**The Sphingolipidome of the model organism *Caenorhabditis elegans***

Victoria Hänel1, Christian Pendleton1, Michael Witting1,2,\*

1 Research Unit Analytical BioGeoChemistry, Helmholtz Zentrum München, Ingolstädter Landstraße 1, 85674 Neuherberg

2 Chair of Analytical Food Chemistry, Technische Universität München, Maximus-von-Imhof-Forum 2, 85354 Freising

\* corresponding author: Dr. Michael Witting, michael.witting@helmholtz-muenchen.de

**Abstract**

Sphingolipids are important lipids and integral members of membranes, where they form small microdomains called lipid rafts. These rafts are enriched in cholesterol and sphingolipids, which influences biophysical properties. Interestingly, the membranes of the biomedical model organism *Caenorhabditis elegans* contain only low amounts of cholesterol. Sphingolipids in C. elegans are based on an unusual C17iso branched sphingoid base, which potentially compensates for the lack of cholesterol. In order to analyze and the sphingolipidome of C. elegans in more detail, we performed fractionation of lipid extracts and depletion of glycero- and glycerophospholipids together with in-depth analysis using UPLC-UHR-ToF-MS. In total we were able to detect 82 different sphingolipids from different classes, including several isomeric species.

**Abbreviations**

Dihydroceramides (DhCers)

Ceramides (Cers)

Dihydrosphingomyelins (DhSMs)

Sphingomyelins (SMs)

Glucosylceramides (GlcCers)

Hexosylceramides (HexCers)

Ultrahigh Performance Liquid Chromatography – Ultrahigh Resolution – Time of Flight – Mass Spectrometry (UPLC-UHR-ToF-MS)

Methanol (MeOH)

Acetonitrile (ACN)

2-Propanol (iPrOH)

Chloroform (CHCl3)

Monomethyl branched chain fatty acid (mmBCFA)

**Introduction**

Sphingolipids are important lipids and integral members of membranes, where they are included in so called lipid rafts, small microdomains within the membrane [1, 2]. Lipid rafts from the biomedical model organism *Caenorhabditis elegans* have been isolated and proteins contained in these rafts have been analyzed, but so far no detailed analysis of the lipid composition has been conducted [3, 4]. In contrast to mammals, *C. elegans* membranes contain only low amounts of cholesterol, another important part of lipid rafts [5].

Sphingolipids have been linked to different important aspects in the biology of *C. elegans*. Nomura et al. have shown that ceramide glucosyltransferases are involved in the formation of oocytes and in early embryonic cell division. *C. elegans* harbors three genes encoding ceramide glucosyltransferases, names *cgt-1*, *-2*, and *-3*, of which CGT-3 shows the highest enzymatic activity. RNAi of *cgt-1/cgt-3* is lethal at the L1 stage [6]. The results showed that glucosylceramides (GlcCers) are required for postembryonic development. Monomethyl branched chain fatty acids (mmBCFAs) have been also linked to *C. elegans* development [7]. Sphingolipids in *C. elegans* are based on a unusual C17iso branched chain sphingoid base [8]. This sphingoid base is derived from the reaction of the mmBCFA 13-methyl myristic acid (C15iso) with Serine. Hannich et al. have shown by isotope labeling that the sphingoid bases are derived from Leucine [9]. Indeed, >99% of mmBCFAs are produced by C. elegans itself [10]. The N-acyl bound fatty acid are usually 2-hydroxy modified and have a length between C20 and C26 in glucosylceramides [8, 11]. Zhu et al. have shown that GlcCers form together with TORC1, a signaling pathway, to coordinate nutrient status and metabolism during growth and development [12].

Sphingolipids have been also linked to autophagy-dependent lifespan extension. Mosbech et al. found that loss of *hyl-1* and *lagr-1* extend the lifespan in an *atg-12* dependent manner. Additionally, *pha-4*, *daf-16* and *skn-1* are also required. *hyl-1*, *-2* and *lagr-1* are ceramide synthases catalyzing the transfer of an acyl group from acyl-CoA to a N-acyl. In this reaction *hyl-1* seems to have preference for acyl chains with 24 or more carbons, while *hyl-2* prefers 22 carbons and shorter [13]. Additionally, *hyl-2* was shown to be protective in anoxia, because loss of *hyl-2* increases sensitivity of *C. elegans* to anoxia. In contrast to this *hyl-1* loss of function lead to more resistance [14].

These examples demonstrate different species of a certain class play important and often opposing roles and that sphingolipid classes cannot be treated as homogenous entities. A putative biosynthetic pathway of different sphingolipids is shown in Figure 1A, adapted from Watts and Ristow [15]. So far, no definite number of sphingolipids in *C. elegans* is known. Given the need for more detailed (routine) analysis of sphingolipids in *C. elegans* it is important to define the sphingolipidome of this organism.

Here we describe structural analysis of sphingolipids from wildtype *C. elegans*. We performed UPLC-UHR-ToF-MS analysis to detect members of the sphingolipid class and perform partial structural elucidation of intact sphingolipids. By combining data independent and date dependent fragmentation together with *in silico* prediction of potential sphingolipid structures we could putatively identify 82 sphingolipids, including several isomeric species, in wild type *C. elegans* extracts. Putative identifications can be used in future sphingolipidomics approaches in the worm and will advance our knowledge on the regulation of sphingolipid metabolism and potentially lipid rafts formation and function in *C. elegans*.

**Material and Methods**

*Chemicals*

Methanol (MeOH), 2-Propanol (iPrOH), Acetonitril (ACN) were of LC-MS grade (Sigma-Aldrich). All other solvents and chemicals were of the highest available purity, usually analytical grade. Water was purified on Merck Millipore Integral 3 water purification system with TOC < 3 ppb, 18 MOhm.

*C. elegans cultivation*

*C. elegans* were grown in liquid culture to obtain sufficient biomass. Worms were grown in S-medium at 20°C and fed with concentrated *Escherichia coli* NA22. Worms were regularly checked and *E. coli* NA22 was added to prevent starvation. After one week, worms were harvested and separated from bacteria by filtration. After two times of washing with cold M9, worms were frozen at -80°C until extraction and analysis.

*Lipid Extraction*

Lipids were extracted according to the Folch method [16]. A pellet of about 750 mg (wet weight) of mixed stage worm samples was mixed with 1 mL of MeOH and homogenized in a Precellys Evolution bead beater (Bertin Instruments, Montigny-le-Bretonneux, France) at about 0°C and 8000 rpm. After addition of 2 mL CHCl3 the sample was shaken for one hour at room temperature and 500 rpm. Phase separation was induced by addition of 1 mL H2O and centrifugation at 12,000 rpm and 4°C for 15 minutes. The polar phase was re-extracted with 2 mL of CHCl3 / MeOH / H2O (v/v, 86 / 14 / 1) for 15 minutes. After phase separation organic phases were combined and dried in two aliquots in a SpeedVac Savant centrifugal evaporator (Thermo Scientific, Dreieich, Germany). One aliquot was re-dissolved in iPrOH / ACN / H2O (v/v, 60 / 35 / 5) prior to analysis with UPLC-UHR-ToF-MS and the other in CHCl3 for lipid fractionation.

*Sphingolipid fractionation*

Sphingolipids were fractionated according to Bodennec [17]. Briefly, different sphingolipids were eluted from either separated or piggy-backed LC-NH2 (Supelclean LC-NH2 SPE tubes, 1 mL, 100mg sorbent, Supelco) and LC-WCX (Supelclean LC-WCX SPE tubes, 1 mL, 100 mg sorbent) SPE columns. 8 different solvent mixtures were used to obtain 7 fractions. Fractionation was exactly performed as described in [17].

*Glycero- and Glycerophospholipid depletion*

A 50 µL aliquot of lipidome fractions was dried and resuspended in 50 µL 1 M KOH in MeOH. The samples were incubated at 37°C for 2 hours in a Thermomixer and shaken at 1000 rpm. After this time samples were neutralized with glacial acetic acid (~ 1µL) and 100 µL CHCl3 were added and the sample shaken at 1000 rpm and 20°C for 1 hour. After addition of 50 µL H2O, samples were vortexed and phases separated by centrifugation. The lower organic phase was dried in a SpeedVac and redisolved in 50 µL iPrOH / ACN / H2O (v/v, 60 / 35 / 5) for UPLC-UHR-ToF-MS analysis.

*UPLC-UHR-ToF-MS analysis*

Analysis of lipids was performed according to Witting et al. [18]. Briefly, a Waters Acquity UPLC (Waters, Eschborn, Germany) was coupled to a Bruker maXis UHR-ToF-MS (Bruker Daltonic, Bremen, Germany). Separation of lipids was achieved on a Waters Cortecs C18 column using a gradient from eluent A (60% ACN / 40% H2O + 10 mM NH4OOCH + 0.1% formic acid) to eluent B (90% iPrOH / 10% ACN + 10 mM NH4OOCH + 0.1% formic acid). Analysis was performed in positive and negative ionization mode. In contrast to the described method, the gradient time was doubled for high resolving power to detected individual lipids and increase separation of isomeric species. Gradient conditions were as followed: After an isocratic step of 32% B for 3 minutes a linear increase to 97% B in 39 minutes was performed with an isocratic hold of 97% B for 7 minutes. After return to initial conditions the column was re-equilibrated for 5 minutes with the starting conditions.

Mass spectrometric settings were as followed: Nebulizer 2.0 bar, Dry Gas 10.0 L/min, Dry Temp 200°C, Capillary Voltage 4000V and End Plate Offset 500 V. Mass range was set from m/z 100 to m/z 2000. 1:4 diluted Low Concentration Tuning Mix (Agilent Technologies, Waldbronn Germany) was injected via a 6-port valve prior to each analysis for individual recalibration of m/z axis.

Fragmentation data was either collected in data-independent (broad band Collision Induced Dissociation, bbCID) or data-dependent (AutoMSn) acquisition mode. 40 eV were used as collision energy for bbCID, while for AutoMSn composite spectra of 10 and 40 eV were collected.

*In silico sphingolipid structure generation*

Sphingolipid structures have been generated using the JChem for Excel Plugin (Chemaxon, Budapest, Hungary). Template reactions have been manually drawn and the JCReactProductStructure() reaction function was used to generate product structures. SMILES representation of fatty acid and sphingoid bases were used as input (SI Table 1 and 2). SMILES, InChIs and InChIKeys were generated using the JCMolFormat() function. Where available, ChEBI, LipidMaps and SwissLipids IDs have been added. The full table of structures is available in the SI (SI Tables 3-8).

**Results and Discussion**

*Screening for sphingoid backbones*

Sphingolipids in *C. elegans* contain an unusual d17:1 iso sphingoid base [8]. This sphingoid base is prodduced from 15:0iso fatty acid (FA(14:0(13Me), 13-methyl myristic acid) and serine. Additionally, Hannich et al have shown that the enzyme can also accept alanine, which produces a 1-deoxy sphingoid base [9]. In order to get an impression on the sphingoid bases in complex lipids we performed data independent fragmentation (broad band Collision Induced Dissociation, bbCID). Typical fragmentation of sphingolipids yields a sphingoid based fragment and the respective fragment plus an additional loss of H2O (-18 Da) and loss of water and CH2O (-48Da). We generated extracted ion chromatograms (EICs) with an error of 0.005 Da for the MS2 trace on the respective masses for d17:0iso, d17:1iso, -m17:0iso, m17:1iso sphingoid bases. As negative control we also searched for d18:0 and d18:1 sphingoid bases, which are absent in *C. elegans*. We only observed peaks for d17:1iso sphingoid base, derived from the loss of N-acyls as ketene. In our data the highest observed peak is the sphingoid base fragment – 2 x H2O (m/z 250.2529), followed by the fragment – H2O and CH2O (m/z 238.2529) and the sphingoid base fragment – H2O (m/z 268.2635) (Figure 1B). Our results confirm previous findings that C. elegans only uses C17 sphingoid bases.



Figure 1: (A) Biosynthetic pathway of sphingolipids in C. elegans, adapted from [15]. Known genes that participate in the synthesis of different sphingolipids are indicated. For simplification cofactors and other participating metabolites, e.g. fatty acyls CoAs are not shown. (B) hrEICs of common fragment masses indicating the presence of C17 sphingoid bases using bbCID fragmentation. Arrows indicate the co-elution of three fragment masses attributed to the C17 sphingoid base.

*Generation of structures*

Next, we investigated potential structures for *C. elegans* sphingolipids. At the current stage, LipidMaps does not contain ceramides with a C17iso branched chain sphingoid base [19]. We generated *in silico* all possible structures based on d17:0iso and d17:1iso sphingoid backbones. *C. elegans* is able to synthesize a large range of fatty acids on its own, including branched chain, mono- and polyunsaturated fatty acids. Different publications have investigated the fatty acid composition of *C. elegans* [20, 21]. However, most of these works focused on the total lipid composition and not specifically on sphingolipids. Chitwood et al. and Gerdt et al. specifically analyzed the composition of glycosphingolipids and found that many of them contain even and odd long chain 2-hydroxy fatty acids [8, 11]. Zhu et al. also reported the dependence of a sphingolipid dependent phenotype on *fath-1*, which produces 2-hydroxy fatty acids [12, 22].

We therefore generated sphingolipids from two different sets of input fatty acids. The first one, called “standard fatty acids”, is based on commonly observed fatty acids described in literature and usually make up glycero- and glycerophospholipids, and the second one is called “2-hydroxy and related fatty acids”. The second group contained non-hydroxylated and 2-hydroxy fatty acids of length as described by Chitwood et al. and Gerdt et al. Table 1 summarizes the used fatty acids with their shorthand notation. A full list with all molecular formulae and, InChIKeys and ChEBI identifiers are found in SI Tables 1 and 2.

Table 1: Table of shorthand notations of fatty acids used for in silico generation of sphingolipid structures.

|  |  |
| --- | --- |
| **Group** | **Fatty acids** |
| Standard fatty acids | FA(12:0), FA(12:0(11Me)), FA(13:0), FA(13:0(12Me)), FA(14:0), FA(14:0(12Me)), FA(14:0(13Me)), FA(15:0), FA(15:1(9Z,14Me)), FA(16:1(11Z)), FA(16:1(9Z)), FA(15:0(14Me)), FA(16:0), FA(17:1(10Z)), FA(16:0(14Me)), FA(16:0(15Me)), FA(17:0), FA(18:4(6Z,9Z,12Z,15Z)), FA(18:3(6Z,9Z,12Z)), FA(18:3(9Z,12Z,15Z)), FA(18:2(9Z,12Z)), FA(18:1(11Z)), FA(18:1(9Z)), FA(17:0(16Me)), FA(18:0), FA(19:1(10Z)), FA(19:0), FA(20:5(5Z,8Z,11Z,14Z,17Z)), FA(20:4(5Z,8Z,11Z,14Z)), FA(20:4(8Z,11Z,14Z,17Z)), FA(20:3(8Z,11Z,14Z)), FA(20:2(11Z,14Z)), FA(20:1(11Z)), FA(20:1(13Z)), FA(20:1(9Z)), FA(20:0), FA(22:0) |
| 2-hydroxy and related fatty acids | FA(16:0), FA(18:0), FA(20:0), FA(22:0), FA(24:0), FA(26:0), FA(28:0),FA(30:0), FA(16:0(2OH)), FA(18:0(2OH)), FA(20:0(2OH)), FA(22:0(2OH)), FA(24:0(2OH)), FA(26:0(2OH)), FA(28:0(2OH)), FA(30:0(2OH)), FA(14:0(13Me)), FA(16:0(15Me)), FA(18:0(17Me)), FA(20:0(19Me)), FA(22:0(21Me)), FA(24:0(23Me)), FA(26:0(25Me)), FA(28:0(27Me)), FA(14:0(13Me,2OH)), FA(16:0(15Me,2OH)), FA(18:0(17Me,2OH)), FA(20:0(19Me,2OH)), FA(22:0(21Me,2OH)), FA(24:0(23Me,2OH)), FA(26:0(25Me,2OH)), FA(28:0(27Me,2OH)), FA(15:0), FA(17:0), FA(19:0), FA(21:0), FA(23:0), FA(25:0), FA(27:0), FA(29:0), FA(15:0(2OH)), FA(17:0(2OH)), FA(19:0(2OH)), FA(21:0(2OH)), FA(23:0(2OH)), FA(25:0(2OH)), FA(27:0(2OH)), FA(29:0(2OH)) |

We generated structures for dihydroceramides (DhCers), ceramides (Cers), dihydrosphingomyelins (DhSMs), sphingomyelins (SMs), ceramide-1-phosphates (C1Ps) and glucosylceramides (GlcCers). In total we obtained 510 sphingolipids at a full structural level, 324 at the fatty acyl level and 282 at the hydroxyl group level, according to the nomenclature by Liebisch et al. [23]. Initial screening of the data obtained data showed that we were not able to detect DiHexCer or TriHexCers and were therefore ignored in structure generation and further analysis. Structures of all *in silico* generated sphingolipids are found in SI Table 3 to 8.

*MS1 screening for sphingolipid masses*

Using the generated list of potential sphingolipids we next checked for the presences of fitting masses on the MS1 level. We filtered all sphingolipids based on the sum composition (e.g. Cer(d41:1) and unique masses and created hrEICs for the [M+H]+ and [M-H2O+H]+ adducts. Since the loss of H2O is a prominent feature we used the coelution of the [M+H]+ and [M-H2O+H]+ masses criterium towards positive identification as sphingolipid. However, for DhSM and SM species this does not hold true. We used the odd masses of [M+H]+ adducts for identification of these sphingolipids and checked that they are not part of an isotope pattern of glycerophospholipids. Instead of bbCID we used data dependent acquisition of tandem MS to potentially further verify the identity of detected masses. The number of putative sphingolipids identified by exact mass are summarized in Table 2. Interestingly, we detected a high number of DhSMs and SMs with fatty acids from the standard fatty acids. However, they were very low in intensity close to the noise level.

Table 2: Number of detected peaks with fitting masses for different predicted sphingolipids based on MS1 level.

|  |  |  |  |
| --- | --- | --- | --- |
| **Class** | **Fatty acyl group** | **Theoretical** | **Found** |
| DhCer | Std Fatty acids | 22 | 0 |
| 2-OH fatty acids | 32 | 4 |
| Cer | Std Fatty acids | 22 | 1 |
| 2-OH fatty acids | 32 | 13 |
| DhSM | Std Fatty acids | 22 | 7 |
| 2-OH fatty acids | 32 | 6 |
| SM | Std Fatty acids | 22 | 8 |
| 2-OH fatty acids | 32 | 16 |
| HexCer | Std Fatty acids | 22 | 0 |
| 2-OH fatty acids | 32 | 3 |

We were not able to detect C1Ps as well as di- and trihexosylceramides. All of them might be too low in concentration and were excluded from further analysis. While Gerdt et al. detected di- and trihexosyl ceramides they used 10 g of dried biological material and obtained about 550 mg of lipid extract. We conclude that either a larger number of worms or more sensitive methods are required for the detection of these sphingolipids. The most detected sphingolipids had fatty acids derived from fatty acids from the 2-hydroxy and related fatty acids group. We therefore decided to further investigate these species, since also previous studies have shown that *C. elegans* sphingolipids mostly contain these fatty acyls.

*Fragmentation pattern of different sphingolipid classes*

Fragmentation of sphingolipids have been extensively studied, mostly in negative ionization mode or from alkali adducts [24-28]. To further identify sphingolipids of *C. elegans* we compared fragmentation pattern of sphingolipid candidates detected so far with known fragmentation patterns of different sphingolipids to derive fragmentation pathways and fragments that can be used for identification. Due to higher sensitivity we focused on the fragmentation in positive ionization mode.

Typical fragments for Cers are the loss of a water molecule, which is possible for both hydroxyl groups. This fragmentation requires low energy, since it is often already observed as in-source fragment. Both obtained structures can lose the N-Acyl, again yielding fragments of similar masses. One of these fragments is losing an additional water molecule, while the other is losing a formaldehyde. The fragments are yielding a typical peak triplet with the mass’s m/z 238.2529, 250.2529 and 268.2635. These three fragments can identify a molecule as a sphingolipid containing a C17:1 sphingoid base. The N-Acyl can be identified by the difference between the [M-H2O+H]+ fragment and m/z 268.2635. Figure 2A shows a typical MS2 spectrum from a peak identified as Cer(d17:1/16:0) and the structure of the proposed fragments (Figure 2B).

In contrast to Cers, DhCers show the typical water loss to a lesser extent. This suggests that the loss of the hydroxyl group at position 3 is the favored one in ceramides, because of the stabilizing effect of the neighboring double bond. Additionally, m/z 288 is observed for DhCers, which represents the loss of the fatty acyl as ketene directly from the [M+H]+. In DhCers this double bond is missing, therefore DhCers tend to show lower intensities for the [M-H2O+H]+ fragment. Interestingly, DhCers show a N-Acyl fragment, which correspond to a N-Acyl amide. These fragments can be also found in of highly abundant Cer fragmentation spectra, but only at low intensities.



Figure 2: (A) Example MS/MS spectrum of Cer(d17:1/16:0). Major peaks are the [M-H2O+H]+ fragment as well as m/z 250.2530. (B) Proposed fragmentation pathway for C. elegans ceramides. Two possibilities for loss of water from the parent ion are possible, yielding two different fragmentation trees.

Similar N-Acyl amide fragments as the ones observed for DhCers were found in GlcCers. GlcCers showed different fragments related to the loss of the hexosyl headgroup. Neutral losses of 162 were observed from the [M+H]+ and [M-H2O+H]+ peak.

DhSMs and SMs yield a fragment of m/z 184.07 corresponding the phosphocholine headgroup. This was the only observed fragment and therefore no further structural characterization was possible for SMs.

*Fractionation of lipidomes*

In order to analyze the sphingolipids in more detail and to perform detailed tandem MS for identification without interference from overlapping glycerol- or glycerophospholipids we fractionated our lipid extract according to Bodennec et al. [17]. This methodology uses two SPE column, NH2 and WCX, to fractionate lipids into 7 fractions. For this study fractions 2, 4, 5, 6 and 7 were of particular interest containing free DhCers and Cers, neutral glycosphingolipids (HexCer, DiHexCer and TriHexCer), sphingosines, DhSMs and SMs and C1Ps respectively. To further remove overlapping interreferences from remaining glycerophospholipids they were saponified and the dried fractions were reconstituted with methanolic KOH. Since we were not able to detect C1Ps in neither the raw extract nor the fractions, we focused on the fractions 2, 4 and 6 and searched for peaks corresponding to sphingolipids and then used the mass list derived from the 2-OH fatty acids. Additionally, we included these masses in the preference list for data dependent fragmentation to obtain MS2 fragmentation data to confirm the identity of sphingolipids. In order to allow for relative quantification within classes we used the peak area of the detected and confirmed peaks.

With the fractionation and saponification we were able to detect more sphingolipid species compared to the full Folch extract, especially low abundant species suppressed by other co-eluting lipids. Already with the MS1 screen we detected several double peaks for the same mass which are mostly different by about 0.3 minutes in their retention time. We are assuming that the different peaks are derived from either straight or branched chain fatty acid isomers with the same number of carbons. Calculating the logP value for the respective species, branched chain derivates have always the smaller logP, although the difference is not large. The earlier eluting peak would represent the branched chain version and it usually represents the small peak when peak areas are compared. Using MS2 we could not find any differences in the fragmentation behavior of the two different peaks. Chitwood et al. and Gerdt et al. detected different iso branched chain fatty acids in glucosylceramides, including even chained iso fatty acids along with straight chain fatty acids. Particularly, an iso C24:0(OH) fatty acid accounted for 3.3% relative amount, while the straight chain isomer C24:0(OH) for 17.5%. In our measurement these fatty acids were 4.2% and 23.2%, respectively. Similar trends were found for HexCers containing C23:0(OH) and C25:0(OH). Due to missing standards no verification was possible.

In order to get an impression on the distribution of different N-acyls in the different lipid classes, we estimated the relative amounts of each detected sphingolipid species within its class. Since no internal standards area available for *C. elegans* sphingolipids no absolute quantities can be determined. However, the goal is to compare the relative composition of the different classes to determine specificity of fatty acid profiles. We used relative peaks areas for each class to determine the relative composition. In order to allow a comparison between the different classes, peaks areas for individual isomers were summed up. Although the amount of different sphingolipid classes cannot be compared to each other due to different ionization efficiencies, still when comparing the total peak areas of DhCers with the Cers and DhSMs with SMs they are only 5.1% and 2.3% respectively and were neglected in the further discussion.



Figure 3: (A) Extracted ion chromatograms of detected sphingolipids. Peaks are annotated with their putative annotation on the MS1 level. MS/MS identified that all the detected peaks shown in this figure contain a C17 sphingoid base. (B) Comparison of sphingolipid composition based on the relative peak area. Each sphingolipid is normalized to the sum of all sphingolipids from the same class. HexCers show a unique pattern with only hydroxy fatty acids (C) Venn diagram showing the overlap between sphingolipid species detected by Mosbech et al., Menuz et al. and this paper.

Compared to the other detected sphinoglipids, HexCers show a very specific fatty acid profile. We found that hexosylceramides contain exclusively hydroxy fatty acids. While Gerdt et al. detected also C16, C18:1 and C18:0 fatty acids in the glucosylceramides, we were not able to find them. Consistent with previous findings the most prominent lipid contains a C22:0(OH) fatty acyl with almost 46% [11, 13]. Likewise, Mosbech et al. also detected only HexCers containing three hydroxyl groups [13]. Only C21-C6 fatty acids were detected. It was shown that HexCers in *C. elegans* contain a β-D-Glucose [11]. Mosbech et al. have shown that *hyl-1* is required for synthesis of very long chain fatty acids (>= C24) and *hyl-2* produces ceramides with shorter chain acyl (<= C22). A similar substrate specificity might exist for glucosyltransferases, accepting only 2-hydroxy fatty acyl containing sphingolipids of a specific length with the highest activity towards C22. The worm harbors three ceramide glucosyltransferases, *cgt-1* to *-3*, whereas *cgt-3* was shown to have the highest activity [6]. No substrate specificity regarding bound n-acyls have been shown so far for these three enzymes but looking at the fatty acid distribution it might be possible that they only use specific Cers, the fatty acid distribution of sphingomyelins mirrors the distribution of Cers with a few exceptions. A list with all detected sphingolipids is available in SI Tables 9 and 10.

It will be of great importance in future to determine the substrate specificity of different enzymes involved in biosynthesis and degradation of sphingolipids. Several publications treat the individual classes as single entities. However, each class contains several individual species, each with slightly different physicochemical properties. These properties influence the activity of individual lipids and their function in defined lipid rafts. Furthermore, it is interesting why *C. elegans* uses d17:1iso sphingoid bases and why their membranes contain only low levels of cholesterol [5].

*Comparison against previous detected sphingolipids*

In total we were able to detect 82 different sphingolipids, including several isomeric species, in our sample based on extensive fractionation, saponification and analysis with UPLC-UHR-ToF-MS. Sphingolipids have been previously analyzed in different conditions in *C. elegans*. Mosbech et al. found that different ceramide synthetases have opposing roles on the lifespan of *C. elegans*. They detected in total 46 sphingolipids from Cers, SMs and HexCers [13]. Menuz et al. have shown that *hyl-2* is protective against anoxia in *C. elegans*. They quantified 12 Cers and 12 SMs [14]. Since only nominal masses are presented, we selected the closest matching sphingolipid from our list. Furthermore, Cutler et al. performed analysis of sphingolipids in *C. elegans*. However, from their presented results it is not possible to deduce which lipid species were used as a basis (d17iso or d18 sphingoid bases). We therefore neglected their results for a comparative analysis [29].

We compared the sphingolipids previously detected with our obtained list. For this comparison the sum composition, e.g. Cer(t44:1) was used. This reduced the number of sphingolipids to 60 for this publication. From all the publications 12 sphingolipids were detected in all (Figure 3C), mostly Cers and SMs. Shared species between Mosbech and this publication are different HexCers and SMs, while with Menuz mostly Cers and SMs are common. Consistent with our own and previous finding, HexCers were found to mostly contain 3 hydroxyl groups, with one most likely located in the N-Acyl. Assuming a C17 sphingoid bases N-Acyl side chains had a length of 21-26 carbons. HexCers containing a C22:0(OH) N-Acyl group were the highest found in this group.

In comparison to previous published papers we performed UPLC-UHR-ToF-MS, which allowed to separate potential isomers and increases ionization efficiency by separating lipid species. Additionally, performed fractionation and saponification of base sensitive lipids further enhanced efficiency. This allowed us to describe the *C. elegans* sphingolipidome in more detail. However, it remains elusive what the exact structure of the minor isomers is and if they play specific roles in lipid raft formation. So far this publication in the most comprehensive description of sphinoglipids in *C. elegans*.

**Conclusion**

Sphingolipids play important roles in the biology of *C. elegans*. In this work we describe a detailed analysis of intact sphingolipids from different classes. In total we were able to detect and confirm 82 sphingolipids, including different isomeric species. Although several genes are known, we are still missing parts of the biosynthetic pathways, e.g. which enzymes produce the very long chain fatty acids. *C. elegans* was shown to also contain ascarosides, glycolipids important in signaling, with chain length up to 30 carbons [30]. Likewise, Gao et al. were able to detect a C30 fatty acid [31, 32]. So far no biosynthetic pathway for this fatty acid has been established in *C. elegans* and no sphingolipid with such a N-acyl side chain has been detected.

Since *C. elegans* contains, compared to other mammals and normalized to PC, 20 times less cholesterol in the membrane, it will be interesting in the future to analyze sphingolipids from isolated lipid rafts. It might be possible that the unusual branched chain sphingoid base compensates for the lack of cholesterol. We believe that UPLC-UHR-ToF-MS will be a valuable tool for the in-depth analysis of the *C. elegans* sphingolipidome in future, since it allows a more detailed snapshot.

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**The Sphingolipidome of the model organism *Caenorhabditis elegans – Supplementary Information***

Victoria Hänel1, Christian Pendleton1, Michael Witting1,2,\*

1 Research Unit Analytical BioGeoChemistry, Helmholtz Zentrum München, Ingolstädter Landstraße 1, 85674 Neuherberg

2 Chair of Analytical Food Chemistry, Technische Universität München, Maximus-von-Imhof-Forum 2, 85354 Freising

\* corresponding author: Dr. Michael Witting, michael.witting@helmholtz-muenchen.de

SI Table 1: Names, shorthand notations, InChIKey, formulae, exact masses and ChEBI IDs of fatty acids related to the group “2-hydroxy and related fatty acids” in Table 1.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Name** | **Shorthand** | **InChIKey** | **Formula** | **exact Mass** | **ChEBI** |
| Palmitic acid | FA(16:0) | IPCSVZSSVZVIGE-UHFFFAOYSA-N | C16H32O2 | 256.24023 | CHEBI:15756 |
| Stearic acid | FA(18:0) | QIQXTHQIDYTFRH-UHFFFAOYSA-N | C18H36O2 | 284.27153 | CHEBI:28842 |
| Arachidic cid | FA(20:0) | VKOBVWXKNCXXDE-UHFFFAOYSA-N | C20H40O2 | 312.302831 | CHEBI:28822 |
| Behenic acid | FA(22:0) | UKMSUNONTOPOIO-UHFFFAOYSA-N | C22H44O2 | 340.334131 | CHEBI:28941 |
| Lignoceric acid | FA(24:0) | QZZGJDVWLFXDLK-UHFFFAOYSA-N | C24H48O2 | 368.365431 | CHEBI:28866 |
| Cerotic acid | FA(26:0) | XMHIUKTWLZUKEX-UHFFFAOYSA-N | C26H52O2 | 396.396731 | CHEBI:31009 |
| Montanic acid | FA(28:0) | UTOPWMOLSKOLTQ-UHFFFAOYSA-N | C28H56O2 | 424.428031 | CHEBI:31001 |
| Melissic acid | FA(30:0) | VHOCUJPBKOZGJD-UHFFFAOYSA-N | C30H60O2 | 452.459331 | CHEBI:31003 |
| 2-Hydroxy palmitic acid | FA(16:0(2OH)) | JGHSBPIZNUXPLA-UHFFFAOYSA-N | C16H32O3 | 272.235145 | CHEBI:65101 |
| 2-Hydroxy stearic acid | FA(18:0(2OH)) | KIHBGTRZFAVZRV-UHFFFAOYSA-N | C18H36O3 | 300.266445 | CHEBI:19660 |
| 2-Hydroxy arachidic acid | FA(20:0(2OH)) | CPLYLXYEVLGWFJ-UHFFFAOYSA-N | C20H40O3 | 328.297745 | CHEBI:76992 |
| 2-Hydroxy behenic acid | FA(22:0(2OH)) | RPGJJWLCCOPDAZ-UHFFFAOYSA-N | C22H44O3 | 356.329045 | CHEBI:76980 |
| 2-Hydroxy lignoceric acid | FA(24:0(2OH)) | MSUOLNSQHLHDAS-UHFFFAOYSA-N | C24H48O3 | 384.360345 | CHEBI:61302 |
| 2-Hydroxy cerotic acid | FA(26:0(2OH)) | IFYDZTDBJZWEPK-UHFFFAOYSA-N | C26H52O3 | 412.391646 | CHEBI:76986 |
| 2-Hydroxy montanic acid | FA(28:0(2OH)) | FYDAGLSVOSNEBV-UHFFFAOYSA-N | C28H56O3 | 440.422946 | CHEBI:85340 |
| 2-Hydroxy mellisic acid | FA(30:0(2OH)) | FOPDLHYJCVRBAQ-UHFFFAOYSA-N | C30H60O3 | 468.454246 |  |
| 13-Methyl myrstic acid | FA(14:0(13Me)) | ZOCYQVNGROEVLU-UHFFFAOYSA-N | C15H30O2 | 242.22458 | CHEBI:39250 |
| 15-Methyl palmitic acid | FA(16:0(15Me)) | IIUXHTGBZYEGHI-UHFFFAOYSA-N | C17H34O2 | 270.25588 | CHEBI:70850 |
| 17-Methyl stearic acid | FA(18:0(17Me)) | YETXGSGCWODRAA-UHFFFAOYSA-N | C19H38O2 | 298.28718 | CHEBI:133136 |
| 19-Methyl arachidic acid | FA(20:0(19Me)) | BDGYZTCVQAZQFG-UHFFFAOYSA-N | C21H42O2 | 326.318481 |  |
| 21-Methyl behenic acid | FA(22:0(21Me)) | BQKXBLINRIKSGC-UHFFFAOYSA-N | C23H46O2 | 354.349781 |  |
| 23-Methyl lignoceric acid | FA(24:0(23Me)) | MLLNAEIUHLSUPB-UHFFFAOYSA-N | C25H50O2 | 382.381081 |  |
| 25-Methyl cerotic acid | FA(26:0(25Me)) | ZAQKRFKZRXRVLJ-UHFFFAOYSA-N | C27H54O2 | 410.412381 |  |
| 27-Methyl montanic acid | FA(28:0(27Me)) | OVBLOKZRGDSDGY-UHFFFAOYSA-N | C29H58O2 | 438.443681 |  |
| 2-Hydroxy-13-Methyl myristic acid | FA(14:0(13Me,2OH)) | VORFIVRUGMJWAE-UHFFFAOYSA-N | C15H30O3 | 258.219495 |  |
| 2-Hydrxoy-15-Methyl palmitic acid | FA(16:0(15Me,2OH)) | XGUBRJWBFWOVAP-UHFFFAOYSA-N | C17H34O3 | 286.250795 |  |
| 2-Hydrxoy-17-Methyl stearic acid | FA(18:0(17Me,2OH)) | NKGLGTMODIPSNJ-UHFFFAOYSA-N | C19H38O3 | 314.282095 |  |
| 2-Hydroxy-19-Methyl arachdic acid | FA(20:0(19Me,2OH)) | VSNAKZBBOKBVPK-UHFFFAOYSA-N | C21H42O3 | 342.313395 |  |
| 2-Hydroxy-21-Methyl behenic acid | FA(22:0(21Me,2OH)) | NNLYJQNQENRGBO-UHFFFAOYSA-N | C23H46O3 | 370.344695 |  |
| 2-Hydroxy-23-Methyl lignoceric acid | FA(24:0(23Me,2OH)) | ZMMHVKLVXJXDTC-UHFFFAOYSA-N | C25H50O3 | 398.375995 |  |
| 2-Hydroxy-25-Methyl cerotic acid | FA(26:0(25Me,2OH)) | SKDKJYYMHFZYAY-UHFFFAOYSA-N | C27H54O3 | 426.407296 |  |
| 2-Hydroxy-27-Methyl montanic acid | FA(28:0(27Me,2OH)) | PTAGDOOSBIWBDB-UHFFFAOYSA-N | C29H58O3 | 454.438596 |  |
| Pentadecanoic acid | FA(15:0) | WQEPLUUGTLDZJY-UHFFFAOYSA-N | C15H30O2 | 242.22458 | CHEBI:42504 |
| Heptadecanoic acid | FA(17:0) | KEMQGTRYUADPNZ-UHFFFAOYSA-N | C17H34O2 | 270.25588 | CHEBI:32365 |
| Nonadecanoic acid | FA(19:0) | ISYWECDDZWTKFF-UHFFFAOYSA-N | C19H38O2 | 298.28718 | CHEBI:39246 |
| Henicosanoic acid | FA(21:0) | CKDDRHZIAZRDBW-UHFFFAOYSA-N | C21H42O2 | 326.318481 | CHEBI:39248 |
| Tricosanoic acid | FA(23:0) | XEZVDURJDFGERA-UHFFFAOYSA-N | C23H46O2 | 354.349781 |  |
| Pentacosanoic acid | FA(25:0) | MWMPEAHGUXCSMY-UHFFFAOYSA-N | C25H50O2 | 382.381081 |  |
| Heptacosanoic acid | FA(27:0) | VXZBFBRLRNDJCS-UHFFFAOYSA-N | C27H54O2 | 410.412381 |  |
| Nonacosanoic acid | FA(29:0) | IHEJEKZAKSNRLY-UHFFFAOYSA-N | C29H58O2 | 438.443681 |  |
| 2-Hydroxy pentadecanoic acid | FA(15:0(2OH)) | NKASEPJANRVKDD-UHFFFAOYSA-N | C15H30O3 | 258.219495 | CHEBI:84853 |
| 2-Hydroxy heptadecanoic acid | FA(17:0(2OH)) | KUZABABLVHWUGR-UHFFFAOYSA-N | C17H34O3 | 286.250795 | CHEBI:84854 |
| 2-Hydroxy nonadecanoic acid | FA(19:0(2OH)) | PYNCEZHRMYECCB-UHFFFAOYSA-N | C19H38O3 | 314.282095 | CHEBI:84855 |
| 2-Hydroxy henicosanoic acid | FA(21:0(2OH)) | DHCJPXKWDPRJAX-UHFFFAOYSA-N | C21H42O3 | 342.313395 | CHEBI:84857 |
| 2-Hydroxy tricosanoic acid | FA(23:0(2OH)) | JZWLIRVAYJRWLN-UHFFFAOYSA-N | C23H46O3 | 370.344695 | CHEBI:84858 |
| 2-Hydroxy pentacosanoic acid | FA(25:0(2OH)) | CUSDWPFQTXVFJL-UHFFFAOYSA-N | C25H50O3 | 398.375995 | CHEBI:84860 |
| 2-Hydroxy heptacosanoic acid | FA(27:0(2OH)) | ZFPIEBUFHZZBPX-UHFFFAOYSA-N | C27H54O3 | 426.407296 |  |
| 2-Hydroxy nonacosanoic acid | FA(29:0(2OH)) | YHMOKJUCCUSAKW-UHFFFAOYSA-N | C29H58O3 | 454.438596 |  |

SI Table 2: Names, shorthand notations, InChIKey, formulae, exact masses and ChEBI IDs of fatty acids related to the group “standard fatty acids” in Table 1.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Name** | **Shorthand** | **InChIKey** | **Formula** | **exact Mass** | **ChEBI** |
| Lauric acid | FA(12:0) | POULHZVOKOAJMA-UHFFFAOYSA-N | C12H24O2 | 200.177630 | CHEBI:30805 |
| 11-Methyl lauric acid | FA(12:0(11Me)) | SIOLDWZBFABPJU-UHFFFAOYSA-N | C13H26O2 | 214.193280 | CHEBI:77359 |
| Tridecanoic acid | FA(13:0) | SZHOJFHSIKHZHA-UHFFFAOYSA-N | C13H26O2 | 214.193280 | CHEBI:45919 |
| 12-Methyl tridecanoic acid | FA(13:0(12Me)) | YYVJAABUJYRQJO-UHFFFAOYSA-N | C14H28O2 | 228.208930 | CHEBI:43722 |
| Myristic acid | FA(14:0) | TUNFSRHWOTWDNC-UHFFFAOYSA-N | C14H28O2 | 228.208930 | CHEBI:28875 |
| 12-Methyl myristic acid | FA(14:0(12Me)) | XKLJLHAPJBUBNL-UHFFFAOYSA-N | C15H30O2 | 242.224580 | CHEBI:39251 |
| Pentadecanoic acid | FA(15:0) | WQEPLUUGTLDZJY-UHFFFAOYSA-N | C15H30O2 | 242.224580 | CHEBI:42504 |
| 9Z-14-Methyl pentadecenoic acid | FA(15:1(9Z,14Me)) | YRYADTKRRRPFOA-ALCCZGGFSA-N | C16H30O2 | 254.224580 |  |
| 11Z-palmitoleic acid | FA(16:1(11Z)) | JGMYDQCXGIMHLL-WAYWQWQTSA-N | C16H30O2 | 254.224580 | CHEBI:35464 |
| 9Z-palmitoleic acid | FA(16:1(9Z)) | SECPZKHBENQXJG-FPLPWBNLSA-N | C16H30O2 | 254.224580 | CHEBI:28716 |
| 14-Methyl pentadecanoic acid | FA(15:0(14Me)) | ZONJATNKKGGVSU-UHFFFAOYSA-N | C16H32O2 | 256.240230 | CHEBI:84890 |
| Palmitic acid | FA(16:0) | IPCSVZSSVZVIGE-UHFFFAOYSA-N | C16H32O2 | 256.240230 | CHEBI:15756 |
| 10Z-heptadecenoic acid | FA(17:1(10Z)) | GDTXICBNEOEPAZ-FPLPWBNLSA-N | C17H32O2 | 268.240230 | CHEBI:75094 |
| 14-Methyl palmitic acid | FA(16:0(14Me)) | FXUKWLSZZHVEJD-UHFFFAOYSA-N | C17H34O2 | 270.255880 | CHEBI:84874 |
| 15-Methyl palmitic acid | FA(16:0(15Me)) | IIUXHTGBZYEGHI-UHFFFAOYSA-N | C17H34O2 | 270.255880 | CHEBI:70850 |
| Heptadecanoic acid | FA(17:0) | KEMQGTRYUADPNZ-UHFFFAOYSA-N | C17H34O2 | 270.255880 | CHEBI:32365 |
| Stearidonic acid | FA(18:4(6Z,9Z,12Z,15Z)) | JIWBIWFOSCKQMA-LTKCOYKYSA-N | C18H28O2 | 276.208930 | CHEBI:32389 |
| Gamma-Linolenic acid | FA(18:3(6Z,9Z,12Z)) | VZCCETWTMQHEPK-QNEBEIHSSA-N | C18H30O2 | 278.224580 | CHEBI:28661 |
| Alpha-Linolenic acid | FA(18:3(9Z,12Z,15Z)) | DTOSIQBPPRVQHS-PDBXOOCHSA-N | C18H30O2 | 278.224580 | CHEBI:27432 |
| Linoleic acid | FA(18:2(9Z,12Z)) | OYHQOLUKZRVURQ-HZJYTTRNSA-N | C18H32O2 | 280.240230 | CHEBI:17351 |
| Vaccenic acid | FA(18:1(11Z)) | UWHZIFQPPBDJPM-FPLPWBNLSA-N | C18H34O2 | 282.255880 | CHEBI:50464 |
| Oleic acid | FA(18:1(9Z)) | ZQPPMHVWECSIRJ-KTKRTIGZSA-N | C18H34O2 | 282.255880 | CHEBI:16196 |
| 16-Methyl heptadecanoic acid | FA(17:0(16Me)) | XDOFQFKRPWOURC-UHFFFAOYSA-N | C18H36O2 | 284.271530 | CHEBI:84896 |
| Stearic acid | FA(18:0) | QIQXTHQIDYTFRH-UHFFFAOYSA-N | C18H36O2 | 284.271530 | CHEBI:28842 |
| 10Z-nonadecenoic acid | FA(19:1(10Z)) | BBOWBNGUEWHNQZ-KTKRTIGZSA-N | C19H36O2 | 296.271530 | CHEBI:83051 |
| Nonadecanoic acid | FA(19:0) | ISYWECDDZWTKFF-UHFFFAOYSA-N | C19H38O2 | 298.287180 | CHEBI:39246 |
| Eicosapentaenoic acid | FA(20:5(5Z,8Z,11Z,14Z,17Z)) | JAZBEHYOTPTENJ-JLNKQSITSA-N | C20H30O2 | 302.224580 | CHEBI:28364 |
| Arachidonic acid | FA(20:4(5Z,8Z,11Z,14Z)) | YZXBAPSDXZZRGB-DOFZRALJSA-N | C20H32O2 | 304.240230 | CHEBI:15843 |
| 8Z,11Z,14Z,17Z-icosatetraenoic acid | FA(20:4(8Z,11Z,14Z,17Z)) | HQPCSDADVLFHHO-LTKCOYKYSA-N | C20H32O2 | 304.240230 | CHEBI:71488 |
| Dihomo-gamma-linolenic acid | FA(20:3(8Z,11Z,14Z)) | HOBAELRKJCKHQD-QNEBEIHSSA-N | C20H34O2 | 306.255880 | CHEBI:53486 |
| 11Z,14Z-icosadienoic acid | FA(20:2(11Z,14Z)) | XSXIVVZCUAHUJO-HZJYTTRNSA-N | C20H36O2 | 308.271530 | CHEBI:73731 |
| 11Z-icosaenoic acid | FA(20:1(11Z)) | BITHHVVYSMSWAG-KTKRTIGZSA-N | C20H38O2 | 310.287180 | CHEBI:32425 |
| 13Z-icosaenoic acid | FA(20:1(13Z)) | URXZXNYJPAJJOQ-FPLPWBNLSA-N | C20H38O2 | 310.287180 | CHEBI:134479 |
| 9Z-icosanenoic acid | FA(20:1(9Z)) | LQJBNNIYVWPHFW-QXMHVHEDSA-N | C20H38O2 | 310.287180 |  |
| Arachidic acid | FA(20:0) | VKOBVWXKNCXXDE-UHFFFAOYSA-N | C20H40O2 | 312.302831 | CHEBI:28822 |
| Behenic acid | FA(22:0) | UKMSUNONTOPOIO-UHFFFAOYSA-N | C22H44O2 | 340.334131 | CHEBI:28941 |



SI Figure 1: Tandem MS spectra of DhCers detected in the fractionated samples.



SI Figure 2: Tandem MS spectra of Cers with non-hydroxylated, even-numbered fatty acids detected in the fractionated samples.



SI Figure 3: Tandem MS spectra of Cers with 2-hydroxylated, even-numbered fatty acids detected in the fractionated samples.



SI Figure 4: Tandem MS spectra of Cers with non-hydroxylated, odd-numbered fatty acids detected in the fractionated samples.



SI Figure 5: Tandem MS spectra of Cers with 2-hydroxylated, odd-numbered fatty acids detected in the fractionated samples.



SI Figure 6: Tandem MS spectra of DhSMs detected in the fractionated samples.



SI Figure 7: Tandem MS spectra of SMs with non-hydroxylated, even-numbered fatty acids detected in the fractionated samples.



SI Figure 8: Tandem MS spectra of SMs with 2-hydroxylated, even-numbered fatty acids detected in the fractionated samples.



SI Figure 9: Tandem MS spectra of SMs with non-hydroxylated, odd-numbered fatty acids detected in the fractionated samples.



SI Figure 10: Tandem MS spectra of SMs with 2-hydroxylated, odd-numbered fatty acids detected in the fractionated samples.



SI Figure 11: Tandem MS spectra of HexCers detected in the fractionated samples.



SI Figure 12: Comparison of Cer(d17:0/22:0(OH)), Cer(d17:1/22:0(OH)) and HexCer(d17:1/22:0(OH))