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Structural Profiling and Quantification of Sphingomyelin in Human Breast Milk by HPLC-MS/MS

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Supporting Information

ABSTRACT: The sphingolipid composition of food as well as of physiological samples has received considerable interest due to their positive biological activities. This study quantified the total amount of sphingomyelin (SM) in 20 human breast milk samples from healthy volunteers and determined the structures of SM by detailed mass spectrometric studies in combination with enzymatic cleavage. The quantification of SM was performed by hydrophilic interaction liquid chromatography coupled to electrospray ionization−tandem mass spectrometry (HILIC-HPLC-ESI-MS/MS) measuring the characteristic fragment ion of the phosphorylcholine group at m/z 184.2 and by using hexanoylsphingomyelin (C6-SM) and heptadecanoylsphingomyelin (C17-SM) as internal standards. The structures of SM species were identified after enzymatic cleavage with alkaline sphingomyelinase (SMase) to the corresponding ceramides. Structure elucidation of the sphingoid base and fatty acid backbone was performed by reversed-phase HPLC-ESI-MS/MS. The method includes the sphingoid bases dihydrosphingosine (d18:0), sphingosine (d18:1(Δ4)), 4,8-sphingadienine (d18:2(Δ4,8)), 4-hydroxysphinganine (phytosphingosine (t18:0)), and 4-hydroxy-8-sphingenine (t18:1(Δ8)) and fatty acids with even-numbered carbon atoms (C12−C26) as well as their (poly)unsaturated and monohydroxylated analogues. The total amount of SM in human breast milk varied from 3.87 to 9.07 mg/100 g fresh weight. Sphingosine (d18:1) was the predominant sphingoid base, with 83.6 ± 3.5% in human breast milk, followed by 4,8-sphingadienine (d18:2) (7.2 ± 1.9%) and 4-hydroxysphinganine (t18:0) (5.7 ± 0.7%). The main SM species contained sphingosine and palmitic acid (14.9 ± 2.2%), stearic acid (12.7 ± 1.5%), docosanoic acid (16.2 ± 3.6%), and tetracosenoic acid (15.0 ± 3.1%). Interestingly, the fatty acid composition of SM species in this study differs from the total fatty acids in human breast milk, and the fatty acids are not consistently distributed among the different sphingoid bases.

KEYWORDS: sphingolipid, sphingomyelin, human breast milk, ESI-MS/MS, SMase

INTRODUCTION

Sphingolipid comprise a complex class of lipids that occur in all eukaryotes and in some prokaryotic organisms and viruses.[1] The sphingolipid composition of physiological samples as well as of food has received considerable interest due to their positive biological activities.[2] Sphingolipid metabolism is clearly involved in the regulation of cell growth, differentiation, and programmed cell death (apoptosis). Sphingosine mediates as a pro-apoptotic molecule, whereas sphingosine-1-phosphate, which is formed from sphingosine by sphingosine kinase, does not induce apoptosis.[3,4] Ceramide promotes cell-stress responses such as the regulation of apoptosis.[5] Cell migration and inflammation are mediated by sphingosine-1-phosphate,[6] and ceramide-1-phosphate is well-known to induce inflammatory responses.[7] For a summary of the biological activities of sphingolipids, see the recent reviews.[8−10]

The common structural element of sphingolipids is a sphingoid base backbone to which an amide-linked long-chain fatty acid can be attached, leading to the ceramides (*N*-acyl-sphingoid bases).[11] The primary hydroxyl group of the sphingoid base can be linked to a polar headgroup, such as phosphorylcholine in the case of sphingomyelin (Figure 1).

Sphingoid bases and attached fatty acids vary in alkyl chain length and position and number of double bonds and hydroxyl groups. Sphingolipids of mammals mainly consist of sphingosine (d18:1(Δ4)) as backbone, which refers to (2*S*,3*R*,4*E*)-2-amino-octadec-4-ene-1,3-diol (Figure 2).[12]

Ceramide is the substrate for the biosynthesis of sphingomyelin. Phosphorylcholine is transferred from phosphatidylcholine (Figure 3) by sphingomyelin synthases (SMS), whereas SMS1 is localized in the Golgi and SMS2 primarily at the plasma membrane.[13]

Human breast milk is supposed to be the ideal food for infants. The ingested lipids serve as an energy store in adipose tissue, and they play important roles in all biological membranes. Polyunsaturated fatty acids are essential components, and properties such as permeability, fluidity, and the activity of membrane-bound enzymes as well as of receptors are regulated by the membrane constitution.[14−16]

The fatty acid composition of human breast milk has been analyzed in various studies, including samples from different continents, showing a great variety in the fatty acid composition. Samples from Australia, Europe, and the United States consisted of constant levels of saturated and monounsaturated fatty acids, whereas high variations were observed for polyunsaturated fatty

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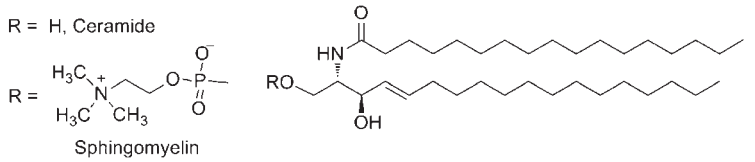

Figure 1. Structures of sphingolipids: ceramide and sphingomyelin.

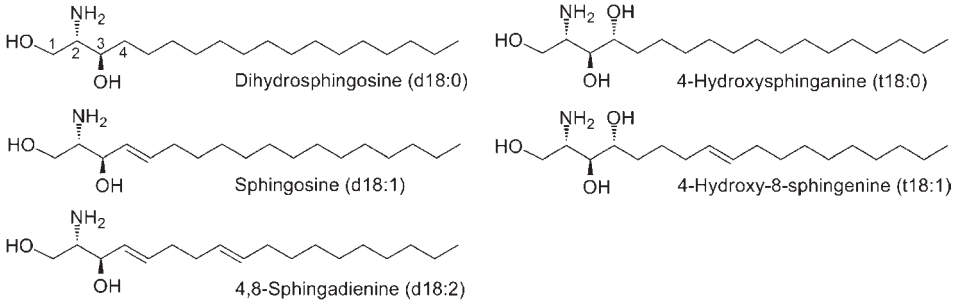

Figure 2. Overview of naturally occurring sphingoid bases.

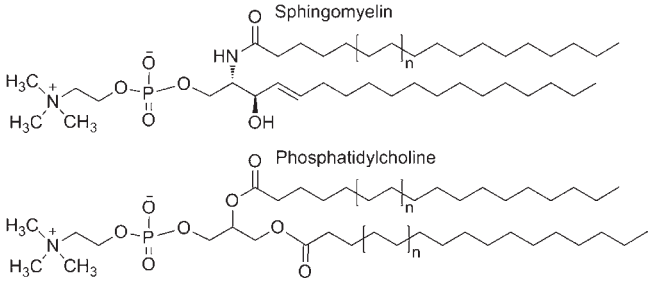

Figure 3. Structure similarity of phospholipids: sphingomyelin and phosphatidylcholine.

acid levels.[17] The analysis of human breast milk reflects fat sources of the maternal diet 12−36 h after consumption. The composition is directly associated with food intake and its preparation; for example, the use of refined and unsaturated vegetable oil for stir-frying caused significantly higher amounts of trans fatty acids in human breast milk.[18] According to Koletzko *et al.*[19] saturated even-chain fatty acids are the major group in hindmilk, with 42.76% (w/w) of the total fatty acids, with palmitic acid accounting for half of it. Cis-monounsaturated acids represented 37.98%. Furthermore, six saturated odd-chain fatty acids have been identified with only 1.16% (w/w) of the total fatty acids.[19] They can be produced from microbial metabolism and be taken up with the diet.[20]

Human breast milk is a dynamic system. Its composition is influenced by diverse maternal regional diets;[21] other associated factors might be geography, cultural traditions, and socioeconomic status.[22]

The concentration of phosphatidylcholine (Figure 3) in human breast milk is in the range of 107 nmol/mL, whereas sphingomyelin occurs in lower yield with 67 nmol/mL. Both phospholipids are important membrane constituents and serve as choline and fatty acid sources.[23]

The biological importance of sphingomyelin in human breast milk for the growth and development of infants is still unknown. In the gut intestinal alkaline sphingomyelinase hydrolyzes sphingomyelin to ceramide, which is further cleaved to sphingosine. Sphingomyelin itself and its metabolites can influence the cholesterol absorption, lipoprotein formation, and mucosal growth.[24] The activity of alkaline sphingomyelinase was analyzed in the meconium of preterm and term infants; significant amounts were observed in all gestational ages.[25] However, the sphingomyelin composition and content are of considerable interest with regard to their nutritional and functional properties. Up to now, data about the sphingomyelin content are given in total amounts without any information of the composition of the ceramide backbone. Most of the existing studies examined sphingomyelin species with sphingosine (d18:1) (Figure 2) as sphingoid base, but other amino alcohols such as 4-hydroxysphinganine (t18:0), which might reflect a vegetable-based diet, have not been considered.

In 1988 it was reported that the sphingomyelin level in human breast milk is in the range of 20−30 mg/100 mL 4−6 weeks before delivery and that the content decreases abruptly 2 weeks before delivery to 10 mg/100 mL (HPLC-UV).[26]

A later study focused on the sphingomyelin content during lactation. Milk production is divided into three phases: colostrum (1−5 days postpartum), transitional milk (6−15 days postpartum), and mature milk (after 15 days). The quantification of sphingomyelin was performed by high-performance liquid chromatography−evaporative light scattering detection (HPLC-ELSD). The content in colostrum was 40.49 ± 3.57 wt % of the total phospholipid amount (in mature milk, 124 ± 23 nmol/mL). The total phospholipid amount was slightly different in the three phases, but the sphingomyelin content remained constant.[27]

Sphingomyelin can be analyzed by conventional methods such as thin-layer chromatography (TLC) or HPLC mainly in combination with mass spectrometry (MS), which is the method of choice to obtain structural information and to perform a robust, specific, and sensitive quantification. Nowadays, high-performance liquid chromatography electrospray ionization−tandem mass spectrometry (HPLC-ESI-MS/MS) is the most commonly used method. The high specificity and sensitivity of mass spectrometry is essential, due to the large structural diversity of sphingomyelin species and their relatively low occurrence in biological samples.[28] In 2009 our group published a method for the determination of

the total sphingomyelin content of various meat samples by hydrophilic interaction liquid chromatography coupled to electrospray ionization−tandem mass spectrometry (HILIC-HPLC-ESI-MS/MS).[29] We applied this method for the analysis of sphingomyelin in human breast milk samples. Sample cleanup is based on lipid extraction followed by solid phase purification using silica gel columns. The structures of sphingomyelin species were identified after enzymatic cleavage with alkaline sphingomyelinase to the corresponding ceramides, which were separated by reversed-phase HPLC and analyzed via MS/MS experiments. Our method includes five different sphingoid bases, dihydrosphingosine (d18:0), sphingosine (d18:1(Δ4)), 4,8-sphingadienine (d18:2(Δ 4,8)), 4-hydroxysphinganine (phytosphingosine (t18:0)), 4-hydroxy-8-sphingenine (t18:1(Δ8)), and fatty acids with even-numbered carbon atoms (C12−C26) as well as their (poly)unsaturated and monohydroxylated analogues.

MATERIALS AND METHODS

Materials. Human breast milk samples of healthy volunteers were stored at −18 °C until analysis. The participants were informed in detail about the aim of this project.

Reagents. Solvents used for sample extraction and chromatography were obtained from Merck (Darmstadt, Germany), VWR (Darmstadt, Germany), and Carl Roth (Karlsruhe, Germany) in gradient grade quality. Water was purified with a Milli-Q Gradient A 10 system (Millipore, Schwalbach, Germany). The reference standards hexanoylsphingomyelin (C6-sphingomyelin) and heptadecanoylsphingomyelin (C17-sphingomyelin) were obtained from Avanti Polar Lipids (Alabaster, AL). Sphingomyelinase (from *Bacillus cereus*) for enzymatic cleavage of sphingomyelin was obtained from Sigma-Aldrich (Steinheim, Germany) and silica gel (0.061−0.100 mm) from Merck.

Stock Solutions. C6-sphingomyelin and C17-sphingomyelin were each dissolved in chloroform to a concentration of 1 μg/mL and stored at −18 °C. These solutions were further diluted according to requirements with tetrahydrofuran/methanol (3:2, v/v). C6-sphingomyelin is not present in human breast milk and can therefore be used as internal standard.

Sample Preparation. Samples were freeze-dried and thoroughly homogenized. For extraction of sphingomyelin, 10 mL of chloroform/methanol/water (60:30:8, v/v/v) was added to 100 mg of milk powder (corresponding to ca. 0.8 g fresh weight of milk). Each sample was extracted in duplicate. The homogenate was placed on a mechanical shaker for 60 min at ambient temperature and afterward vacuum filtered through a Büchner funnel until dryness. The procedure was repeated twice. The extracts were combined, evaporated to dryness, and dissolved in 1 mL of chloroform/methanol (2:1, v/v). For hydrolysis of phosphatidylcholine, 25 μL of methanolic sodium hydroxide (1 mol/L) was added to 250 μL of the obtained extract, yielding a final concentration of 0.1 mol/L, and incubated at 37 °C for 4 h. Phosphatidylcholine is mainly cleaved under this alkaline condition, whereas sphingomyelin is stable as was shown by HPLC-ESI-MS/MS measurements (data not shown). After neutralization with hydrochloric acid (1 mol/L), the solvents were evaporated to dryness. The residue was dissolved in 250 μL of chloroform/methanol (2:1, v/v). An aliquot was diluted 25-fold with tetrahydrofuran/methanol (3:2, v/v) and spiked with the reference standard C6-sphingomyelin (final concentration = 50 ng/mL) for the quantification of sphingomyelin. The calibration curve contained 1−150 ng/mL C17-sphingomyelin (as analyte) and 50 ng/mL of C6-sphingomyelin (as quantification standard). The standards were dissolved in tetrahydrofuran/methanol (3:2, v/v). The obtained samples were directly analyzed by HILIC-HPLC-ESI-MS/MS. For recovery experiments 100 mg of freeze-dried bovine milk was spiked with 5 μg of C17-sphingomyelin and treated as described above.

For structure elucidation a further cleanup was performed to separate sphingomyelin from ceramide. After extraction with chloroform/methanol/water (60:30:8, v/v/v) as described before, the solvents were evaporated to dryness and the residue was dissolved in 1 mL of chloroform. The crude lipid extract without hydrolysis of glycerophospholipids was applied to a conditioned silica gel column (Varian Bond Elute HF MEGA BE-Si 5 g, Varian, Palo Alto, CA). The columns were conditioned before by washing with 100 mL of chloroform/methanol (60:40, v/v) and 100 mL of chloroform. Fraction I contained neutral lipids and was obtained by elution with 120 mL of chloroform; 120 mL of chloroform/methanol (95:5, v/v) and 120 mL of chloroform/methanol (1:2, v/v) resulted in ceramide- and glycolipid-containing fractions II and III, respectively, and 200 mL of chloroform/methanol (1:3, v/v) and the final elution with 100 mL of methanol yielded the sphingomyelin containing fractions (fractions IV and V). For method development all fractions were analyzed by TLC and HPLC-MS/MS. The sphingomyelin-containing fractions IV and V were combined, the solvent was evaporated to dryness on a rotary evaporator, and the residue was dissolved in 1 mL of chloroform/methanol (2:1, v/v). The extract was analyzed by RP-HPLC-MS/MS to verify the absence of free ceramide.

Enzymatic Cleavage. Seven hundred and fifty microliters of the sphingomyelin-containing extract (obtained from fractions IV and V as described above) were transferred into 4 mL borosilicate glass vials with screw caps and evaporated to dryness under a stream of nitrogen at 37 °C. The residue was resuspended in 0.02 mL of 10% Triton X-100 solution and 0.08 mL of Tris-HCl solution I (200 mM, pH 7.4). After sonication for 1 min and incubation for 1 min at 37 °C, 0.08 mL of Tris-HCl solution II (200 mM, 5 mM magnesium chloride, pH 7.4) and 0.01 mL of sphingomyelinase solution (1 unit/0.01 mL) were added. This mixture was incubated for 2 h at 37 °C in a water bath. HPLC-MS/MS measurements revealed an almost complete cleavage of sphingomyelin as only traces were detectable after incubation. The reaction was stopped by the addition of 1.5 mL of chloroform/methanol (2:1, v/v) and 0.2 mL of purified water. After vortexing and centrifugation, the upper aqueous phase was removed. The lower organic phase was dried at 37 °C under a stream of nitrogen. After the residue had been dissolved in 0.1 mL of methanol/tetrahydrofuran/water (0.2% formic acid, 1 mM ammonium formate) (48:32:20, v/v/v), the solution was used for mass spectrometric investigation by RP-HPLC-MS/MS.

HILIC-HPLC-ESI-MS/MS Analysis for Quantification. Mass spectrometric experiments for quantification were performed on an API 4000 QTrap mass spectrometer (ABI Sciex, Darmstadt, Germany) coupled to an Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany). Data acquisition was performed with Analyst 1.4.2 software (ABI Sciex). For chromatographic separation a 250 × 4 mm i.d., 10 μm, LiChroCART LiChrospher Si 60 column (Merck) was used, applying a binary gradient consisting of water (1% formic acid) and acetonitrile (1% formic acid) as follows: isocratic step at 95% acetonitrile (1% formic acid) for 5 min, followed by a linear gradient to 38% acetonitrile (1% formic acid) in 15 min. The flow rate was 1 mL/min, whereas the eluate was split at the ratio of 1:1 before entering the mass spectrometer. After each run, the column was equilibrated for 10 min at the starting conditions, and the injection volume was 0.025 mL. The mass spectrometer was operated in the positive multiple reaction mode (+MRM). The resolution for Q1 and Q3 was set at ±0.35 amu. For fragmentation of the $[M + H]^+$ molecular ions into the specific fragment ion m/z 184.2, nitrogen (4.5×10^{-5} Torr) served as collision gas. Zero-grade air served as nebulizer gas (35 psi), heated at 350 °C, and as turbo gas for solvent drying (45 psi); the ion spray voltage was set at 5500 V, DP (declustering potential) at 96 V, CE (collision energy) at 29 V, and CXP (cell exit potential) at 12 V. The transition reactions monitored for a duration of 20 ms each are shown in Table 1 of the Supporting

Information. For quantification purposes, a calibration curve of six standard solutions containing 1−150 ng/mL C17-sphingomyelin (as analyte) and 50 ng/mL of C6-sphingomyelin (as quantification standard) were compiled. Therefore, the ratios of the peak area of the analyte to that of the standard were plotted against their concentration ratios.

Phosphatidylcholine and sphingomyelin can be monitored by the MRM transition of m/z 184.2, which corresponds to the loss of the phosphorylcholine headgroup. Both lipid classes have very similar properties with regard to their polarity, and phosphatidylcholine elutes closely before sphingomyelin under HILIC conditions. If the concentration of phosphatidylcholine is too high, the peaks of sphingomyelin and phosphatidylcholine are not baseline separated anymore; therefore, the hydrolysis of glycerophospholipids is essential. By using reversed-phase conditions, phosphatidylcholine and sphingomyelin elute at the same time and it is not possible to distinguish between the lipid classes.

RP-HPLC-ESI-MS/MS Analysis for Structure Elucidation. After enzymatic cleavage (see above), analysis of the obtained ceramides was performed on an API 4000 QTrap mass spectrometer (ABI Sciex) coupled to an Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany). Data acquisition was performed with Analyst 1.4.2 software (ABI Sciex). For chromatographic separation a 150 × 2 mm i.d., 3 μm, Varian Polaris C_8 column (Varian) with a 2 × 4 mm Varian Polaris C_8 precolumn was used with binary gradient. Eluents were (A) methanol/tetrahydrofuran (60:40, v/v) (1 mM ammonium formate and 0.2% formic acid) and (B) water (1 mM ammonium formate and 0.2% formic acid). An isocratic step at 80% A for 4 min was followed by a linear gradient to 100% A at 9 min with a flow rate of 0.2 mL/min; the column oven temperature was set at 40 °C. After each run, the column was equilibrated for 6 min at the starting conditions, and the injection volume was 0.025 mL. The mass spectrometer was operated in the positive multiple reaction mode (+MRM). The resolution for Q1 and Q3 was set at ±0.35 amu. For fragmentation of the $[M + H]^+$ molecular ions of ceramides into the specific fragment ions, nitrogen (4.5×10^{-5} Torr) served as collision gas. Zero-grade air served as the nebulizer gas (35 psi) and was heated at 350 °C and as turbo gas for solvent drying (45 psi); the ion spray voltage was set at 5500 V, DP at 56 V, CE at 39 V, and CXP at 15 V. The transition reactions monitored for a duration of 20 ms each are shown in Table 2 of the Supporting Information. The fragment ion m/z 262.2 is specific for the bases d18:2 and t18:1,[30,31] m/z 264.2 is reported for d18:1 and t18:0,[30,32] and d18:0 results in m/z 266.2.[32] To identify the ceramide structures three different chromatographic runs were performed corresponding to the characteristic fragment ions of the sphingoid bases at m/z 262.2 (d18:2 and t18:1), m/z 264.6 (d18:1 and t18:0), and m/z 266.2 (d18:0). In each run 102 theoretical precursor ions (Table 2 of the Supporting Information) were analyzed in MRM experiments. The structure elucidation was done via tabulating the potential base and even-chain fatty acid combinations to distinguish, for example, between ceramide species with d18:2 and t18:1, which are both monitored by the fragment ion m/z 262.2. Quantification is based on the peak area ratios of the MRM transitions of the ceramide structures referring to the amount of the corresponding sphingomyelin species. All identified sphingomyelin species of the analyzed human breast milk samples 7, 8, 10, and 15 together with their content are shown in Tables 6-1 and 6-2 of the Supporting Information.

Isotope Correction. Due to the natural abundance of isotopes of H, C, N, and O, the precursor ions $[M + H]^+$ of sphingomyelin species generate isotope signals. The highest impact has C^{13} as sphingomyelin consists of at least of 35 carbon atoms in the case of short-chain fatty acids such as lauric acid. Two sphingomyelin species with their precursor ions and isotopic signals are shown in Figure 4. The chemical formula $C_{47}H_{94}N_2O_6P$ corresponds to d18:0/C24:2; d18:1/C24:1, or d18:2/C24:0 (Figure 4A). The mass spectrum shows the molecular ion at m/z 813.7 and an isotope signal at m/z 815.7 in 20% intensity. Figure 4B shows the isotope pattern for $C_{47}H_{96}N_2O_6P$ with the molecular ion at

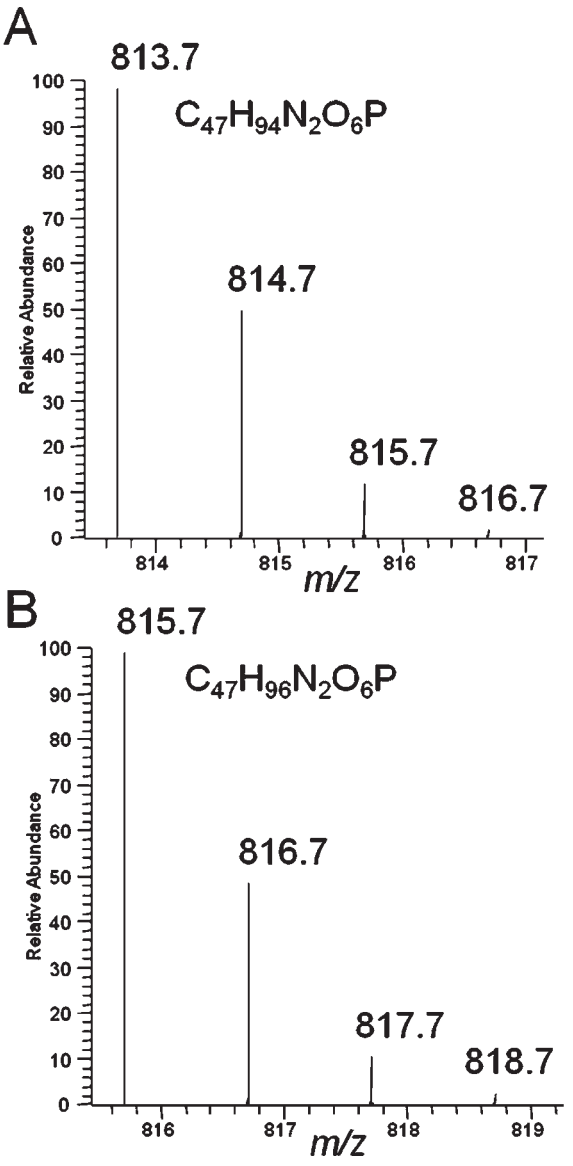

Figure 4. Natural isotope distribution of sphingomyelin species with the chemical formulas $C_{47}H_{94}N_2O_6P$ (A) and $C_{47}H_{96}N_2O_6P$ (B).

m/z 815.7 corresponding to the sphingomyelin species d18:0/C24:1 or d18:1/C24:0. It can be clearly seen from the mass spectra in Figure 4 that the isotope peak of $C_{47}H_{94}N_2O_6P$ at m/z 815.7 (Figure 4A) is overlapping with the molecular ion of $C_{47}H_{96}N_2O_6P$ at m/z 815.7 (Figure 4B). Due to this overlapping, the result of the MRM experiment for the precursor ion m/z 815.7 → 184.2 is generated by $C_{47}H_{96}N_2O_6P$ (Figure 4B) and additionally by the isotope signal of the precursor ion m/z 813.7, which belongs to $C_{47}H_{94}N_2O_6P$ (Figure 4A). For this reason the peak area of the transition m/z 815.7 → 184.2 was corrected by the peak area of the isotope signal at m/z 813.7. The correction of the peak area was achieved by the use of the calculated isotope distribution for each species. The relative abundance of isotopes was calculated by using Qual Browser and was embedded into Excel Macros to correct the corresponding peak areas.

RESULTS AND DISCUSSION

Quantification of Sphingomyelin in Human Breast Milk. For the quantification of sphingomyelin, human breast milk

Table 1. Total Amount of Sphingomyelin before and after Isotope Correction in Human Breast Milk Samples 7, 8, 10, and 15

	isotope correction sphingomyelin (mg/100 g)		
sample	before	after	difference (%)
7	4.92 ± 0.17	3.70 ± 0.13	24.8
8	3.90 ± 0.03	3.03 ± 0.03	22.3
10	2.94 ± 0.29	2.19 ± 0.21	25.5
15	2.29 ± 0.22	1.79 ± 0.16	21.8

samples were extracted with chloroform/methanol/water, glycerophospholipids such as phosphatidylcholine were hydrolyzed under alkaline condition, and the samples were analyzed using HILIC-HPLC-ESI-MS/MS (see Materials and Methods for details).

Precursor ions of sphingomyelin species were calculated for five different sphingoid bases: dihydrosphingosine (d18:0), sphingosine (d18:1(Δ4)), 4,8-sphingadienine (d18:2(Δ 4,8)), 4-hydroxysphinganine (phytosphingosine (t18:0)), and 4-hydroxy-8-sphingenine (t18:1(Δ8)). Fatty acids with even-numbered carbon atoms (C12−C26) as well as their (poly)unsaturated and monohydroxylated analogues in combination with the five sphingoid bases resulted in 600 theoretical precursor ions (see Tables 3-1−3-8 in the Supporting Information). Odd-chain fatty acids were not included in this study, due to their minor occurrence in human breast milk.[19] The overlap of precursor ions of different sphingomyelin species with the same molecular mass, for example, $[M + H]^+$ of m/z 701.5, belonging to d18:2/C16:0, d18:1/C16:1, and d18:0/C16:2, reduced the analyzed transitions from 600 in total to 102, which are given in Table 1 of the Supporting Information. As glycerophospholipids such as phosphatidylcholine (Figure 3) exhibited the same fragment as sphingomyelin at m/z 184.2 (loss of phosphorylcholine) and would therefore interfere with the quantification, glycerophospholipids were removed by alkaline hydrolysis during the sample cleanup (see Materials and Methods for details). Small concentrations of phosphatidylcholine remaining after alkaline hydrolysis could be chromatographically separated from the sphingomyelin and did not interfere with the interpretation of the results.

Another challenge occurring during the quantification of sphingomyelin is the overlapping of isotope signals. In the example shown in Figure 4, the $[M + H]^+$ signal of the sphingomyelin with the chemical formula $C_{47}H_{96}N_2O_6P$ at m/z 815.7 (Figure 4B) overlaps with the isotope signal at m/z 815.7 of the sphingomyelin with the chemical formula $C_{47}H_{94}N_2O_6P$ (Figure 4A). For this reason an isotope correction was performed as described under Materials and Methods.

Due to the time-consuming and complex calculation, the isotope correction was exemplarily performed only for samples 7, 8, 10, and 15. Table 1 shows the total amount of sphingomyelin of these samples before and after isotope correction (detailed tables of all monitored transitions can be found in the Supporting Information, Tables 5-1 and 5-2). As can be seen from Table 1 the results are not much affected by the overlapping isotope signals, and 22−25% lower concentrations were observed after isotope correction.

The total amount of sphingomyelin (without isotope correction) in 20 human breast milk samples varied from 3.08 to 9.07 mg/100 g fresh weight (Table 2), taking into account the recovery rate of 58.2 ± 4.45%. As the dry weights of the analyzed human breast milk samples varied over a broad range from 11 to 16%, all results are calculated for the fresh weights.

Table 2. Total Amount of Sphingomyelin in Human Breast Milk

sample	dry weight (%)	total amount of SM (mg/100 g)	total amount of SM (mg/100 g) corrected by recovery rate (58.2 ± 4.45%)
1	13.4	4.85[a]	8.33
2	12.3	4.42 (±7.1%)	7.59
3	12.6	4.38 (±7.2%)	7.53
4	13.5	**4.00**/6.1[b]	6.87
5	13.3	5.28 (±0.0%)	9.07
6	13.5	2.88 (±0.7%)	4.95
7	14.3	3.70 (±0.1%)[c]	6.36
8	13.3	3.03 (±0.0%)[c]	5.21
9	12.5	2.90 (±7.1%)	4.98
10	16.1	2.19 (±0.2%)[c]	3.76
11	11.6	2.78 (±9.8%)	4.78
12	12.1	2.37 (±9.0%)	4.07
13	12.6	2.75 (±5.7%)	4.73
14	12.9	3.56 (±5.3%)	6.12
15	13.0	1.79 (±0.2%)[c]	3.08
16	11.6	**2.04**/1.4[b]	3.51
17	13.4	**3.32**/1.4[b]	5.70
18	12.8	2.25 (±14.7%)	3.87
19	12.5	3.42 (±8.4%)	5.88
20 (pool sample)	13.0	2.60 (±10.9%)	4.47

[a] Without repeat determination due to insufficient sample quantity. [b] Deviation >15%: the boldface entry was chosen as the final amount. [c] After isotope correction.

Structure Elucidation of Sphingomyelin in Human Breast Milk. For structure elucidation of sphingomyelin, the extracted milk samples were purified by solid-phase extraction using silica gel columns to separate sphingomyelin and ceramide. Human breast milk samples 7, 8, 10, and 15 were chosen randomly for detailed analysis regarding fatty acid and sphingoid base composition. The obtained sphingomyelin fractions were treated with sphingomyelinase and therefore cleaved to the corresponding ceramides, which were then analyzed using RP-HPLC-ESI-MS/MS by monitoring the characteristic MRM transitions for the five different sphingoid bases (see Materials and Methods for details).

Sphingosine (d18:1) was determined as the predominant sphingoid base at 83.6 ± 3.5% in human breast milk, followed by 4,8-sphingadienine (d18:2) at 7.2 ± 1.9% and 4-hydroxysphinganine (t18:0) at 5.7 ± 0.7%. Dihydrosphingosine (d18:0) and 4-hydroxy-8-sphingenine (t18:1) appeared in the range of 0.6−1.0% (Figure 5).

The analyzed samples 7, 8, 10, and 15 showed a comparable distribution of sphingoid bases, the major and minor sphingoid bases occurr in the same ratio. The distribution of saturated and unsaturated fatty acids with up to four double bonds in the analyzed sphingomyelin species is demonstrated in Figure 6. These data were calculated from all sphingomyelin species including the five mentioned sphingoid bases in four breast milk

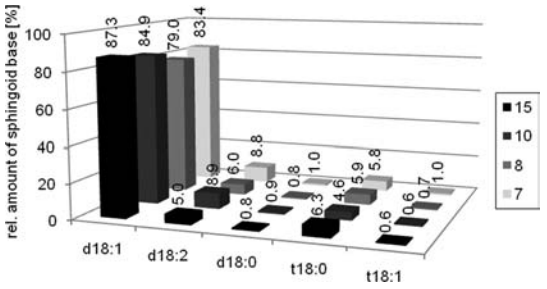

Figure 5. Pattern of detected sphingoid bases in human breast milk samples 7, 8, 10, and 15.

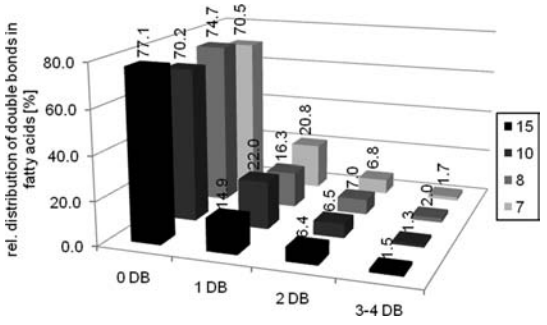

Figure 6. Distribution of double bonds (DB) in fatty acids demonstrated for all sphingomyelin species in human breast milk samples 7, 8, 10, and 15.

samples. The content of saturated fatty acids with 73.1 ± 3.3% exceeded the amount of unsaturated species.

The amount of monosaturated fatty acids was 18.5 ± 3.4%; the concentration of those with two double bonds was lower at 6.7 ± 0.3%. The fatty acids with three to four double bonds or monohydroxylated species were only minor components in the analyzed samples.

As sphingosine was determined as the predominant sphingoid base in human breast milk, we compared the fatty acid composition for this single base in detail (Figure 7).

The main fatty acids were palmitic acid at 14.9 ± 2.2%, stearic acid at 12.7 ± 1.5%, docosanoic acid at 16.2 ± 3.6%, and tetracosenoic acid (C24:1) at 15.0 ± 3.1%. Fatty acids with C12 and C26 are only minor components. As shown in Figure 6 the unsaturated fatty acids appeared in lower yields than the corresponding saturated fatty acids, for example, stearic acid at 12.7 ± 1.5% and oleic acid at 0.4 ± 0.1% or eicosanoic acid at 10.9 ± 2.0% and eicosenoic acid at 0.3 ± 0.1%. The only exception is tetracosenoic acid (C24:1) at 15.0 ± 3.1%, which had a higher content than the saturated tetracosanoic acid (C24:0) at 8.1 ± 0.9%.

Interestingly, the fatty acid compositions of sphingomyelin species with different sphingoid bases are not identical. We compared the distribution of fatty acids linked to sphingosine and the second main base 4,8-sphingadienine (d18:2). The predominant acids for 4,8-sphingadienine were eicosanoic acid and tetracosanoic acid; palmitic acid occurred only in lower yields (figure not shown, data are given in the Supporting Information, Tables 6-1 and 6-2). The high concentration of saturated fatty acids in comparison to unsaturated species can be applied for both main bases, but the main fatty acids are not identical. A big difference for 4,8-sphingadienine-linked fatty acids was the lower concentration of tetracosenoic acid (C24:1)

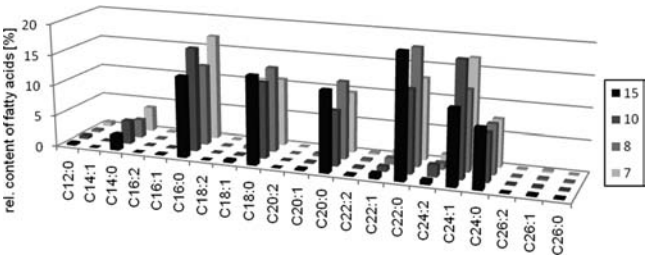

Figure 7. Detailed fatty acid composition of sphingomyelin species with the predominant sphingoid base sphingosine in human breast milk samples 7, 8, 10, and 15.

compared to tetracosanoic acid (C24:0), which was opposite for sphingosine. These results indicate that the formation of ceramides is somehow regulated and the fatty acids are not consistently distributed to the five sphingoid bases in the same manner.

In the Supporting Information (Tables 6-1 and 6-2) detailed information about all analyzed sphingomyelin species of the four exemplary samples is available.

The fatty acid composition of sphingomyelin species in this study differs from the total fatty acids in human breast milk. The predominant fatty acids in human breast milk are saturated, such as myristic acid, palmitic acid, and stearic acid; among the unsaturated fatty acids, oleic acid (18:1ω9) and the essential linoleic acid (18:2ω6) occur in higher concentrations.[18] The fatty acid composition of the ceramide backbone of sphingomyelin is not affected by fatty acids, which are predominantly available in human breast milk. The main fatty acids in sphingomyelin have longer chain length compared to the total fatty acid composition in human breast milk (see Figure 7).

This is the first report about the structural variety of sphingomyelin species in human breast milk. For further conclusions a larger number of characterized study samples with regard to background information such as time of lactation, dietary habits, socioeconomic factors, and health status of the volunteering donor mothers are required.

■ ASSOCIATED CONTENT

Supporting Information. The 102 analyzed precursor ions of sphingomyelin are presented in Table 1, and the corresponding precursor ions for the ceramide backbone are listed in Table 2. The theoretical precursor ions for sphingomyelin species: fatty acids with even-numbered carbon atoms (C12−C26) as well as their (poly)unsaturated and monohydroxylated analogues in combination with the 5 sphingoid bases resulted in 600 theoretical precursor ions, which are shown in Tables 3-1−3-8. The theoretical precursor ions for the ceramide backbone are given in Tables 4-1−4-8. The results of isotope correction of four samples are listed in Tables 5-1 and 5-2. All identified sphingomyelin species are shown in Tables 6-1 and 6-2. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

CE, collision energy; CXP, cell exit potential; d18:0, dihydrosphingosine; d18:1, sphingosine; d18:2, 4,8-sphingadienine; DP, declustering potential; ELSD, evaporative light scattering detector; ESI, electrospray ionization; HILIC, hydrophilic interaction liquid chromatography; HPLC, high-performance liquid chromatography; i.d., inner diameter; IS, internal standard; MRM, multiple reaction monitoring; MS, mass spectrometer; MS/MS, tandem mass spectrometer; *m*/*z*, mass-to-charge ratio; rel, relative; t18:0, 4-hydroxysphinganine; t18:1, 4-hydroxy-8-sphingenine; TLC, thin layer chromatography; V, volt; v/v, volume to volume; w/w, weight to weight.

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