Tandem Mass Spectrometry of Sphingolipids: Application in Metabolic Studies and Diagnosis of Inherited Disorders of Sphingolipid Metabolism

Ladislav Kuchař, Befekadu Asfaw and Jana Ledvinová

Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague and General University Hospital in Prague

Czech Republic

1. Introduction

Sphingolipids are an amazingly diverse category of lipids found in all eukaryotes and in some prokaryotes and viruses. They are primarily a component of plasma membranes and of intracellular organelle membranes, including those of the nucleus, mitochondria, endosomes, and lysosomes (Hirabayashi, et al., 2006; Kaushik, et al., 2006; R. Ledeen & Wu, 2011; R. W. Ledeen & Wu, 2008; Prinetti, et al., 2009; van Meer, et al., 2008). Sphingolipids are also an important constituent of plasma lipoprotein classes (Schweppe, et al., 2010; Wiesner, et al., 2009) and of the multilamellar water barrier of the skin (Holleran, et al., 2006). In addition, they are excreted in urine, mostly in the cellular debris of urinary sediment. Urinary sediment analysis, or “indirect biopsy,” of kidney cellular elements (Desnick, et al., 1970) can provide information that helps to diagnose certain lysosomal storage diseases (Kitagawa, et al., 2005; Kuchar, et al., 2009; Whitfield, et al., 2001).

Sphingolipids are a heterogenous group. Amide bonds link long-chain fatty acids to aminoalcohols from the sphingoid group, of which sphing-4-enin (2S,3R,4R)-2-aminooctadec-4-ene-1,3-diol, historically called sphingosine) and its saturated derivative (sphinganine) are the most abundant. Longer or shorter sphingoids, which may be saturated or hydroxylated, also occur in lesser quantities.

The name sphingosine was chosen by German clinician and chemist J. L. W. Tchudichum in 1884 to reflect the enigmatic, “Sphinx-like” properties of the sphingolipid compounds first isolated from the brain. Fatty acid variations include mostly C16-C24 acyl chains, which are often saturated but can also exhibit a degree of unsaturation or hydroxylation (e.g., C24:1, C24:1-OH fatty acids). The general name for N-acylated sphingoids is ceramide.

Sphingomyelin and glycosphingolipids have a headgroup in the phosphodiester or glycosyl linkage to the hydroxyl on the carbon-1. The latter compounds are classified as either neutral glycosphingolipids with uncharged sugars (glucose, galactose, N-acetylglucosamine, N-acetylgalactosamine and fucose) or acidic glycosphingolipids with ionized functional groups (sulfates) or charged sugar moieties (N-acetylneuraminic acid or “sialic” acid).

Two examples of sphingolipid structures are shown in Fig. 1.
The simplest glycosphingolipids are the monohexosylceramides glucosylceramide (GlcCer, Glcβ1-1´Cer) and galactosylceramide (galactocerebrosides, GalCer, Galβ1-1´Cer). The latter is less abundant but is specific to neural tissue, where it is also present in a sulfated form (sulfatide). Galactosylceramide also gives rise to small Gala-series of glycosphingolipids (Tab. 1). Glucosylceramide is a key compound in sphingolipid metabolic pathways; more complex glycosphingolipids are derived from the stepwise elongation of the oligosaccharide chain in the Golgi compartment. The addition of β-linked galactose yields lactosylceramide (LacCer, Galβ1-4GlcβCer), whose oligosaccharide chain is the precursor to the different core structures of more complex glycosphingolipids (Tab. 1). These structures are specific to certain tissues: e.g., neolacto-series predominate in leukocytes, lacto-series in secretory organs, globo-series in erythrocytes and ganglio-series in nervous tissue (Schnaar, et al., 2009). This diversity is related to the functional differences between the individual glycosphingolipids.

The catabolism of sphingolipids occurs in acidic cell compartments, i.e. late endosomes and lysosomes. Degraded lipids are embedded in inner membranes rich in negatively charged lipids such as bis(monoacylglycerol)phosphate (BMP) (Kolter & Sandhoff, 2010). Sequential degradation steps proceed from the non-reducing end of the oligosaccharide chain catalyzed by soluble lysosomal hydrolases. For lipids with oligosaccharide chains shorter than four sugars, the assistance of small sphingolipid activator proteins (the saposins A, B, C, or D or the GM2 activator protein) is required (Sandhoff, et al., 2001). The sphingoids and fatty acids produced can be degraded in the cytoplasm and processed through the salvage pathway, where they become the building blocks of new membranes (Kitatani, et al., 2008). They can also be used in the regulation systems that control cell function (Kolter & Sandhoff, 2010).

Inherited defects in gene-coding enzymes or proteins involved in sphingolipid degradation result in the accumulation of non-degraded substrates in the lysosomes. “Traffic jams” in the endolysosomal system caused by the accumulation of lipids co-precipitated with other
hydrophobic substances severely impair cell function and lead to lysosomal storage diseases (sphingolipidoses) (Desnick, et al., 2001; Liscum, 2000; Sandhoff, et al., 2001; Schulze & Sandhoff, 2011; von Figura, et al., 2001). Studies of these defects, however, may unveil the complicated mechanisms of cell function and regulation. Recent information learned about the role of NPC1 and NPC2 proteins in the intracellular transport of cholesterol can serve as an example (Infante, et al., 2008; Kwon, et al., 2009; Storch & Xu, 2009; Xu, et al., 2008). Hitherto, more than 400 structurally distinct sphingolipid variants in mammals have been listed in SphinGOMAP© (http://www.sphingomap.org and http://www.glycoforum.gr.jp) (Aug 2011). A detailed sphingolipid nomenclature is available at http://www.chem.qmul.ac.uk/iupac/lipid/lip1n2.html (Aug 2011) and http://www.chem.qmul.ac.uk/iupac/lipid/lip3n4.html (Aug 2011).

Table 1. The major root structures of vertebrate glycosphingolipids.

<table>
<thead>
<tr>
<th>Root names of series</th>
<th>Core structures</th>
<th>Abbreviations</th>
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<tbody>
<tr>
<td>Lacto-</td>
<td>Gal(β1→3)GlcNAc(β1→3)Gal(β1→4)Glc(β1→1)Cer</td>
<td>Lc</td>
</tr>
<tr>
<td>Ncolacto-</td>
<td>Gal(β1→4)GlcNAc(β1→3)Gal(β1→4)Glc(β1→1)Cer</td>
<td>nLc</td>
</tr>
<tr>
<td>Globo-</td>
<td>GalNAc(β1→3)Gal(α1→4)Gal(β1→4)Glc(β1→1)Cer</td>
<td>Gb</td>
</tr>
<tr>
<td>Isoglobo-</td>
<td>GalNAc(β1→3)Gal(α1→3)Gal(β1→4)Glc(β1→1)Cer</td>
<td>iGb</td>
</tr>
<tr>
<td>Ganglic-</td>
<td>Gal(β1→3)GaLNAC(β1→4)Gal(β1→4)Glc(β1→1)Cer</td>
<td>Gg</td>
</tr>
<tr>
<td>Gaia-</td>
<td>GaL(α1→4)Gal(β1→1)Cer</td>
<td>Ga</td>
</tr>
</tbody>
</table>

*Note: key structures characteristic for each series are underlined.*

Physiological sphingolipid function at the cellular level is highly complex. Sphingolipids participate in many cellular events, including cell-cell recognition, the modulation of membrane protein functions, adhesions, intra- and extra-cellular signaling and many still undiscovered processes (Kitatani, et al., 2008). Their rigid, highly saturated character and ability to undergo hydrogen bonding and dipolar interactions predetermines them to cluster into semiordered structures called lipid microdomains – rafts, together with cholesterol and specific set of proteins (Goldschmidt-Arzi, et al., 2011; Helms & Zurzolo, 2004; Holthuis, et al., 2003). They function as important mediators of membrane transport and signaling.

In recent years, investigation of the metabolism and biological functions of sphingolipid biomolecules has increased (Wennekes, et al., 2009). As a result, more accurate methods of analyzing sphingolipids have been developed. A leading method of analysis, tandem mass spectrometry (tandem MS or MS/MS), provides high selectivity and sensitivity of measurement. Indisputable advantage of this technique is a possibility to identify various molecular species of different sphingolipid classes in crude biological samples.

We will focus on the contribution of tandem mass spectrometry to the study of sphingolipids and the usefulness of the technique in diagnosing inherited disorders of sphingolipid degradation. The topics discussed will include the following:

- Tandem mass spectrometry used to analyze sphingolipids in tissues, cells and urine (both the general approach used and its applications in the diagnosis of sphingolipidoses)
- The investigation of the sphingolipid degradation pathway in living cells using stable isotopes or atypical fatty acid labeling
- Sphingolipid isoform profiling, which is a useful tool for diagnosing disorders associated with Gb3Cer and sulfatide storage.
2. Electrospray ionization tandem mass spectrometry of sphingolipids

The equipment used was an AB/MDS SCIEX API 3200 triple quadrupole mass spectrometer. Multiple reaction monitoring was used in positive (neutral glycosphingolipids) and negative (acidic glycosphingolipids) ion mode. Superior sensitivity and selectivity were exhibited when the technique was used in conjunction with normal phase HPLC separation.

2.1 Electrospray ionization

Electrospray ionization abbreviated as ESI is one of the soft ionization methods used in mass spectrometry (Cole, 2010; de Hoffman & Stroobant, 2002). A strong electric field is applied to the analyte solution as it passes through a metal capillary at atmospheric pressure. This field is created using a high voltage (a voltage of up to 6 kV) between the capillary tip and the counterelectrode. Droplets of analyte are formed under these conditions on the capillary tip, with ions generated and sorted on the surface. Cations are formed in positive ion mode and anions in negative ion mode. The generation of ions results from the electrochemical processes that occur on the capillary tip and the strong electric field used.

Fig. 2. Scheme of electrospray ionization process.

Droplets flow in the direction of the electric field and shrink in size as the solvent evaporates. When the accumulated surface charge of a droplet exceeds the surface tension force, the droplet breaks into smaller droplets via Rayleigh fission. This process is repeated until the ions on the surface of the droplet are able to overcome the forces holding them, at which point molecular ions are formed (Fig. 2) (Cole, 2010; de Hoffman & Stroobant, 2002; Dulcks & Juraschek, 1999).
The distribution of compounds on the droplet surface results from their relative concentration and solubility. Less soluble compounds tend to be on the surface rather than in the bulk of the solution, which affects their ionization efficiency. The surface of each droplet is limited; thus, the concentration is more important than the total amount of the compound injected in the source (Cole, 2010; de Hoffman & Stroobant, 2002). Matrix effects occur when additional compound ions are generated on the surface. These compounds can completely mask the analyte (Fig. 3). The salt concentration has a similar effect on ionization; the maximum tolerable concentration of salts is approximately $10^{-3}$M (Cole, 2010; de Hoffman & Stroobant, 2002). The matrix effect can negatively influence electrospray ionization. It is important to choose a sample preparation technique that removes interfering compounds. The use of HPLC or capillary electrophoresis separation prior to electrospray ionization can minimize the matrix effect (Cole, 2010; Micova, et al., 2010); indeed, we saw a 10-fold increase in signal intensity when HPLC was used (data not shown). Established methods of evaluating matrix effects are described in the literature (Taylor, 2005).

![Matrix effect interference in electrospray ionization conducted on a urinary sample.](image)

**Fig. 3.** Matrix effect interference in electrospray ionization conducted on a urinary sample. Total ion current (TIC) of ceramide, ceramide dihexoside and sphingomyelin. Red line: urinary sample after Folch extraction without purification. Visible signal decrease was caused by matrix effect. Blue line: sample after the purification process, which removed the matrix effects.

### 2.2 Quadrupole mass spectrometer

The quadrupole is a device that uses the stability of ion trajectories in oscillating electric fields to separate ions according to their m/z ratio (de Hoffman & Stroobant, 2002). The device consists of four parallel circular or hyperbolic metal rods to which radiofrequency voltage (RF) and direct current (DC) are applied. The total electric field is composed of quadrupolar alternative fields superposed on a constant field resulting from
the application of potential on the rods. The result is a mass filter used to separate ions according to their m/z ratio (de Hoffman & Stroobant, 2002; Douglas, 2009). The mathematical description of the total electric field is based on Mathieu equations (de Hoffman & Stroobant, 2002; Douglas, 2009). The equations can be used to generate a stability diagram for ions in the field. Direct current voltage (U) and radiofrequency voltage amplitude (V) are the only variables in the equations used to define ion position in these diagrams. The other parameters are constants, including the mass and the charge. Thus, changes in U and V determine a given ion’s position in the diagram. Only ions in the stable region are able to pass the quadrupole mass filter through the stable trajectories. By continually changing U and V, the quadrupole mass spectrometer is able to scan for ions with different m/z ratios (Fig. 4).

Fig. 4. A quadrupolar mass filter and the trajectories of two ions. Red line: an ion in an unstable region of the stability diagram on an unstable trajectory. Blue line: an ion in a stable region of the stability diagram passing the quadrupole on a stable trajectory. A) Section of the quadrupole illustrating radiofrequency voltage; B) Upper side view of the ion trajectories in the quadrupole.

Practically speaking, quadrupole mass spectrometers are hampered by mass resolution and mass range limitations. The useful level of resolution is one mass unit, and the highest detectable m/z is 4000 (de Hoffman & Stroobant, 2002; McLuckey & Wells, 2001).

2.3 Triple quadrupole tandem mass spectrometry

Triple quadrupole tandem mass spectrometry uses three quadrupoles in a series. The first and third quadrupoles function as mass analyzers, whereas the middle quadrupole, with only the radio frequency voltage used, is employed as a collision cell that does not separate ions according to their m/z ratios. Instead, it works as an ion channel that systematically returns ions to the center of the rods (de Hoffman & Stroobant, 2002; McLuckey & Wells, 2001).

Collision induced dissociation is a process of changing the accelerated ion kinetic energy to internal energy using collisions in a collision cell filled with inert gas (N₂, Ar, or He). Analyte fragments are produced if the volume of accumulated internal energy is greater than the energy of the chemical bonds in the molecule (Cole, 2010; de Hoffman & Stroobant, 2002; McLuckey & Wells, 2001; Sleno & Volmer, 2004).
The static mode (in which only one selected m/z is measured) and the scanning mode (in which a range of m/z ratios is measured) are both used with the first and third quadrupole to attain four specific types of measurement (Cole, 2010; de Hoffman & Stroobant, 2002; McLuckey & Wells, 2001; Sleno & Volmer, 2004):

1. **Product ion scans.** To attain these measurements, the first quadrupole is used in the static mode, whereas the third quadrupole is used to scan products of collision induced dissociation.

2. **Precursor ion scans.** To attain these measurements, the first quadrupole is used in scanning mode to determine the mass range of precursor ions which are fragmented in the process of collision induced dissociation. The third quadrupole functions in static mode and is set on the m/z value of the selected fragment which is usually structure specific.

3. **Neutral loss scans.** The technique used for this purpose monitors the loss of neutral fragments. The first and third quadrupoles are used for scanning with a constant mass offset that represents the neutral fragment loss.

4. **Single or multiple reaction monitoring.** Here, the first and third quadrupoles work in the static mode and scan the m/z values of the analyte and its selected fragment. A pair of precursor-product ions of this type is called a transition pair. This technique is used extensively in quantitative analysis because it yields the greatest possible measurement sensitivity.

Tandem mass spectrometry exhibits higher selectivity and sensitivity (with a higher signal-to-noise ratio because noise is suppressed) than does simple mass spectrometry. This advantage also makes this technique useful when tandem mass spectrometry is coupled with HPLC separation (Haynes, et al., 2009; Sullards, et al., 2011).

### 2.4 Tandem mass spectrometry of sphingolipids: ionisation, fragmentation and specificity of sphingolipid analysis

#### 2.4.1 Ionization

The ionization of sphingolipids by electrospray ionization varies for different classes of sphingolipids. This variation can be used in intrasource separation in lipid analyses (Han & Gross, 2005; Haynes, et al., 2009).

Neutral sphingolipids are usually protonated by ammonium formiate, ammonium acetate, formic or acetic acid. Ammonium salts exhibit the highest degree of ionization efficiency and are widely used (Mano, et al., 1997). Neutral salts of Li⁺ or Na⁺ are also used for ionization and generate ion-lipid adducts (Boscaro, et al., 2002; Olling, et al., 1998).

Acids are generally used for H⁺ transfer in solution to basic groups on analyte or to creation of cluster of protonated solvents which later transfer H⁺ to analyte in the process of charge separation. On the other hand neutral amonium salts are usually added to the solution to facilitate the analysis of polar and neutral analytes by adduct formation and later protonation in the process of ionization through gas-phase reactions. Sodium and lithium adducts are also aded in form of neutral salts; the ionisation process has the character of specific charge separation (Cech & Enke, 2001; Kebarle, 2000).

We found that for positive ion mode measurements, 5 mM ammonium formiate is a more efficient additive for use in sphingolipid ionization than is ammonium acetate. However, when using HPLC combined with mass spectrometry, we prefer to use ammonium acetate and acetic acid because of their better solubility in methanol. The ions generated during the electrospray ionization process were [M+H]⁺.
Acidic sphingolipids, such as sulfatides or gangliosides, have acidic groups that lose H\(^+\) even in pure methanol. It is also possible to create chloride adducts (Han & Gross, 2005) using halogenated solvents that can also abstract protons (Cech & Enke, 2001). For measurements in negative ion mode, we used pure methanol solvent to generate [M-H\(^-\)] ions.

2.4.2 Fragmentation

Fragmentation studies of sphingolipids revealed characteristic fragments useful for tandem mass spectrometric analysis (Domon & Costello, 1988; Fuller, et al., 2005; Gu, et al., 1997; Hsu, et al., 1998; Hsu & Turk, 2000; Li, et al., 1995; Kerwin, et al., 1994; Liebisch, et al., 1999; Mano, et al., 1997; Murphy, et al., 2001; Olling, et al., 1998; Whitfield, et al., 2001). The most common fragments used for sphingolipid analysis in positive ion mode are structurally derived from ceramide with C18:1 sphingosine. When the amide bond is broken, followed by the formation of ion derived from sphingoid structure minus one water molecule, the fragment with the m/z value of 282 is produced. If another molecule of water is lost, the fragment with the m/z value of 264 is generated (Fig. 5A) (Gu, et al., 1997; Liebisch, et al., 1999; Murphy, et al., 2001; Olling, et al., 1998). These product ions have different uses. The 264 m/z fragments are used to analyze sphingolipids, whereas the 282 m/z fragments are used to analyze N-deacylated sphingolipids (lysoderivatives) (Gu, et al., 1997; Lieser, et al., 2003; Olling, et al., 1998; Scherer, et al., 2010).

The ceramide fragments mentioned above are commonly used in tandem mass analysis, but other specific sphingolipid structures can also be used for this purpose. Sphingomyelin has characteristic phosphocholines that generate fragments with an m/z value of 184 (Fig. 5B) (Hsu & Turk, 2000; Kerwin, et al., 1994; Murphy, et al., 2001). Sialic acid is another example of a specific sphingolipid structure that is a component of gangliosides. Fragments of sialic acid have m/z values of 290 and 308 (Fig. 5C) (Domon & Costello, 1988; Li, et al., 1995). A structure that is specific to sulfatides is the sulfate group, which exhibit an m/z value of 97 (Fig. 5D) (Hsu, et al., 1998; Whitfield, et al., 2001).

Another analytical approach involves scanning for the neutral loss of saccharides from oligosaccharide chains of glycosphingolipids (Fig. 5E and 6) (Boscaro, et al., 2002; Domon &
Costello, 1988; Olling, et al., 1998). In this approach, transition pairs consisting of analyzed glycosphingolipids and their products with shorter or missing oligosaccharides are measured (Fig. 6).

Neutral loss scanning is the technique of choice for glycosphingolipids containing dihydroceramide or sphinganine. Saturating the double bonds of sphing-4-enine reduces the production of sphingoid base fragments (Fig. 5A) in the positive mode, yielding a fragmentation (Fig. 6) efficiency of approximately 2-3%. In contrast, neutral loss scan fragmentation efficiency for the above-mentioned molecules is 10-15%. It is therefore crucial to select the best fragment to conduct a successful tandem mass analysis of sphingolipids.

![Diagram showing the principle of neutral loss measurements of sphingolipids with deuterated dihydroceramide and sphing-4-anine in the ceramide region of the molecule.](image)

2.4.3 Specificity of mass spectrometry analysis of sphingolipids
Mass spectrometry analyzes molecules according to their m/z values. Different classes of sphingolipids are not represented by one specific molecule; rather, they are a heterogeneous group of molecules with different molecular masses. Their variability is mostly represented by a spectrum of fatty acids that form ceramide structures. Molecular species of individual sphingolipids are called isoforms, and their profiles are usually cell- and tissue-specific (Fig. 7). Therefore, it is important to determine the specific isoform profiles of sphingolipids in biological material before conducting a quantitative analysis.

2.5 Sample preparation for tandem mass spectrometric analysis
A common step in the processing of different biological samples (urine, plasma, cerebrospinal fluid, cells, bioptic or autopic tissues, etc.) prior to tandem mass spectrometric analysis is the preparation of a lipid extract. Cells and tissues are homogenized, and aliquots of homogenate are usually taken for protein determination. Many extraction procedures have been introduced over the years, most of which have been
based on chloroform-methanol mixtures (Bligh & Dyer, 1959; Folch, et al., 1957) or less harmful solvents such as 2-propanol, ethylacetate, hexane or tetrahydrofuran (Heitmann, et al., 1996). The first approach remains the most popular and efficient. Widely used variations on the above-mentioned procedures and recommended methods of removing contaminants from total lipid extract were summarized by Schnaar R (Schnaar, 1994) and van Echten-Deckerd G (van Echten-Deckert, 2000).

2.5.1 Preparation of lipid extract from urine
Extracts were prepared as previously published (Kuchar, et al., 2009). First, 150 µl of sonicated urine was extracted with 700 µl of chloroform:methanol (2:1, v:v) containing internal standards in a polypropylene Eppendorf tube. Then, after 15 min of repeated vortexing at 5 min intervals, 150 µl of MilliQ water was added. The vortex mixing procedure was repeated for another 15 min. After a 20 min pause, the samples were centrifuged for 5 min at 14 000 x g. The lower organic layer was isolated using a Hamilton syringe and filtered using hydrophilic polytetrafluorethylene (PTFE) syringe filters. The samples were purified after the addition of 500 µl of MilliQ water and 15 min of vortexing. The organic and aqueous phases were separated by 5 min of centrifugation at 14 000 x g, and interfering salts and small organic molecules were removed with the upper water layer. The lower
organic layer containing the sphingolipids was collected, dried under a stream of nitrogen and stored in a freezer (-20°C).

2.5.2 Preparation of lipid extract from cultured fibroblasts
Fibroblast pellets were extracted by standard procedures using a chloroform:methanol:water mixtures as previously described (Asfaw, et al., 1998). The harvested cells from the 75 cm² cultivation flask were homogenized in 250 µl of MilliQ water by sonication. Next, 50 µl of homogenate was used for protein determination (Hartree, 1972). The remaining 200 µl of cell homogenate was mixed with 800 µl of chloroform:methanol (2:1, v/v) in a 15 ml glass tube. The mixture was rigorously vortexed twice for 1 min followed by a 15 min settling time at laboratory temperature. The organic and water layers were then separated during 10 min of centrifugation at 400 x g. The upper water and lower organic layers were collected, and the precipitated protein was left in the tube. The protein debris was washed with 500 µl of chloroform:methanol (2:1, v/v), and the organic layer without protein was added to the previously collected phases. The lipid extracts were dried under a stream of nitrogen and redissolved in 400 µl of chloroform:methanol (2:1, v/v). The extracts were then filtered using hydrophilic polytetrafluorethylene syringe pump filters. The filtrates were dried under a stream of nitrogen and stored in a freezer (-20°C) for processing.

2.5.3 Preparation of lipid extract from tissues
The basic procedures used in the tissue extraction process were generally the same as those used by Natomi H (Natomi, et al., 1988), though minor modifications were made. Tissue samples with a wet weight of up to 0.5 g were weighed and homogenized in water or methanol:water (10:1, v/v). Small portions of the homogenate were stored for protein quantification (Hartree, 1972), and the remaining portion was used to prepare the lipid extracts. Chloroform was added to the methanol:water homogenate to get a ratio of chloroform:methanol:water (20:10:1 v/v/v). After vortexing and sonicating, the extracted samples were centrifuged and the supernatant collected. The sediment was reextracted with a more polar solvent mixture of chloroform, methanol, and water (10:20:1, v/v/v) and then with chloroform:methanol (1:1, v/v). The total volume of the extraction mixture corresponding to 20 volumes of the original tissue sample was added during every step in the extraction process. The supernatants were combined, filtered and dried under a stream of nitrogen. Dried samples were stored at freezer (-20°C) prior to processing and analysis.

2.5.4 Processing of extracted lipid samples prior to tandem mass analysis
Corresponding internal standards were added to the urinary samples during the extraction process (Kuchar, et al., 2009). The same volume of internal standards as was used for the urine samples was added to the appropriate aliquots of purified lipid extracts: 5 µg of cellular protein or 150 µg of tissue protein. Loading experiments with specifically labeled sphingolipid isoforms required 50 µg protein aliquots. Finally, the samples were dried under a stream of nitrogen and dissolved in methanol with 5 mM ammonium formiate for measurement in the positive ion mode or in pure methanol for measurement in the negative ion mode.
2.6 Quantitative analysis of sphingolipids by tandem mass spectrometry

We used an AB/MDS SCIEX API 3200 triple quadrupole mass spectrometer equipped with an Agilent 1100 series LC system with an autosampler. The Analyst software version 1.5 was used to operate the hardware and process the measured data. Optimizations of electrospray ionization and tandem mass spectrometry conditions were conducted for each analyzed sphingolipid (Tab. 2). A standard lipid solution with a sphingolipid concentration of 5 µg/ml, was used in the optimization process. For positive ion measurement, 5 mM ammonium formiate in methanol was used to produce [M+H]+ ions. To generate [M-H]- ions in negative ion mode, pure methanol was used.

We measured lipids using a flow injection analysis of 20 µl sample aliquots samples. We used pure methanol as the mobile phase with a flow rate of 50 µl/min. One lipid was analyzed during one injection to provide the best possible quantitative data. The scan time for a transition pair was usually 100 ms but in some cases was increased to 500 ms for higher sensitivity. The settling time was usually 0 ms. However, it was necessary to increase this parameter to 500-700 ms when the ion optics setting was changed to measure more than one class of sphingolipid in one injection. The resolution was generally set to unit (±1 m/z), but in some cases, we used a high resolution setting for the first quadrupole, as using such a setting can improve mass spectrometer sensitivity.

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The Table 2. Example of electrospray ionization and ion optics parameters used in tandem mass spectrometry to analyze selected sphingolipids (Sciex API 3200, product ion m/z 264, 5 mM ammonium formiate in methanol). CTH – ceramidetrihexoside; CDH – ceramidedihexoside, CMH and lyso-CMH – ceramidemonohexoside and its N-deacylated derivative.

Procedure of our quantitative analysis was described in a previous study (Kuchar, et al., 2009). Problems associated with the matrix effect were addressed using internal standards, whereas the calibration of the method was based on an external standard. Quantification was performed via single-point calibration using an external calibration point with a standard lipid concentration (an external calibration standard) corrected by the signal ratio toward internal standard (mostly C17:0 isoform which is not naturally
abundant) isoform (mostly with C17:0 fatty acid). All standard lipid concentrations were within the broad range of linear response. The internal standard concentration at the external calibration point and in the measured samples was the same. For the quantification procedure, molecular species of sphingolipids with fatty acids of chain lengths from C16 to C26 were selected.

2.7 Preparation of sphingolipids internal standards using enzymatic semi-synthesis

Not all internal standards are commercially available. Thus, we developed a method of enzymatic semi-synthesis using immobilized sphingolipid ceramide N-deacylase (*Pseudomonas* sp, TK4) on porous magnetic cellulose (Kuchar, et al., 2010). Magnetic macroporous bead cellulose was used as carrier for sphingolipid ceramide N-deacylase (SCDase) which was immobilized using a standard procedure (Bilkova, et al., 2005; Korecka, et al., 2005). A 100 µl aliquot of washed settled particles was activated with 0.2 M freshly prepared NaIO₄. The activated particles were then washed with 0.1 M phosphate buffer with a pH 7. Binding of 250 mI.U. of sphingolipid ceramide N-deacylase on activated magnetic macroporous bead cellulose was achieved by 10 min incubation in phosphate buffer with a pH 7. The formed Schiff base was stabilized via overnight incubation in a NaCNBH₃ solution. The final step consisted of washing particles in phosphate buffer with a pH 7. Immobilized sphingolipid ceramide N-deacylase was stored in phosphate buffer with a pH 7 with 0.1% Triton X-100 at 4°C.

Under specific conditions, this enzyme also catalyzes the reverse reaction (Kita, et al., 2001). Thus, lysoderivates can be reacylated with a specific fatty acid. Using this procedure, we prepared several internal standards with C17:0 fatty acids, e.g. sulfatides, glucosylceramides, and GM1 gangliosides. In this process, we incubated 50 nmol of lyso-glycosphingolipid, 50 nmol of C17:0 fatty acid and immobilized sphingolipid ceramide N-deacylase in 300 µl of pH 7 phosphate buffer with 0.1% Triton X-100 for 20 hrs at 37°C while mixing it on a rotator. The magnetic particles were separated, and the supernatant was transferred and dried under a stream of nitrogen. The quality of the prepared lipids was monitored by HPTLC and tandem mass spectrometry.

2.8 Standardization of quantitative data in urine

The results of sphingolipid quantification in cellular material are often related to protein concentration, a well-established standardization parameter (Liebisch, et al., 1999). Urinary sphingolipids mostly originate from desquamated renal tubular cells. The standardizing parameter commonly used for urinary metabolites is creatinine, but creatinine does not reflect the cellular origin of sphingolipids. Therefore, in urinary samples with a creatinine level lower than 1 mM, the concentration of excreted sphingolipids is artificially inflated (Fig. 8A), which may encourage the incorrect diagnosis of some patients with lysosomal storage diseases (e.g., Fabry disease, prosaposin and saposin B deficiencies, and sulfatidoses). Regarding Fabry disease, this issue has already been pointed out (Forni, et al., 2009). In our experience, such diagnostic errors are especially likely to occur with newborns or children up to six years of age, whose normal concentration of creatinine is generally low (≤ 4 mM). Surprisingly, urinary volume has been found to be much more convenient for use as a standardization parameter (Fig. 8B). It is also possible to use the ratio of the analyzed compound to sphingomyelin (Berna, et al., 1999; Kuchar, et al., 2009) or phosphatidylcholine
(Fuller, et al., 2005; Whitfield, et al., 2001), which are membrane-bound lipids that can be measured simultaneously in the same sample.

Fig. 8. Comparison of two methods of standardization of urinary Gb3Cer in three groups of samples: in controls with low creatinine (creatinine $\leq 1$ mM), in controls with creatinine within a normal range (creatinine $>1$ mM-15 mM) and in Fabry patients. It is most critical to appropriately evaluate urine control samples with low creatinine concentrations, which are indistinguishable from samples from patients with Fabry disease (A). The use of urine volume as a standardization parameter makes it possible to differentiate more appropriately between controls and Fabry patients (B).

3. **Sphingolipids in lysosomal storage disorders – mass spectrometric data useful for diagnosis and research**

Flow injection analysis (FIA) combined with electrospray ionization tandem mass spectrometry makes it possible to determine the concentration of sphingolipids in various biological materials: e.g. urine, cultured fibroblasts and autoptic or bioptic tissue samples from different lysosomal storage disorders.

Examples of these analyses are presented in following paragraphs.

3.1 **Sphingolipids in urine**

Urine is a non-invasive diagnostic material that is of practical importance in diagnosing lysosomal disorders in which the storage of non-degraded substrate causes pathological processes in the kidneys. These disorders are characterized by the massive excretion of specific sphingolipids, e.g., Gb3Cer in Fabry disease ($\alpha$-galactosidase A deficiency due to mutations of the GLA gene); multiple hydrophobic sphingolipids in complex sphingolipidoses, in which the defect is caused by mutations in the prosaposin gene (sphingolipids with a saccharide chain that is shorter than four monosaccharide units and ceramides are not degraded in prosaposin deficiency and Gb3Cer and sulfatides in saposin B deficiency due to defective activator proteins); sulfatides in metachromatic leukodystrophy (arylsulfatase A deficiency due to mutations of the ARSA gene) (Fuller, et al., 2005; Kuchar, et al., 2009; Whitfield, et al., 2001).

We developed a method of tandem mass spectrometry quantification of urinary sphingolipids that can be used in pre-diagnostic screening for the lysosomal disorders mentioned above (Kuchar, et al., 2009). Our data are presented in Table 3.
Table 3. Massive excretion of urinary sphingolipids in the case of saposin-B and prosaposin deficiencies and in patients with Fabry disease and metachromatic leukodystrophy.

<table>
<thead>
<tr>
<th></th>
<th>µg/l</th>
<th>sulfatide</th>
<th>CDH</th>
<th>Gb3Cer</th>
<th>CMH</th>
<th>Cer</th>
<th>SM</th>
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<tr>
<td>pSap-d</td>
<td>756</td>
<td>507</td>
<td>2322</td>
<td>289</td>
<td>184</td>
<td>1323</td>
<td></td>
</tr>
<tr>
<td>44-day-old</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SapB-d</td>
<td>2149</td>
<td>513</td>
<td>755</td>
<td>204</td>
<td>101</td>
<td>1480</td>
<td></td>
</tr>
<tr>
<td>50-month-old</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MLD</td>
<td>3566</td>
<td>397</td>
<td>261</td>
<td>191</td>
<td>113</td>
<td>3417</td>
<td></td>
</tr>
<tr>
<td>late infantile</td>
<td>±1855</td>
<td>±220</td>
<td>±188</td>
<td>±115</td>
<td>±67</td>
<td>±2448</td>
<td></td>
</tr>
<tr>
<td>(n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fabry disease (male)</td>
<td>88</td>
<td>460</td>
<td>2615</td>
<td>45</td>
<td>62</td>
<td>1602</td>
<td></td>
</tr>
<tr>
<td>(n = 10)</td>
<td>±17</td>
<td>±199</td>
<td>±915</td>
<td>±13</td>
<td>±11</td>
<td>±1081</td>
<td></td>
</tr>
<tr>
<td>Controls</td>
<td>155</td>
<td>113</td>
<td>152</td>
<td>52</td>
<td>45</td>
<td>1181</td>
<td></td>
</tr>
<tr>
<td>infantile/juvenile</td>
<td>±48</td>
<td>±37</td>
<td>±68</td>
<td>±23</td>
<td>±12</td>
<td>±437</td>
<td></td>
</tr>
<tr>
<td>(n = 16)</td>
<td></td>
<td></td>
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</table>

Table 3. Massive excretion of urinary sphingolipids in the case of saposin-B and prosaposin deficiencies and in patients with Fabry disease and metachromatic leukodystrophy. pSap-d - prosaposin deficiency, SapB-d - saposin B deficiency, MLD - metachromatic leukodystrophy; Cer - ceramide; CTH – ceramidetrihexoside; CDH – ceramidedihexoside, CMH – ceramidemonohexoside SM – sphingomyelin; Gb3Cer - globotriaosylceramide

Values are in ng/µg of protein (mean±SD). Non-degraded sphingolipids related to particular lysosomal storage disorders are bolded.

Analyzing non-degraded metabolites can be very helpful in the pre-diagnosis of sphingolipid activator deficiencies in which routine enzymology fails to indicate deficient enzyme activity due to the detergents commonly used in the assays.

Urinary Gb3Cer has been suggested as biomarker for monitoring efficiency of enzyme replacement therapy of Fabry disease. Our tests in a group of Fabry male-patients showed however, that monitoring of this marker is not informative for all treated patients in general but for individual patients only (data not shown). Although excreted Gb3Cer is useful parameter for diagnosis, it is not reliable biomarker for clinical trials as also confirmed by another studies (Schiffmann, et al., 2010). Biological basis of urinary Gb3Cer and its isoforms is still subject of research.

3.2 Sphingolipids in cultured fibroblasts

Although cultured fibroblasts are not typical “storage cells,” the concentration of non-degraded lipids increases significantly in some lysosomal storage disorders as documented in Table 4. Investigating sphingolipid profile can help in laboratory diagnosis of these rare diseases, especially among those suspected of having defective activators of lysosomal hydrolases.

3.3 Sphingolipids in tissues: Gb3Cer and lyso-Gb3Cer in Fabry myocardium and kidney

In some cases, a postmortem analysis of autopic tissue has revealed metabolic defects. Here, we give two examples of tissue analysis that led to a final diagnosis confirmed by DNA analysis later on.
Table 4. Increased concentration of sphingolipids in cultured skin fibroblasts in patients with sphingolipid activator deficiencies (saposin-B and prosaposin deficiencies) and in patients with defective enzyme proteins (in Fabry disease and metachromatic leukodystrophy). Values are in ng/µg of protein. Cer - ceramide; CDH – ceramidedihexoside, CMH – ceramidemonohexoside; SM – sphingomyelin; Gb3Cer - globotriaosylceramide. Non-degraded sphingolipids corresponding to particular lysosomal storage disorders are bolded.

The first example shows the accumulation of Gb3Cer in the kidneys of patients with Fabry disease and in cases of prosaposin deficiency (Tab. 5).

Table 5. Concentration of sphingolipids in the kidneys of Fabry male patient and in a case of prosaposin deficiency. Values are in ng/µg of protein, Control is represented by the mean value. Cer - ceramide; CDH – ceramidedihexoside, CMH – ceramidemonohexoside, SM – sphingomyelin; Gb3Cer - globotriaosylceramide. Non-degraded sphingolipids related to particular lysosomal storage disorders are bolded.

Another example demonstrates the storage of Gb3Cer and lyso-Gb3Cer (globotriaosylsphingosine) in the myocardium of Fabry patient (Fig. 9). It is possible that the role of these derivates has been underrated (Dekker, et al., 2011). The role of lyso-Gb3Cer as a molecule that stimulates smooth muscle cell proliferation is now known. These findings indicate the possible role of lyso-Gb3Cer as a signal molecule (Aerts, et al., 2008).

4. The investigation of sphingolipid degradation pathways

4.1 Loading experiments on living cells using lipid substrates labeled with a stable isotope or containing atypical fatty acids

Loading experiments in cell cultures (also called feeding experiments) are frequently used to investigate the metabolic fate of exogenous compounds in living model systems. The main advantage of such experiments is that they assess the entire degradation system, including any nonenzymatic cofactors.

This method can be used to conduct a general analysis of metabolic pathways (Schwarzmann, et al., 1983; Sonderfeld, et al., 1985), intracellular transport or a distribution (Martin & Pagano, 1994) assessment of residual activity in enzyme-deficient cells.
Fig. 9. Gb3Cer and lyso-Gb3Cer (globotriaosylsphingosine) in the myocardium of Fabry patient. A) Precursor ion spectrum of Gb3Cer molecular species in control myocardium. The spectrum was measured by a 1 min scan of a 3 mg protein aliquot of lipid extract dissolved in methanol with 5 mM ammonium formiate. The increased concentration of B) Gb3Cer and C) lyso-Gb3Cer is visible in the autopic myocardium of Fabry patient in comparison to that of an age-matched control. Quantity was measured by flow injection analysis electrospray ionization tandem mass spectrometry using an multiple reaction monitoring scan. C17:0 Gb3Cer was used as the internal standard for lyso-Gb3Cer quantification similarly as described in (Mills, et al., 2005). Concentrations were measured by method with coefficient of variation - CV <7%.
The method can also be used to diagnose storage disorders. This technique has been used to distinguish between metachromatic leukodystrophy and arylsulfatase A pseudodeficiency (Kihara, et al., 1980) and to identify deficiencies in nonenzymatic protein cofactors of lysosomal glycolipid catabolism (sphingolipid activator proteins) (Klein, et al., 1994; Schepers, et al., 1996; Schmid, et al., 1992; Sonderfeld, et al., 1985; Wrobe, et al., 2000), including prosaposin deficiency (Harzer, et al., 1989; Chatelut, et al., 1997).

In these experiments, the degradation products of labeled exogenous lipid substrates are determined using specific analytical methods. Traditionally, sphingolipids are labeled with radioisotopes, and their degradation products are separated chromatographically and traced using radioactivity assays. Recently, radiolabeled sphingolipids are often replaced by non-radioactive analogues with atypical molecular masses that can be analyzed by tandem mass analysis.

Sphingolipids can be labeled on different parts of the molecule using non-natural fatty acids, creating molecules with atypical m/z values. However, only a few labeled standards are commercially available. Sphingoids can also be deuterium labeled at the double bond to increase mass, but the fragmentation patterns will be altered (see Fragmentation, Fig. 6). We compared the radioisotope and mass labeling methods in loading experiments involving fibroblast cultures from patients with inherited lysosomal storage diseases such as GM1 gangliosidosis. In this study, genetic variants of the GLB1 (β-galactosidase; β-gal) gene were selected. Both approaches, the use of [3H]GM1 ganglioside and the use of its C18:0-D3 analogue, clearly showed that the impaired degradation of critical glycosphingolipids resulted from defects in β-gal function, as indicated in Fig. 10.

The experiments conducted with stable isotope-labeled substrates and tandem mass spectrometry facilitated a more accurate quantification analysis of the lipids, and the results were better correlated with the clinical and biochemical phenotypes of the samples. The procedure used to prepare the cellular lipids for tandem mass spectrometry analysis was simple and relatively rapid; unlike radioisotope assays, it did not require separation during the pre-analytical phase (Asfaw, et al., 2002; 1998). However, experiments with radiolabeled glycolipid substrates indicate the entire metabolic pattern (Fig 10A) and can thus make it possible to identify relevant metabolites to be further analyzed via tandem mass spectrometry.

Results similar to those obtained in analyzing the GM1 gangliosidosis were obtained by loading experiments using Gaucher fibroblasts and fibroblasts from patients with prosaposin deficiencies (data not shown).

4.2 Tandem mass determination of in vitro acid glycosidase activity

Enzymology, in combination with tandem mass spectrometry, is useful in lysosomal storage disorders screening and in evaluations of enzyme activity (Kasper, et al., 2010; Li, et al., 2004; Spacil, et al., 2011; Turecek, et al., 2007). One practical application of this technique is the analysis of lysosomal β-glucocerebrosidase activity using glucosylceramide with C12:0 fatty acid as the enzyme substrate. Tandem mass spectrometry evaluation techniques can be used with cells and tissue homogenates but also with dried blood spots as the screening material.

We followed Turecek’s method (Turecek, et al., 2007) in measuring lysosomal β-glucocerebrosidase activity in homogenates of cultured skin fibroblasts. The reaction
Fig. 10. In situ degradation of GM1 ganglioside by skin fibroblasts from control and β-galactosidase deficient patients. Tritium-labeled glycolipid GM1 ganglioside or a C18:0-D3 analogue was added to the culture of skin fibroblasts in 25 cm² flasks. After 5 days, cells were harvested and lipids extracted. The radio-labeled lipid extracts were separated via TLC and analyzed using a linear scanner (Asfaw, et al., 1998), whereas the GM1 ganglioside with C18:0-D3 fatty acid and its degradation products were extracted and directly analyzed directly in tandem mass spectrometry (details in Materials and Methods). A: Degradation pattern of [³H]GM1 ganglioside in control and β-galactosidase-deficient cells. The chromatographic positions of the products are indicated by the number of sugar residues on the glycolipid, which range from 0 (ceramide) to 4 (tetrahexosylceramide). B: Quantification of degradation products of [³H]GM1 ganglioside (sum of all products on the TLC plate) in skin fibroblasts from the control and the different β-galactosidase-deficient genetic variants. The cell lines are arranged according to clinical phenotypes of GM1 gangliosidoses: a-control, b-adult GM1 gangliosidosis, c-Morquio B, d-adult GM1 gangliosidosis /Morquio B, e-juvenile GM1 gangliosidosis, f-infantile GM1 gangliosidosis, and g-infantile GM1 gangliosidosis. C: Quantification of GlcCer product formed from stable isotope-labeled GM1 ganglioside (C18:0-D₃) in the same cell lines as the radioactive analogue (B). Values are average of two samples.
mixture contained 0.5 µg of sample protein and 0.05% inactivated bovine serum albumin (BSA) to stabilize the enzyme. The mass spectrometry settings were optimized to prevent the artificial conversion of the substrate into the enzyme reaction product. An example of this analysis is presented in Figure 11.

![Activity of lysosomal acid β-glucocerebrosidase in Gaucher and control fibroblasts](image)

Fig. 11. Activity of lysosomal acid β-glucocerebrosidase in Gaucher and control fibroblasts as measured with natural substrate C12:0 glucosylceramide. The reaction product was analyzed by flow injection analysis electrospray ionization tandem mass spectrometry using a multiple reaction monitoring.

Nowadays, procedures are simplified by skipping laborious process of extraction of reaction mixture after incubation. Instead, HPLC is combined with mass spectrometry. The HPLC step purifies the sample, removing any potential interfering compounds (Kasper, et al., 2010; Spacil, et al., 2011).

5. Sphingolipid isoform profiling – a useful metabolomic approach to disorders involving Gb3Cer and sulfatide storage

Tandem mass spectrometry has advantages over HPLC and other analytical methods in helping to determine the individual molecular species (isoforms) of sphingolipids. Changes in isoform profiles may provide diagnostically important information and indicate specific pathological processes (Fauler, et al., 2005; Paschke, et al., 2011). For example, an analysis of urinary lipid extracts in a case of metachromatic leukodystrophy showed significant differences in sulfatide isoform profiles; such differences were also evident in cases of prosaposin and saposin B deficiency, two other sulfatide storage disorders. We also found changed patterns of globothriaosylceramide species in the urine of Fabry patients and patients with prosaposin gene defects. A shift in the isoform pattern to species with longer chain fatty acids was characteristic of both prosaposin and saposin B deficiencies. The results presented in Table 6 were evaluated by determining the ratio of the various isoforms to the C18:0 isoform, which is invariable in the profile. The major advantages of
this procedure include simple sample preparation without internal standard and a simple data collection process; only a small number of transitions must be measured. The elevation of certain molecular species, particularly those with longer, hydroxylated chains (in the case of the sulfatides), is clearly demonstrated.

Table 6. Changed signal ratios of Gb3Cer isoforms (A) and sulfatide isoforms (B) (to the C18:0 species) in the urine of patients with lysosomal storage disorders. The C18:0 species were selected as the standard invariable parameter in the isoform pattern. Changes in the levels of specific isoforms in patients with lysosomal storage disorders are highlighted. SapB-d – saposin B deficiency; pSap-d – prosaposin deficiency; Fabry – Fabry disease; MLD - metachromatic leukodystrophy.

6. Conclusions

In this chapter, we have introduced a methods of complex sphingolipid analysis, covering sample preparation and final tandem mass spectrometry analysis for various biological materials. This approach has been used in various studies of lysosomal storage disorders and examples showing a range of applications are presented.

Findings in urine are very important in the pre-diagnosis of lysosomal storage disorders and especially in identifying defects in the protein activators of sphingolipid hydrolases. We have also drawn attention to the problems with evaluating urinary sphingolipids using creatinine and have thereby suggested a more reliable approach to standardize sphingolipid excretion.

The tandem mass analysis of sphingolipids in cells and tissues is useful in diagnosing unresolved cases (examples shown in this chapter). This method can also contribute
important information to lipidomic studies of the cellular function of these molecules and their bioactive derivatives.

The use of tandem mass spectrometry in loading experiments using labeled sphingolipids can increase quantification accuracy and throughput while eliminating working risk and restrictions by eliminating the need for radioactive analysis. Although tandem mass spectrometry cannot yet fully replace radioisotope methods, using this technique can improve the precision and specificity of the results of metabolic experiments. The demand for useful screening methods for lysosomal storage disorders has led to the use of tandem mass spectrometry in enzymology. Analyses of enzyme activity using mass spectrometry performed on dried blood spots are highly sensitive and specific, and dried samples are easy to transport. Some methods use natural substrates, which is helpful in research studies of enzyme function and characteristics.

The aforementioned advantage of tandem mass spectrometry is the ability to analyze individual sphingolipid molecules (isoforms). Evaluation of isoform profiles can have diagnostic value in disorders involving storage of Gb3Cer or sulfatides. Metabolomic principles have a tremendous number of research applications, especially in the investigation of various cellular events.

In conclusion, tandem mass spectrometry is robust and sensitive analytical procedure that is still evolving. The method is efficient for determining the composition of endogenous sphingolipid classes in various biological materials and following their metabolic fate. Its ability to establish the metabolomic profiles of sphingolipids under normal and abnormal conditions contributes to a better understanding of the biological significance of sphingolipid molecules.

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Tandem Mass Spectrometry - Applications and Principles presents comprehensive coverage of theory, instrumentation and major applications of tandem mass spectrometry. The areas covered range from the analysis of drug metabolites, proteins and complex lipids to clinical diagnosis. This book serves multiple groups of audiences; professional (academic and industry), graduate students and general readers interested in the use of modern mass spectrometry in solving critical questions of chemical and biological sciences.

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中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821