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Mass Spectrometric Analysis of Bioactive Sphingolipids in Fungi

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Abstract

As biomolecules, sphingolipids represent a broad spectrum of structures ranging from simple long chain bases to complex glycosphingolipids. While several different mass spectrometry based approaches have been proven to be useful in qualitative and quantitative analysis of sphingolipids, we find that electrospray ionization tandem mass spectrometry (ESI-MS/MS) in the multiple-reaction monitoring (MRM) mode using a triple quadrupole instrument, coupled to high-performance liquid chromatography (HPLC), is the most suitable approach for the analysis. In this chapter, we describe the method in a step-by-step manner toward the targeted analysis of sphingolipids in fungi. With optimized HPLC separation and instrument settings, this MRM approach affords detection of many sphingolipid species simultaneously with good sensitivity.

Keywords

Sphingolipids; Multiple-reaction monitoring; Mass spectrometry; High-performance liquid chromatography

1 Introduction

Sphingolipids are a unique cluster of biomolecules reported in a wide variety of fungi, including the dimorphic fungi, molds and yeasts [1]. Several researches over the last two decades have shown that sphingolipids play key roles in fungal biology [2–13]. Specifically, sphingolipids are an important regulator of fungal pathogenicity and affect the processes like hyphae formation, cell division, germination, pH tolerance among many others [2–13]. Specific sphingolipid structures have been shown to be synthesized by specific enzymes [14]. With an increased thrust in sphingolipid research in fungi, it has become important to characterize various sphingolipid structures. Therefore, continuous emphasis is being put to develop simple and accurate methods of sphingolipid analysis. While the traditional platforms like radiolabeled thin layer chromatography (TLC) is still in practice, an advanced

technique like electrospray ionization tandem mass spectrometry (ESI-MS/MS) provides a better alternative [15]. ESI-MS/MS allows the detection and quantification of various sphingolipid structures. For example, using ESI-MS/MS platform we could categorically analyze different long chain bases (LCB), ceramides (Cer), dihydroceramides (dhCer), phytoceramides (PCer), glucosylceramides (GlcCer), inositol phosphorylceramides (IPC) among several others [15–18]. Notwithstanding identification and quantification of closely related sphingolipid structures requires a prior separation of the sphingolipid mixture on a high performance liquid chromatography (HPLC) system, before analysis on ESI-MS/MS [15–18]. Recently, several studies have focused on improving the methods of sphingolipid extraction, purification, separation, structure determination, and quantification [16, 19–22]. Further, using a wide variety of commercially available internal and external standards, it has become much easier to validate the structural identities of various sphingolipids as well as their absolute quantity [23]. In the present chapter we describe the key aspects of sphingolipid extraction, HPLC separation, ESI-MS/MS based identification, and semi-quantitation with addition of internal standard.

2 Materials

2.1 Fungal Strains

Reference fungal strains are available at the standard repositories of American Type Culture Collection (ATCC), US (www.atcc.org) and CBS-KNAW culture collection, The Netherlands (<http://www.wi.knaw.nl/>).

2.2 Cell Culture

1. YPD-agar (yeast extract, peptone and dextrose, Difco).
2. SAB (sabouraud's dextrose broth, Difco).
3. RPMI 1640 medium (Roswell Park Memorial Institute 1640 Medium, Gibco).
4. YNB (yeast nitrogen base, HiMedia).
5. Pyrex® culture tube with PTFE lined screw cap (Sigma Aldrich, USA).
6. Glass disrupter beads (0.5 mm, Fisher Scientific, UK).

2.3 Chemicals

All solvents are LC-MS grade unless otherwise stated and can be procured from Fisher Scientific, UK and Sigma Aldrich, USA.

1. Ultrapure distilled water (dH₂O).
2. Diethylether.
3. Pyridine.
4. Methanol.
5. Trichloroacetic acid (TCA).
6. Trifluoroacetic acid (TFA).

7. Ammonium formate.
8. Formic acid.
9. Phosphate Buffer Saline (PBS).
10. HEPES Buffer.
11. Ascorbic acid.
12. Butylated hydroxytoluene (BHT).
13. Ammonium molybdate.
14. Ethanol.
15. NH_4OH .
16. Chloroform.
17. H_2SO_4 .
18. Perchloric acid.
19. NaH_2PO_4 .
20. KOH.
21. HCl.
22. Internal standards (IS) (All from Avanti Polar Lipids, Inc., Alabaster, USA).
 - a. Cer(d17:1/18:0) (N-stearoyl-D-erythro-sphingosine (C17 base)).
 - b. SPH(d17:1) (D-erythro-sphingosine (C17 base)).
 - c. Cer(d18:1/17:0) (N-heptadecanoyl-D-erythro-sphingosine).
 - d. Cer/Sph Mixture I (LM6002) comprising SPH(d17:1), DHS(d17:0), S1P(d17:1), DHS1P(d17:0), lactosylceramide(d18:1/12:0), SM(d18:1/12:0), GlcCer(d18:1/12:0), Cer(d18:1/12:0), Cer1P(d18:1/12:0) and Cer (d18:1/25:0) (see Notes 1 and 2).

2.4 Equipments

1. TSQ Quantum Ultra™ Triple Quadrupole Mass Spectrometer (Thermo Scientific, USA) fitted with Accela Pump/Autosampler (Thermo Finnigan, USA).
2. HPLC column: 3 μm C8SR, 150 $\times$ 3.0 mm column (Peeke Scientific, USA).
3. SpeedVac (SPD210, Thermo Fisher Scientific, Waltham, MA).

¹-Other sphingolipid standards required for quantification of sphingolipid species contents are also available at Avanti Polar Lipids, Inc., Alabaster, USA.

²-External sphingolipid standards are required to generate concentration-based calibration curves. At least two standards are required for each class of sphingolipid. One low molecular weight and the other high molecular weight, to cover for entire mass range of sphingolipid species under analysis [23, 34]. Quantification is done in the linear range of the calibration curve. Quantification in lower concentration ranges has been found to be more accurate.

4. Centrifuge (Eppendorf, USA).

3 Methods

3.1 Culture Collections

1. Inoculate fungal cell on YPD-agar plates at 30 °C.
2. Re-culture fungal cells in limited YNB or RPMI media buffered with HEPES or MOPS [3-(N-morpholino)propanesulfonic acid], pH 7.4) for 16 h at 30 °C (*see* Note 3) [16].
3. Centrifuge the culture at $1300 \times g$ at 25 °C (10 min), and aspirate the supernatant.
4. Wash the cell pellet with sterile dH₂O thrice using pellet disruption by vortexing and re-centrifugation ($1300 \times g$ at 25 °C, 10 min), and resuspend the pellets in PBS.
5. Count the cells with a hemocytometer under the microscope, and aliquot 5×10^8 cells in PBS to a separate tube and centrifuge at $1300 \times g$ at 25 °C for 10 min to obtain the cell pellet (*see* Notes 4 and 5).
6. Re-wash twice with sterile dH₂O and transfer the cell pellets to a Pyrex® culture tube with PTFE lined screw cap (*see* Note 6).

3.2 Lipid Extraction

Several approaches have been used for sphingolipid extraction from fungal cells [24–28]. For sphingolipid analysis pyridine based extraction using Mandala buffer extraction followed by Bligh and Dyer extraction and mild alkaline hydrolysis is routinely used [26–28].

3.2.1 Mandala Extraction [27]

1. Suspend cell pellet (5×10^8 cells) in 1.5 mL Mandala buffer (dH₂O:ethanol:diethyl ether:pyridine:NH₄OH (15:15:5:1:0.018; v/v)).
2. Add 50 mg glass disrupter beads and 50 pmol internal standards (*see* Notes 7 and 8).

³.Studies have used YPD, YNB, or PDA (potato dextrose agar) at varying temperature and pH conditions to culture cells [16, 35–38]. The culture conditions may be altered as per requirement of the study. It is important to note that YPD or SAB should only be used for medium in the start-up culture and not for the medium to grow cells that then will be used for lipid analysis. These rich media contain lots of lipids, including sphingolipids. The limited media like RPMI or YNB should be used instead [36, 39].

⁴.At this stage cells can be kept on 5% TCA on ice for 15 min.

⁵.During analysis, presence of varied enzymatic activities within the sample results in significant noise in the data and often replicates of individual samples don't show consistency. Stopping enzymatic activity by addition of TCA improves data reproducibility [40]. Addition of TCA is recommended during the analysis of phosphorylated sphingolipid LCBs [21, 41]. However, caution must be exercised by monitoring the extraction efficiencies. Some sphingolipid species might show better response without the addition of TCA.

⁶.Sphingolipids are quite sticky lipids. Use of Pyrex® culture tube with PTFE lined screw cap during lipid extraction allows maximum sample recovery while transferring from one tube to another. This overall reduces data variability.

⁷.Homogenization using the glass beads is crucial for efficient cell breaking of the prominent fungal cell wall.

⁸.Cer(d17:1/18:0), SPH(d17:1), and Cer(d18:1/17:0) are the frequently used IS. However, Cer/Sph Mixture I covers much broad range on sphingolipid classes. The physicochemical properties of unnatural C17-Sph and C17-Cer are shared with the naturally occurring

3. Homogenize the sample by alternate sonication and vortexing of 30 s each. Repeat this step at least three times (*see* Note 9).
4. Incubate the sample at 60 °C water bath for 15 min.
5. Homogenize the sample again by alternate sonication and vortexing of 30 s each. Repeat this step at least three times.
6. Incubate the sample again at 60 °C water bath for 15 min.
7. Sonicate the sample for 30 s.
8. Centrifuge at $1300 \times g$ at 4 °C for 10 min.
9. Transfer the clear supernatant into a fresh tube using a Pasteur pipette (Fisher Scientific, UK) and dry in SpeedVac system (*see* Note 10).
10. Re-dissolve the pellet in 2 mL methanol by vortexing and sonication, if necessary.
11. Incubate the sample at 37 °C for 60 min. Vortex twice for 30 s in between this process.
12. Centrifuge at $1300 \times g$ at 25 °C for 10 min.
13. Transfer the clear supernatant using a Pasteur pipette into a fresh tube.
14. Add 1 mL chloroform and 1 mL sterile dH₂O and vortex twice for 30 s.
15. Centrifuge at $1300 \times g$ at 25 °C for 5 min.
16. Transfer the lower hydrophobic layer using a Pasteur pipette into a fresh tube. At this point 1/10th of the sample is kept for the determination of inorganic phosphate (Pi) content (described later).
17. Dry sample in SpeedVac system, flush with nitrogen and store at –20 °C until processed further. Dry weight of extracted lipids is also recorded at this stage and is used for data normalization.

3.2.2 Post Mandala Extraction—This extraction method involves a mild alkaline hydrolysis to remove polar glycerophospholipid contaminants and enrich the sphingolipid contents [28, 29] (*see* Note 11). Steps are described as below (*see* Notes 10 and 11):

1. Dissolve the dry Mandala extract pellet in 1 mL chloroform.
2. Add 1 mL 0.6 M KOH in methanol and vortex.

18-carbon counterparts and reliably consistent (these include diagnostic fragmentation pattern and retention time and elution order between species) [23, 34]. Further, the sphingolipid species present in Cer/Sph Mixture I are below detection in our analysis of fungal lipid mixtures. Therefore, these IS can be used to spike the sample for sphingolipid analysis. Any pre-spiking sphingolipid levels that are detected are considered as background noise and subtracted from the sample data.

⁹Alternatively, depending upon the sample type, cell homogenization step can also be performed by using a Daum's homogenizer or a Fast prep (MP biomedical) [35–37]. Quick handling of the sample is required at this stage as cell breakage releases active phospholipases which can easily digest lipids.

¹⁰Avoid excessive drying of the sample. At this stage sample can be flushed with nitrogen and stored at –20 °C.

¹¹At an optimum concentration of the base, glycerophospholipids are deacylated [29]. This results in a cleaner sphingolipid extract. Further, during the analysis we *see* a minimal interference due to glycerophospholipids, which can occur during HPLC separation or MS ionization steps.

3. Incubate the sample at 25 °C for 60 min.
4. Thereafter add 0.65 mL HCl and 0.25 mL sterile dH₂O and vortex well.
5. Centrifuge at 1300 × *g* at 25 °C for 10 min.
6. Transfer the lower hydrophobic layer using a Pasteur pipette into a fresh tube.
7. Dry sample in SpeedVac system, flush with nitrogen and store at −20 °C until processed further.

Apart from the abovementioned procedure for sphingolipid extraction, often a separate extraction procedure adapted for the analysis of free sphingoid base and sphingoid base phosphate. For example, the extraction efficiency of S1P can be as low as 20%, where the majority of the content is lost in aqueous phase during extraction. Addition of 0.1% TFA (by volume) to the extraction solvent improves the S1P recovery to over 80% (*see* Note 12) [30]. Additionally, for samples that contain polyunsaturated fatty acids, it is recommended that 0.01% BHT must be added to the solvents (*see* Note 13) [31].

3.3 Estimation of Phosphate (Pi) Content

Estimation of Pi content is critical to sphingolipid profiling as the quantities are used to normalize the lipid data sets [23, 28]. Steps involved in Pi estimation are as below:

1. Take the 1/10th aliquot of the lipid extract stored earlier for Pi estimation (*see* Subheading 3.2.1, **step 16**).
2. Add 0.6 mL wet acid digestion buffer (10% perchloric acid:10 N H₂SO₄:dH₂O (1:9:40;v/v).
3. Incubate overnight at 150 °C.
4. Cool down the sample to room temperature and add 0.9 mL sterile dH₂O and vortex well.
5. Add 0.5 mL of 0.9% ammonium molybdate and vortex well.
6. Add 0.2 mL of 9% ascorbic acid and vortex well.
7. Incubate the sample at 45 °C for 30 min.
8. Record the absorbance at 820 nm.
9. Plot a standard curve using varying concentrations of NaH₂PO₄ to estimate the unknown Pi concentration of the sample.

3.4 HPLC Separations

1. Dissolve the sample in a buffer containing 1 mM ammonium formate +0.2% formic acid in methanol (buffer B).

¹²Due to high volatility and ion-pairing properties, TFA causes a forced protonation of both the positively charged amino group and the negatively charged phosphate group. This decreases the span in the protonation states and improves recovery rates of S1P [30].

¹³Addition of BHT inhibits the enzymatic hydrolysis of lipids [31].

2. Deliver 10 μ L sample by Accela Pump/Autosampler to the HPLC column (3 μ m C8SR, 150 $\times$ 3.0 mm) (*see* Note 14) [15, 16].
3. Use the two buffer system as the mobile phase: 2 mM ammonium formate +0.2% formic acid in sterile dH₂O (buffer A) and buffer B.
4. Resolve the sphingolipid species using the following buffer gradient:
 - a. Hold the buffer B at 70% for 30 s.
 - b. Gradually increase the concentration of buffer B to 90% in next 4.5 min.
 - c. Increase the concentration of buffer B further to 99% in next 12 min.
 - d. Hold buffer B at 99% for next 11 min.
 - e. Lower the concentration of buffer B to 70% in next 30 s.
 - f. Hold buffer B at 70% for the next 7.5 min.
5. Record the elution time (in min) for each sphingolipid species as the analyte is detected by the MS [23].
6. The total run time for this gradient is 34 min and a constant flow rate of 500 μ L is maintained.

3.5 MS Analysis

In our lab, detection of various sphingolipid species is performed using a TSQ Quantum Ultra™ Triple Quadrupole Mass Spectrometer. Other groups have used similar platforms for these analyses [17, 19, 32]. Prior to analysis of complex sphingolipid mixtures, it is important to understand the process of method development.

In targeted sphingolipidomics by MRM (multiple-reaction monitoring) approach using triple quadrupole MS, it is important to identify the SRM (Single Reaction Monitoring) transition that can be used to detect any particular sphingolipid species. The intent is to record the m/z (mass to charge ratio) of parent ion (or molecular ion) of sphingolipid species of interest in the first quadrupole (Q1), and its specific daughter ions (or fragmentation products) in third quadrupole (Q3). The fragmentation or collision induced dissociation (CID) of the parent ion is achieved by applying collision gas in the collision cell (q2) (Fig. 1) [15]. Steps involved in developing a SRM transition are explained below using GlcCer(d18:2/16:0(2OH) standard (Fig. 2), as an example:

1. Set up the MS instrument operating in positive ion mode with Heated Electrospray Ionization (HESI) probe as the ion source.

¹⁴. A good separation of sphingolipid species can also be obtained using the Kinetex® 1.7 μ m C8 100 Å, 50 $\times$ 2.1 mm column (Phenomenex, USA). Other groups have used C18 columns like Agilent poroshell 120 EC-C18 column, 2.7 μ m (50 $\times$ 4.6 mm), specifically for LCB analysis [21]. Further, sphingolipids with long chain fatty acyls are sticky and difficult to elute. Therefore, a column jacket may be used to maintain a specific temperature that benefits the elution process.

2. Set the vaporizer temperature at 400 °C, capillary temperature at 300 °C, aux gas pressure at 15 (a.u.), sweep gas pressure at 2.0 (a.u.), and sheath gas pressure at 60 (a.u.) [16].
3. Prepare GlcCer(d18:2/16:0(2OH) standard in Buffer B.
4. Introduce the sample into MS by direct syringe loop injection.
5. Obtain Q1-MS spectrum and identify the monoisotopic peak. The m/z 714.61 represents the monoisotopic peak for GlcCer (d18:2/16:0(2OH) standard (Fig. 2b) (*see* Note 15).
6. Record the fragmentation pattern of m/z 714.61 parent ion in Q3-MS. We can *see* that three daughter ions of m/z 262.3, 696.9, and 534.8 are abundant (Fig. 2c, d) (*see* Note 16).
7. Optimize the CE to maximize the daughter ion intensities. The ions of m/z 262.3, 696.9, and 534.8 show best intensities at the CE of 47, 19, and 26 V, respectively (Fig. 2c).
8. Subsequently, use the SRM transitions 714.6 $\rightarrow$ 262.3 (CE 47 V) and 714.6 $\rightarrow$ 696.9 (CE 26 V) to detect and quantify GlcCer(d18:2/16:0(2OH) standard on HPLC-ESI-MS/MS (Fig. 2d, inset).

Using this approach, “Parent ion $\rightarrow$ Product ion” transitions (SRMs) can be identified for analyzing a select sphingolipid species. Several SRMs can be used simultaneously in MRM mode to analyze a complex lipid mixture [15]. For example, separation and detection of ten individual sphingolipid species present in Cer/Sph Mixture I (LM6002) using MRM approach is represented in Fig. 3. In the SRM, the product ion can represent:

1. Loss of a H₂O molecule from the parent ion. For example,
 - a. 286.4 $\rightarrow$ 268.3, SPH(d17:1).
 - b. 288.5 $\rightarrow$ 270.4, DHS (d17:0).
 - c. 482.5 $\rightarrow$ 464.4, Cer(d18:1/12:0).
 - d. 664.7 $\rightarrow$ 646.6, Cer(d18:1/25:0).
2. Loss of phosphate moiety and two H₂O molecules from the parent ion. For example,
 - a. 366.4 $\rightarrow$ 250.3, S1P(d17:1).
 - b. 368.5 $\rightarrow$ 252.3, DHS1P(d17:0).
3. Loss of double dehydrated sphingoid base from the parent ion. For example,
 - a. 806.6 $\rightarrow$ 264.3, LacCer(d18:1/12:0).

¹⁵Calculate the theoretical m/z of the parent ion, which $[M+H]^+$ in this case. Putative structure can be sorted using a lipid database like www.lipidmaps.org (Fig. 2a).

¹⁶The fragment m/z 262.3 corresponds to the double dehydrated sphingoid base. The fragment m/z 696.9 corresponds to the loss of one H₂O molecule from the parent ion.

- b. 644.6 $\rightarrow$ 264.3, GlcCer(d18:1/12:0).
- c. 562.6 $\rightarrow$ 264.3, Cer1P(d18:1/12:0).
- 4. Phosphocholine head group. For example,
 - a. 647.7 $\rightarrow$ 184.1, SM(d18:1/12:0). Of note, sphingomyelin is a mammalian lipid [20].

This MRM approach is commonly used for targeted sphingolipidomics in fungi [15–19, 21]. Some common fungal sphingolipids and their MS based detection parameters are depicted in Table 1.

3.6 Data Analysis

3.6.1 Lipid Quantification—Data is processed using the Xcaliber and LCquan software for the TSQ Quantum Ultra™ triple quadrupole MS or similar software in other MS platforms. However, there can be variations in the obtained signals due to differences in ionization efficiencies, solvent systems, instrument detection limits, among others. Therefore, our quantification potential is largely dependent on the availability of natural and internal standards [16, 17, 23]. Determination of linear working ranges is the key to nullify any signal variations and for accurate quantification. Sphingolipid species are identified by matching the observed retention time (RT). The RT of the available standards can be used to predict the theoretical RT of the sphingolipid species for which a standard is not available, referred as mass and relative elution time (MRET) profiling [33]. The underlying principle behind MRET is that a sphingolipid class (for example Cer(d18:1)) containing molecular species of varying fatty acyl chain lengths (for example C12–C26), in a set isocratic gradient on HPLC, resolve and elute in a specific pattern. This information can be used to determine theoretical RT (*see ref. 33 for details*). Together, the RT and mass spectrometry data is sufficient for sphingolipid species authentication.

For quantification of sphingolipids, we have come to the use of six to eight point calibration curves, in standardized dilution ranges (Fig. 4). The dilutions of calibration standards are spiked with the known concentration of IS, and these are analyzed using the HPLC-MRM-ESI-MS/MS methods discussed earlier (*see Note 17*). To generate the calibration curve “area of analyte/area of IS” ratios are plotted against the “concentration (or amount) of analyte/concentration of IS” ratios.

The fungal lipid extracts are pre-spiked with known concentrations of IS. To calculate the concentration of any specific sphingolipid species (for example Cer(d18:1/18:0) target species and Cer (d18:1/17:0) IS), the concentration ratio of “Cer(d18:1/18:0)/Cer(d18:1/17:0)” corresponding to the peak area ratio of “Cer (d18:1/18:0)/Cer(d18:1/17:0)” is multiplied by the added concentration of IS (Fig. 4) [34].

¹⁷For many sphingolipid species, an authentic external standard is not available. In such cases, a closely related sphingolipid species standard can be used. For example, Cer(d18:0/24:0) standard can be used to quantify Cer(d18:0/22:0), standard for which is unavailable [34].

3.6.2 Data Normalization—The quantitative data for a specific sphingolipid species is obtained in pmol. The data is required to be normalized to a parameter that shows minimum variation and conserves the overall changes in the sphingolipid levels, across samples [23]. Following can be used:

1. Pi content (*see* Subheading 6) is the most commonly used parameter to report the sphingolipid data (as pmol/nmol Pi).
2. Total protein content (in mg), reported as “pmol/mg protein.” Protein concentration can be determined by BCA assay using Pierce BCA protein assay kit (Thermo Fisher Scientific) and BSA (Bovine serum albumin) as standard [21].
3. Cell count (O.D. cells) reported as “pmol/OD cells.”
4. Lipid dry weight (in mg), reported as “pmol/mg lipid dry weight.”

It is recommended to establish a set normalization parameter and continue the usage of the same across all studies. This will allow an efficient comparison of different datasets from various follow-up studies (*see* Note 18).

We have routinely used the lipid data sets for fungi that are normalized to the dry weight of extracted lipids [13, 16, 35–38]. Compared to the other normalization parameters, we found that normalization to lipid dry weight allowed us to draw accurate conclusions and comparison of various lipid datasets across different fungal studies [13, 16, 35–38].

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18. Normalization with a different parameter may result in different numbers in terms of quantification. However, the trends in the data sets may be retained. The data can also be reported as the fold change or relative abundance (mol%) for the comparison of trends [23].

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Fig. 1. Schematics of ion detection in ESI-MS/MS setup. HESI probe ionizes the sample. Q1 and Q3 act as mass analyzers and collision induced dissociation of ion occurs at q2

Fig. 2. Development of SRM transitions for detection of sphingolipids by HPLC-ESI-MS/MS. (a) Structure of GlcCer(d18:2/16:0(2OH)). (b) Q1 spectrum shows GlcCer(d18:2/16:0(2OH)) as $[M+H]^+$ ion with m/z 714.61. (c) The breakdown curve of m/z 714.61 shows three most abundant ions: m/z 262.3 at CE 47 V, m/z 696.9 at CE 19 V, and m/z 534.8 at CE 26 V. (d) Product ion analysis of the monoisotopic peak m/z 714.61. Inset depicts the representative chromatogram for detection of GlcCer(d18:2/16:0(2OH)) using the SRM transitions: 714.6 $\rightarrow$ 262.3 or 714.6 $\rightarrow$ 696.9

Fig. 3.

Detection of sphingolipid mixture using HPLC-ESI-MS/MS by MRM approach. Separation and detection of Cer/Sph Mixture I (LM6002, Avanti Polar Lipids) is shown. The SRM mass transitions used for detection, CE (in V) and R.T. (in min) are depicted. (i) Sph(d17:1), (ii) Sph(d17:0), (iii) S1P(d17:1), (iv) dhS1P(d17:0), (v) LacCer (d18:1/12:0), (vi) GlcCer(d18:1/12:0), (vii) Cer(d18:1/12:0), (viii) SM(d18:1/12:0), (ix) Cer1P(d18:1/12:0), (x) Cer(d18:1/25:0)

Fig. 4.

Representative calibration curve for the quantification of Cer(d18:1/18:0). The *y*-axis shows the peak area ratio of analyte to Cer(d18:1/17:0) IS. The *x*-axis shows the amount ratios of analyte to Cer(d18:1/17:0) IS. The equation and regression coefficient for the curve is also shown

Table 1

Optimized parameters for detection of various sphingolipid classes in a fungal lipid extract

Sphingolipid class	Product ion	Fragment	Fatty acyls	CE (V)
DHS(d18:0)	<i>m/z</i> 284	<i>Sphingoid base</i> —1H ₂ O	–	12
	<i>m/z</i> 266	<i>Sphingoid base</i> —2H ₂ O	–	12
SPH(d18:1)	<i>m/z</i> 282	<i>Sphingoid base</i> —1H ₂ O	–	12
	<i>m/z</i> 264	<i>Sphingoid base</i> —2H ₂ O	–	12
PHS(t18:0)	<i>m/z</i> 300	<i>Sphingoid base</i> —1H ₂ O	–	12
	<i>m/z</i> 282	<i>Sphingoid base</i> —2H ₂ O	–	12
DHS1P(d18:0)	<i>m/z</i> 284	<i>Parent ion</i> —phosphate moiety—1H ₂ O	–	12
S1P(d18:1)	<i>m/z</i> 264	<i>Parent ion</i> —phosphate moiety—2H ₂ O	–	12
PHS1P(t18:0)	<i>m/z</i> 282	<i>Parent ion</i> —phosphate moiety—2H ₂ O	–	19
Cer(d18:1)	<i>m/z</i> 264	<i>Parent ion</i> — <i>Sphingoid base</i> —2H ₂ O	12–26	10–25
αOH-Cer(d18:1)	<i>m/z</i> 264	<i>Parent ion</i> — <i>Sphingoid base</i> —2H ₂ O	12–26	10–25
dhCer(d18:0)	<i>m/z</i> 266	<i>Parent ion</i> — <i>Sphingoid base</i> —2H ₂ O	12–26	12–25
PCer(t18:0)	<i>m/z</i> 282	<i>Parent ion</i> — <i>Sphingoid base</i> —2H ₂ O	14–28	19–28
αOH-PCer(t18:0)	<i>m/z</i> 282	<i>Parent ion</i> — <i>Sphingoid base</i> —2H ₂ O	14–28	30
IPC(t18:0)	<i>m/z</i> 282	<i>Parent ion</i> — <i>Sphingoid base</i> —2H ₂ O	16–28	30–45
IPC(t20:0)	<i>m/z</i> 310	<i>Parent ion</i> — <i>Sphingoid base</i> —2H ₂ O	16–28	30–45
GlcCer(d18:1)	<i>m/z</i> 264	<i>Parent ion</i> — <i>Sphingoid base</i> —2H ₂ O	18–26	45
GlcCer(d18:2)	<i>m/z</i> 262	<i>Parent ion</i> — <i>Sphingoid base</i> —2H ₂ O	18–26	45
GlcCer(d19:2)	<i>m/z</i> 276	<i>Parent ion</i> — <i>Sphingoid base</i> —2H ₂ O	18–26	45