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Liquid Chromatography–Tandem Mass Spectrometry for the Determination of Sphingomyelin Species from Calf Brain, Ox Liver, Egg Yolk, and Krill Oil

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ABSTRACT: In this study, molecular species of sphingomyelin (SM) in egg yolk, calf brain, ox liver, and krill oil were investigated. Classes of phospholipids (PLs) were purified, identified, and quantified by normal phase semipreparative high-performance liquid chromatography (HPLC) combined with evaporative light scattering detectors (ELSD). For SM molecular species identification, pure SM collected through a flow splitter was loaded to HPLC–electrospray ionization–tandem mass spectrometry (LC-ESI-MS^2), with 100% methanol containing 5 mM ammonium formate as mobile phase. In addition to classes of PLs, the used approach allowed the determination of profiles of SM species in egg yolk, ox liver, and calf brain, whereas krill oil turned out not to contain any SM. It also allowed the separation and identification of SM subclasses, as well as tentative identification of species with the same molecular mass, including isomers. The results showed that egg yolk contained the highest proportion of (d18:1–16:0)SM (94.1%). The major SM molecular species in ox liver were (d18:1–16:0)SM (25.5%), (d18:1–23:0)SM (19.7%), (d18:1–24:0)SM (13.2%), and (d18:1–22:0)SM (12.5%). Calf brain SM was rich in species such as (d18:1–18:0)SM (40.7%), (d18:1–24:1)SM (17.1%), and (d18:1–20:0)SM (10.8%).

KEYWORDS: *sphingolipids, sphingomyelin, PLE, LC-ESI-MS^2*

INTRODUCTION

In contrast to the more prevalent glycerophospholipids, complex sphingolipids are composed of only a single fatty acid esterified to a sphingoid backbone. Yet they present a large degree of structural diversity due to variations in the degree of unsaturation, hydroxylation, and methylation of long-chain sphingoid base (sphingosine, sphinganine, etc.) in the nature of the fatty acid, as well as in the nature of the polar headgroup.[1] Over 300 sphingolipids of different classes have in fact been identified and structurally characterized.[2] To a certain extent, classes of sphingolipids are characteristic of the organisms in which they are synthesized. In plants, the predominant sphingolipids are glucosylceramides.[3] Within this class, inositol-containing glycosphingolipids are restricted to plants and fungi; galactosylceramide occurs only in fungi and animals. Glucosylceramide is, however, the unique glycosphingolipid that plants, fungi, and animals have in common. The predominant sphingolipid in mammals is sphingomyelin (SM). The latter contains sphingosine (d18:1), which is an aminodiol with 18 carbon atoms in its chain and an *E*-double bond in position 4. It is the most abundant among the sphingoid constituents of sphingolipids in human and animal cells.[4] Other sphingoid bases, such as sphinganine (d18:0) and 4-hydroxysphinganine (t18:0) in mammals, are reported in smaller amounts.[5]

Sphingolipids are present in eukaryotes as components of the biological membrane and are involved in cell proliferation, differentiation, cellular membrane traffic, stress response, signal transduction, and apoptotic cell death.[6,7] Sphingolipid metabolites, especially ceramide and sphingosine, are known as bioactive molecules involved in cell growth and death[8] and had been found to posess antitumor[2] and atherosclerosis activities,[9] to reduce cholesterol absorption,[10] and to protect the skin.[11]

Despite the wide interest surrounding sphingolipids, information about the molecular classes and species and their amounts in foods is still limited. Such determination is often hampered by the complexity of the food matrix, as well as the relatively small amounts of phospholipids (PLs) present as compared to the total lipids (TLs). For humans, SM is the principal source of free ceramide and sphingosine, yet its presence in food is limited, for example, 0.2% in egg yolk and 2.2% in ox liver (Table 1). Among the limited number of investigations addressing SM presence in food, none tried to purify SM prior to LC-MS analysis.[12−14] In fact, the high contents of neutral lipids (NLs) make it impossible to obtain pure PLs by simple extraction, so preliminary cleanup and concentration procedures have to be developed.

Table 1. SM Presence as a Percentage (Mean ± SD) of TLs and PLs in Various Food Matrices (n = 3)

food matrix	content in TLs	content in PLs
egg yolk	0.2 ± 0.0	0.9 ± 0.1
calf brain	33.4 ± 0.6	56.8 ± 1.1
ox liver	2.2 ± 0.5	5.4 ± 0.7
krill oil	nd[a]	nd

[a]Not detected.

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One of the most powerful techniques used today in the analysis of PLs is LC-MS. Among MS systems, electrospray ionization−tandem mass spectrometry (ESI-MS^2) provides an improved sensitivity, low spectral background signals, and a good compatibility with LC.[15] Consequently, analysis of molecular species of PLs using LC-ESI-MS^2 presents a low level of artifact formation and a high specificity for PLs.[16−18]

In the present work, an LC-ESI-MS^2 method was developed to separate and quantitate molecular species of SM from four animal food matrices: egg yolk, calf brain, ox liver, and krill oil. Egg yolk was chosen because it contains high fat (>30%, w/w), whereas ox liver and calf brain contain low fat (<10%, w/w). Krill oil represents a feature of marine oils. TLs were first extracted by pressurized liquid extraction (PLE), the polar lipid fraction was purified by column chromatography, and the PL classes were separated by semipreparative HPLC combined with evaporative light scattering detectors (ELSD). SM was collected via a split system placed ahead of the ELSD, before its molecular species profiles were determined by LC-ESI-MS^2.

MATERIALS AND METHODS

Materials. All solvents used for LC analyses were of HPLC grade. Chloroform was obtained from VWR (Strasbourg, France), and methanol was obtained from Carlo Erba (Val de Reuil, France). Chloroform (Sigma-Aldrich, Steinheim, Germany), methanol, used for lipid extraction and SM purification were of analytical grade. Standard egg yolk SM, (d18:1−18:1)SM, and PL standards including phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylserine (PS) were purchased from Sigma-Aldrich (St. Louis, MO). Hen's eggs, calf brain, and ox liver were freshly purchased at a local retailer. Krill oil was kindly provided by Nestec SA (Lausanne, Switzerland).

Sample Preparation. Ox liver and calf brain were ground under cryogenic conditions (three steps of 5 min each) using liquid nitrogen and a 6870 freezer/mill (Spex CertiPrep, Stanmore, U.K.). The obtained frozen powder was freeze-dried (Alpha 2-4, Bioblock Scientific, Illkirch, France). Egg yolk powder was obtained by removing egg white from fresh eggs, homogenizing, and freeze-drying. Krill oil was used for PL extraction and analysis without any preparation. All food samples were stored at −20 °C until use.

PLE. PLE was performed as previously published[19] in a Dionex PLE 350 (Dionex, Sunnyvale, CA) extractor using $CHCl_3/CH_3OH$ (2:1, v/v) for each extraction. Two grams of each sample powder was mixed homogenously with sand so as to fill the 10 mL stainless steel extraction cell (L × ⌀, 52 mm × 15 mm). The use of a dispersion agent is intended to enhance solute exchange between phases and reduce the solvent volume used by filling the void in the cell. The extraction temperature, time, and pressure were set at 140 °C, 5 min, and 10.34 MPa, respectively, according to the method of Zhou et al.[19] The mobile phase containing the lipid fraction was collected, evaporated under vacuum using a rotary evaporator (40 °C, 300 kPa), and dried under a gentle stream of N_2.

PL Purification. PLs were separated from TLs according to the procedure of Hernàndez et al.,[20] with small modifications. Briefly, 75 mg of TL extract, dissolved in 1 mL of $CHCl_3$, was transferred onto a preparative silica gel column (35 cm × 2 cm i.d., 15 g of Si 60 silica gel, particle size 40−63 μm, Geduran, Merck), which was preactivated with 10 mL of $CHCl_3$. Elution was performed at a flow rate of 4 mL/min. First NLs such as triacylglycerols and carotenoids were removed by 250 mL of $CHCl_3$. Then, PLs were eluted with 200 mL of CH_3OH/1 M aqueous formic acid (adjusted to pH 3 with triethylamine) (98:2, v/v) mixture. The solvent was removed by rotary evaporation, and the residue containing pure PLs was stored at −20 °C for the SM purification.

Determination of the Content of SM by HPLC-ELSD. A chromatographic system, made of a 616 controller, a 2424 ELS detector, and a 717 Plus autosampler (Waters, Saint-Quentin-Fallavier, France) controlled with Empower 2 software (Waters), was used to determine the content of SM in each purified PL samples obtained by PLE. High-purity nitrogen from a nitrogen generator (Domnik Hunter, Villefranche-sur-Saône, France) was used as a nebulizing gas at a pressure of 310 kPa. The drift tube temperature was set at 45 °C.[15] SM was separated from other PL classes by using a Luna normal phase column (150 × 3 mm, 3 μm, Phenomenex, Le Pecq, France). The flow rate of mobile phase was 0.5 mL/min, and separations were performed at room temperature using a 20 min linear gradient ranging from $CHCl_3/CH_3OH$ (88:12, v/v) to $CHCl_3/CH_3OH$/1 M aqueous formic acid (adjusted to pH 3 with triethylamine) (28:60:12, v/v/v).[21] Each purified PL was dissolved in a mixture of $CHCl_3/CH_3OH$ (2:1, v/v), filtered on a 0.45 μm filter (Macherey-Nagel, Hoerdt, France), and injected (20 μL) in the chromatographic system. SM was identified by comparison of its retention time with that of its standard obtained in the same analytical conditions. The content of SM was evaluated on the basis of a quadratic model of external calibration.

Semipreparative Chromatography. Semipreparative separation was achieved using a silica column (Uptisphere, 250 × 10 mm, 5 μm, Interchim, Montluçon, France). The mobile phase was made of $CHCl_3/CH_3OH$ (88:12, v/v) (solvent A) and CH_3OH/1 M aqueous formic acid (adjusted to pH 3 with triethylamine) (82.4:17.6, v/v) (solvent B). Solvents were delivered at 4 mL/min according to the following gradient: 0−8 min, 100−80% A; 8−10 min, 80−60% A; 10−13 min, 60% A; 13−18 min, 60−40% A; 18−20 min, 40−100% A; 20−30 min, 100% A. A flow splitter was used ahead of the ELSD inlet, allowing 97.5% of the flow (3.9 mL/min) to be diverted to the collector and 2.5% (0.1 mL/min) to enter the ELSD. The autosampler was set to inject 50 μL of purified PL solution in $CHCl_3$ (about 200 mg/mL). The SM peak was identified in comparison of its retention time (RT) to the standard one, and the time window was set (RT ± 0.75 min) to collect the SM peak.

The purity (P) of collected SM from different matrices was checked by the analytical HPLC-ELSD procedure described above. It was calculated as the ratio of SM peak area against the sum of all peak areas in the HPLC chromatogram.

Separation and Identification of SM Molecular Species by LC-ESI-MS^2. Molecular species of SM were determined using a Prostar HPLC system made of two 210 solvent delivery modules, a 410 autosampler, and a 1200 L triple-quadrupole mass spectrometer fitted with an ESI source (Varian, Les Ulis, France). High-purity nitrogen (Domnik Hunter) was used as nebulizing gas, set at 317 kPa, and as drying gas, set at 250 °C. A sample volume of 10 μL was injected to a Nucleodur C18HTec (250 × 4.6 mm, 3 μm, Macherey-Nagel) using an isocratic flow of 100% CH_3OH containing 5 mM ammonium formate at a rate of 1 mL/min. A flow splitter allowed, however, the HPLC effluent to enter the mass spectrometer at a flow rate of 0.2 mL/min. Full-scan acquisition was performed in positive mode in a mass range between m/z 400 and 900, and $[M + H]^+$ molecular ions were selected by the first quadrupole and fragmented in the collision cell. Argon was used as a collision gas (31.72 Pa), and the collision energy was set to 25 V. For fragmentation products, the mass range was set between m/z 200 and 900. The dwell time was set to 0.2 s for each m/z.

RESULTS AND DISCUSSION

SM Content. Direct injection of TLs in LC-MS without prior PL purification does allow the identification of different PL classes.[22] However, considering their high level of structural similarity, the molecular species within a given class cannot be completely resolved and the isomers cannot be distinguished. In addition, quantification of the different molecular species within each PL class can be only carried out by SIM mode. Another impediment in this case is the predominance of NLs in TL extracts of most foods, which affects the ionization in ESI sources and the desolvation of the liquid chromatography effluent droplets. This calls for preliminary cleanup procedures to be developed, prior to the analysis of polar lipids.

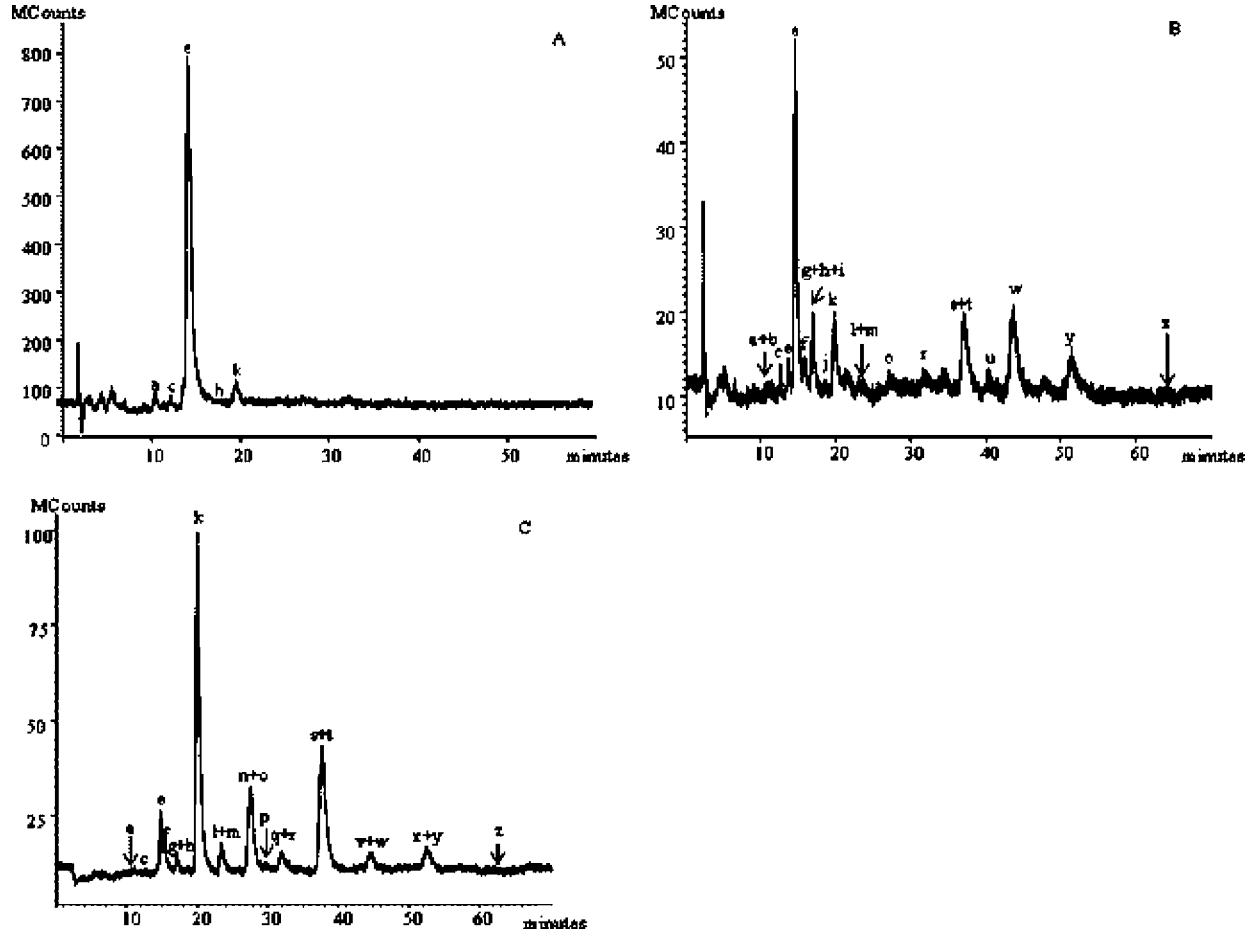

Figure 1. Chromatograms of purified SM: (A) egg yolk, (B) ox liver, and (C) calf brain, using LC-ESI-MS. Separation was performed using isocratic conditions: CH_3OH containing 5 mM ammonium formate at a rate of 1 mL/min onto a Nucleodur, C18HTec (250 mm × 4.6 mm, 3 μm). Peaks: a, d18:1−14:0; b, d18:1−16:1; c, d18:1−15:0; d, d18:1−17:1; e, d18:1−16:0; f, d18:1−18:1; g, d18:0−16:0; h, d18:1−17:0; i, d18:1−19:1; j, d18:1−18:0; k, d18:1−18:0; l, d18:0−18:0; m, d18:1−19:0; n, d18:1−22:1; o, d18:1−20:0; p, d18:1−24:2; q, d18:1−23:1; r, d18:1−21:0; s, d18:1−24:1; t, d18:1−22:0; u, d18:1−23:0; v, d18:1−25:1; w, d18:1−23:0; x, d18:1−26:1; y, d18:1−24:0; z, d18:1−25:0.

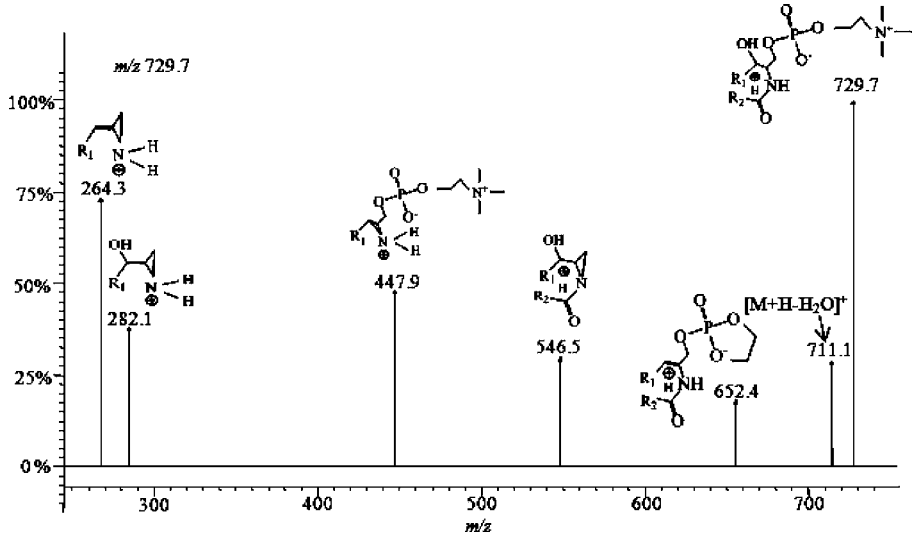

Figure 2. Ions obtained in positive mode in ESI-MS^2 between m/z 200 and 750 from fragmentation of reference SM (d18:1−18:1) at m/z 729.7. R_1 = $C_{15}H_{29}$; R_2 = $C_{17}H_{33}$.

In the present study, NLs of all food matrices were removed by column chromatography to obtain pure PLs, prior to the purification of SMs using semipreparative chromatography and its injection into LC-MS. SM contents varied largely depending on the food matrix considered. Calf brain contained the largest proportions of SM with 33.4% of TLs and 56.8% of PLs (Table

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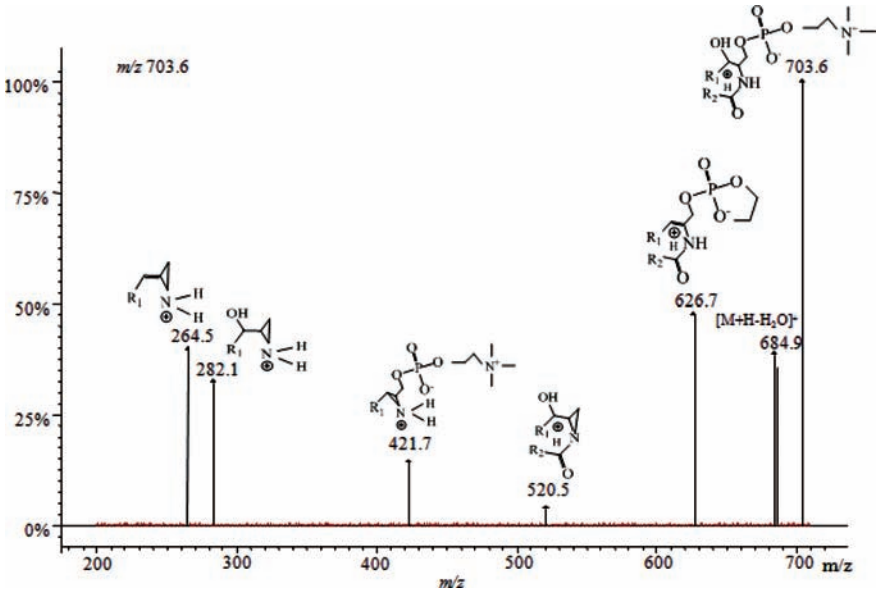

Figure 3. Ions obtained in positive mode in ESI-MS^2 between m/z 200 and 750 from fragmentation of egg yolk SM (d18:1−16:0) at m/z 703.6. R_1 = $C_{15}H_{29}$; R_2 = $C_{15}H_{31}$.

Table 2. Molecular Species Profiles of Each Purified SM

			SM (%)			
m/z $[M + H]^+$	RT (min)	molecular species[a]	egg yolk[b]	ox liver[b]	calf brain[b]	reference (d18:1−18:1)SM
675.7	11.0	d18:1−14:0	1.4	0.5	0.1	nd[c]
689.7	12.8	d18:1−15:0	1.0	1.6	0.1	nd
701.7	11.5	d18:1−16:1	nd[c]	0.8	nd	6.6
703.6	14.8	d18:1−16:0	94.1	25.5	4.4	nd
705.7	17.2	d18:0−16:0	nd	2.7	0.6	nd
715.7	13.4	d18:1−17:1	nd	0.1	nd	2.9
717.7	17.3	d18:1−17:0	0.5	2.3	0.5	nd
727.7	12.0	d18:1−18:2	nd	nd	nd	4.1
729.7	15.4	d18:1−18:1	nd	0.5	2.0	72.7
731.7	17.9	d18:0−18:1	nd	nd	nd	4.7
	18.7	d18:1−18:0	nd	0.5	0.2	nd
	20.0	d18:1−18:0	3.0	5.6	40.7	nd
733.7	23.5	d18:0−18:0	nd	0.5	2.3	nd
743.7	17.3	d18:1−19:1	nd	0.1	nd	nd
745.7	23.5	d18:1−19:0	nd	0.4	0.6	nd
757.7	20.8	d18:1−20:1	nd	nd	nd	9.0
759.7	27.5	d18:1−20:0	nd	1.8	10.8	nd
773.7	32.1	d18:0−21:0	nd	2.3	0.3	nd
785.7	27.1	d18:1−22:1	nd	nd	3.0	nd
787.7	37.9	d18:1−22:0	nd	12.5	6.8	nd
799.7	32.0	d18:1−23:1	nd	nd	2.2	nd
801.7	41.3	d18:1−23:0	nd	3.1	nd	nd
	44.4	d18:1−23:0	nd	19.7	2.5	nd
811.7	29.0	d18:1−24:2	nd	nd	0.6	nd
813.7	37.5	d18:1−24:1	nd	4.9	17.1	nd
815.7	52.5	d18:1−24:0	nd	13.2	4.2	nd
827.7	44.3	d18:1−25:1	nd	nd	0.6	nd
829.7	62.3	d18:1−25:0	nd	1.9	0.2	nd
841.7	52.1	d18:1−26:1	nd	nd	0.3	nd

[a]The position of fatty acids in the glycerol moiety has not been determined. [b]Percentages of all identified molecular species. [c]Not detected.

1). In egg yolk and ox liver, SM accounted for no more than 0.2 and 2.2% of TLs and 0.9 and 5.4% of PLs, respectively, whereas in krill oil no SM was found, which is in accordance with the findings of Albessard et al.,[23] who investigated the variation of PL classes of krill and did not find SM among different organs of krill.

The purity of isolated SM above 98% was obtained from each food matrices analyzed.

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SM Molecular Species. The method proposed was very simple by using an isocratic mobile phase made of a single solvent (methanol) containing 5 mM ammonium formate. The molecular mass peaks for SM were detected in the four food matrices using positive ion full-scan ESI-MS analysis (Figure 1). Overall, the molecular species of SM, having different polarities, were very well separated. Figure 1A shows a chromatogram of egg yolk SM, revealing a simple composition with few molecular species eluting between 11 and 20 min. In contrast, ox liver SM (Figure 1B) and calf brain SM (Figure 1C) showed complex compositions, with at least 13 molecular species peaks. Whereas all molecular species of calf brain SM were separated during 0−62 min, some of the molecular species in ox liver SM had very close retention times, mainly between 10 and 20 min.

Identification of SM Molecular Species. Under our experimental conditions, the ESI tandem mass spectra of SM $[M + H]^+$ ions yielded six fragments: (1) A fragment ion at m/z 264 is characteristic of the sphingosine long-chain base (d18:1).[24] This fragment represented a loss of the polar headgroup $[HPO_4(CH_2)_2N(CH_3)_3]^+$, H_2O, and the acyl group $[RCO]^+$ corresponding to the fat acid (2) An $[M + H - 183]^+$ ion corresponds to the loss of the polar headgroup. (3) A fragment ion at m/z 282 corresponds to the loss of the polar headgroup and acyl group. (4) An $[M + H - 18]^+$ ion results from the loss of H_2O. (5) An $[M + H - 77]^+$ ion corresponds to the loss of trimethylamine and H_2O. (6) An $[M + H - 282]^+$ ion corresponds to the loss of the acyl group and a hydroxyl group.

Representative MS^2 fragmentation spectra of SM molecular species are presented in Figure 2. The product ion spectra of reference (d18:1−18:1)SM at m/z 729.7 (RT = 15.4 min) clearly showed the fragment ion at m/z 264.3, reflecting an 18:1 long-chain base. Two fragment ions at m/z 711.1 and 546.5 presumably reflected the loss of H_2O and the polar headgroup, respectively. The tandem mass spectrum also contained fragment ions at m/z 447.9, 282.1, and 652.4, which were related to the loss of the fatty acid, of both the polar headgroup and acyl group and of both the trimethylamine and H_2O molecule, respectively. However, the analysis of the reference (d18:1−18:1)SM used showed that this standard is not pure (72.7% pure), with a chromatographic profile clearly revealing the presence of other SM species, such as (d18:1−20:1)SM (9.0%), (d18:1−16:1)SM (6.6%), (d18:0−18:1)SM (4.7%), (d18:1−18:2)SM (4.1%), and (d18:1−17:1)SM (2.9%).

Another representative MS^2 fragmentation spectrum of (d18:1−16:0)SM from egg yolk (m/z 703.6, RT = 14.8 min) is given in Figure 3. It also clearly showed six fragments apart from $[M + H]^+$, and the fracture mode was the same as in Figure 2.

Profiles of SM Molecular Species. The approach used for the reference SM was applied to each m/z detected from the purified SM samples, which allowed the identification of a number of constitutive SM molecular species for each food investigated (Table 2). As far as egg yolk SM is concerned, the major species was (d18:1−16:0)SM (94.1%). Other SM species were also identified: (d18:1−18:0)SM (3.0%); (d18:1−14:0)SM (1.4%); (d18:1−15:0)SM (1.0%); and (d18:1−17:0)SM (0.5%). The results obtained here for egg yolk are, to a certain extent, in agreement with our study carried out under the same conditions with the standard egg yolk SM purchased from Sigma-Aldrich (see above). In the latter, the main SM molecular species were also (d18:1−16:0)SM (86.8%); the minor molecular species were (d18:1−18:0)SM (6.9%) and (d18:0−16:0)SM (6.3%). Pacetti et al.[25] also showed that (d18:1−16:0)SM was the predominant species in egg yolk, representing approximately 95% of the total SM molecular species.

Within ox liver, 21 SM species were clearly identified. Saturated fatty acids (SFA) were the most abundant and accounted for 94.1% of total fatty acids, the rest being made of monounsaturated fatty acids (MUFA) (6.4%) (Table 2). Consequently, most SM species contained SFA, with (d18:1−16:0)SM representing the largest proportion (25.5%) of the total SM (Table 2). This result is consistent with the fact that C16:0 (28.2%) is the predominant fatty acid in ox liver. Other SFA-containing SM species included (d18:1−22:0)SM (12.5%), (d18:1−24:0)SM (13.2%), and (d18:1−23:0)SM (19.7%). The most abundant MUFA-containing species was (d18:1−24:1)SM, with a proportion of 4.9%. To our knowledge, this is the first report of SM molecular species in ox liver. A similar study exists only for rat liver, with an SM composition showing a predominance of (d18:1−16:0)SM, (d18:1−22:0)SM, and (d18:1−24:0)SM and two minor species, (d18:1−24:2)SM and (d18:1−20:0)SM, which were not present in our determinations.[26]

It is known that different SM subclasses exist depending on the sphingoid base component.[3] One of these subclasses is based on sphinganine (d18:0)SM, with an m/z 266 characteristic fragment ion. In ox liver, this fragment ion was present among others in the chromatographic peak at 17.2 min ($[M + H]^+$ = 705.7), which allowed the identification of (d18:0−16:0)SM (2.7%) (Table 2). Other (d18:0)SM species were also identified: (d18:0−21:0)SM (2.3%) and (d18:0−18:0)SM (0.5%). When applied to the used (d18:1−18:1)SM reference, this analysis showed that it contained as high as 4.7% of (d18:0−18:1)SM (m/z 731.7).

As mentioned above, one of the most important SM species in ox liver is (d18:1−23:0)SM, which corresponded to a clear chromatographic peak at 44.4 min (m/z 801.7). Another peak with the same mass was also observed at 41.3 min. The presence of the fragment ion m/z 264.3 showed clearly that this peak corresponded to (d18:1)SM. In addition, a m/z 433.4 fragment ion, corresponding to $[M + H - RCO^- - OH]^+$, confirmed the presence of C23:0 as fatty acid. These results suggested that the two molecules observed at 41.3 and 44.4 min were isomers of (d18:1−23:0)SM, the difference between them being probably due to the relative position of the double bond or its trans/cis forms in sphingosine (d18:1). Unfortunately, the technique used in this study did not allow a more precise determination of the chemical structure of the sphingosine base. A similar observation was also made for (d18:1−18:0)SM, with two possible isomers at 18.7 min (0.5%) and 20.0 min (5.6%) (Table 2). It is noteworthy that the chromatographic separation allowed three different SM species (two (d18:1−18:0)SM isomers and (d18:0−18:1)SM) of the same mass (m/z 731.7) to be separated, although their chemical structures could not be completely resolved.

As opposed to ox liver, calf brain showed a high proportion of MUFA-containing SM species, with (d18:1−24:1)SM (17.1%) being second to (d18:1−18:0)SM (40.7%) (Table 2). Results show that 18:0, 24:1, and 24:0 are the most abundant fatty acids in bovine brain SM.[11,27] Whereas this is consistent with our results regarding 18:0 and 24:1, the content of (d18:1−24:0)SM in our case was only 4.2%, which is less than (d18:1−20:0)SM (10.8%), (d18:1−22:0)SM (6.8%), and (d18:1−16:0)SM (4.4%). As with ox liver, (d18:0)SM species

were also identified: (d18:0−18:0)SM (2.3%), (d18:0−16:0)SM (0.6%), and (d18:0−21:0)SM (0.3%). This is in agreement with previous studies showing the presence of (d18:0)SM in bovine brain.[11,27] Furthermore, the suggested (d18:1−18:0)SM isomers at 18.7 and 20.0 min discussed previously for ox liver were also identified in calf brain.

This study used various food matrices to develop a purification procedure for PLs from TL extracts by column chromatography and for SM from PLs by normal phase semipreparative chromatography and, finally, a separation procedure of SM species by reverse phase HPLC, coupled to ESI-MS^2 analysis for identification. In addition to classes of PLs, the used approach allowed the determination of profiles of SM species in egg yolk, ox liver, and calf brain, whereas krill oil turned out not to contain any SM. It also allowed the separation and identification of SM subclasses, as well as tentative identification of species with the same molecular mass, including isomers.

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