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Analysis of Sphingomyelin in Meat Based on Hydrophilic Interaction Liquid Chromatography Coupled to Electrospray Ionization−Tandem Mass Spectrometry (HILIC-HPLC-ESI-MS/MS)

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The amount of sphingomyelin in different kinds of meat was analyzed by hydrophilic interaction liquid chromatography (HILIC-HPLC) coupled with electrospray ionization−tandem mass spectrometry (ESI-MS/MS). Analysis comprised sphingomyelin species with the five different sphingoid bases dihydrosphingosine (d18:0), sphingosine (d18:1$^{\Delta4}$), 4,8-sphingadienine (d18:2$^{\Delta4,8}$), 4-hydroxysphinganine (phytosphingosine (t18:0)), and 4-hydroxy-8-sphingenine (t18:1), and fatty acids with 12−26 carbon atoms as well as their (poly)unsaturated (up to four double bonds) and monohydroxylated analogues. Most sphingolipids contained sphingosine (d18:1) as the predominant sphingoid base, while stearic acid and palmitic acid were found as prevalent fatty acids. Total amounts vary from 361−471 mg/kg, whereas the meat of the wild animals showed considerably lower amounts.

KEYWORDS: Sphingolipids; sphingomyelin; ESI-MS/MS; hydrophilic interaction chromatography; HILIC-HPLC

INTRODUCTION

Since their first description by the German medic J. L. W. Thudichum in 1884 (*1*), sphingolipids have attracted more and more attention in research as they are highly bioactive compounds and do not only serve as components of biological structures such as membranes and lipoproteins but also participate in diverse cellular processes, for example, cell proliferation, differentiation, cell−cell and cell−matrix interactions, cell migration, membrane trafficking, intra- and extracellular signaling, autophagy, and apoptotic cell death (*2*−*5*).

Sphingolipds are the structurally most diverse class of lipids. Their common structural element is a long-chain amino-alcohol, the so-called sphingoid base. Complex sphingolipids such as sphingomyelin are composed of this sphingoid base, an amide-linked fatty-acid, as well as an additional polar headgroup attached to the hydroxyl group in position 1 of the sphingoid base (**Figure 1**). They are present in essentially all eukaryotes and a few prokaryotes and therefore are ubiquitous constituents of food. Variations in chain length and degree of saturation and/or hydroxylation in the sphingoid base (**Figure 2**) as well as in the fatty acid lead to their huge complexity. Furthermore, additional variations in the polar headgroup also contribute to the enormous variety.

Sphingolipids of mammalian origin mainly contain sphingosine (d18:1), whereas the double bond is enzymatically introduced between the carbon atoms C4 and C5. Besides, smaller amounts of sphinganine (d18:0) and 4-hydroxysphinganine (t18:0) are reported (*6*−*8*). Abbreviations of the sphingoid bases are used according to that of the fatty acids: the numbers quote the chain length as well as the number of double bonds. The prefix d or t indicates the number of hydroxyl groups of the di- and trihydroxy bases.

The amide-linked fatty acids are mostly long-chain or very long-chain and of monounsaturated or saturated character. Apart from variations due to tissue type, diet, and age, the predominant fatty acid of mammalian sphingomyelin is palmitic acid (C16:0), accompanied by smaller amounts of nervonic acid (C24:1), lignoceric acid (C24:0), and behenic acid (C22:0) (*6*, *9*). Appreciable amounts of sphingomyelin, possessing phosphorylcholine as the polar headgroup (**Figure 1**), are described for dairy products, meat, fish, and egg, while it has not been reported in plants yet (*10*).

Although sphingolipids occur in considerable amounts in foods such as soy and dairy products, which are considered to be protective against carcinogenesis, they have not tracked as much attention as other food constituents such as, for example, isoflavones. Physiological effects of their consumption in humans are still elusive, but several animal studies have shown that administration of, for example, sphingomyelin inhibited colon carcinogenesis (*11*−*17*), reduced cholesterol absorption (*18*−*20*). For the inhibition of carcinogenesis, the effects are attributed to the hydrolysis products of sphingomyelin. In contrast, reduction of cholesterol is attributed to sphingomyelin itself (*19*, *21*). For distinct biological activities, the presence or absence of the trans double bond in the sphingoid base is crucial (*22*, *23*), and animal studies revealed an increasing potency in the reduction of cholesterol levels for sphingomyelin species with an increasing chain

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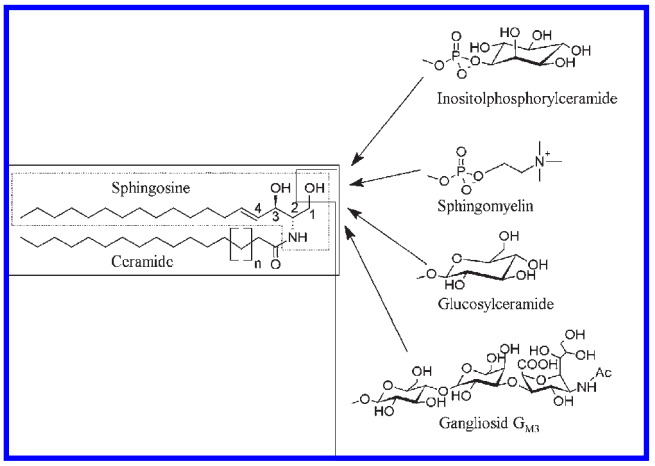

Figure 1. Representative structures of complex sphingolipids in mammals and plants.

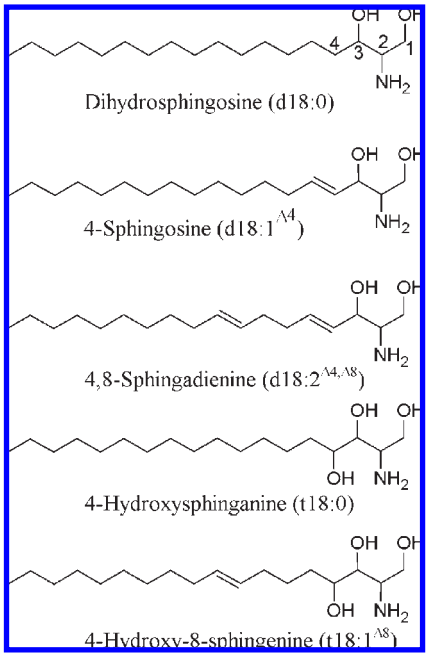

Figure 2. Structure of the five investigated sphingoid bases, differing in the number of hydroxyl groups and double bonds.

length and degree of saturation of the fatty acid moiety (*19*). Therefore, information about the nature of the contained sphingoid bases and fatty acids is important for the evaluation of possible biological effects. Hence, structural elucidation on the intact, not hydrolyzed sphingomyelin species is necessary.

Although hints for a protective role of sphingolipids against several diseases accrue, information about amounts and structural composition of sphingolipids in foods is still rather limited (*21*, *24*). This lack of knowledge is due to several obstacles in the analysis of sphingolipids. The complex food matrix often hampers a simple detection of their relatively small amounts. Therefore, preliminary cleanup procedures have to be developed. Besides, the enormous structural diversity and missing reference standards pose special problems. Systematic studies concerning the occurrence and composition of sphingolipids in food have hardly been performed yet, and only a very limited number of publications concerning sphingomyelin in food is available (*25*−*31*). Often, the sphingolipids are submitted to hydrolysis

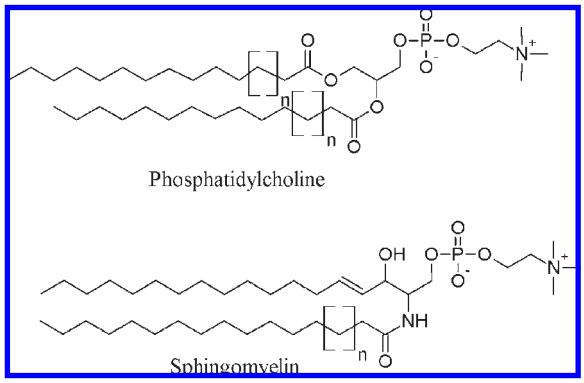

Figure 3. Structural similarity of sphingomyelin and phosphatidylcholine. Both possess the zwitterionic phosphorylcholine group.

before their components such as fatty acids and sphingoid bases are analyzed using techniques such as thin layer chromatography (TLC), high performance liquid chromatography−evaporative light scattering detection (HPLC-ELSD), gas chromatography−flame ionization detection (GC-FID), or gas chromatography−mass spectrometry (GC-MS). However, these procedures may introduce artifacts, imply the loss of structural information due to the hydrolysis step, and are less sensitive compared to the HPLC-MS/MS based methods. For these reasons, much data of sphingolipid contents in food rely on estimations (*21*).

For the past few years, electrospray ionization−tandem mass spectrometry (ESI-MS/MS) has played an emerging role in the analysis of sphingolipids. However, recent mass spectrometric investigations of sphingolipids have mainly been used for cell culture studies and not for the analysis of complex food samples. They provide information about intact known and unknown sphingolipid species and allow their simultaneous quantification (*32*, *33*).

Therefore, a new hydrophilic interaction chromatography (HILIC)-HPLC-ESI-MS/MS method for the quantification of sphingomyelin in mammalian tissue has been developed. In order to maximize sensitivity, the transition of the precursor ions to the fragment ion formed by the phosphatidylcholine moiety has been monitored. Because of the large structural similarity, phosphatidylcholine yields the same fragment ion, and its isotopes would influence the quantification of sphingomyelin without chromatographic separation (**Figure 3**). By using a chromatographic method based on the principles of hydrophilic interaction chromatography (HILIC), we achieved the separation of sphingomyelin and phosphatidylcholine (**Figure 4**). HILIC conditions were obtained by running a silica gel normal phase column under reversed phase conditions.

Subsequent to the quantification, the polar phosphorylcholine group was cleaved enzymatically since otherwise the comprehensive structural determination of sphingomyelins is hampered because of the very dominant fragment ion formed by the phosphorylcholine group. Mass spectrometric investigations of the obtained corresponding ceramides allowed structural elucidation of sphingomyelin species present in food samples. Prior to the enzymatic hydrolysis, we ensured by HPLC-MS/MS-analysis that the sphingomyelin fractions did not contain ceramides.

MATERIALS AND METHODS

Materials. Meat from pigs (*Sus scrofa domestica*) and beef (*Bos primigenius f. taurus*) was freshly bought in local supermarkets. Meat of wild boar (*Sus scrofa*) and roe deer (*Capreolus capreolus*) came from animals of the regional wild areas and were obtained from local hunters.

Reagents. Solvents used for sample extraction and chromatography were obtained from Merck (Darmstadt, Germany), VWR (Darmstadt,

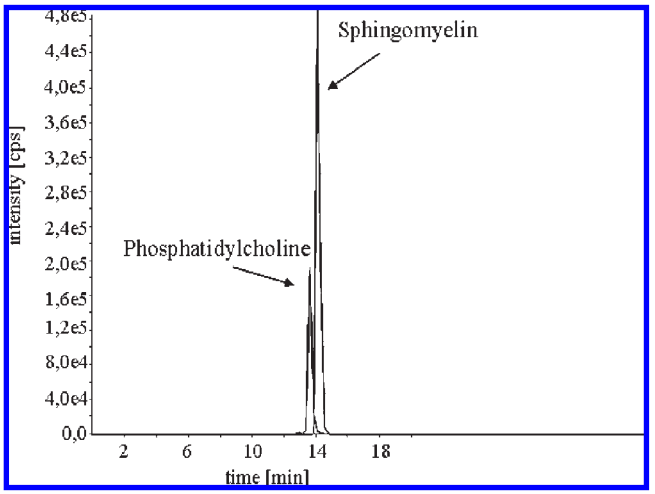

Figure 4. Chromatographic separation of phosphatidylcholine and sphingomyelin using HILIC conditions.

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 hexanoyl-sphingomyelin (C6-sphingomyelin) and acetyl-sphingomyelin (C2-sphingomyelin) were obtained from Avanti Polar Lipids (Alabaster, USA). Bovine brain sphingomyelin and sphingomyelinase (from *Bacillus cereus*) for enzymatic cleavage of sphingomyelin was from Sigma-Aldrich (Steinheim, Germany). Silica gel (0.061−0.100 mm) was obtained from Merck (Darmstadt, Germany).

Stock Solutions. C2-sphingomyelin and C6-sphingomyelin were each dissolved in chloroform to a concentration of 1 μg/mL and stored at −18 °C to increase stability. These solutions were diluted according to requirements with tetrahydrofuran/methanol (3/2, v/v).

Sample Preparation. Samples were freeze-dried and thoroughly homogenized. For extraction of sphingomyelin, 100 mL of chloroform/methanol/water (60/30/8, v/v) was added to an amount corresponding to 25 g of fresh weight. The homogenate was placed on a mechanical shaker for 60 min at ambient temperature and afterward vacuum filtered through a Buechner funnel until dryness. The procedure was repeated twice. The extracts were combined and evaporated to dryness.

For a further cleanup, the crude lipid extract was dissolved in 20 mL of chloroform and applied to a silica gel column. Therefore, silica gel (about 10 g) was poured into a glass column plugged with glass wool, washed with chloroform/methanol (40/60, v/v), and finally conditioned with 100 mL of chloroform. Elution with chloroform yielded the simple lipids (fraction I), chloroform/methanol (95/5, v/v) gave a ceramide and glycolipid containing fraction (fraction II), and chloroform/methanol (40/60, v/v) and chloroform/methanol (1/2, v/v) yielded the sphingomyelin containing fractions (fractions III and IV). A final methanol fraction (fraction V) was tested negative for the presence of sphingomyelin. Conditioning and elution volume were 100 mL each. The solvents were evaporated to dryness on a rotary evaporator.

The sphingomyelin containing fractions were combined and subsequently submitted to an alkaline hydrolysis. Therefore, methanolic sodium hydroxide (1 mol/L) was added to an aliquot of the corresponding fraction, yielding a final concentration of 0.1 mol/L and incubated at 37 °C for 3 h. Under these conditions, phosphatidylcholine is mainly cleaved, while sphingomyelin is stable. After neutralization, the solvents were evaporated to dryness. The residue was dissolved in chloroform/methanol (2/1, v/v). An aliquot was spiked with the reference standard C2-sphingomyelin (final concentration 50 ng/mL) and diluted 100-fold with tetrahydrofuran/methanol (3/2, v/v) for the quantification of sphingomyelin. The analysis was performed in duplicate. For recovery experiments, beef was spiked with 2.5 mg/kg and 12 mg/kg C6-SM. The samples were treated as described above.

Enzymatic Cleavage. The sphingomyelin containing fractions (0.1 mL) (tested negative for ceramides by HPLC-MS/MS) were transferred into 4-mL borosilicate glass vials with screw caps. The solvent was evaporated to absolute dryness under a stream of nitrogen at 40 °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 0.02 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. 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, the phases were separated by centrifugation, and the upper aqueous phase was removed. The lower, organic phase was dried at 37 °C under a stream of nitrogen. After dissolving the residue in 0.1 mL of tetrahydrofuran/methanol (3/2, v/v), the solution was used for mass spectrometric investigation.

Table 1. Measured Transition Reactions of Sphingomyelin Species in Positive MRM-Mode for the Quantification of Sphingomyelin in Meat

fragment ion					
m/z = 184.2					
m/z precursor $[M + H]^+$					
643.5	689.5	731.5	771.6	807.6	843.5
645.5	691.5	733.5	773.5	811.5	845.5
647.5	693.5	735.6	775.5	813.6	847.7
649.5	699.5	737.6	777.6	815.5	849.7
659.5	701.6	743.5	783.5	817.5	855.5
661.5	703.5	745.6	785.6	819.6	857.7
663.5	705.5	747.6	787.5	821.6	859.7
665.5	707.5	749.6	789.5	825.7	861.7
671.5	709.5	755.5	791.6	827.7	863.7
673.5	715.5	757.6	793.6	829.5	873.7
675.5	717.6	759.5	797.6	831.5	875.7
677.5	719.6	761.5	799.7	833.7	877.7
679.5	721.6	763.6	801.5	835.6	
681.5	727.5	765.6	803.5	839.5	
687.5	729.6	769.6	805.6	841.6	

calibration curve		
	m/z precursor $[M + H]^+$	*m/z* fragment ion
C2-SM	507.3	184.2
C6-SM	563.4	184.2

HILIC-HPLC-ESI-MS/MS Analysis for Quantification. Mass spectrometric experiments for quantification were performed on an API 4000 QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany). Data acquisition was performed with Analyst 1.4.2 software (Applied Biosystems, Darmstadt, Germany). For chromatographic separation, a 250 × 4 mm i.d., 10 μm, LiChroCART LiChrospher Si 60 column (Merck, Darmstadt, Germany) 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 1:2 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). For fragmentation of the $[M + H]^+$ molecular ions into the specific fragment ions, nitrogen (4.5×10^{-5} Torr) served as collision gas. Zero-grade air served as nebulizer gas (35 psi) and was heated at 350 °C, 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 150 ms each are shown in **Table 1**. For quantification purposes, a calibration curve of seven standard solutions containing 5−250 ng/mL C6-sphingomyelin (as analyte) and 50 ng/mL of C2-sphingomyelin (as quantification standard) was compiled. Therefore, the peak area ratios of the analyte to that of the standard were plotted against their concentration ratios.

Table 2. Measured Transition Reactions of Corresponding Ceramide Species in Positive MRM-Mode for Structural Elucidation of Sphingomyelin in Meat

1. fragment ion	m/z = 266.2 (d18:0, t18:0)				
2. fragment ion	m/z = 264.2 (d18:1, t18:1)				
3. fragment ion	m/z = 262.2 (d18:2)				
	m/z precursor $[M + H]^+$				
478.5	524.5	566.5	606.6	642.6	678.5
480.5	526.5	568.5	608.5	646.5	680.5
482.5	528.5	570.6	610.5	648.6	682.7
484.5	534.5	572.6	612.6	650.5	684.7
494.5	536.6	578.5	618.5	652.5	690.5
496.5	538.5	580.6	620.6	654.6	692.7
498.5	540.5	582.6	622.5	656.6	694.7
500.5	542.5	584.6	624.5	660.7	696.7
506.5	544.5	590.5	626.6	662.7	698.7
508.5	550.5	592.6	628.6	664.5	708.7
510.5	552.6	594.5	632.6	666.5	710.7
512.5	554.6	596.5	634.7	668.7	712.7
514.5	556.6	598.6	636.5	670.6	
516.5	562.5	600.6	638.5	674.5	
522.5	564.6	604.6	640.6	676.6	

HILIC-HPLC-ESI-MS/MS Analysis for Structural Elucidation. After enzymatic cleavage, analysis of the obtained ceramides was performed on an API 4000 QTrap mass spectrometer (Applied Biosystems, Darmstadt, Germany) coupled to an Agilent 1100 series HPLC (Agilent Technologies, Waldbronn, Germany). Data acquisition was performed with Analyst 1.4.2 software (Applied Biosystems, Darmstadt, Germany). For chromatographic separation, a 250 × 4 mm i. d., 10 μm, LiChroCART LiChrospher Si 60 column (Merck, Darmstadt, Germany) was used under isocratic conditions. Eluents were water (1% formic acid) and acetonitrile (1% formic acid) as follows: 95% acetonitrile (1% formic acid) for 7 min, with a flow rate of 1 mL/min, whereas the eluate was split at the ratio of 1:2 before entering the mass spectrometer. After each run, the column was equilibrated for 10 min at the starting conditions, and the injection volume was 0.020 mL. The mass spectrometer was operated in the positive multiple reaction mode (+MRM). For fragmentation of the $[M + H]^+$ molecular ions 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, as turbo gas for solvent drying (45 psi), the ion spray voltage was set at 5500 V, DP at 96 V, CE at 29 V, and CXP at 12 V. The transition reactions monitored for a duration of 150 ms each are shown in **Table 2**.

Isotopic Correction. A common obstacle in the analysis of complex mixtures of lipids is the possible variation in the number of contained double bonds leading to a series of molecular masses only differing by two mass units. Furthermore, every sphingomyelin species gives not only its monoisotopic peak but also a whole cluster of peaks because of the natural isotopic distribution of their constituting elements carbon, hydrogen, oxygen, phosphate, and nitrogen. In particular, the large number of carbon atoms yields highly abundant isotopic peaks. This results in overlapping signals which may give errors in quantification of a single sphingomyelin species.

To counteract this effect, an isotopic correction has been performed. For each molecule with a molecular mass M, the contribution of the molecule with M-2 to the total peak area was calculated from their naturally occurring isotopic distribution. The obtained relative amounts were then used as a correction factor for the experimental data to evaluate the revised peak area.

RESULTS AND DISCUSSION

Quantification of Sphingomyelin in Meat Samples. The amount of sphingomyelin was investigated in pig (*Sus scrofa domestica*), beef (*Bos primigenius f. taurus*), wild boar (*Sus scrofa*), and roe deer (*Capreolus capreolus*) considering the five different sphingoid bases dihydrosphingosine (d18:0), sphingosine (d18:1$^{\Delta 4}$), 4,8-sphingadienine (d18:2$^{\Delta 4,8}$), 4-hydroxysphinganine (phytosphingosine (t18:0)), and 4-hydroxy-8-sphingenine (t18:1). For each sphingoid base, the linkage with fatty acids of 12−26 carbon atoms with up to four double bonds as well as their hydroxylated analogues has been taken into account leading to a number of 720 theoretical precursor ions (cf Table 3−14 in Supporting Information). In order to maximize sensitivity, the transitions of the precursor ions to the fragment ion formed by the phosphatidylcholine moiety have been monitored for the quantification of total amounts. Different sphingomyelin species can show identical molecular masses. For example, the sphingomyelin species d18:0, C18:2, d18:1, C18:1 as well as d18:2, and C18:0 have the same molecular mass and therefore form identical precursor ions. Because of this overlap, the effective number of measured transitions for quantification of sphingomyelin in meat was reduced to 87 as shown in **Table 1**.

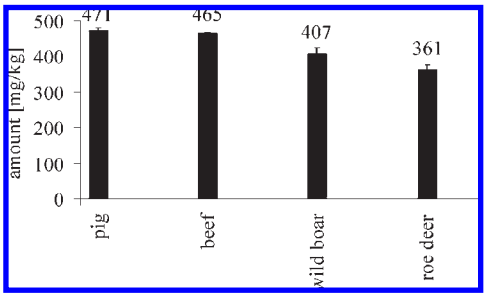

Figure 5. Total amounts of sphingomyelin in meat after isotopic correction.

Recovery rates were determined by spiking beef with 2.5 mg/kg and 12 mg/kg C6-SM. For each concentration, two samples were extracted, cleaned up, and subsequently measured using the developed HILIC-HPLC-ESI-MS/MS method. Recovery rates of 91.3% ± 5.3% and 80.4% ± 3.2% were obtained, respectively. The limit of quantification is < 1 μg/kg, whereas a signal-to-noise ratio of 9/1 was not undercut. Using a smaller dilution factor or a higher initial sample weight would even increase sensitivity.

As shown in **Figure 5**, total amounts of sphingomyelin vary from 361 to 471 mg/kg after isotopic correction. While these amounts are distributed over 39 and 40 transitions in pigs and beef, respectively, wild boar and roe deer showed signals for 46 and 54 transitions, respectively. This indicates a more diverse sphingomyelin pattern in wild animals than in livestock. Beyond this, the total amounts are clearly lower. Both effects are probably due to the different diets of feral animals.

Structural Investigation of Sphingomyelin in Meat Samples. As described above, the contained phosphorylcholine moiety hampers a comprehensive mass spectrometric structural elucidation of sphingomyelins by forming a very dominant fragment ion. Therefore, the sphingomyelin fraction was submitted to an enzymatic cleavage with a bacterial sphingomyelinase. The obtained ceramides made further structural investigation accessible.

Hence, a number of sphingomyelin species were clearly identified. For pigs, 27 species have been found in 17 transitions comprising an amount of 344 mg/kg or 73% of the total amount. For beef, 24 species (329 mg/kg) have been clearly identified in 16 different transitions, while for wild boar and roe deer 29 (280 mg/kg) and 35 (256 mg/kg) sphingomyelin species in 20 and 23 transitions, respectively, have been elucidated. This corresponds to a portion of 71% of the determined total amount in all three cases. The lacking 27−29%, making an absolute amount of 105−136 mg/kg, could not be clearly identified. In case of pigs and beef, these amounts were distributed over 22 and 24 transitions, respectively, while for wild boar and roe deer, it comprised 26 and 31 transitions, respectively. For this reason, the measured intensities were too small, and further structural investigations became impossible.

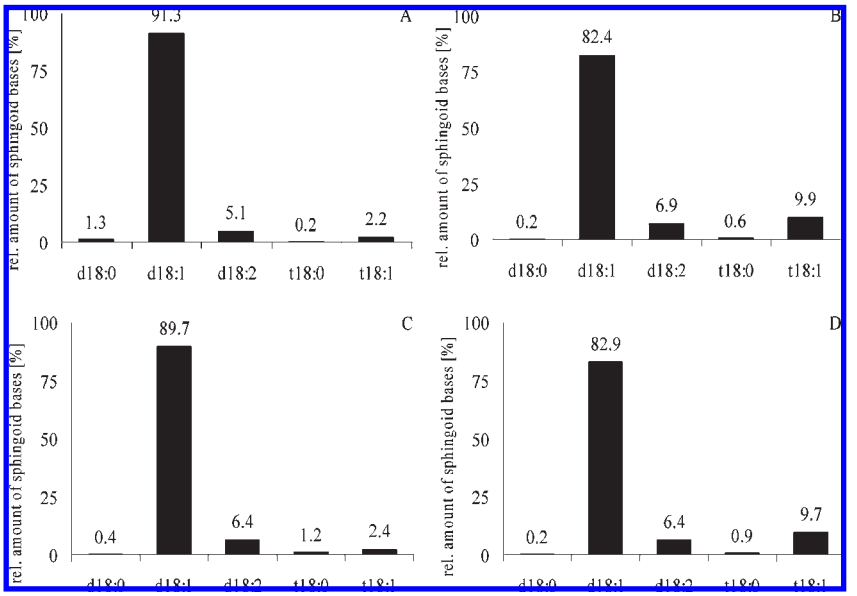

Figure 6. Pattern of sphingoid bases dihydrosphingosine (d18:0), sphingosine (d18:1$^{\Delta 4}$), 4,8-sphingadienine (d18:2$^{\Delta 4,8}$), 4-hydroxyspinganine (phytosphingosine (t18:0)), and 4-hydroxy-8-sphingenine (t18:1) in sphingomyelin of pig (**A**), beef (**B**), wild boar (**C**), and roe deer (**D**).

All five sphingoid bases have been detected. Their relative amount with respect to the identified sphingomyelin species is depicted in **Figure 6**. It is clearly visible that in all cases, sphingosine (d18:1$^{\Delta 4}$) forms the most prevalent sphingoid base. Interestingly, meat of the ruminants beef and roe deer shows slightly increased levels of t18:1. **Figure 7** shows the relative distribution of fatty acids of different chain length with respect to the identified sphingomyelin species. The most important role is played by fatty acids with 16−18 carbon atoms (palmitic and stearic acids) with a portion of about 70% in all of the investigated samples, followed by the long chain fatty acids up to 26 carbon atoms comprising about 30%. Short chain fatty acids with 12 or 14 carbon atoms account for only about 1% in pigs and wild boar, while their relative amounts in the ruminants beef and roe deer account for about 3%. The differences in the pattern of fatty acids are only marginal. A sphingomyelin consisting of sphingosine (d18:1$^{\Delta 4}$) as sphingoid base linked to stearic acid (C18:0) forms the most abundant sphingomyelin in pigs (43%), beef (36%), and roe deer (31%), while it is the second most abundant in wild boar (31%). The most prevalent sphingomyelin in wild boar consists of sphingosine (d18:1$^{\Delta 4}$) linked to palmitic acid (C16:0), representing a portion of 32% (with respect to the total amount). This sphingomyelin again (d18:1, C16:0) is the second most prevalent in pigs (23%), beef (21%), and roe deer (25%). These numbers show that 50−60% of the contained sphingomyelins in the investigated meat samples are formed by only two different sphingomyelin species.

With a total amount of 361 to 471 mg/kg, the sphingomyelin content of meat is relatively high and contributes considerably to the estimated daily intake of 0.3−0.4 g/day (*21*). Since the referred study took significantly lower amounts for meat as the basis, these data suggest an even higher actual daily intake. According to an early study of Nilsson et al. (*34*), sphingomyelin hydrolysis takes place in the small intestine and the colon. Although the cleavage and absorption was incomplete, 25% of the administered dose (5 mg) was found in the feces, mainly as ceramide, a considerable amount of the dietary sphingomyelin remained in the intestine. In several animal studies, sphingomyelin supplementation at 0.1% of the diet (w/w) has reduced the number of aberrant colonic crypt foci (an early marker of colon carcinogenesis) by ∼70% as well as the number of adenocarcinomas in mice

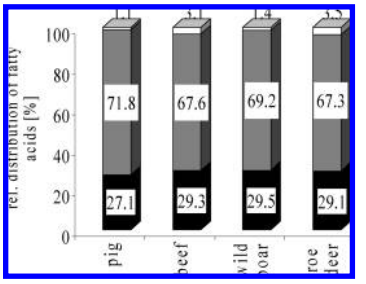

Figure 7. Distribution of fatty acids according to their chain length in sphingomyelin species of pig, beef, wild boar, and roe deer. The white segments show the relative amount of fatty acids with 12−14 carbon atoms, the gray segments the amount of C16−18, and the black segments represent the portion of fatty acids with C > 18 (−26).

treated with 1,2-dimethylhydrazine (DMH) to induce colon tumors (*11*, *15*, *23*). Since the estimated daily intake of 0.3−0.4 g/day is comparable to the administered relative amounts in the animal studies, the determined amounts give strong hints that dietary sphingomyelin might have similar effects in human nutrition.

The newly developed method for quantification and structural elucidation of sphingomyelin in food samples delivers a sensitive and powerful tool for an overdue systematic investigation which enables a new estimation of daily intake of these functional food ingredients. The HILIC-HPLC-ESI-MS/MS based method is more sensitive and selective than the formerly used methods based on GC-FID, GC-MS, or HPLC-ELSD.

ABBREVIATIONS USED

°C, degree Celsius; C, carbon; C2-SM, acetyl-sphingomyelin; C6-SM, hexanoyl-sphingomyelin; CE, collision energy; cps, counts per second; CXP, cell exit potential; d18:0, dihydrosphingosine; d18:1, sphingosine; d18:2, 4,8-sphingadienine; DMH, 1,2-dimethylhydrazine; DP, declustering potential; DSS, dextran sulfate sodium; ELSD, evaporative light scattering detector; ESI, electrospray ionization; FID, flame ionization detector; g, gram; GC, gas chromatography; h, hour; HDL cholesterol, high-density lipoprotein cholesterol; HILIC, hydrophilic interaction

liquid chromatography; HPLC, high performance liquid chromatography; i.d., inner diameter; IS, internal standard; LDL cholesterol, low-density lipoprotein cholesterol; mg/kg, milligram per kilogram; min, minute; mL, milliliter; mM, millimole; mm, millimeter; mol/L, mole per liter; MRM, multiple reaction monitoring; MS, mass spectrometer; MS/MS, tandem mass spectrometer; ms, millisecond; m/z, mass-to-charge-ratio; μm, micrometer; ng/mL, nanogram per milliliter; rel., relative; t18:0, 4-hydroxysphinganine (phytosphingosine); t18:1, 4-hydroxy-8-sphingenine; TLC, thin layer chromatography; V, volt; v/v, volume to volume; wt/wt, weight to weight.

Supporting Information Available: As described before, for each sphingoid base, the linkage with fatty acids of 12−26 carbon atoms with up to four double bonds as well as their hydroxylated analogues has been taken into account prior to the quantification, and the molecular masses of these possible precursor ions were calculated, leading to a number of 720 theoretical precursor ions shown in Tables 3−14. This material is available free of charge via the Internet at http://pubs.acs.org.

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