

Simultaneous quantitative analysis of bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry

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Abstract

There has been a recent explosion in research concerning novel bioactive sphingolipids (SPLs) such as ceramide (Cer), sphingosine (Sph) and sphingosine 1-phosphate (Sph-1P) that necessitates development of accurate and user-friendly methodology for analyzing and quantitating the endogenous levels of these molecules. ESI/MS/MS methodology provides a universal tool used for detecting and monitoring changes in SPL levels and composition from biological materials. Simultaneous ESI/MS/MS analysis of sphingoid bases (SBs), sphingoid base 1-phosphates (SB-1Ps), Cers and sphingomyelins (SMs) is performed on a Thermo Finnigan TSQ 7000 triple quadrupole mass spectrometer operating in a multiple reaction monitoring (MRM) positive ionization mode. Biological materials (cells, tissues or physiological fluids) are fortified with internal standards (ISs), extracted into a one-phase neutral organic solvent system, and analyzed by a Surveyor/TSQ 7000 LC/MS system. Qualitative analysis of SPLs is performed by a Parent Ion scan of a common fragment ion characteristic for a particular class of SPLs. Quantitative analysis is based on calibration curves generated by spiking an artificial matrix with known amounts of target synthetic standards and an equal amount of IS. The calibration curves are constructed by plotting the peak area ratios of analyte to the respective IS against concentration using a linear regression model. This robust analytical procedure can determine the composition of endogenous sphingolipids (ESPLs) in varied biological materials and achieve a detection limit at 1 pmol or lower level. This and related methodology are already defining unexpected specialization and specificity in the metabolism and function of distinct subspecies of individual bioactive SPLs.

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1. Introduction

Prevalent complex sphingolipids (SPLs): phosphosphingolipids (PSPLs) and glycosphingolipids (GSPLs) are found in all eukaryotes, some prokaryotes and viruses, mainly as components of the plasma membrane and related organelles. SPLs constitute about 30% of the total lipid of plasma membranes, but these percentages can be considerably higher because SPLs are asymmetrically distributed, and can spontaneously aggregate to form liquid ordered

micro domains termed “rafts”. SPLs form specialized structures, mediate cell–cell and cell-substratum interactions, modulate the behavior of cellular proteins and receptors, and participate in signal transduction. Several SPL metabolites, especially ceramide (Cer), sphingosine (Sph) and sphingosine-1-phosphate (Sph-1P), have been identified as bioactive key molecules that control cell growth and death [1–9]. This discovery emphasizes the need for examination of SPL metabolic pathways [1–9]. To understand how SPL biosynthesis and turnover regulates cell behavior under normal and abnormal conditions, how perturbations in SPL of one type may enhance or interfere with the action of another, and where and how all these SPLs are made and removed, we must be able to establish the metabolomic

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profile of SPLs. Mass spectrometry (MS) methodology offers an efficient tool to monitor changes in the composition of all these bioactive species under different environments, and may provide a missing link in the search for novel and effective health therapy.

A variety of sample preparation, ionization modes and instrumental design have been developed so far to analyze particular SPL classes by MS technology [10]. Design for this methodology has been based on the fact that different SPL subclasses dissociate into structurally distinctive patterns corresponding to their sphingoid bases, *N*-acyl chain and polar head group [11–21]. Recent advances in electrospray ionization (ESI) have provided a new approach to successfully examine total SPL components in crude lipid extracts [15,18,19,21]. ESI methodology allows generation of intact molecular ions of molecules directly from solution by coupling a high-performance liquid chromatography (HPLC) column directly to the mass spectrometer. Further improvements in instrumentation, such as the triple quadrupole with robust ion sources, fast scanning mass analyzers and reduced chemical noise (possible in MS/MS technique), allow the identification and quantitation of SPLs with great sensitivity (sub-picomole detection limit) in a highly reproducible manner. SPL identification is accomplished by tandem mass spectrometry (MS/MS) with precursor ion scans to distinguish various molecular species in crude lipid extract by taking advantage of the unique molecular decomposition pattern [15,21] for each SPL class. SPL quantitation is performed by using positive ionization and multiple reaction monitoring (MRM) in conjunction with HPLC separation [21].

In this paper, we describe method for simultaneous analysis of the following SPLs: sphingoid bases (SB): Sph, dhSph; sphingoid base phosphates (SB-1P): Sph-1P, dhSph-1P, ceramides (Cers), dihydroceramides (dhCers), and sphingomyelins (SMs) using state-of-the-art HPLC-MS/MS techniques. The developed protocol describes a new and efficient one-phase neutral organic solvent system used for the preparation of lipid extract containing the above SPLs, and their identification and quantitation.

In general, the correct, sufficient, simple, and safe method for lipid extract preparation is one of the major considerations in lipid analysis, because analytical methods are applied specifically to the SPL components present in the particular lipid extract. In this protocol lipid extracts are prepared under a safe and neutral condition to avoid destruction of the parent “soft” SPLs (e.g. SPLs containing *O*-acyl group) and to efficiently and quantitatively extract the SB-1Ps from biological material since the latter are notoriously difficult to recover quantitatively [22,23].

Our ultimate goal is to provide a total metabolomic profile of SPLs. SPL components missing in this protocol are ceramide 1-phosphate (Cer-1P), psychosine (GlcSph), glucosylceramide (GlcCer), lactosylceramide (LacCer) and inositolceramide 1-phosphate (IPC). The missing SPL components are under development and a full protocol will be

presented after completion of the synthetic project for the unavailable analytical standards necessary for reliable quantitation of these SPLs. A semiquantitative LC/MS method for analysis of GlcCer, LacCer, and other glycosphingolipids has been described by Sullards et al. [21].

1.1. SPL structural diversity and nomenclature

General structures and nomenclature for SPLs cited and described in this paper are shown in Fig. 1. SPLs constitute one of the most structurally diversified classes of amphipathic lipids abundant in all living organisms. Variations in the nature of the head group attached to the primary hydroxyl group (carbohydrates, phosphorylcholine, phosphate or phosphoinositol), *N*-acyl group, and sphingoid-base backbone result in a great number of chemically distinct SPLs, where Sph, dhSph or phytoSph are the core structural moieties. Some 3-*O*-alkyl, 3-*O*-alkenyl (vinyl ether linkage) and 1- or 3-*O*-acyl-analogs of natural components have also been identified. Thousands of natural, complex SPLs have been isolated based on almost 60 distinct species of SBs, although most of them are very minor components. SBs, the backbone of all SPLs, encompass a wide array of (2S, 3R, 4E)-2-amino-1,3-dihydroxyalkenes (Sphs), (2S, 3R)-2-amino-1,3-dihydroxyalkanes (dhSphs), and (2S, 3S, 4R)-2-amino-1,3,4-trihydroxyalkanes (phytoSphs) with alkyl chain lengths from 14 to 22 carbon atoms and variations in the number and position of the double bonds, hydroxyl groups and branching methyl groups. Mammal SPLs are predominantly composed of 2-amino-1,3-dihydroxy-octadecene (18Sph, abbreviated here as Sph) and 2-amino-1,3-dihydroxy-octadecane (18dhSph, abbreviated here as dhSph), yeast and plant SBs are mainly composed of 2-amino-1,3,4-trihydroxy-octadecane (18phytoSph), 18dhSph and their eicosa-homologs (20phytoSph and 20dhSph). Additionally, some SPLs may contain a double bond in position 8 or have double bonds in positions 4 and 8 (which can be found in plant SPLs). Ceramides are *N*-acyl-derivatives of SBs. Combinations of different SBs with different fatty acids (including their hydroxy-analogs) generates a huge variety of Cer, dhCer, and phytoCer. These basic SPLs are modified at the 1-hydroxyl group to: (i) phosphates (e.g. Sph-1P and Cer-1P), (ii) phosphocholine-analogs (e.g. SM and lysosphingomyelin, *lyso*-SM), and (iii) glucosyl- and galactosyl-analogs (e.g. glucosylceramide and galactosylceramide, known as cerebrosides, and their *lyso*-form: psychosine). The latter group also serve as precursors to hundreds of different species of complex GSPLs, with lactosylceramide (containing only two sugar residues) being the simplest. Additionally, some Cers and GSPLs can be modified on their hydroxyl groups (e.g. *O*-acyl-Cers), and some SBs can be *N*-methylated.

The structural diversity of SPLs dictates that every step in analysis of these natural products must be carefully evaluated.

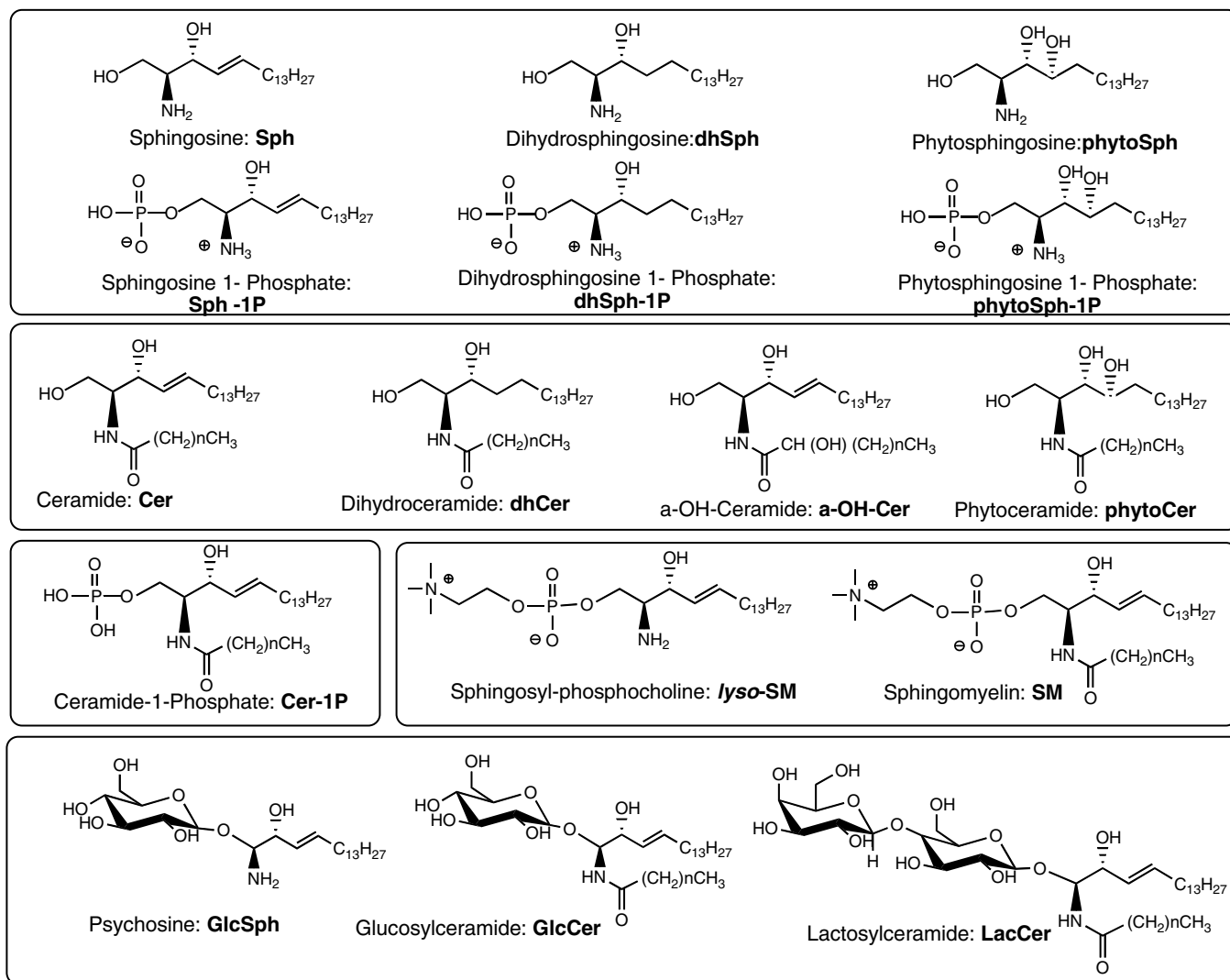


Fig. 1. General structures, nomenclature, and abbreviations for SPLs cited and described in this protocol. Cn, chain length of *N*-acyl part of SPLs; 18SB, SPLs containing 2-amino-1, 3-dihydroxy-octadecene-4E or 2-amino-1,3-dihydroxy-octadecane; 17SB, SPLs containing 2-amino-1,3-dihydroxy-heptadecene-4E (not shown).

2. Description of method

2.1. Biological material

Cells in culture: (1×10^6 – 10×10^6 cells) and biological samples: tissues (mass equivalent to 0.5–1.0 mg of protein), serum (50–100 μ l), culture media (2.0–5.0 ml).

2.2. Internal standards (ISs)

Internal standards (ISs) are prepared by the Lipidomics Core, MUSC or are purchased from a commercial source, if available (Metreya Inc., AVANTI Polar Lipids Inc.). Additional SPL standards are under development by the Lipidomics Core, MUSC.

1 μ M IS “Solution A” containing: 17Sph, 17Sph-1P, 17C16-Cer, and 18C17-Cer in methanol.

5 μ M IS “Solution B” containing 18C6-SM and 18C17-SM in methanol.

Note: Spike sample and/or calibration standards with 50 μ l of the appropriate IS solution(s).

2.3. Endogenous sphingolipid calibration standards (ESCSs)

Endogenous sphingolipid calibration standards (ESCSs) are prepared by the Lipidomics Core, MUSC or purchased from commercial source, if available (Metreya Inc., Avanti Polar Lipids Inc.). Additional SPL standards are under development by the Lipidomics Core, MUSC.

2.3.1. Primary screen

ESCS for SBs and Cers: 0.25 μ M solution of “Calibration Mix A” and 1 μ M solution of “Calibration Mix B” in methanol containing SPLs with 18C-SB: Sph, dhSph, Sph-

1P, dhSph-1P, C14-Cer, C16-Cer, C18-Cer, C18:1-Cer, C20-Cer, C24-Cer, and C24:1-Cer.

ESCS for dhCers: 0.25 μM solution of “Calibration Mix A” and 1 μM solution of “Calibration Mix B” in methanol containing SPLs with 18C-dhSB: dhC14-Cer, dhC16-Cer, dhC18-Cer, dhC18:1-Cer, dhC24-Cer, and dhC24:1-Cer.

ESCS for SM: 1.0 μM solution of “Calibration Mix A” and 5.0 μM solution of “Calibration Mix B” in methanol containing SPLs with 18C-SB: lyso-SM, C16-SM, C18-SM, C18:1-SM, and C24:1-SM.

Note: Prepare standard curves in the range of 1.0–200.0 pmol for SBs and SB-1Ps; 2.5–400 pmol for Cers and 12.5–2000.0 pmol for SMs.

2.4. Lipid extraction

Solvents: HPLC grade: chloroform (passed through a column of basic alumina), methanol, ethyl acetate, iso-propanol, and DI water, 1 M NaOH solution in methanol, 1 M NaCl solution in water.

Equipment: Solvent dispensers (Repipet Dispensers & Dilutors, Lab Industries, Inc.), ultra sonicator (bath type), vortex, centrifuge, tissue homogenizer (e.g. Polytron PT 1200C), vacuum centrifuge for evaporating solvents under reduced pressure (e.g. Speed Vac from Savant), nitrogen evaporator (e.g. N-Vap from Organomation Associate, Inc.) for evaporating solvents under nitrogen stream, 15 ml polyethylene vials, 13 \times 100 mm glass screw cap vials, 1.8 ml autosampler vials with 200 μl insert.

2.5. Tandem mass spectrometry

Solvents and reagents: DI water, methanol, formic acid, ammonium formate.

Equipment: Triple quadrupole mass spectrometer equipped with Electrospray Ion Source (ESI) (e.g. Thermo Finnigan, PE Sciex), syringe pump, syringes, (5 μl –1 ml), nitrogen generator (Parker Hannifin Corp.).

2.6. High-performance liquid chromatography

Solvents: Methanol, water, formic acid, ammonium formate.

Equipment: “Surveyor” quaternary HPLC pump, “Surveyor” autosampler, column BDS Hypersil C8 150 \times 3.2 mm; 3 μm particle size (Phenomenex).

3. Recipes

Note: All the following solutions should be prepared in volumetric flasks

Recipe 1: 1 M Ammonium formate. Prepare 1 M ammonium formate in water.

Recipe 2: 1 M NaOH. Prepare 1 M NaOH in methanol.

Recipe 3: Tissue homogenation buffer. Prepare buffer containing: 0.25 M sucrose, 25 mM KCl, 50 mM Tris, and 0.5 mM EDTA, pH 7.4.

Recipe 4: Cell or tissue extraction mixture. Prepare a solution of iso-propanol:water:ethyl acetate (30:10:60; v:v:v).

Recipe 5: Media extraction mixture. Prepare a solution of iso-propanol:ethyl acetate (15:85; v:v).

Recipe 6: Positive ion MS spray solution. Prepare a solution of methanol:water:formic acid (70:29.8:0.2; v:v:v).

Recipe 7: HPLC Mobile Phase “A”. Prepare 1 mM ammonium formate in methanol containing 0.2% formic acid.

Recipe 8: HPLC Mobile Phase “B”. Prepare 2 mM ammonium formate in water containing 0.2% formic acid.

4. Instructions

4.1. Preparation of biological materials for MS analysis

4.1.1. Cells in culture

Cells grown in suspension: Transfer cell suspension to 15 ml polyethylene vials kept on ice, separate cell pellet from media by centrifugation at 1000 rpm for 5 min at 5–10 $^{\circ}\text{C}$, aspirate media, wash cell pellet twice with ice-cold phosphate-buffered saline (1 \times PBS), separate pellet from PBS wash, use cell pellet for lipid extraction.

Adherent cells: Remove media, wash cells twice with ice-cold PBS, scrape cells with ice-cold PBS and transfer to 15 ml polyethylene vial placed on ice (repeat this process), separate cell pellet by centrifugation at 1000 rpm for 5 min at 5–10 $^{\circ}\text{C}$, use cell pellet for lipid extraction.

Note: Cell pellets can be collected and stored at -80°C before use or cell pellets can be lyophilized and stored as a dry material.

Tissue samples: To prepare tissue homogenate use a frozen tissue and keep ratio of tissue weight to buffer volume as 10% (w/v). Homogenize frozen tissue (3–4 times) with ice-cold buffer (Recipe 3). After each homogenization put homogenate on ice to rest and filter homogenate through the layers of gauzes using Pasteur pipette. The efficient amount of tissue homogenate for MS analysis is established through the protein concentration measurement (Bio-Rad Protein Assay, 24) [24].

Note: Practically, homogenize ~ 100 mg wet tissue in 1 ml of buffer and use ~ 100 μl of tissue homogenate for lipid extraction. This volume of tissue homogenate will correspond to 0.5–1.0 mg of protein and ~ 150 –300 nmols of Pi (level of phospholipids extracted from tissue homogenate by Bligh & Dyer extraction method [25]). Smaller size of tissue can be used if analytical request is for Cers or SM only (~ 50 μg of protein).

Biological fluids: Dilute 100 μl of the biological fluids (whole blood, serum, plasma) with serum free media (SFM) to the volume of 2 ml, and then proceed with extraction as for the media samples (below).

4.2. Extraction of sphingolipids

Cells or tissues: (i) Fortify cell pellets or tissue homogenate with 50 μl of IS solution(s), (ii) add 2 ml of extraction

mixture (Recipe 4), vortex, sonicate 3 × periodically for 30 s, and centrifuge for 10 min at 4000 rpm, (iii) transfer supernatant to a new vial, (iv) re-extract sample following step “ii”, (v) combine supernatants or upper layers from the extracts (should be 4 ml total), (vi) aliquot 1 ml of lipid extract from steps “v” for SM determination, and (vii) use the remaining lipid extract from step “v” for MS/MS analysis of SBs and Cers.

Note: If the Pi determination is also required, aliquot 2 ml of extract from step “v”. Will be used for MS/MS analysis of SM species, as shown in below steps, and Pi determination [26].

Culture media: (i) Fortify 2 ml of media with 50 µl of IS solution(s), (ii) add 2.0 ml of extraction mixture (Recipe 5), vortex, centrifuge for 10 min at 4000 rpm, transfer upper phase to a new vial, (iii) acidify the aqueous phase with 100 µl of formic acid, (iv) repeat extraction process from step “ii”, (v) combine the upper organic phases, (vi) aliquot 1 ml of lipid extract from steps “v” for SM determination, (vii) use the remaining lipid extract from step “v” for MS/MS analysis of SBs and Cers.

4.3. Preparation of sample for SBs, SBPs, and Cer analysis

(i) Evaporate organic extracts from steps “v” to dryness under reduced pressure in a Speed Vac or under N₂ gas in N-Vap, (ii) reconstitute the dry residue from step “i” in 150 µl of the Mobile Phase “A” (Recipe 7) vortex, centrifuge for 5 min at 4000 rpm, (iii) transfer supernatant to an autosampler HPLC vial with 200 µl insert, and (iv) inject 20 µl on the HPLC system.

4.4. Preparation of sample for SM analysis

(i) Evaporate organic extracts from step “v” to dryness under reduced pressure in a Speed Vac or under N₂ gas in N-Vap, (ii) add 3 ml of chloroform:methanol 1:2 (v/v) and continue Bligh & Dyer extraction [25] by adding 0.8 ml of water, 1 ml of chloroform and again 1 ml of water, vortex well after every step and centrifuge for 5 min at 3000 rpm, (iii) separate the lower organic phase (should be 2 ml of volume), (iv) aliquot 0.5 ml of lipid extract (in duplicate) for Pi determination [25] (v) evaporate remaining extract, re-suspend in 1 ml of methanol and add 10 µl of 1 M MeONa (Recipe 2), vortex well and keep at room temperature for 2 h (vortex occasionally), (vi) evaporate extract from step “v” to dryness under reduced pressure in a Speed Vac or under N₂ gas in N-Vap, and (vii) reconstitute residue from step “vi” in 150 µl of the Mobile Phase “A”, (Recipe 7). This will be sample for determination of SM components and lyso-SM.

4.5. Preparation of calibrator standard samples (CSS)

CSS are processed in identical manner as regular samples, substituting cell/tissue samples with bovine serum albumin (BSA) and media/biological fluids with serum free media (SFM), respectively. Spiking these artificial matrices, with a known amount, encompassing the dynamic range of

analysis, of the target analyte, and a constant amount of corresponding IS will generate calibration curves for quantitative analysis.

Procedure: (i) Fortify 1 mg of BSA or 2 ml of SFM with an appropriate volume of the SB/Cer or SM calibration mix “A” or “B”, (see Section 2.3) (typical is an eight-point calibration ranging 2.5–400 pmol) and 50 µl of IS (see Section 2.2), (ii) follow the cell/tissue extraction procedure (above), and (iii) use a total extract for HPLC/MS/MS analysis.

4.6. Identification of SPLs by tandem mass spectrometry

Qualitative analysis of SPLs from crude extracts is performed by analysis of their unique molecular decomposition products using a Parent Ion scan of common fragment ions characteristic for the particular class of SPLs (Fig. 2). Ionization conditions and collision energy are optimized for individual molecular species to achieve maximum sensitivity and quantitative accuracy. Each target analyte is uniquely identified by the Parent-to-Daughter Ion mass transition and the specific retention time (Tables 1 and 2). SPLs composition has to be established for every new matrix.

Ceramides' composition: (C18-SB) is established by the Parent Ion scan, performed for the common Daughter Ion ($m/z = 264.2$ and 266.1 for Cers and dhCers, respectively) at the high collision energy (35–55 eV), operating in positive ionization mode (Fig. 2). A representative sample extract is infused directly into ESI source, and is scanned for molecular ions of the potential Cers. Further confirmation of Cers' identity is achieved through MRM analysis with “soft” fragmentation (15–20 eV). Running sample through the HPLC system also confirms a reasonable retention time. Only Cers that satisfy identification criteria in both analyses are considered truly present in the sample and are included in the quantitative determinations.

SM composition (18C-SB): Identification of the SM components is performed similarly, employing common Daughter Ion ($m/z = 183.9$) at 40 eV collision energy (Fig. 2).

Procedure: (i) Infuse reconstituted samples (from step “v” in Section 4.3 or 4.4 from Article Outline) at 5–10 µl per min along with 200 µl per min MS spray solution (Recipe 6), (ii) perform precursor ion scans for the following Daughter Ions: 264.1 (collision energy 35 eV); 266.1 (collision energy 55 eV), and 183.9 (collision energy 40 eV) for Cers, dhCers, and SMs, respectively.

Note: Optimize the ionization conditions for each class of SPLs and collision energy for each individual molecular sub-species, including ISs and ESCSs, to be applied for quantitative MRM analysis.

4.7. Quantitation of SPLs by HPLC-MS/MS using multiple reaction monitoring (MRM)

Quantitative analyses of SPLs are based on the calibration curves generated for each CSS as shown above. The CSS are then subjected to the identical extraction proce-

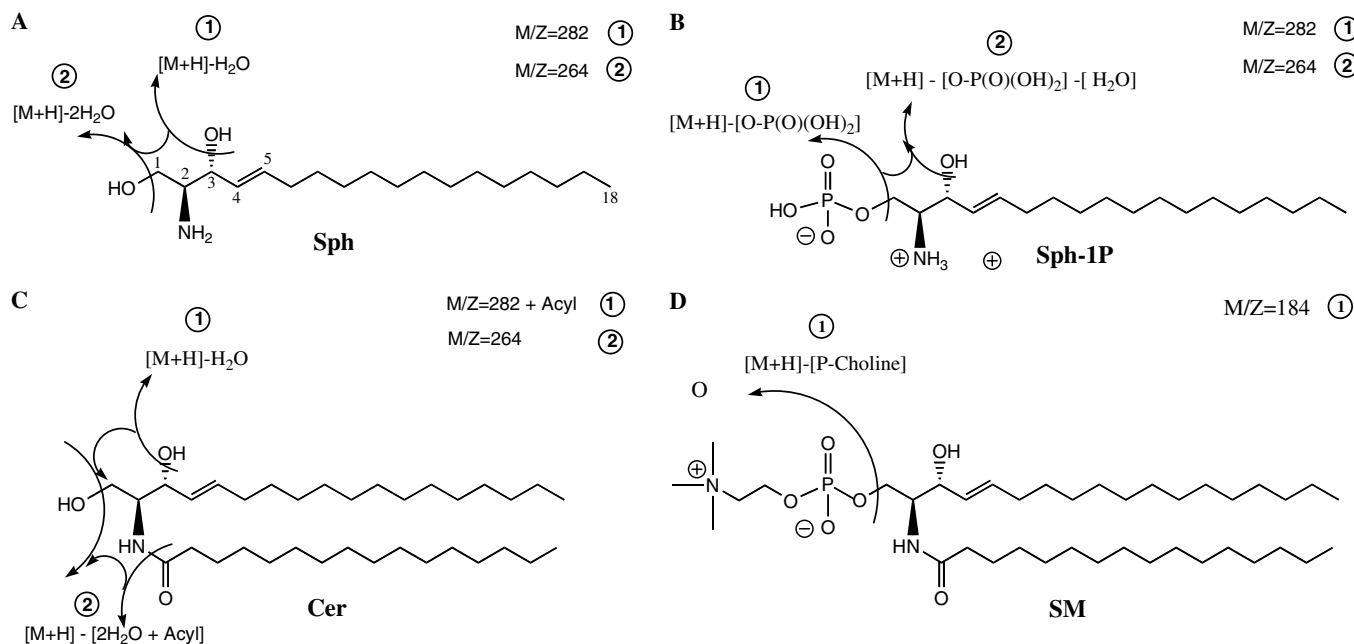


Fig. 2. MS/MS fragmentation patterns of the initial positive molecular ions, generated upon ESI of: Sph (A), Sph-1P (B), Cer (C), and SM (D). The specific common fragment ion of $m/z = 264$ (2, C) for Cers, and $m/z = 184$ (1, D) for SM are used in the Parent Ion scan experiments for determination of molecular species composition prior to quantitative analysis with the MRM experiments.

dures as the “biological” samples and analyzed by the HPLC/MS/MS system operating in positive MRM mode, employing HPLC conditions described in footnotes of Tables 1 and 2. Peaks for the target analytes and IS are recorded and processed using the Xcalibur software system. Plotting the CSS/IS peak area ratios against CSS concentration and applying linear regression model with the 1/X weighting factor generates the CSS-specific calibration curves. Any SPL for which no standard is available is quantitated using the calibration curve of the closest counterpart from Tables 1 or 2.

SBs, SB-1Ps and Cers analyses: (i) Load reconstituted samples (step “v” in 4.3 Article Outline) into the autosampler, (ii) pre-equilibrate HPLC column, (iii) set the autosampler to inject 20 μ l of reconstituted extracts onto column, (iv) start the gradient elution according to the HPLC conditions specified in footnote of Table 1, (v) start MS/MS, monitoring Parent-to-Daughter Ion transitions for all SBs and Cers sequentially, and (vi) determine the areas under the peaks for quantitation using extracted ion chromatograms. Fig. 3 shows a typical total ion chromatogram (TIC) from simultaneous analysis of SBs, SB-1Ps, Cers, and dhCers.

SM analysis: (i) Load reconstituted samples (step “v” in 4.4 Article Outline) into the autosampler, (ii) pre-equilibrate HPLC column, (iii) set the autosampler to inject 10 μ l of reconstituted extracts onto column, (iv) start the gradient elution according to HPLC condition specified in the footnote of Table 2, (v) start MS/MS, monitoring Parent-to-Daughter ion transitions for all SMs, and (vi) determine the peak areas for quantitation using extracted ion chromatograms. Fig. 4 shows a typical total ion chromatogram (TIC) from simultaneous analysis of SMs.

5. Concluding remarks and hints for troubleshooting

Very low sub-picomole detection limits have been achieved by applying rigorous sample preparation procedures, excellent HPLC separation and MRM experiments, which greatly reduce chemical noise in the final LC/MS chromatograms. We have selected ISs for particular SPL classes based on C17-SB as the closest ‘unnatural’ sphingoid base to the natural C18-Sph counterpart. This selection gives us a confidence that physicochemical properties such as the elution order and mass fragmentation pattern accurately reflect natural SPLs, but are not present in the analyzed sample.

Finally, we want to draw attention to a few very important issues that reflect on the final analytical results and share our findings with the readers. These include the following:

Effects of lipid extract preparation: In some protocols [21,27,28], the organic extract of lipids is subjected to mild alkaline hydrolysis in order to remove diglycerides and other glycerolipids by cleaving ester linkages. However, 1-*O*-acyl-ceramides [29] will also be hydrolyzed, thus artificially increasing the level of Cers. Comparison of the Cer level calculated from lipid extracts that were prepared with and without the base hydrolysis step can provide important data about the level of *O*-acyl-ceramides. Our results show some (20–40%) increase in Cers after this step. Nevertheless, this simple approach is recommended in the preparation of samples for SM analysis to allow elimination of phosphatidylcholine (PC) from the lipid extract. If simultaneous analysis of SM and PC is required, then different approaches should be employed [30–32].

Table 1
Quantitative LC/MS/MS parameters for SBs and Cers analysis

SPL	Mass transition	Collision energy (eV)	Retention time (min)
17CSph	286.1–268.0	17.0	2.16
Sph	300.4–282.2	18.0	2.44
dhSph	302.5–284.2	22.0	2.66
17CSph-1P	366.1–250.1	20.0	4.30
Sph-1P	380.4–264.2	23.0	4.92
dhSph-1P	382.3–266.1	45.0	5.44
17C16-Cer	524.8–506.4	15.0	14.23
C14Cer	510.7–492.6	15.0	13.23
C14-dhCer	512.5–494.2	36.0	13.45
C16-Cer	638.8–520.4	15.0	15.21
C16-dhCer	540.8–522.4	33.0	15.93
C18:1-Cer	564.5–546.4	15.0	16.01
C18:1-dhCer	566.5–548.2	37.0	16.77
C18-Cer	566.4–548.3	15.0	17.44
C18-dhCer	568.3–550.1	37.0	18.21
18C17-Cer	552.8–534.5	15.0	16.33
C20-Cer	594.4–576.5	15.0	19.78
C20-dhCer	596.4–578.2	40.0	20.74
C24:1-Cer	649.8–631.6	15.0	22.68
C24:1-dhCer	651.9–633.8	40.0	24.46
C24-Cer	651.6–633.7	15.0	25.02
C24-dhCer	652.0–634.2	40.0	25.41
C26:1-Cer	680.1–662.3	15.0	26.02
C26:1-dhCer	682.0–664.1	40.0	26.56
C26-Cer	681.9–664.0	15.0	26.93
C26-dhCer	684.0–666.1	40.0	27.26

Quantitative mass transitions along with collision energy, applied for the MRM experiments of simultaneous analysis of the SBs, SB-1Ps, Cers, and dhCers. Each panel begins with the relevant IS (bold print) used for quantitation of the associated target analytes. The last column lists a typical HPLC retention time obtained using the Reverse Phase C8 150 × 3.0 mm, column, flow rate .5ml/min and the following elution gradient: A/B over time (Mobile Phase A: 1 mM ammonium formate in methanol with .2% formic acid, Mobile Phase B: 2 mM ammonium formate in water with .2% formic acid), 0–4.5 min: 80/20–90/10; 4.4–7.0 min: 90/10; 7.0–28.0 min: 90/10–99/1; 28.0–29.0 min: 99/1–80/20; 29.0–33.0 min: 80/20.

Contribution of ¹³C isotope: Some natural SPLs differ by only 2 mass units (e.g. Cers vs. their respective dhCers, mono-unsaturated Cers vs. saturated counterparts), therefore ¹³C isotope contribution from the lower to higher molecular masses has to be evaluated. Usually, due to the low ¹³C natural abundance this effect can be negligible. However, with the large differences in the concentrations (a common situation for Cers vs. dhCers in most of the mammalian cells), the isotope contribution from Cer to respective dhCer becomes significant. Consequently, without sufficient chromatographic separation, the common case when normal phase HPLC is employed, dhCer concentration may be artificially elevated, even by several-folds (e.g. contribution of C16-Cer to the C16-dhCer mass transition channel, Fig. 5). Applying rigorous decomposition conditions in the MRM experiment and monitoring the molecular ion-to-mono-dehydrated mass transitions can decrease this effect even further. Cers yield a mono-dehydrated fragment uniformly at a collision energy of 15-to-20 eV, whereas the dihydro-counterparts require much higher energy (35–45 eV) at which Cers undergo further fragmen-

Table 2
Quantitative LC/MS/MS parameters for SM analysis

SM	Mass transition	Collision energy (eV)	Retention time (min)
18C6-SM	563.3–183.7	35.0	3.65
Lyso-SM	465.2–183.7	35.0	3.21
C14-SM	675.4–183.8	35.0	6.71
18C17-SM	717.3–183.8	35.0	8.33
C16-SM	703.4–183.7	35.0	8.17
C18:1-SM	729.4–183.8	35.0	8.57
C18-Cer	731.4–183.7	35.0	9.72
C20:1-SM	757.4–183.8	35.0	11.02
C20-SM	799.5–183.8	35.0	11.75
C22:1-SM	785.4–183.8	35.0	12.61
C22-SM	787.5–183.9	35.0	12.96
C24:1-SM	813.4–183.8	35.0	13.15
C24-SM	815.3–183.8	35.0	14.47

Quantitative mass transitions along with collision energy, applied for the MRM experiments of simultaneous analysis of SMs. Each panel begins with the relevant internal standard (bold print) used for quantitation of the associated target analytes. The last column lists a typical HPLC retention time obtained using the Reverse Phase C8 150 × 3.0 mm, column, flow rate .5 ml/min and the following elution gradient A/B over time (Mobile Phase A: 1 mM ammonium formate in methanol with .2% formic acid; Mobile Phase B: 2 mM ammonium formate in water with .2% formic acid): 0–12.0 min: 90/10–99/1; 12.0–25.0 min: 99/1; 25.0–26.0 min: 99/1–90/10; 26.0–30.0 min: 90/10.

tation, predominantly to the double dehydrated/deacylated fragment “2” (Fig. 2C). Applying this diversified collision energy, supported with an excellent HPLC separation, we have been able to perform quantitative determination of all Cers and dhCers molecular species simultaneously.

Quantitation: To achieve reliable quantitation of all molecular species, calibration curves should be generated for as many representative components of SPL as possible, due to diversified MS responses, as reflected by calibration curve slopes (Fig. 6A and B). Our typical calibration ranges are 2.5–400 pmol and 12.5–2000 pmol, for Cers and SMs, respectively. Examination of extended calibration ranges indicates (data not shown) that linear MS response can be extended up to 2000 and 10,000 pmol for Cers and SMs, respectively.

Sample delivery for MS analysis: Cell and tissue samples can be delivered as freshly harvested, shipped on dry ice, or can be lyophilized and shipped at room temperature. We have performed comparable analysis for the newly harvested and lyophilized MCF-7 cells and liver tissue samples, and both examples yielded virtually identical results (data available upon request).

Data normalization: Results from the MS analysis represent the mass level of particular SPLs (in pmol) per total sample used for lipid extract preparation and quantitative analysis. In general, treatment with exogenous agents causes changes in SPL levels and compositions. For the final data presentation, MS results should be normalized to some stable parameters (which are considered not affected by that particular treatment). Total protein (mg) [24], or phospholipid Pi (nmol) [26] present in the Bligh & Dyer extract [25], which corresponds to the amount of the biological material used for MS analysis, can be used as the

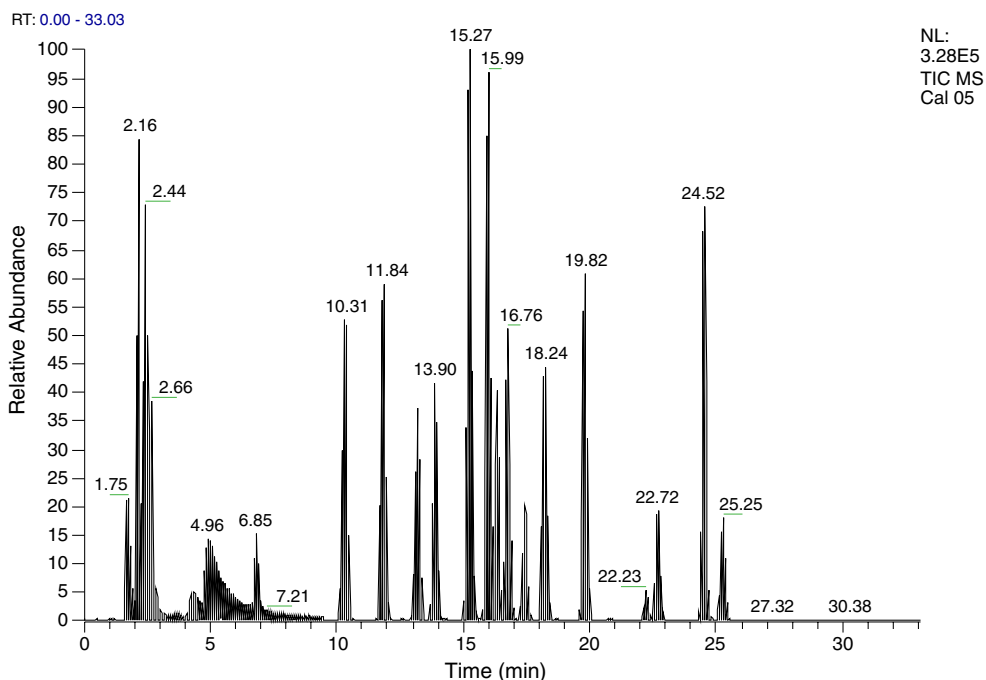


Fig. 3. A typical total ion chromatogram (TIC) composed from MRM simultaneous analysis of SBs, SB-IPs, Cers, and dhCers. A total of 26 individual species has been monitored. For detailed information about compound identification and LC/MS/MS conditions, please refer to Table 2.

normalization parameters [28,33,34]. Also normalization to the total cell number is used [35]. Final results should be shown as changes in the relation to the control (% control).

From our experience, data normalized to the protein or to the Pi (shown as % of the control) are not exactly the same. The normalization issue needs a lot of attention.

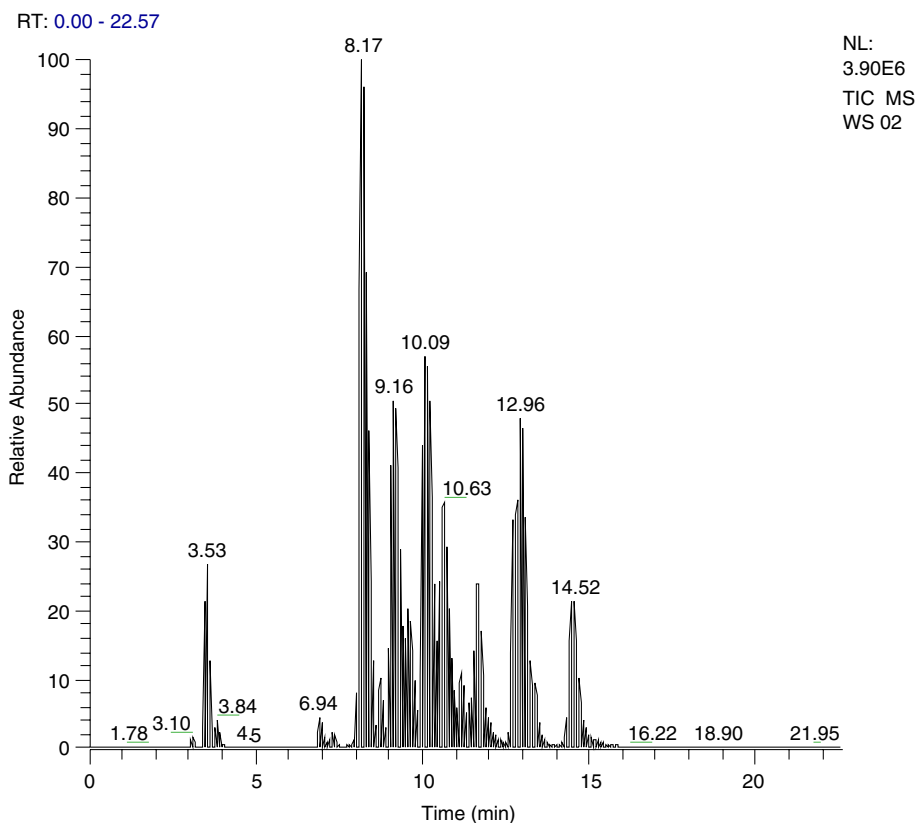


Fig. 4. A typical total ion chromatogram (TIC) composed from MRM simultaneous analysis of SMs. A total of 14 individual species have been monitored. For detailed information about compound identification and LC/MS/MS conditions, please refer to footnotes of Tables 1 and 2.

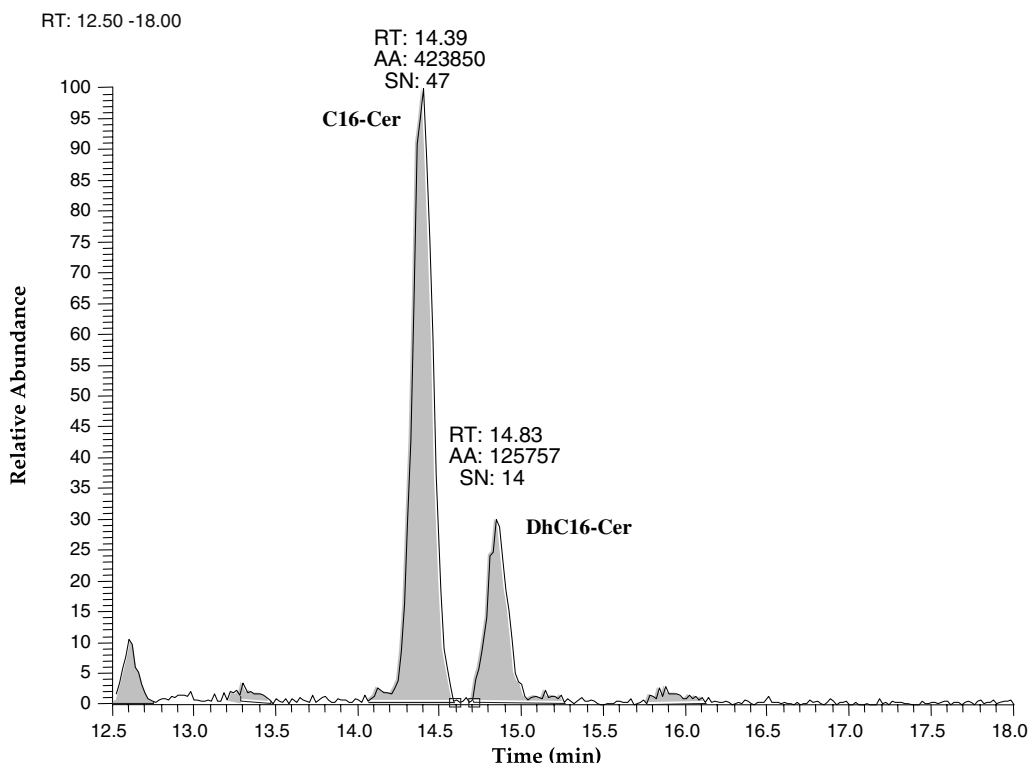


Fig. 5. An example of ^{13}C isotope contribution from ceramide-to-dihydroceramide specific mass transition channel. With much higher concentration of ceramide than dihydroceramide, even the small ^{13}C natural abundance may cause a significant contribution to the dhCer. Such contribution (C16-Cer) may artificially increase dhC16-Cer concentration, unless sufficient HPLC separation is achieved. In this particular case, if C16-Cer would not be effectively resolved from dhC16-Cer, it could artificially increase the dihydroceramide concentration from the real 8.6–37.9 pmol/sample.

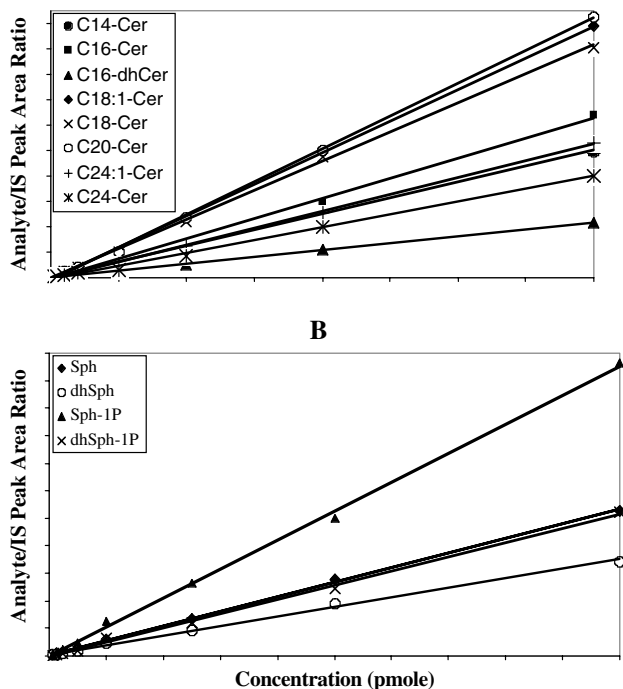


Fig. 6. The MS response varies for molecular species even within the particular SPL class, as indicated by the calibration curve slopes; therefore, individual calibrations should be generated for as many target analytes as possible. A linear instrument response (R^2 value of .99) is obtained for the typical calibration ranges: 1.0–200.0 pmol for SBs and SB-1Ps (B), as well as for all ceramide species (A).

6. Related techniques

A variety of different techniques (mostly radio-labeling, HPLC analysis of fluorescent analogs, and enzymatic methods) in addition to MS methodology are used for SPLs measurement. Up to now the enzymatic method employing diacylglycerol kinase and [^{32}P] ATP has been the most commonly used procedure for total Cer quantitation in the range of 25 pmol to 2 nmol [34,36]. Cellular SBs are most often analyzed by the HPLC technique developed for their fluorescent derivatives [37]. Cellular SB-1Ps are analyzed via their derivatization to [^3H] C2-ceramide phosphate, by enzymatic method (employs alkaline phosphatase, followed by action of recombinant sphingosine kinase and [^{32}P] ATP) after separation of Sph-1P from the cellular Sph, or by employing HPLC analysis of OPA-derivated Sph-1P [39–41]. These procedures require less expensive equipment than mass spectrometry but are not as informative. SM may be determined by several different approaches including TLC analysis, GC analysis of silylated derivatives and MS techniques [21,30–33]. Cer and SM molecular species can be determined following hydrolysis and analysis of the liberated and derivated SBs by means of HPLC [37–42] and fatty acids by means of GC [43] or GC/MS [44] and can base on normal or reverse phase HPLC separation of their fluorescent analogs [45,46], or GC analysis of silylated derivatives [30–32]. Moreover in

the last few years, MS methodologies have been developed for the detection of Cers and SM molecular species [15,18–21].

Further improvement in the MS technology to the ESI-MS/MS has allowed the simultaneous analysis of SPLs subclasses [18,19,21] and this method should become a major analytical methodology in the modern analysis of SPLs.

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