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Improved Quantitative Approach for Monitorization of Gangliosides Structural Diversity in Fungal Cell Factories by LC-MS/MS

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Abstract: Gangliosides are glycolipids occurring in higher animals, with a sphingoid core in the form of ceramide, bound to a glycan moiety including several units of sialic acid. Gangliosides are involved in important (patho)-physiological processes as components of cell membranes in humans, which has led to intensive study and interest in production strategies. Their structural variability depends on the combination of a sphingoid base, a fatty acyl chain, and an attached oligosaccharide. The combinatorial diversity differs and grows exponentially in synthetic biology approaches, e.g., use of microbial cell factories. A specific analytical platform accounting for this complexity is not available to date. However, quantification of the intermediates of the whole biosynthetic route is needed to boost projects on biotechnological ganglioside production. In this study, a fast high-throughput quantitative LC-MS/MS methodology was developed to cover analysis of gangliosides, with a wider structural perspective adapted to fungal organisms. This work was achieved using metabolically engineered strains that further allowed to test detection in biological complex matrixes. Ganglioside backbones—hitherto uncharacterized—with the five most common fungal sphingoid bases and both simple and hydroxylated fatty acids were subjected to characterization. The addition of glycans to the polar head was also successfully monitored with up to 4 units—corresponding to GD3 which bears two sialic acid units and furthermore represents the common precursor for the whole ganglio-series. This platform represents an improved methodology to study the biochemical diversity associated to gangliosides for natural and metabolically engineered biosynthetic pathways.

Keywords: LC; MS/MS; mass spectrometry; liquid chromatography; glycosphingolipids; ceramides; gangliosides; GM3; GD3

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1. Introduction

Ganglio-series sphingolipids, or gangliosides, are glycosphingolipids occurring in higher animals, consisting of a ceramide core and an anchored glycan moiety containing neuraminic acid. Gangliosides accumulate predominantly in central and peripheral nervous system—up to 10% of total lipid content, but they occur in all tissues in vertebrate animal cells [1]. Gangliosides have not been observed in lower animals and other kingdoms such as plants, fungi, or bacteria. They accumulate in the outer leaflet of the cell membrane, whereby they form part of the glycocalyx, constituting lipid rafts and microdomains of functional relevance. They operate as antigens/receptors of specific molecules, mediators in cell-to-cell interactions, and modulators of the charge density at the membrane surface. Accordingly, they show physiological and patho-physiological roles in mammalian cells [2] and ganglioside metabolism is implicated in human health and disease [3]. Recent studies pinpoint diagnostic and therapeutic properties in neurodegenerative diseases [4,5], cancer [6], or autoimmune diseases [7]. Monosialogangliosides and disialogangliosides with

short saccharide forms—such as GM3 and GD3 herein studied—have shown beneficial effects in infant neurologic development [8,9].

Obtention in laboratory of isolated gangliosides has been essential for applied and fundamental studies. Gangliosides have also attracted interest as high-added value products due to their biological properties [10]. Synthetic methodologies are mainly based on chemical procedures [11], semisynthetic assembly [12], or more recently, complete enzymatic in vitro approaches [13,14]. However, scarce work has been performed to produce gangliosides via bioprocessing in the classical microbial biotechnological workhorses. It seems plausible, through synthetic biology, to engineer metabolism of microorganisms towards production of gangliosides, e.g., in eukaryotic fungal species. This work should account for the particular structural diversity associated to the variability of the sphingoid base, fatty acyl chain and attached oligosaccharide [15]. Remarkably, the dissimilar and more extensive sphingolipid structural basis associated to organisms such as fungi is well known [16]. An adapted analytical quantitative platform becomes of the utmost importance when dealing with these potential microbial producers. In fact, this may be one of the main factors that hampers, so far, biotechnological production of gangliosides. An efficient analysis for the putative biosynthetic pathway must consider several structural details.

The common glycan moiety pattern for mammalian gangliosides is based on a binding lactose upon the hydroxy group in carbon number one of the sphingoid base, with one or more sialic acid units linked to this core. A variable number of sialic acids and other neutral monosaccharides including glucose, galactose, and N-acetyl-galactose are sequentially added in the most complex forms [15]. In this context, monosialogangliosides GM3 and disialogangliosides GD3 are especially relevant. These species can be found ubiquitously but they predominate in extra-neural tissues. Enriched quantities of GM3 and GD3 are present in tissues as digestive system, kidney, heart, or liver—in the range of nanomols per milligram of dry weight [17], but most importantly, they represent the precursors of most of gangliosides structural diversity [15]. Regarding the sphingoid backbone, fatty acyl chains bound to the sphingoid base vary in length depending on the organism—from 16 to up to 26 carbons, and unsaturations—one or none most commonly [18]. The sphingoid base is most often C18-sphingosine (d18:1), as corresponds to higher animals sphingolipid biosynthesis [15]. Nonetheless, recent studies reveal the importance of gangliosides based on non-canonical sphingoid bases such as 3-ketosphinganine [19] or eicosphingosine [20]. In the context of synthetic biology, an emphasis on revisiting structural diversity is needed. In the case of fungal microorganisms, other sphingoid bases such as sphinganine (d18:0), 4-hydroxysphinganine (t18:0), sphingadienine (d18:2), or 4-methylsphingadienine (dm18:2) must be considered to evaluate production [16]. Moreover, the existence of alpha-hydroxylated fatty acids must not be overlooked [21].

Available analytical methodologies to monitor ganglioside profiles mainly rely on mass spectrometry hyphenated to liquid chromatography. They focus on the most common structures, namely sphingosine-containing gangliosides [17,22,23]. They also encompass analysis of the most complex forms including isomeric resolution [24,25]. With respect to glycosphingolipids in fungal species, some works have already elucidated the glycosphingolipid structural base [26] and help to propose plausible biosynthetic options. An approach that considers the analysis of putative gangliosides species in non-mammalian engineered organisms remains unexplored to date.

In this work, we expanded the analytical coverage of the structural diversity of gangliosides with a focus on the sphingoid base and fatty acyl variability in fungal organisms. We designed the analytical platform to offer a comprehensive and technically transferable analysis. Data from LC, MS and MS/MS have been gathered to rationalize the detection of ganglioside species based on non-canonical bases and chemical modifications such as extension of the aliphatic chains, hydroxylation, and degree of saturation. This was accomplished making use of metabolically engineered fungal strains (unpublished results) to generate an adequate expanded structural diversity and demonstrate detection in biological complex matrixes. The fast high-throughput quantitative LC-MS/MS methodology developed pro-

vides quantitative values of fungal glycosphingolipids—hitherto uncharacterized—with the five most common sphingoid bases and both simple and hydroxylated fatty acids. Sequential addition of monosaccharides to the bare ceramide was successfully analyzed — up to 4 units corresponding to GD3, which bears two sialic acid monomers. This monitorization platform represents an improved quantitative methodology to study the chemical diversity associated to the ganglio-series glycosphingolipids for natural and metabolically engineered biosynthetic pathways.

2. Materials and Methods

2.1. Chemicals

Internal standards D-erythro-sphingosine (C17 base), N-heptadecanoyl-D-erythro-sphingosine, N-(2′-(R)-hydroxyheptadecanoyl)-D-erythro-sphingosine, D-glucosyl-b-1,1-N-heptadecanoyl-D-erythro-sphingosine, D-lactosyl-b-1,1′-N-Heptadecanoyl-D-erythro-Sphingosine, and deuterated C18:0 GM3 Ganglioside-d5 from Avanti® Polar Lipids, Inc. (Alabaster, AL, USA) were used. HPLC grade chloroform, methanol, acetonitrile, formic acid, and ammonium formate were purchased from Fisher Scientific Ltd. (Leicestershire, UK). Milli-Q water was used for all experiments, filtered through a 0.22 µm filter and obtained using a Milli-Q Millipore system (Synergy®, Millipore Corporation, Billerica, MA, USA).

2.2. Standard Preparations

Six commercial standards of high purity (<99%) were dissolved at 200 ppb concentration: D-erythro-sphingosine (C17 base), N-heptadecanoyl-D-erythro-sphingosine, N-(2′-(R)-hydroxyheptadecanoyl)-D-erythro-sphingosine, D-glucosyl-b-1,1-N-heptadecanoyl-D-erythro-sphingosine, and D-lactosyl-b-1,1′-N-Heptadecanoyl-D-erythro-Sphingosine and deuterated C18:0 GM3 Ganglioside-d5, hereafter referred as Sph(17:1), Cer(d18:1/17:0). Cer(d18:1/h17:0), HexCer(d18:1/17:0), Hex2Cer(d18:1/17:0) and GM3(d18:0/18:0-d5), respectively. They were used without further purification in methanol:choloroform:water (4:1:1). The solution was kept at −20 °C until use. For method development, this mixture was injected for analysis. The standards mixture was also used as extraction solvent for biological samples, in order to allow the use of internal standards in known concentrations.

2.3. Extraction of Ceramides and Gangliosides in Biological Samples

Approximately 10 milligram of lyophilized mycelia was suspended in 1 mL of extraction solvant in 2 mL plastic tubes containing 1.4 mm ceramic beads (Precellys—Bertin Techologies, Montigny-le-bretonneux, France). Lyophilizate weight was annotated to correct absolute quantitative values. A benchtop Minilys homogenizer (Bertin Technologies) was used for mycelia lysis with three rounds of 30 s at 4000 rpm agitation. Samples were kept in ice during the process and between disruption rounds. After lysis, samples were centrifuged at 17,000 rpm for two minutes in a benchtop centrifuge. Supernatant was collected in LC-MS vials for analysis.

2.4. LC-MS/MS Analysis of Ceramides and Glycoceramides

Sample separation for ceramides and glycoceramides was performed using an Ascentis Express C8 Solid Core column (30 mm × 2.1 mm, 2.7 µm, 90 Å) from Sigma-Aldrich (St. Louis, MO, USA) in a Vanquish Flex UHPLC with flow rate set at 300 µL/min and column compartment temperature 30 °C. Binary gradient used was 70–100% B in 10 min, 10% B for 5 min, 1000–70% B in 1 min, and re-equilibration at 70% B for 4 min. The solvents used formic acid 0.1% (A) and acetonitrile (B).

The Orbitrap Q-Exactive Focus (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer was operated with electrospray (ESI) voltage −2.75 kV/+3.5 kV, capillary temperature, 250 °C; sheath gas flow 40 units, auxiliary gas flow 10 units, spare gas 1.5 units, probe heater 300 °C). MS analysis was acquired in both polarities with resolving power 70,000 (full width half maximum), m/z = 250–1250 using an automatic gain control (AGC) target of 10^6. Targeted analysis was performed through parallel reaction monitoring

(PRM) with an inclusion list for the species monitored and a m/z window of 1 unit. Two different runs to split the number of transitions for an efficient detection—methods otherwise identical—were used (a) for ceramides and glucosyl-ceramides and (b) their lactosyl ceramides counterparts. MS/MS spectra were obtained using higher-energy collisional dissociation (HCD) fragmentation (stepwise 20, 25, and 30% normalized collision energy), AGC target of 10^6 and maximum injection time of 75 ms at a resolution of 17,500. Data acquisition was carried out using Xcalibur data system (V3.3, Thermo Fisher Scientific, Waltham, MA, USA). 10 µL of standards solution or biological samples were injected per run.

2.5. LC-MS/MS Analysis of Monosialo and Disialo Gangliosides

Sample separation for monosialo- and disioalogangliosides was performed using an Ascentis Express C8 Solid Core column (30 mm × 2.1 mm, 2.7 µm, 90 Å) from Sigma-Aldrich (St. Louis, MO, USA) in a Vanquish Flex UHPLC with flow rate set at 300µL/min and column compartment temperature 30 °C. Binary gradient used was 50–100% B in 10 min, 100% B for 5 min, 100–50% B in 1 min and re-equilibration for 5 min at 50%B. The solvents used were 0.1% formic acid (A) and acetonitrile:methanol:water (79:19:2; $v/v/v$), 20 mM ammonium formate, 20 mM formic acid (B).

The Orbitrap Q-Exactive Focus mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was operated with electrospray voltage −2.75 kV/+3.5 kV, capillary temperature, 250 °C; sheath gas flow 40 units, auxiliary gas flow 10 units, spare gas 1.5 units, probe heater 300 °C). MS analysis was acquired in both polarities with resolving power 70,000 (full width half maximum), m/z = 250–1250 using an automatic gain control (AGC) target of 10^6. Targeted analysis was performed through parallel reaction monitoring (PRM) with an inclusion list for the species monitored and a m/z window of 1 unit. MS/MS spectra were obtained using higher-energy collisional dissociation (HCD) fragmentation (stepwise 20, 25, and 30% normalized collision energy), AGC target of 10^5, and maximum injection time of 50 ms at a resolution of 17,500. Data acquisition was carried out using the Xcalibur data system (V3.3, Thermo Fisher Scientific, Waltham, MA, USA). A measure of 10 µL of standards solution or biological samples was injected per run.

2.6. Quantitative LC-MS/MS Analysis

Quantification was performed by integrating the area under the curve for the corresponding MS/MS fragment ions chromatographic peak chosen for each species, using Quan Browser software (Thermo Fisher Scientific, Waltham, MA, USA). Signals were normalized versus the associated internal standard: Sph(17:1) for free bases, Cer(d18:1/17:0) for ceramides. Cer(d18:1/h17:0) for hydroxylated fatty acid ceramides, HexCer(d18:1/17:0) for glucosyl ceramides, Hex2Cer(d18:1/17:0) for lactosyl ceramides and GM3(d18:0/18:0-d5) for monosialo- and disialogangliosides. The nanograms of each species was extrapolated from the nanograms of internal standards injected (2 nanograms), and further normalized versus the milligrams of mycelia used for extraction.

3. Results

3.1. Optimizatin of MS Ionizatin Parameters

A preliminary analysis based on ESI-MS spectra was performed to evaluate ionization efficiency in both polarities, including the presence of adducts. The corresponding standards mimicking ceramides, glycoceramides—with one or two hexoses, and the free sphingoid bases, showed $[M + H]^+$ ions that clearly outperform other ionization forms (Supplementary Figure S1a). Other ionization forms comprising salt adducts (e.g., sodium or potassium adducts) were not abundant or induced signal suppression. Analysis in negative voltage was rendered useless due to very low sensitivity for these species. In the case of gangliosides, both $[M + H]^+$ and $[M - H]^-$ ions of the corresponding standard yielded an intense comparable signal that dominates the spectrum in each polarity mode

(Supplementary Figure S1b). Eventually, positive mode was selected for the whole analysis, also due to optimization on MS/MS transitions as detailed in the next sections.

3.2. LC-MS/MS Method Development for Ceramides and Glycoceramides

According to the efficient formation of [M + H]+ ions for ceramides and glycoceramides, MS/MS fragmentation produces an intense ion based on the protonated sphingoid base. The doubly dehydrated sphingoid base ion is the most intense ion for all structures, except in the case of the free sphingoid base where it competes with simple dehydration (Supplementary Figure S2). Therefore, $[M + H]^+$ ion based on the doubly dehydrated sphingoid base was chosen for MS/MS transitions. It was optimal to completely elucidate the structures, since it allowed assignation of the sphingoid base and the fatty acid bound, with increased sensitivity for all species in the method (Table 1). To note, the presence of a hydroxylated fatty acid leads to an intense fragment cation associated to water loss from the $[M + H]^+$ precursor ion (Supplementary Figure S2). This may slightly hamper detection by decreasing sensitivity through competence with the production of sphingoid base fragments. In return, this trait can be considered to confirm this kind of species in a MS/MS scan. In the case of glycosylated ceramides, the sequential neutral loss of the hexose units could be observed providing structural information (Supplementary Figure S2).

Table 1. Most abundant observed MS/MS fragments for the five internal standards used in the method development of ceramides and glycoceramides analysis.

Internal Standard	MS/MS Fragment Ions
Sph (d18:1/17:0)	- NL of 2xH_2O, $[M + H]^+$ = 250.252 (equivalent to 264.268 in C18 sphingoid base) - NL of H_2O, $[M + H]^+$ = 268.263
Cer(d18:1/17:0)	- NL of H_2O, $[M + H]^+$ = 534.523 - NL of fatty acyl chain and H_2O $[M + H]^+$ = 264.268
Cer(d18:1/h17:0)	- NL of water, $[M + H]^+$ = 550.518 - NL of 2xH_2O, $[M + H]^+$ = 532.508 - NL of fatty acyl chain and water: $[M + H]^+$ = 264.268
HexCer(d18:1/17:0)	- NL of water, $[M + H]^+$ = 696.577 - NL of 1 hexose, $[M + H]^+$ = 534.524 - NL of fatty acyl chain, hexose, and water: $[M + H]^+$ = 264.268
Hex2Cer(d18:1/17:0)	- NL of water, $[M + H]^+$ = 858.629 - NL of 1 hexose, $[M + H]^+$ = 696.576 - NL of 2 hexoses, $[M + H]^+$ = 534.523 - NL of fatty acyl chain, 2 hexoses, and H_2O: $[M + H]^+$ = 264.268

The most abundant fragment ion for each species is underlined. Annotation is based on the corresponding neutral loss (NL) of different parts of the structure. Experimental m/z values in the MS/MS spectrum are included (Supplementary Figure S2), and match theoretical values with precision within 10 ppm.

Some in-source fragmentation events were found as the main issue regarding method development. These undesired interferences are observed for certain species and can mislead assignation. They are neutral losses from part of the structure in the process of ionization, forming structures that behave as a new precursor that represents a confounding equivalent to other species. Specifically, the water molecule in hydroxylated fatty acids or monosaccharides in glucosyl and lactosyl ceramides are labile and readily removed in the ionization process. Therefore, hydroxylated fatty acids can be confounded with their counterpart without hydroxylation but one more unsaturation. It is also the case of glycosylated species and their non-glycosylated counterparts with one more unsaturation. However, the method developed allowed discrimination of interferences by avoiding coelution in the chromatogram (Supplementary Figure S3).

Free sphingoid bases, ceramides, and glycoceramides with up to 2 neutral monosaccharides were successfully distinguished by LC separation in a unique gradient, with elution order based on their hydrophobicity (Figure 1). Hence, lactosyl-ceramides eluted in the

shortest retention times, followed by glucosyl ceramides and non-glycosylated ceramides. Sphingoid bases eluted in the beginning of the run due to their high hydrophilicity but showed a well-defined peak shape that allowed quantification. The inclusion of a hydroxy group in the fatty acyl chain shortens retention times versus the non-hydroxylated fatty acids.

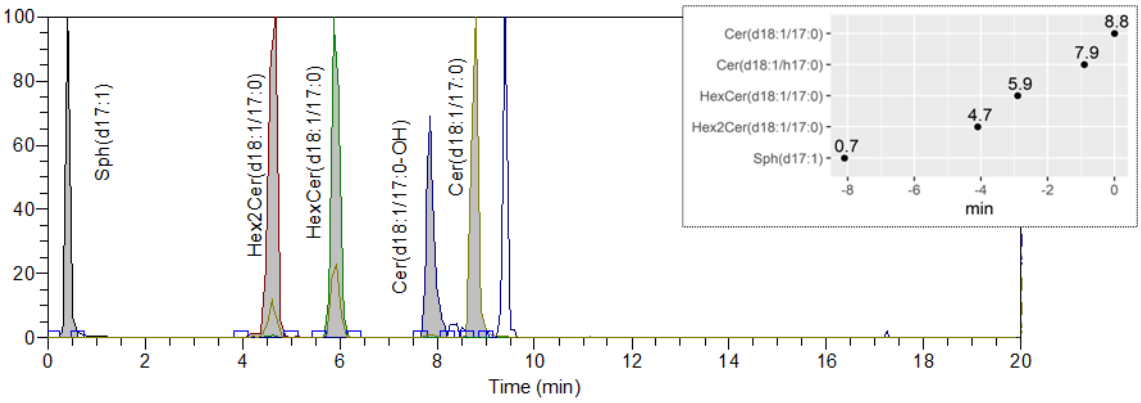

Figure 1. Annotated LC-MS/MS chromatogram for internal standards used for the method development of ceramides and glycoceramides analysis. The inset image presents retention time variations due to the structural differences, i.e., sequential addition of hexoses and hydroxylation of the fatty acyl chain, as well as the free sphingoid base retention time.

3.3. LC-MS/MS Method Development for Gangliosides

Seeking specific MS/MS transitions to unequivocally detect ganglioside structures, we opted for a positive polarity for method development. Even if negative ions provide a good intensity, fragment cations became of great advantage for structural discrimination. Product ions in the negative mode MS/MS spectrum mainly corresponded to the uninformative dehydrated sialic acid anion (Supplementary Figure S4). In the positive MS/MS, the cation corresponding to the sialic acid was observed but disregarded for the same reason. The doubly dehydrated sphingoid base remained the most prominent and structurally relevant fragment cation (Figure 2) and accordingly chosen for MS/MS transitions. Nonetheless, the MS/MS spectrum further provided structurally useful transitions such as the sequential neutral loss of the monosaccharides. (Figure 2).

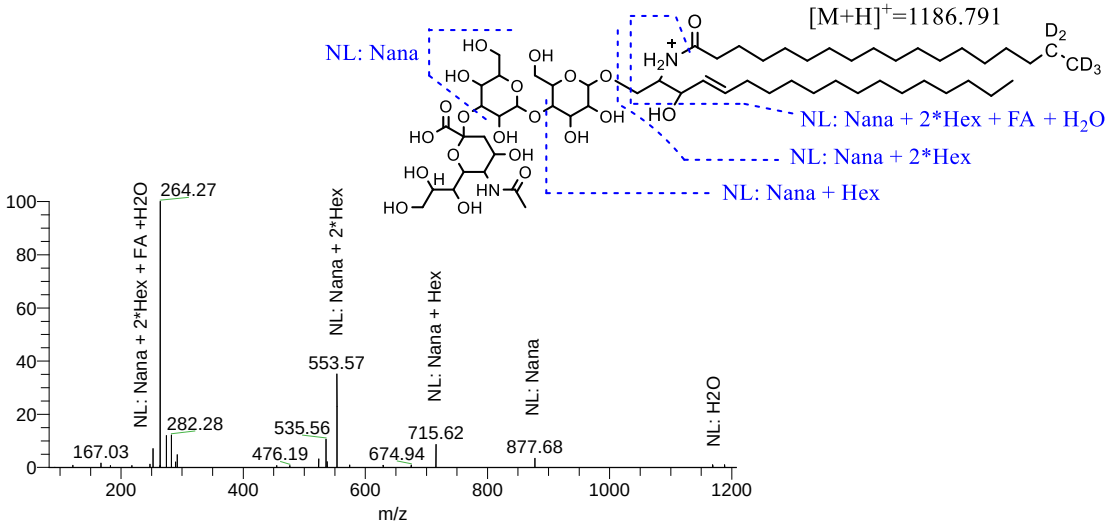

Figure 2. Structure and fragmentation of the deuterated GM3(d18:1/18:0-d5) standard. Schematic representation of the molecular structure and cleavage sites. Annotation is based on the corresponding neutral losses (NL) of different parts of the structure generating fragment ions.

From the chromatographic point of view, the method used for ceramides and glycoceramides was incompatible with detection of gangliosides. A new solvent system with elevated ionic strength—ammonium formate and formic acid in concentration 20 mM—was determined as best performing. After adaptation of the gradient, good peak shapes were obtained for the ganglioside standard. In this new method, the GM3 standard showed the shortest retention regarding ceramides and glycosylated ceramide standards (Figure 3), as expected due to the increased hydrophilicity upon addition of the sialic acid residue.

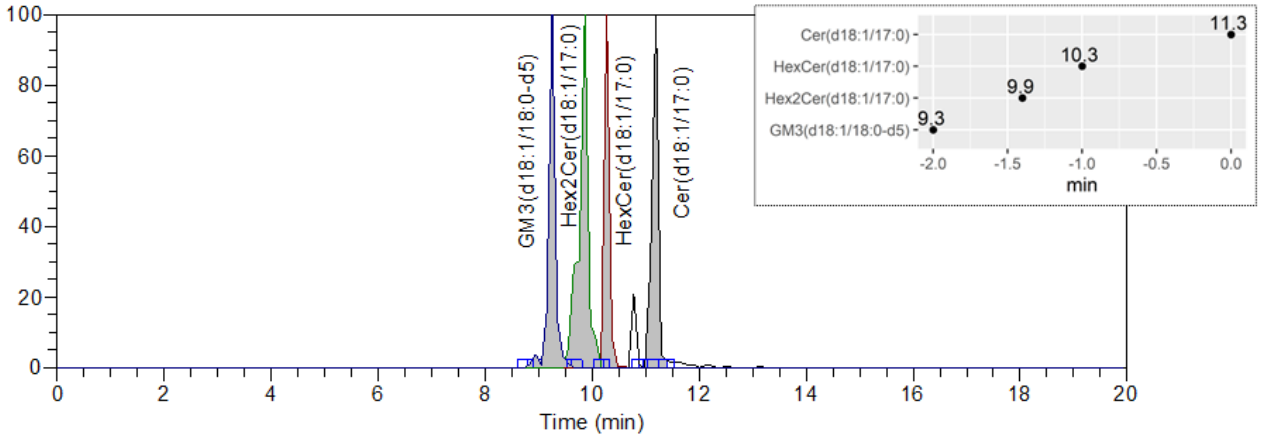

Figure 3. Annotated LC-MS/MS chromatogram for deuterated internal standard GM3(d18:17/18:0-d5) in the method developed for ganglioside analysis, compared with the other standards. The inset image presents variation due to the structural differences regarding the sequential addition of monosaccharides to the glycan moiety, one or two hexoses, and the subsequent sialic acid.

3.4. Expanded Characterization of Species upon Biological Samples

Following method development with standards, detection of species with a wider variety of sphingoid bases was undertaken by utilizing extracts from metabolically engineered organism, that allowed analysis of structures with the bases d18:1, d18:0, d18:2, dm18:2, and t18:0. The monitorization upon detection of the sphingoid base product ion, taking into account the chemical modification, allowed unequivocal assignation of the species. Chromatographic methods developed were efficient for separation of the five corresponding sphingoid backbones (Figure 4b), in both simple ceramides and their glycosylated equivalents. Considering ceramide structures with the same fatty acyl chain, addition of hydroxy groups or unsaturations shortened retention times, while saturation or addition of a methyl group delays elution (Figure 4a).

The increased number in structures generated by the variety in sphingoid bases implied that, even when chromatographic optimization was maximized to the best of our ability/setup capabilities, some of the species could still not be separated. This conveyed detection to the positive mode and more specifically to the use of sphingoid base fragments, whereas the use of sialic acid fragment anions or other potential transitions did not allow discrimination. Such is the case of GM3(d18:1/18:0) and GM3(dm18:2/h16:0)—both isobaric species in terms of m/z value and eluting at similar retention times, that would be indistinguishable if information on the sphingoid base is not available (Supplementary Figure S5). This handicap affects this and other pairs of species, but it was overcome in all cases throughout the developed method either by chromatographic separation or MS/MS information.

Disialogangliosides, namely GD3, were also characterized thanks to their presence in biological samples of the metabolically engineered organism. Same ionization forms and MS/MS transitions relying on the sphingoid base were proven efficient. Other fragments based on the sequential loss of monosaccharides and the protonated sialic acid helped to ascertain structures. The chromatographic behavior observed was a shift, of the whole group of GD3 species, to shorter retention times versus the monosialo- counterparts, due to

the addition of a second hydrophilic sialic acid residue (Figure 5). Within the GD3 group, retention times depend on traits previously explained.

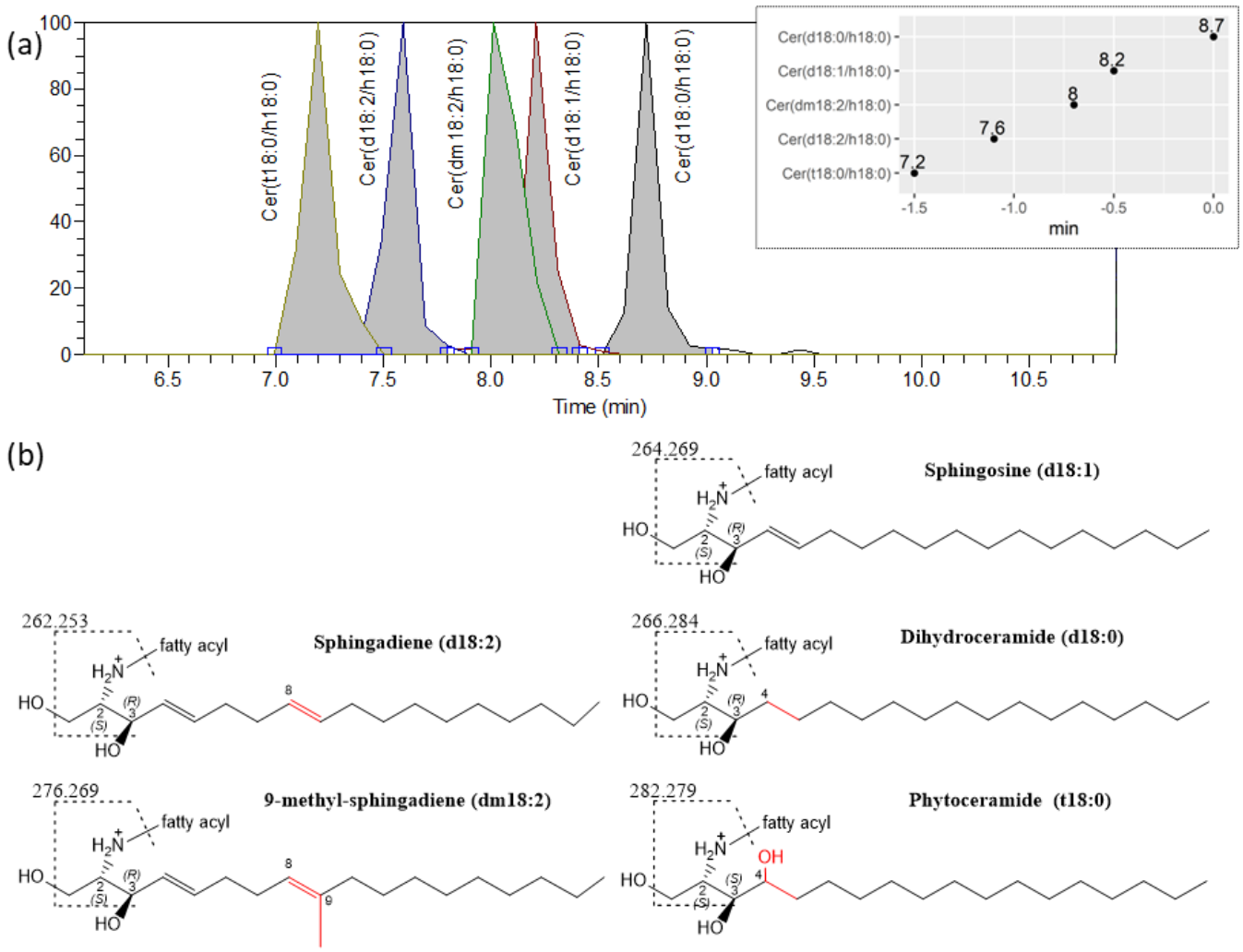

Figure 4. (**a**) Zoomed LC-MS/MS chromatogram for ceramide species containing five different sphingoid bases found in a fungal biological matrix (d18:1, d18:2, d18:0, dm18:2, and t18:0). All ceramide species plotted contain the same fatty acid, allowing variation in retention time due to the sphingoid base to be observed (inset image). (**b**) Structure and MS/MS fragments used for each of the five structures with different sphingoid bases. Mass of the fragment indicated beside the discontinuous line, which represents the most abundant MS/MS fragmentation cleavage.

3.5. Method Validation in Biological Samples

Our method was successfully tested in biological matrixes by analyzing extracts from fungal mycelia. Both wild-type and a mutant organism were analyzed, the latter potentially containing all of the repertory of species targeted in this work. Based on the known naturally occurring array of fatty acids in the wild-type organism—regarding length and degree of unsaturation, all combinations for the corresponding ceramide, glycosylated ceramide, and ganglioside structures were considered. A white list containing more than one hundred MS/MS transitions was generated. We observed that methods in our instrumental setup with more than 50 MS/MS transitions to be monitored led to poor chromatographic power and efficient detection was restored by adjusting number of transitions in unfolded methods.

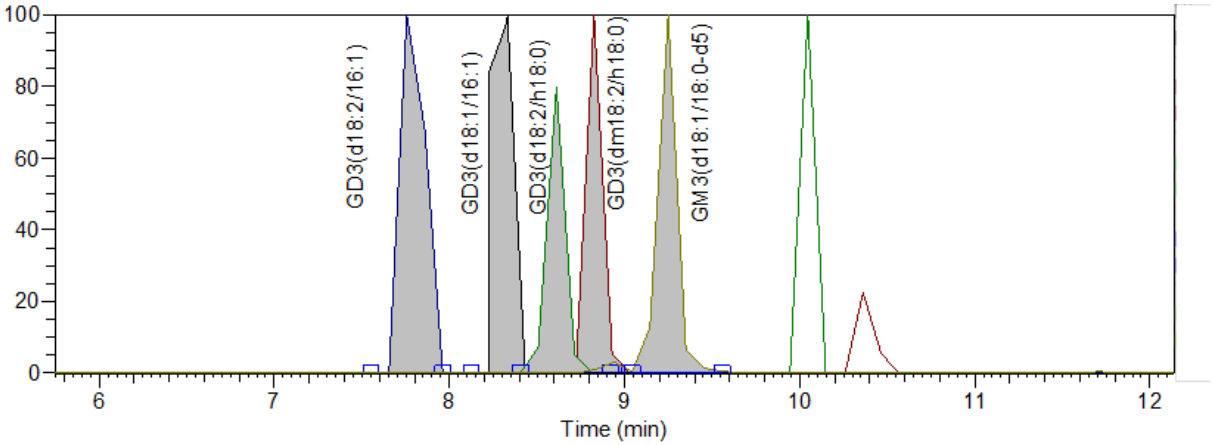

Figure 5. LC-MS/MS chromatogram for disialoganglioside GD3 species containing three different sphingoid bases (d18:1, d18:2, dm18:2) and both hydroxylated and non-hydroxylated fatty acids. GM3 internal standard is included as elution time reference. Comparison of retention times is not straightforward due to the different fatty acids attached, but GD3 species elute as a group before the GM3 internal standard.

Biological replicates showed reproducibility in quantification—extrapolated from the use of internal standards—and retention times (Supplementary Figure S6). Methods developed for ceramides and glycoceramides monitorization provided quantitative values for 42 species in the wild-type organism, including the five sphingoid types and occasionally alpha-hydroxylation in the fatty acyl chain. Concentrations observed ranged between values close to the observed limit of detection—around 1 nanogram of the species per milligram of mycelia, and a few hundreds of nanograms per milligram of mycelia. Species with two hexoses and the corresponding gangliosides were not observed in the wild type, serving therefore as negative control. Analysis of the mutant strain provided quantitative values in a number of species, with a greater structural variety as explained in the previous sections. Hence, methods developed for lactosyl ceramides and GM3 and GD3 gangliosides also yielded detection of species in this case. However, only structures with d18:1, d18:2, and dm18:2 sphingoid backbone were detected and quantified within these groups (unpublished results). These structures predominantly included C18 saturated alpha-hydroxylated acids, in comparison to the alternative unmodified fatty acids, chain lengths, and number of double bonds, present to a lesser extent up to absolute quantification.

4. Discussion

Gangliosides are lipids that form part of membranes in higher animals and own important functionalities rather than just structural roles. They are recognized as mediators of physiological and pathophysiological processes in humans. We believe that production of well-defined ganglioside structures can be of interest for fundamental and applied research. To date, most of the synthetic processes developed are based merely on extraction or in vitro preparation. However, current state-of-the-art in synthetic biology suggests it must be possible to produce these metabolites in microbial cell factories. The most plausible option seems to be, given current knowledge on gangliosides synthesis and natural occurrence, the use of well-established eukaryotic biotechnological chassis. Hence, fungi such as *Pichia pastoris*, *Saccharomyces cerevisiae*, *Eremothecium gossypii* or *Yarrowia lipolytica* ought to become important key players regarding ganglioside production. From a biosynthetic point of view, in such potential microbial factories, it is essential to consider both the heterologous design of the characteristic glycan moiety but also the available fungal structural basis as starting point to build up gangliosides.

Nevertheless, a structurally precise and quantitative method to facilitate this work must be at hand as a prerequisite. High-throughput sensitive and fine structural information provided by LC-MS/MS technique can meet this challenge. In this work we

considered for analysis the five most important sphingoid bases that can be found in this context, namely sphingosine, sphinganine, 4-hydroxy-sphinganine, 4,8-sphingadienine and 9-methyl-4,8-sphingadienine [16]. We also considered the plausibility of fatty acid hydroxylation in alpha position. Accordingly, combination of fatty acids and sphingoid bases leads to exponential increment of the number of structures. Ambiguities for identification, as highlighted in the results section, must be overcome. Even when high resolution MS helped in this work—a few parts per million of mass deviation and resolution of up to 70,000—methods developed are aimed to allow the platform to be used in middle and low-end MS instruments. For this purpose, chromatographic separation and the selection of structurally relevant fragment ions for MS/MS were carefully chosen. All MS/MS transitions were established relying on sphingoid-base related fragments that allow a better structural elucidation. Hence, positive polarity was required for monitorization, including gangliosides—traditionally analyzed as anions [17,22–25]. Efficient ionization in positive polarity is due to proton addition to the secondary amine in the sphingoid bases. However, gangliosides also ionize intensely in negative mode due to the presence of sialic acid units, readily deprotonated. We found positive polarity is the best option, since sensitivity remains high and MS/MS analysis is crucially improved. To note, an Orbitrap HCD collision cell was employed and energy levels needed to be tuned to maximize fragment ion signals. For chromatography, reversed stationary phases, which have demonstrated good chromatographic performance for sphingolipids [17,22–25], were used for separation with some adaptations. For all free sphingoid bases, ceramides, glycoceramides, and gangliosides, a short C8 column was able to retain species and produce appropriate peak shapes within a 20 min run, that includes washing and equilibration. However, an unique chromatographic method to cover all species was not possible. Retention times heavily increased for the ganglioside internal standard when using the same method than for the rest of species. This is probably the reason why even less retentive phases have been previously used [23]. It was solved by using an elevated ionic strength buffering solvent, which most probably avoids secondary interactions via hydrogen bonds with the residual silanols in the stationary phase. This method for gangliosides could not be used, vice versa, for the rest of species, since it provided insufficient chromatographic separation.

We undertook method development on standards commercially available that can successfully represent all structural groups of the study in terms of mass spectrometry and chromatography behavior. Odd chain fatty acid structures containing heptadecanoic acid for ceramides—Cer(d18:1/17:0), and glycoceramides—HexCer(d18:1/17:0), Hex2Cer(d18:1/17:0); hydroxylated heptadecanoic acid for alpha-hydroxylated ceramides—Cer(d18:1/h17:0), an odd chain sphingoid base (d17:1) for free bases, and deuterated species in the case of gangliosides—pentadeuterated GM3 with sphingosine and octadecanoic acid GM3 (d18:1/18:0-d5), were selected. Their use allowed to face the main challenges in the development of a LC-MS/MS method and succeed in the obtention of a method that avoids interferences when combined with the choice of MS/MS transitions. Remarkably, they are all non-endogenous species for the biological cases of study, allowing eventual internal standard correction. Hence, they helped towards reliable quantification by compensating well-known technical caveats such as in-source fragmentation events, ionization efficiency and biological matrix ion suppression, providing good reproducibility. Given that ionization efficiency mainly depends on the polar head—that accommodates the charge—the selection of one internal standard for each structural polar head trait provides full correction. Furthermore, variation in fatty acyl chain length and unsaturation should still keep polar head-related species grouped in terms of elution times, therefore with similar matrix influence and ion suppression that can be corrected by the internal standards chosen. Accordingly, we consider that methods developed should be suitable for species with fatty acyl chains ranging within structural variety in fungal samples (from 14 to up to 26 carbons). In order to offer wider information and due to the lack of commercial standards, we employed genetically engineered fungal strains to expand the coverage of the analytical platform. The use of this platform allowed to separate and identify structures in a

subsequent step, with the most common fungal sphingoid bases and the possibility of fatty acid hydroxylation, regarding the biological matrix of study. Furthermore, we were able to characterize up to GM3 monosialogangliosides and GD3 disialogangliosides with these backbones for the first time. These are fundamental species since they represent the main precursors of all ganglio-series structural diversity [15]. The design of these strains is out of the scope of this article (unpublished results), while being crucial to provide an expanded set of structures for method development and allow demonstration of the capabilities of the method in biological complex matrixes.

5. Conclusions

In this study, an LC-MS/MS method was developed for absolute quantification of ceramides and gangliosides with a wide structural diversity and applied for detection in biological matrixes. Five different sphingoid bases were characterized as part of the backbone: (d18:1), (d18:0), (t18:0), (d18:2), and (dm18:2). Fatty acyl chains with 16, 18, and 20 carbons and none or one unsaturation were also characterized (namely 16:0, 16:1, 18:0, 18:1, 20:1), including alpha-hydroxylated saturated fatty acids (namely h16:0, h18:0). Glycosylated counterparts with glucose, lactose, and subsequent sialic acid units were finally characterized, specifically GM3 monosialogangliosides and GD3 disialogangliosides.

The setup was designed to perform with high sensitivity, high chromatographic selectivity, and high structural discrimination allowing quantification of a broad variety of relevant gangliosides and their precursors. This analytical platform will allow to assess biotechnological production of gangliosides in fungal microbial factories. Nonetheless, it may further help to improve LC and MS analysis of gangliosides and disclose new biological implications or utilities for this important type of glycosphingolipids, with a wider structural perspective.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9120432/s1, Supplementary Figure S1: (a) Zoomed region of the MS spectrum of the commercial standards used for method development in this work. The [M + H]+ is observed as the predominant ion without significant contribution of other adducts, that should be observed along the m/z range represented. (b). Zoomed region of the MS spectrum of GM3 standard in both positive—upper panel, and negative polarity-lower panel. Similar intensities are observed, according to the absolute signal scale used, to this end, along the y axis. Supplementary Figure S2: MS/MS spectrum in positive polarity of the five commercial standards used in method development for ceramides and glycoceramides. Schematic molecular structures, with fragmentation patterns, are depicted on the right and correlated with the masses annotated in the spectrum., matching theoretical values within a 10 ppm tolerance threshold. Supplementary Figure S3: LC-MS/MS chromatogram for the commercial standard Cer(d18:1/17:0). Correct signal appears at 8.8 min (shaded). The other ghost peaks belong to in-source fragmentation of other structures. Hex2Cer(d18:1/17:0) interferes in the chromatogram through in-source loss of the 2 hexoses of the glycan moiety (4.7 min). HexCer(d18:1/17:0) generates a ghost peak by release of its hexose. (5.9 min). Cer(d18:1/h17:0) easily decomposes by loss of a water molecule from the hydroxylated fatty acid, in such a way that the 2nd isotopic peak is present in the chromatogram of the non-hydroxylated counterpart. Supplementary Figure S4: MS/MS spectrum in negative polarity of the GM3(d18:1/18:0-d5) standard used in method development for ganglioside analysis. Mass annotated in the spectrum matches theoretical values, within 10 ppm tolerance, of the fragment ion consisting of the deprotonated sialic acid unit. Supplementary Figure S5: (a) LC-MS/MS chromatogram in positive polarity, based on the sphingoid base fragment ion, for the isobaric species GM3(d18:1/18:0) and GM3(d18:2/h16:0), that elute at similar retention times. (b) Chimeric MS/MS spectrum with information for both species, given that precursor ions have similar masses and retention times. Quantification is only possible when using MS/MS fragments that allow unequivocal identification, such as the annotated ions corresponding to the sphingoid base. Supplementary Figure S6: Quantitative LC-MS/MS analysis of ceramides (Cer) and glucosyl ceramides (HexCer) in a biological matrix extracted from the wild-type fungus *Eremothecium gossypii.* Quantitative values were extrapolated using internal standards for each group of molecules and calculated versus the initial amount of the mycelia of fungus obtained. A total of 42 species in the wild-type organism were detected, including

the five sphingoid types and occasionally alpha-hydroxylation in the fatty acyl chain. Concentrations observed ranged between values close to the observed limit of detection, around one nanogram of the species per milligram of mycelium, and a few hundreds of ng per mg of mycelium.

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