot is irradiated by a laser pulse. Here the matrix serves two purposes: 1) it absorbs the photonic energy of the laser pulse becoming vibrationally excited and vaporizing, taking it and the analyte off the surface and into the gas phase; and 2) in the gas phase, the matrix and the analyte undergo proton exchange reactions whereby the analyte is ionized. After a brief delay to allow neutrals to dissipate as well as to let the ions have a more uniform spatial distribution, the ions are accelerated out of the source. It can be easily seen that matrix choice is critical for desorption from the surface and efficient ionization of intact molecular species with little fragmentation and high sensitivity.

MALDI is typically used with time-of-flight (TOF) mass analyzers because of the timing of discrete events such as the laser pulse and the delayed extraction of ions from the source. MALDI primarily forms singly charged ions, so when coupled with TOF mass analysis it can provide useful information regarding larger SL. Unfortunately, MALDI is not without limitations, the primary of which is background chemical noise arising from the matrix. In MS mode, this precludes observation of free sphingoid bases and other similarly sized SL. A secondary problem arises from in source fragmentation of some fragile species such as Cer or SL having multiple sialic acid residues. However, some of these limitations can be addressed by higher pressure ion sources, alternative matrices to minimize fragmentation, and MS/MS to filter out background chemical noise.

An interesting application of the MALDI technique has been the imaging of molecules on thin tissue slices. Although originally developed to image proteins [53, 54], lipids and SL can be imaged as well [9, 10]. For general lipid imaging, matrix application is the critical step. Here, the matrix in an aqueous/organic solvent mix must be applied in a manner such that the solvent does not solubilize the lipids leading to their migration resulting in a subsequent smearing of the species image. To this end, several very expensive devices have been made commercially available; however, at least two simple, fast, and inexpensive techniques (i.e. sublimation and oscillating capillary nebulizer) have been demonstrated to apply matrix with highly uniform crystal formation without lipid migration resulting in quality lipid images [9, 10].

4. LC-MS/MS MATERIALS AND METHODS

The following methods have been developed for the analysis of SL from the first step of the *de novo* biosynthetic pathway (i.e. all long chain bases) through the metabolic products of

Cer (and their dihydro counterparts) which include: GlcCer, GalCer, Cer1P, LacCer, SM, and sulfatides (ST).

4.1 Internal Standards and Solvents

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

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

4.2 Biological Samples

Many different kinds of samples can be analyzed for their SL content. These include cultured cells, tissue homogenates, blood, plasma, urine, etc. For cells, typically 10^6 cells are sufficient, however, an order of magnitude more or less may be required depending on the abundance of the analyte of interest in that particular cell line. Generally, 1-10 mg of homogenized tissue at 10% wet weight per volume of phosphate buffered saline (PBS) is sufficient for SL analyses. Small volumes of biological fluids such as blood, plasma, or urine, typically on the order of 1-10μL can be effectively extracted as well. Similarly, tissue culture medium may be extracted for SL, but larger volumes should be used (100-500μL), and the medium should be lyophilized prior to extraction to reduce the aqueous volume.

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

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

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

4.3 Sphingolipid Extraction Protocol

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

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

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

Test tube A is now assigned for further work-up as the “Single phase extract,” test tube B will be designated as the “Organic phase extract”, which will be treated as follows:

The single phase extract from test tube A should be centrifuged to pellet any insoluble material and the remaining solvent should then be transferred to a new glass test tube. Next, re-extract the pellet by adding another 1.0mL CH_3OH and 0.5 mL CH_2Cl_2 to the original test tube followed by sonication, vortexing, and then centrifugation. Remove the solvent and combine with the first extraction. The pellet may now be discarded and the combined extracts may be reduced to dryness via speed vac, being careful not to overheat.

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

The dried single phase extraction residue may be reconstituted in 300 μL of the initial condition mobile phase (see Section 4.6.1) used for reverse-phase LC-MS/MS. The dried organic phase extraction residue is similarly reconstituted in 300 μL of the initial condition

mobile phase (see Section 4.6.2) used for normal-phase LC-MS/MS. Both are sonicated for ~15s, then each is transferred to separate 1.5-mL microfuge tubes (organic solvent resistant) and centrifuged for several minutes or until clear. Approximately 70 μL of the clear supernatant is transferred from each into separate 200 μL tapered glass autoinjector sample vials for LCMS/MS analysis. The remainder may be saved for profiling and further future analyses. It has been demonstrated that the recovery of SL using these methods is very high and will be discussed in detail below [4]. A cartoon summary of the extraction protocol is provided (Figure 2).

4.4 Sphingolipid Profiling

A 50-μL aliquot of the reconstituted extract should be diluted to 500 μL with 99:1 CH_3OH/HCOOH (v/v). This solution may then be infused into the mass spectrometer at a flow rate of 5-10 μL/min (or lower if using nanospray). Sphingolipids such as So, Sa, S1P, Sa1P, Cer, GlcCer, LacCer, and SM ionize readily in the positive ion mode and give strong $(M + H)^+$ ions. Alternatively, anionic SL such as Cer1P, ST, and gangliosides ionize more readily in the negative ion mode and give strong $(M - H)^-$ and $(M - nH)^{n-}$ ions. MS/MS on the positive ion species reveals that most SL fragment to give a common product ion of *m/z* 264 indicative of a doubly dehydrated sphingosine base, and SL having a sphinganine backbone give rise to fragment ions of *m/z* 266 (Figure 3). SM is the exception to this, as it fragments primarily to yield an ion of *m/z* 184, which is indicative of the phosphocholine head group (Figure 3). In the negative ion mode Cer1P, ST, and gangliosides fragment to yield highly abundant ions of *m/z* 79, 97, and 290, respectively. These fragmentations are indicative of PO_3^-, HSO_4^-, and a dehydrated N-acetyl neuraminic acid (NANA) residue, respectively.

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

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

4.5 Multiple Reaction Monitoring (MRM) Optimization

Optimal conditions for both ionization and fragmentation are determined for each analyte of interest as well as the internal standards. Standard curves should be generated for the

internal standards to determine the limit of detection (LOD = 3:1 signal to noise, s/n), limit of quantitation (LOQ = 10:1 s/n), and the linear dynamic range of the mass spectrometer. Each SL standard can be dissolved at a concentration of 1-10 pmol/μL in the appropriate solvent mixture in which it elutes from the column. For example, Cer elutes early in the normal phase run so it is infused in the initial high acetonitrile solution, whereas SM elutes late and is infused in the methanol solution.

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

4.6 Liquid Chromatography Separation Conditions

Summarized in Fig. 4 are the types of LC columns and elution conditions that were found to be optimal for analysis of different subcategories of SL [4].

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

Cer1P has proven to be difficult to analyze via the method mentioned above. It has been observed that there is significant carryover of Cer1P on the LC column of >1%. This occurs with reverse phase columns obtained from different vendors as well as with different lots of columns from the same vendor. As an alternative, Cer1P can be analyzed instead using a Supleco 2.1 (i.d.) × 50 mm Ascentis C8 column (Sigma, St. Louis, MO), with the column heated to 60°C and a binary solvent system (based on reference [4]) at a flow rate of 0.5 mL/min. Prior to the injection, the column is equilibrated for 2 min with a solvent mixture of 70% mobile phase A (CH_3OH/H_2O/THF/HCOOH, 68.5/28.5/2/1, v/v/v, with 5 mM ammonium formate) and 30% Mobile phase B (CH_3OH/THF/HCOOH, 97/2/1, v/v/v, with 5 mM ammonium formate). After sample injection (30 μL), the A/B ratio is maintained at 70/30 for 0.4 min, followed by a linear gradient to 100% B over 1.9 min. The flow is held at

100% B for 5.3 min, followed by a 0.5 min wash of the column with 70:30 A/B before the next run.

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

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

4.7 Generation of Standard Curves

Once the LC and MRM protocols have been determined, standard curves should be generated under these conditions. The concentration of the individual components of the Avanti internal standard cocktail is 25 μM. If additional SL subspecies intend to be quantitated they should be dissolved in methanol to produce a stock solution at 0.5 mg/mL concentration. These may subsequently be serially diluted into the appropriate LC solvent immediately before analysis to provide 0.5 to 1000 pmol of each standard per 50 μL injection. [It should be noted the 4000 QTrap may go much lower in concentration range, and new instrumentation and techniques may also as well.] Each may then be analyzed by the appropriate LC-MS/MS protocol to generate the standard curves such as those shown previously, then the linear regression lines and fit may be calculated [4].

4.8 Data Analysis

The elution profiles for each MRM pair are examined to quantify the amounts of the SL analytes of interest in biological sample extracts. The areas under the peaks generated for both analytes and internal standards are integrated via the native mass spectrometer software (i.e. Analyst 1.5.1 or newer for Applied Biosystems instruments). Using identical integration settings (number of peak smooths = 2-3, bunching factor = 5-10, and noise threshold = 1 ×

10^4) to integrate both internal standard and analyte allowed quantitation via measuring the area under the peak, then the pmol of the analyte is calculated using the following formula:

pmol of analyte of interest = $K_{analyte}$ x ($A_{analyte}$ / A_{IS}) x pmol of added internal standard

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

No correction was necessary for differences in extraction recovery because the extraction conditions were chosen to achieve similar recoveries, as described below and in Section 6. Following the LIPID MAPS convention, the quantities of the SL are then expressed as pmol analyte/μg of DNA. For comparison, it has been our experience that 1×10^6 RAW 264.7 cells contain approximately 3 μg of DNA and approximately 0.25 mg of protein.

4.9 Quality Control

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

5. TISSUE IMAGING MASS SPECTROMETRY (TIMS) MATERIALS AND METHODS

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

5.1 Chemicals

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

5.2 Tissue Sectioning

Tissue to be dissected is typically frozen in liquid nitrogen and stored at −80°C. When ready to section the frozen tissue is placed in a sealed dry ice box and allowed to equilibrate for 1 hr. Afterwards the tissue is transferred into the cryostat at −20°C for 1 h, then sectioned into

8-10 μm thick slices. These thin slices are then thaw-mounted onto chilled MALDI plates. The following section may be cut in an identical fashion and placed onto a glass slide for histological staining. This process may be continued alternating between MALDI plate and slide with some slices saved for extraction and analysis via LC-MS/MS as detailed above. The slices on the MALDI plate are then slowly brought to room temperature in a desiccator for matrix application for subsequent analysis.

5.3 Oscillating Capillary Nebulizer (OCN) Matrix Application

A diagram and description of the nebulizer has been previously published [9]. Briefly, a matrix solution of DHB at a concentration of 30 mg/mL in 1:1 (v:v) acetonitrile/water with 0.1% TFA may be delivered to the OCN at a flow rate of 60 μL/min via syringe pump, which is delivered through the inner needle of the apparatus. Ultra high purity nitrogen is then passed at ~50 psi through the outer needle surrounding the inner needle. This gas flow generates a high frequency oscillation of the inner needle at the tip, which extends slightly outside the outer needle. This oscillation serves to nebulize the matrix solution into a fine and uniformly dispersed spray. The size of the spray droplet, which is subsequently deposited on the tissue, can then be controlled by 3 variables: matrix solution flow rate, gas flow rate, and distance from the spray tip to the tissue slice. For SL imaging the smallest droplets provide the most homogenous and uniform crystals and prevent lipid migration. The sample stage can then be used to uniformly apply the matrix to a thickness of 10-20μm. Typical spraying time for a 4 cm^2 sample area is ~5 min.

5.4 MALDI-MS

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

6. EXTRACTION, QUANTITATION, AND LOCALIZATION OF SPHINGOLIPIDS VIA LC-MS/MS AND TIMS

The goal of these protocols was to identify, quantify, and determine the structure and localization of as many SL as possible in a highly rigorous and reproducible fashion with high throughput. The resulting sphingolipidomic data reflects an “inside-out” measurement monitoring the most critical steps of SL biosynthesis and turnover. To achieve this objective, many of the LC-MS/MS conditions from previous publications [4, 5]were used. However, a number of minor modifications were identified and addressed following those studies, such as the observation of potential carryover of Cer1P occurring on some reversed phase columns.

The subsections below describe how each internal standard compares to possible analytes with regard to signal response and linear dynamic range. This starts with validation of the extraction protocol, identification of some new fragmentation profiles for selection of the precursor-product pairs, optimization of the parameters for LC-MS/MS, and application of the methods to the various classes of lipids.

The goal of the tissue imaging was to apply matrix in such a way as to prevent lipid migration and be able to discern the localization of specific individual molecular species of SL. The resulting imaging data indeed showed individual molecular species were highly localized in certain areas of the brain. ESI and MS^n were used to determine the structures of the most abundant SL observed via TIMS [9].

6.1 Analysis of the Extraction Efficiencies

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

6.2 Standard Curves and Linear Dynamic Range

A manuscript detailing the linear dynamic range, overall sensitivity, and the correlation of the internal standard to different chain lengths of SL has been published [4]. There the instruments used were an older ABI 3000 triple quadrupole (QQQ) and a newer ABI 4000 quadrupole linear ion trap (QTrap). They were both operated as QQQ using MRM protocols similar to the ones above. The QTrap used is at least two generations behind currently available instruments. It is therefore expected that state-of-the-art mass spectrometers would have even greater sensitivity for all classes of SL listed below, given the significant improvements generating, sampling, and transmitting ions as well as newer separation techniques such as ultra high pressure liquid chromatography (UHPLC).

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

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

magnitude greater signal response than the QQQ, and has a corresponding 2 to 3 orders of magnitude lower detection limit than the range over which it was evaluated.

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

6.2.3 Ceramide 1-Phosphate—It was shown that the highest recoveries of these SL were found in the “Single Phase Extract” (as discussed later). It was further demonstrated that Cer1P can be analyzed by either reverse or normal phase (RP and NP, respectively) LC. Using HPLC for identification and quantitation of Cer1P was reported to have several advantages. First, it is necessary that these species be strongly retained, de-salted, and chromatographically focused because of the high salt content of the single phase extraction procedure and the low abundance of the analyte. Second, they can be analyzed in the positive ionization mode where these molecules fragment to give the more structure-specific backbone fragment. This was found to preferable to the ubiquitous phosphate fragment ion of *m/z* 79 and 97 that is observed in negative mode, which may arise from a number of other phosphorylated species.

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

It should be pointed out that some lots of C18 packing material gave rise to significant carryover of Cer1P, in some cases as high as 10%. To address this, another LC method using a weaker retention solid phase (C8) was developed for those instances (see Section 4.6.1). Additionally, the normal phase LC method for complex SL may also be used to analyze Cer1P, however, the peaks are broad and diffuse and have reduced sensitivity. Care must also be taken to neutralize the lipid extracts to minimize hydrolysis of SM to Cer1P, as discussed previously [4, 55].

6.2.4 Sphingomyelin—Sphingomyelin was likewise reported to have a linear signal response from 0.5 to 1000 pmol on both instruments, and there was little difference in signal response with regard to chain length for SM (C12:0/C18:0/C24:0) or DHSM (C12:0/C18:0/C24:0) [4]. Unlike the LCB, DHCer and Cer, the difference in signal response between the QQQ and the QTrap for all SM species was an order of magnitude. It was also noted that SM and DHSM are analyzed via loss of the phosphocholine headgroup rather than backbone cleavage. Therefore, the presence or absence of the Δ4 double bond may not have as much an effect on the signal response for these the molecular species. Further, the magnitude of the signal response on the low end of the concentration range was such that both instruments were capable of detecting these species at least 2 orders of magnitude lower concentrations.

Determination of the structures of the Cer core of SM is not possible via these methods. (Again, this is because in the positive ion mode they fragment to yield the charged head group). However, other methods have been developed to unequivocally differentiate the sphingoid base and fatty acid in these molecules and this was done independently. These methods will be covered in Section 7.3.

6.2.5 Monohexosylceramide—The monohexosylceramides like the previous SL species were observed to have a linear signal response from 0.5 to 1000 pmol for all of the chain length variants HexCer and DHHexCer analyzed (C12:0/C16:0/C18:0/C24:1) on both instruments and little variation in signal response relative to chain length was reported [4]. Much like the LCBs, DHCer, and Cer the signal response for GlcCer was reported to be ~2.5 orders of magnitude higher on the QTrap versus the QQQ, and little correction was needed for the DHGlcCer species. This was in contrast to the QQQ, in which an 8-fold correction factor was reportedly needed. As was also the case for the other SL the detection limit for the QTrap is on the order of single digit fmol for these species as well.

Several comparisons of standard GlcCer and GalCer with similar backbones did not reveal any differences in signal response between the two species. It was therefore concluded that the C12-GlcCer internal standard may be used for the quantitation of total HexCer if the species are not to be differentiated. It was noted that this might not be the case, however, for other backbones, such as ones with α-hydroxy-fatty acids. It is worthy of mentioning that many cells do not contain galacto-family SL, therefore, it is not necessary to distinguish GlcCer and GalCer in some cases. However, it has been observed that in some cases galacto-sphingolipids may not be present under initial conditions, but may be greatly abundant after some stimulus. Thus, the ability to differentiate the two species using the modified LC conditions described in Section 4.6.3, provides this more rigorous distinction when needed.

6.2.6 Dihexosylceramide (lactosylceramide)—LacCer (C12:0/C16:0/C24:0) and DHLacCer (C16:0/C24:0) have displayed a linear signal response from 0.5 to 1000 pmol on both instruments and some differences in signal response were attributed to the length of the fatty acyl-chain [4]. It was likewise concluded that the C12:0 LacCer species would be an appropriate internal standard for the quantitation of a wide variety of alkyl-chain lengths of these SL. It was observed that the signal response for LacCer is the lowest of the SL analyzed in the positive ion mode. Furthermore, like the Cer1P and SM species the QTrap was seen to yield approximately an order of magnitude greater signal response than the QQQ. It was also shown that the difference in signal response for DHLacCer was only 20% lower than that of the LacCer when analyzed using the QTrap, but they were ~5 fold lower when analyzed via the QQQ. Given the magnitude of the signal response generated from the QTrap at the lowest concentration in the experiment, it is estimated that the lower limit of detection is approximately one order of magnitude lower than tested.

6.2.7 Analysis of Other Sphingolipids via Protocol Modification: Sulfatide—The ability to analyze additional SL is a continual and growing process. It should be possible to expand the list of analytes extracted and detected by supplementation of the internal standard cocktail with the appropriate reference compound. It was reported that a commercially available C12-sulfatide (ST d18:1/12:0) was used to quantify sulfatides in RAW264.7 cells [4]. The highest recoveries of these compounds were obtained in the “Single Phase Extract” procedure.

A comparison of the signal responses for sulfatides on the QQQ and QTrap revealed that the latter was approximately one order of magnitude more sensitive than the former. It was further demonstrated that the signal response was virtually identical for all chain length

species investigated (C12:0/C16:0/C24:0), and linear from 0.5 to 1000 pmol for both instruments as well. Similar comparisons with DHST and ST containing α-hydroxy-fatty acid were not possible because of a lack of the necessary standards.

6.3 Estimation of the Coefficient of Variation (CV) for RAW264.7 Cells

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

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

6.4 Tissue Imaging Mass Spectrometry of Sphingolipids

Multiple parameters such as matrix solvent flow, N_2 gas flow, and distance from the surface were evaluated for the application of MALDI matrix to a brain tissue slice [9]. Optimal coating conditions produced very fine matrix crystals, which were observed by scanning electron microscopy. It was determined that discrete spraying and drying cycles yielded optimal results for TIMS [9].

TIMS analysis of a mutant Tay/Sachs-Sandhoff (hexb −/−, knockout) mouse brain showed several striking features [9]. The knockout mouse brain slices when ionized in the negative ion mode showed two prominent ions of *m/z* 888.9 and 1383. The image generated from the former showed it to be localized mainly to the myelinated fiber region of the cerebellum and fairly evenly distributed in the brain stem. This was contrasted by the *m/z* 1383 ion, which was more localized to the granular cell region in the cerebellum and was not detected in the brain stem.

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

ESI MS/MS and MS/MS/MS were performed to elucidate the structures and determine the identity of the molecules observed in TIMS. The MS/MS spectrum of the ion of *m/z* 888.9 was determined to be a ST having a sphingoid base and fatty acid combination of d18:1/24:1. The MS/MS spectrum of the ion of *m/z* 1383 was found to be ganglioside GM2 via prominent glycosidic bond cleavages, however, no peaks corresponding to fragmentation of the Cer core were observed. Therefore, MS^3 was subsequently performed on the Y_0 fragment ion, which revealed that the Cer core had a d18:1/18:0 composition. A detailed discussion regarding this is presented in Section 7.3.

The normal mouse brain was observed to have some similarities and some differences when compared to the mutant [9]. For example, both the normal and the mutant mouse brains displayed highly abundant *m/z* 888.9 ions in the negative mode, which were also localized mainly in the myelinated fiber region of the cerebellum. In contrast, neither *m/z* 1383 (GM2) in the negative mode nor *m/z* 1132 (GA2) in the positive mode were detected in the normal mouse brain.

Multiple other prominent ions were observed to be localized to distinct regions of the brain [9]. Several other chain length variants of both sulfatide and GM2 were also seen to have the same localization as the ones detailed above. In addition, other phospholipids such as PC and PI could also be seen. This clearly demonstrates that specific individual molecular species may be localized in specific regions of the brain, and that these experiments provide spatial information that is complimentary to other types of lipidomic analyses.

7. DISCUSSION

7.1 LC-MS/MS Overview

The extraction and LC-MS/MS protocols detailed in this manuscript have been rigorously tested and validated for the quantitative analysis of all of the components of the SL metabolic pathway starting with the acyl CoA [56], backbone Cer, and its initial metabolites free LCB, their phosphates, SM, GlcCer, GalCer, Cer1P, ST, and LacCer [4]. This also includes the sphingoid bases involved in SL turnover and cell signaling such as sphingosine and S1P. It was reassuring to confirm that a relatively small number of internal standards are needed to quantify these compounds using optimized LC ESI-MS/MS conditions. Further, since these standards are commercially available as a single cocktail, this should facilitate "sphingolipidomic" studies of this spectrum of metabolites. It should be noted that when using these internal standards for other applications, care should be taken to ensure that none of the components are already present in the samples (for example, C17-sphingoid bases, which may be found in some organisms). Also, it should be further validated that the internal standards are appropriate for any additional analytes beyond the ones validated in this study (for example, SL with "phyto"Cer backbones, hydroxylated fatty acids, or other modifications).

Initial screening of each new biological material via precursor ion and neutral loss scans are critically important for generating the necessary "parts list" of SL present. This is because multiple reaction monitoring is a targeted method of analysis and only provides information about the specific precursor-product pairs that have been selected. In addition, it is important to also screen the biological material after any treatments or stimulations that might alter the SL composition. This has been illustrated in other studies in which the appearance of quantifiable amounts of sulfatides was observed only after the RAW264.7 cells were treated with Kdo_2-Lipid A [4]. It has been well established that precursor ion scans for *m/z* 264.4 and 266.4 are signatures for the sphingosine (d18:1) and sphinganine (d18:0) based SL, respectively [3-5, 47]. The same principle may be followed in scanning for sphingoid bases that differ in alkyl chain length such as eicosasphingosine, d20:1 (*m/z* 292.4) and/or unsaturation that occurs for sphingadienes (*m/z* 262.4), which have been found in both mammals, and plants [57]. "Phyto" SL (1,3,4-trihydroxy-) often yield fragments with the same product *m/z* as sphingosine. However, these species can be readily differentiated by both their increased mass and shifted retention time by LC [3]. These precursor scans also provide information about the amide-linked fatty acids, including special subcategories such as α-hydroxy species [58]. Once all the sphingoid bases, N-acyl variants, and head groups are known, the corresponding transitions can be incorporated into a new MRM protocol.

The potential for two or more compounds to have identical precursor/product ion pair *m/z* values is quite possible when analyzing such a large number of compounds. Further, the overlap may occur with not just the nominal mass of the compounds, but also isotopologues (for example, the M +2 ^{13}C isotopologue of d18:1 with d18:0) or ions that are produced by in-source degradation—and even the combination of both of these possibilities, as was found for C25-Cer in previous studies [4]. This is a compelling argument for use of a separation method such as LC prior to mass analysis to reduce the likelihood of such artifacts as well as to confirm the identity of the analytes by a third orthogonal measurement, retention time.

LC has additional advantages such as it reduces the complexity of the electrospray droplet itself. This serves to reduce the likelihood that it will be comprised of compounds with greatly different gas phase basicity or acidity, which can suppress the ionization of the analyte(s) of interest [59]. Further, HPLC may separate and remove any salts in the biological extract, which is especially critical for low abundance LCBs, their phosphates, and Cer1P. This also minimizes formation of multiple salt adducts and other sources of ionization suppression [60], which further reduces sensitivity. Additionally, LC serves to concentrate the analytes of interest into a small elution volume thereby increasing sensitivity. Finally, LC may separate isomers (with appropriate choice of separation conditions) that might not be distinguished by MS or MS/MS alone [61, 62].

7.2 Tissue imaging Overview

The push for developing all encompassing SLomic techniques to identify and quantify all SL is driven by the desire to understand how these molecules are interconverted from one species to another and what the resulting biological response is. In order to obtain this information the molecules of interest are extracted and removed from their normal biological environment and any information regarding their cellular or sub-cellular localization is lost. Another example of this is a recent study which has shown how SL distribution in the human lens changes with age [63]. Thus it can be seen that tissue imaging mass spectrometry (TIMS), is an emerging technology that is beginning to address some of the questions that cannot be answered via the analytical techniques discussed previously.

In addition to the MALDI technique, secondary ionization mass spectrometry (SIMS) has been used to directly analyze tissues [64] and cells [65] for their lipid content. Unlike MALDI, this technique uses a high energy beam of primary ions to strike the surface of the biological material deforming the surface and ejecting both neutral species and some secondarily formed ions. The SIMS technique provides greater spatial resolution because the initial ion beam may be tightly focused. The high energy of the primary beam, however, causes extensive fragmentation of the species ablated from the surface. This often prevents unequivocal identification of intact molecular species, and just portions of a molecule are used for analysis [66]. However, development of “soft projectile” ion guns using clusters of ions or “bucky balls” can dissipate this energy by deforming upon striking the sample surface and generate intact molecular ions [67]. For example, SIMS TOF analysis of brain slice showed that chain length variants of GalCer were distributed differentially. The long chain C24 species was observed to be localized to high salt regions, whereas the short chain C18 species was found in cholesterol rich areas.

7.3 Ion Trapping Techniques and MS^n

There still remain many analytical challenges for the identification and quantitation of SL. Methods for the more complex glycosphingolipids are still undergoing development. The primary impediment to quantitative analysis of individual molecular species in this area is a lack of commercially available internal standards. It is hoped that either enzymatic or

synthetic approaches will yield short chain derivatives of these species similar in approach to the other classes of SL.

Fortunately, the identification and structure elucidation of these molecules, while not easy, is possible. Gangliosides ionize readily in the negative ion mode when sprayed from a methanol solution. When analyzed by classic product ion scan in a QQQ, $(M - 2H)^{2-}$ ions of GD species were reported to fragment to primarily yield highly abundant ions of *m/z* 290, which reflect a C1β – H_2O cleavage indicative of the N-acetyl neuraminic acid group [4]. It was shown that when MS/MS was performed using the ion trapping function "enhanced product ion" scan on a 4000 QTrap much more extensive structural information regarding the glycan headgroup could be observed. In addition to the typical Y-type glycosidic bond cleavages, through ring X-type cleavages were also observed, which may reveal glycosidic bond linkages (Figure 5).

The instrument used was capable of performing MS^3 (MS/MS/MS) analyses. These experiments are performed in a similar manner as a product ion scan. Here the first quadrupole (Q1) is set to pass the precursor ion of interest to the collision cell (Q2). The cell is filled with a neutral gas such as N_2 or Ar. In traversing the collision cell the precursor ions undergo multiple collision events and part of its translational energy is converted into internal energy, and they may subsequently fragment. Rather than mass analyzing the resulting product ions as is usually done, the last quadrupole (Q3) can be used as a linear ion trap (LIT). Here, a potential is applied to the ion exit lens and the LIT is set to trap and hold a 2 *m/z* unit wide window centered on the product ion of interest. When the trap is filled with ions for a user defined time, the selected product ion can then be irradiated with a single wavelength, amplitude frequency to induce further fragmentation to secondary product ions. These are then scanned out of the LIT to the detector. The resulting MS^3 spectrum reveals further structural details regarding the fragment ion from which they arose.

Interestingly, no information regarding the lipid core of the ganglioside could be observed in either product ion mode for the ganglioside mentioned in Section 6.4. Only the core Y_0 Cer fragment was detected. However, MS^3 was performed on the Y_0 ion (*m/z* 592.6) to provide critical structural information regarding the Cer portion of the molecule [4]. It was shown that the Y_0 ion subsequently fragmented to yield highly abundant S, T, U, and V + 16 ions of *m/z* 324, 308, 282, and 283, respectively (Figure 5). These species were reported as being indicative of a fatty acid that is 18:0. Also observed in the MS^3 spectrum were the complimentary P and Q ions (*m/z* 265 and 291, respectively) which were indicative of a d20:1 sphingoid base (Figure 5). It is clear that MS^n capabilities are critical to the total structure elucidation of the sphingoid base, fatty acid, and glycan headgroup in higher order glycosphingolipids.

The detailed structural analysis of SM is another area in which the unique capabilities of the MS^3 and ion trapping techniques have proved invaluable. For example, MS/MS on intact SM species in either the positive or negative ionization mode yields primarily fragment ions indicative of the phosphocholine headgroup (*m/z* 184 and 168, respectively). Information regarding the Cer core is not readily observed. In order to address this, an unusual instrumental analysis is performed. Here SM is ionized in the negative ion mode to yield highly abundant $(M - 15)^-$ precursor ions. The QTrap can then be set up to perform a MS^3 scan, however, the same precursor mass is selected for both the precursor ion and the resulting fragment ion, while the collision energy in Q2 is set to 5-10eV. When done in this fashion, the precursor ions do not undergo fragmentation in Q2 and are passed intact into the LIT. The precursors are subsequently induced to fragment in the ion trap as mentioned in the MS^3 experiment above and the product ions are scanned out. When MS/MS is performed in this way, highly abundant peaks of *m/z* 449 were observed, which were indicative of a d18:1

sphingoid base and phosphocholine headgroup. This arises via cleavage of the amide bond and neutral loss of the fatty acid [4]. This ion trap facilitated fragmentation allows for post-run analysis of a sample for structural verification.

7.4 Emerging Techniques

There are some analytical techniques which may greatly aid in the complete elucidation of the sphingolipidome. Although several exist three of the more intriguing are ultra-high resolution MS, ion mobility MS, and ozone induced dissociation. Each of these addresses a unique area of investigation of SL that was inaccessible using the techniques mentioned in Section 3.

7.4.1 Ultra High Resolution Mass Analysis—Ultra-high resolution and accurate mass measurement mass spectrometry has the ability to provide a more detailed and comprehensive profiling of the sphingolipidome. This is because there are many interferences that arise from isobaric and isotopic species. This is especially true for the sulfatides and gangliosides where ^{34}S, double ^{13}C, and double bond differences can often occur at the same nominal mass precluding unequivocal identification and estimation of relative abundance. Mass analyzers such as TOF and Orbitrap systems are not capable of resolving these species. Currently, only Fourier transform mass spectrometry (FTMS) instruments which have resolving powers in excess of 100,000 and mass accuracies in the sub-ppm range are capable of resolving these species.

Typically analyses of SL using FTMS have been performed with either nanospray [68, 69] or MALDI [70, 71]. As mentioned above, most of this work has been focused on the identification and structure elucidation of the higher order glycosphingolipids. Some of the more cutting edge techniques involving nanospray use alternative ion activation routes such as infrared multiphoton dissociation (IRMPD), electron capture dissociation (ECD) and electron detachment dissociation (EDD) to induce different fragmentation pathways for enhanced structure elucidation [68]. Alternatively, MALDI has been used to investigate lipids present in cells and plants [72, 73], and harkening back to the early days of lipid analysis, TLC plates [71]. One of the key elements provided by the accurate mass measurement of FTMS is the ability to differentiate various classes of phospholipids [72] or distinguish fragment ions as being either carbohydrate or lipid in nature based solely on mass defect plots [68].

7.4.2 Ion Mobility Separations—Ion mobility separations provide a tremendous new and different dimension to the comprehensive analysis of the sphingolipidome. Here separations occur in the gas-phase based on the migration and diffusion of ions through a neutral gas under the influence of an applied field [74]. It has been observed that many broadly defined classes of molecules such as lipids, carbohydrates, and peptides can be differentiated based on their drift times and *m/z* values when measured in a coarse manner [74]. More interesting is the consideration of high resolution “fine” separations for lipids because SM and HexCer adopt more extended structures, whereas glycerophospholipids such as PC, PS, and PE adopt a more compact structure [74]. One could envision the possibility of separation of isomers such as GD1a/GD1b or GlcCer/GalCer, or cis/trans isomers, or double bond positional variants should their cross sections be different. Such separations add an extra dimensional space, drift time, to that of retention time on LC, mass, and structure, for further structural characterization.

7.4.3 Ozone Induced Dissociation—The technique of ozone induced dissociation (OzID) addresses at least one and possibly two critical missing parts in the complete structural elucidation of the sphingolipidome: double bond location and conformation. In

this technique ozone and unsaturated lipids undergo gas-phase ion-molecule reactions at the site of unsaturation to form the primary ozonide [75]. Upon collisional activation the ion fragments to yield two distinct product ions for each double bond: an aldehyde ion and a Criegee ion [76, 77]. Therefore, the position of the double bond is determined by the masses of the two fragments or the neutral lost. It has recently been reported that this technique has been successfully modified such that the gas phase reactions may occur in the collision cell [77]. It is reported that differences in the reactivity between isomeric unsaturated lipids may now be observed [77]. This technique affords the ability to identify isomeric lipids with regard to double bond position in complex mixtures and holds much promise for future analyses.

8. SUMMARY

These methods provide a useful platform for analysis of essentially all of the SL in the early steps of the metabolic pathway (Figure 1) and have been applied to several dozen types of biological samples. Nonetheless, each new application of this methodology will require some degree of re-validation of these parameters, and they should be periodically re-checked with even routine analyses. It is anticipated that adjustments will be minimal. Very importantly, the availability of an internal standard cocktail that is applicable to such a wide profile of compounds is also critical for sphingolipidomic analyses because it not only facilitates such studies but helps ensure that the results from different laboratories can be compared.

Acknowledgments

This work by was supported by the Lipid MAPS Consortium “Glue” grant (U54 GM069338). The authors thank Avanti Polar Lipids for many of the compounds used to validate both the extraction and LC-MS/MS protocols. The authors also thank current and former lab members Drs. Jeremy C. Allegood, Christopher A. Haynes, and Hyejung Park; Ms. Rebecca L. Shaner, Ms. Elaine Wang, and Mr. Samuel Kelly, who all contributed a great deal of time and effort into establishing and validating these techniques.

Abbreviations

So	sphingosine
S1P	sphingosine 1-phosphate
Sa	sphinganine
Sa1P	sphinganine 1-phosphate
1-doxSo	1-deoxysphingosine
1-doxSa	1-deoxysphinganine
1-demeoxSo	1-desmethoxysphingosine
1-demeoxSa	1-desmethoxysphinganine
Cer	ceramide
Cer1P	ceramide 1-phosphate
GalCer	galactosylceramide
GlcCer	glucosylceramide
HexCer	monohexosylceramide
LacCer	lactosylceramide

ST	sulfatide
GM, GD, GT	gangliosides
SM	sphingomyelin
LC-MS/MS	liquid chromatography tandem mass spectrometry
MRM	multiple reaction monitoring
Q	quadrupole
QQQ	triple quadrupole
QTrap	quadrupole linearion trap
CE	collision energy

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- Classical methods for the analysis of sphingolipids are reviewed.
- Modern LC-MS/MS methods for the identification, structure determination, and quantitation of sphingolipids are presented.
- Tissue Imaging Mass Spectrometry of sphingolipids for determination of their localization is presented.
- Applications of new technologies to the analysis of sphingolipids is discussed.

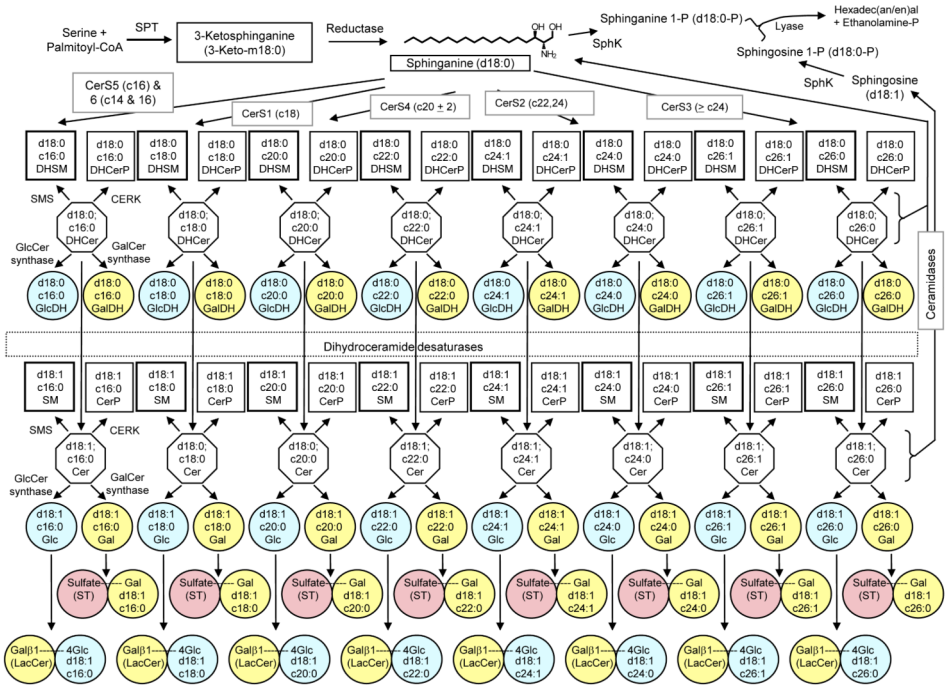

Figure 1.

Biosynthetic pathway of sphingolipids consisting of free long chain bases (top row), which are N-acylated by several CerS enzymes to form dihydroceramides (upper row of octagons). These DHCer may then processed by dihydroceramide desaturases to introduce a 4, 5 double bond to form ceramide (lower row of octagons). Collectively both the DHCer and Cer may be converted to more complex via addition of a polar headgroup at the 1′-OH position via sphingomyelin synthase to form sphingomyelin (thick lined squares), or ceramide kinase to form ceramide-1-phosphate (thin lined squares). Carbohydrates may also be linked to this position as well via glucosylceramide or galactosylceramide synthase to form glucosylceramides (light blue circles) and galactosylceramides (yellow circles), respectively. The former may have additional carbohydrates such as galactose complexed to it to form lactosylceramide (bottom row of light blue and yellow linked circles). The latter may also be sulfated to form the sulfatide species (pink circles). The far right and upper left corners show the degradative pathway of sphingolipids via ceramidases, sphingosine/sphinganine kinases, and sphingosine/sphinganine phosphate lyases, which constitutes the only known exit from the sphingolipid metabolic pathway.

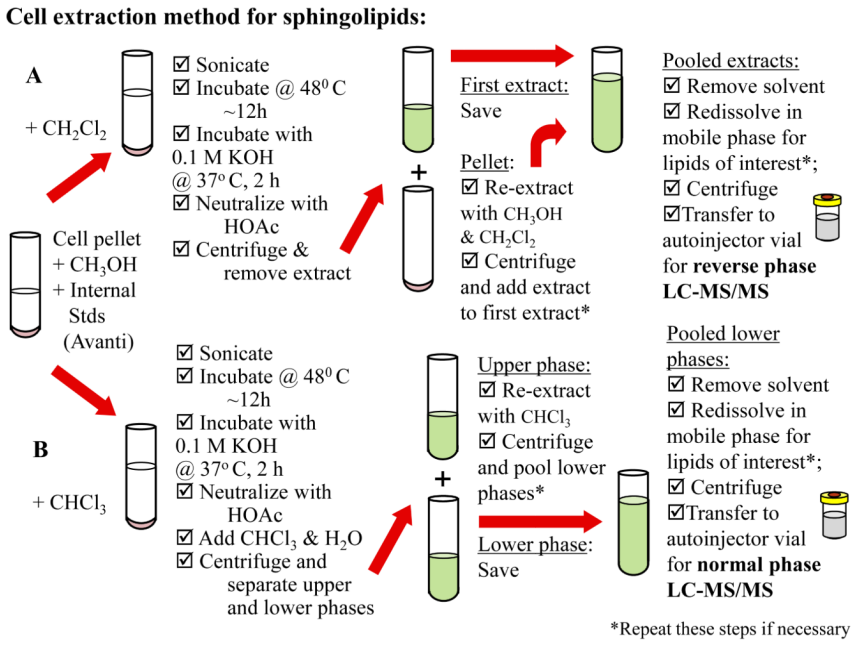

Figure 2.

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

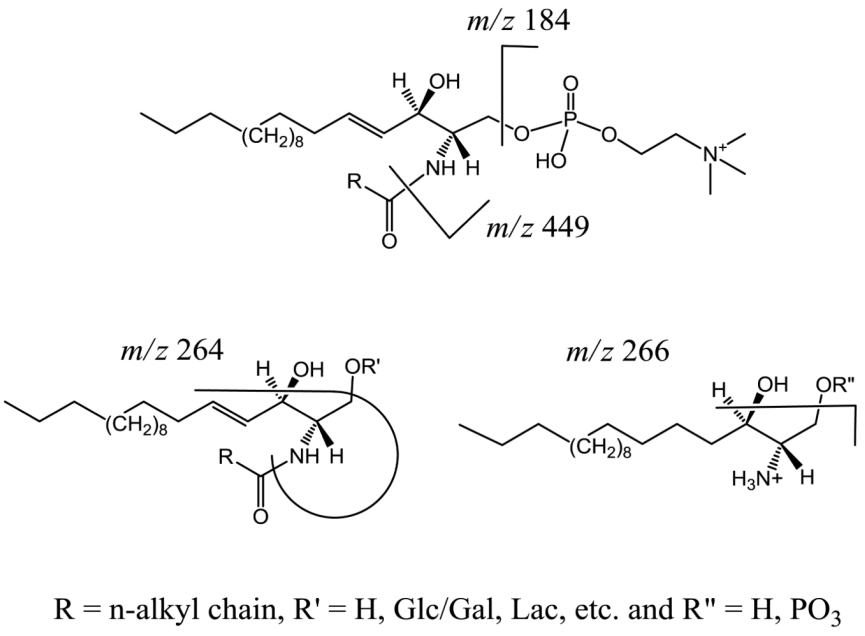

Figure 3.
Common fragmentations of sphingomyelin (top center) showing cleave of the phosphocholine headgroup in the positive mode and neutral loss of the fatty acid in the negative mode. Complex sphingolipids fragment via neutral loss of the fatty acid and dehydration of the sphingoid base (lower left), as do the long chain sphingoid bases (lower right). These unique species are used for determination of sphingoid base, fatty acid, and head group combinations for building a “parts list” for LC-MS/MS.

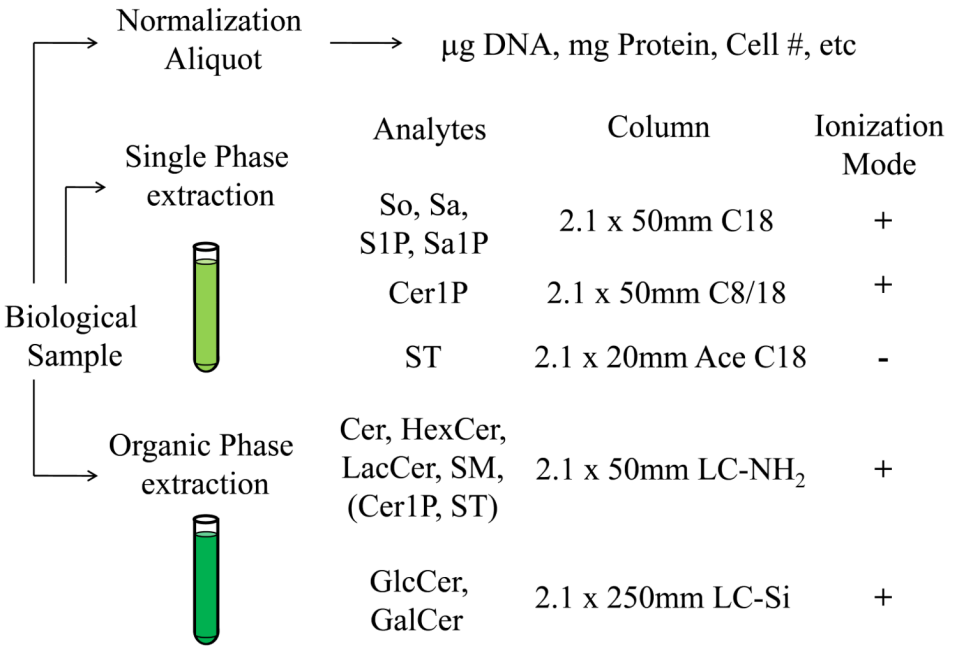

Figure 4.
The sphingolipid analysis pipeline detailing the sample normalization protocol and which classes of sphingolipids may be analyzed using a specific extraction procedure, HPLC column, and ionization mode.

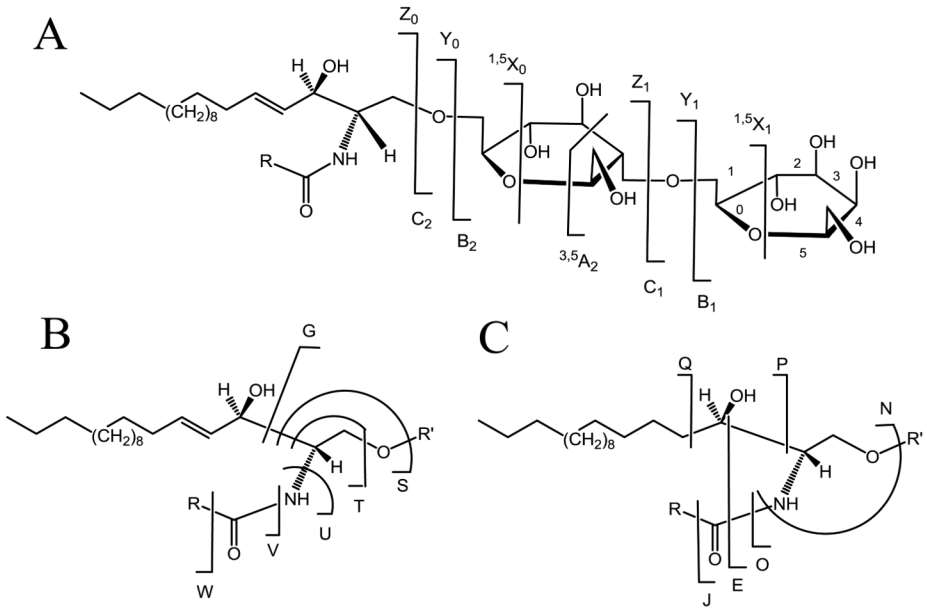

Figure 5.
A detailed nomenclature for glycosphingolipids corresponding to the (A) glycan, (B) fatty acid, and (C) sphingoid base fragment ions.