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Preparation and gas chromatographic–mass spectrometric analysis of N-acetyl-O-trimethylsilyl derivatives of long-chain base residues of natural sphingomyelin

Nils U. Olsson[a,*], Peter Kaufmann[a], Susanna Dzeletović[b]

[a]*Scotia LipidTeknik AB, P.O. Box 6686, S-113 84 Stockholm, Sweden*
[b]*University of Stockholm, Department of Analytical Chemistry, S-106 91 Stockholm, Sweden*

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Abstract

A procedure for the preparation of long-chain base residues of egg yolk, bovine milk and bovine brain sphingomyelin was developed. The bases were converted to N-acetyl-O-trimethylsilyl (TMS) derivatives before being submitted to gas chromatography and mass spectrometry. The chromatographic profile of the milk sample was complex with thirteen peaks, whereas the profiles of brain and egg yolk long-chain bases were simple and remarkably similar. © 1997 Elsevier Science B.V.

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1. Introduction

Sphingomyelins (SPHs) [1] are structural and functional components of animal biomembranes [2,3]. Their molecular species pattern is believed to influence the physico-chemical properties in vivo. Natural SPHs are composed of three different structural units; a polar head group (a phosphorylated substituent), an N-acetylated fatty acid and a long-chain base (LCB), also called a sphingoid base. The polar head group in sphingomyelins is by definition phosphorylcholine [3] but sphingolipids with a phosphorylethanolamine polar head group have also been found in nature [4]. The long-chain bases are generally di-hydroxy analogs, either saturated or mono-unsaturated. A nomenclature which designates di-hydroxy bases with "d" and chain length and number of double bonds similar to fatty acids (i.e., sphingosine is d18:1 and sphinganine is d18:0) is oftentimes employed. Natural sphingomyelins and the ceramide structural units thereof, i.e., fatty acid and LCB without the phosphoryl head-group, have a large commercial potential in the cosmetic and pharmaceutical industries.

The fatty acid composition of sphingomyelins has been determined as methyl ester derivatives by gas

*Corresponding author. Corresponding address: Laboratory of Membrane Biochemistry and Biophysics, NIAAA, National Institutes of Health, 12501 Washington Avenue, Rockville, MD 20852, USA.

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chromatography (GC). Acidic conditions are usually employed in order for the reaction to proceed at a reasonable speed. LCBs of, e.g., porcine and sheep erythrocyte membranes, chicken egg yolk and bovine brain have been determined by GC or high-performance liquid chromatography (HPLC) after derivatization procedures [5–18] intended to lower the polar character of free LCBs in order to improve chromatographic performance. Bovine milk LCBs have been characterized by the combined use of thin-layer chromatography (TLC) fractionation of their DNP-derivatives into saturated and mono-unsaturated sub-groups, followed by GC analysis of aldehyde reaction products after cleavage with periodate [19–21].

Reversed-phase (RP) HPLC has been used for the separation of intact sphingomyelins utilizing light-scattering detection [22], and in a derivatized form with UV-absorbance detection [5,18,23]. Identification is generally difficult due to a lack of suitable standards for retention-time comparisons. LC–MS has been used for identification of individual molecular species [24] but many laboratories may be excluded from this technology due to prohibitive pricing.

Experimental design and multivariate evaluation methods have been successfully used in chromatography [25] to achieve optimal performance. In this work some of these methods were utilized to conserve experimental economy and to ensure the localization of optimal reaction conditions.

Knowledge about molecular species composition, besides the fatty acid determination, plays an important role in the characterization of individual polar lipid classes. Routinely this is done by either RP-HPLC [22], silver ion chromatography or by high-temperature GC after derivatization [26]. Nowadays, state-of-the-art technology permits the extraction of highly purified individual polar lipid classes, such as sphingomyelin, from complex mixtures of natural origin. This investigation was undertaken to test the utility of N-acetylated-O-trimethylsilyl (TMS) derivatives of the long-chain residues of natural sphingomyelin in GC–MS analysis. The identification of the LCBs was intended to serve as a basis for the more convenient RP-HPLC separation [22] of intact sphingomyelins on a routine basis.

2. Experimental

2.1. Materials

Bovine brain and chicken egg yolk sphingomyelin standards were obtained from Sigma (St. Louis, MO, USA). SPH from bovine milk was prepared in-laboratory at Scotia LipidTeknik (Stockholm, Sweden). Sphingomyelinase (EC 3.1.4.12) from *Bacillus cereus* was purchased from Sigma. Fresh N-(trimethylsilyl)-imidazole was obtained from Merck (Darmstadt, Germany) All solvents were of analytical-reagent quality and purchased from Merck. Pyridine was dried over $KOH_{(s)}$ before use.

2.2. Chemometrics

Experimental design and response surface modeling [25] was used for the evaluation of the N-acetylation reaction of the LCB residues.

2.3. Preparation of LCB derivatives

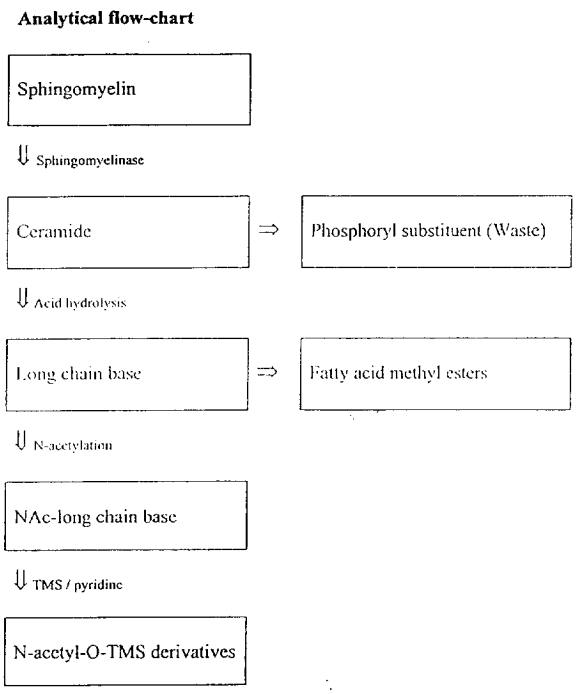
Four consecutive reactions steps were required in order to obtain LCB derivatives amenable for GC–MS analysis (Scheme 1).

2.3.1. Enzymatic hydrolysis

A suspension of milk–SPH (2 ml) in 0.1 *M* Tris buffer at a concentration of 8 mg/ml was added to a test-tube together with 2 ml diethyl ether. After temperature equilibration at 24°C for 5 min, 5 units of sphingomyelinase were added. The test-tube was placed in an ultrasonic water-bath (24°C) and sonicated for 20 min. The hydrolysis was discontinued by extraction with 3×2 ml diethyl ether. The three organic phases, containing the ceramide hydrolysis products, were combined, washed once with 1 ml deionized water and evaporated to dryness under a stream of nitrogen.

2.3.2. Acidic hydrolysis

An acid catalyzed methanolysis was designed [12,27,28] to liberate LCBs from the ceramide. The ceramide residues (4 mg) were dissolved in 4 ml of a freshly prepared solution of: methanol–water–hydrochloric acid (conc.) (82:9.4:8.6, v/v/v) in a



Scheme 1. Preparation of LCB derivatives.

test-tube with a PTFE lined screw cap and placed in 70°C for 18 h. The methanolic solution, containing the liberated LCBs was adjusted to pH>12 with 10 *M* NaOH(aq). A saturated NaCl solution was added to double the volume. The LCBs were extracted by 3×2 ml of diethyl ether. The combined organic phases were washed once with 0.5 ml of the saturated NaCl solution, evaporated to dryness under nitrogen, dissolved in *n*-hexane–2-propanol and checked for purity on the HPLC system.

2.3.3. N-Acetylation derivatization

The free long chain bases, approx. 2 mg, were reacted with 2 ml of a freshly prepared solution of 20% acetic acid anhydride in methanol at 20°C for 20 min. The optimum reaction conditions were ascertained by the use of statistical experimental design. The response (reaction performance) was measured by HPLC (an aliquot of the reaction mixtures was injected) and the experiments were evaluated by response surface modeling. The remaining reaction mixture, containing the N-acetylated long-chain bases, was evaporated to dryness under nitrogen.

2.3.4. TMS derivatization

The N-acetylated long chain bases, approx. 2 mg, were washed with 2 ml methanol in a test-tube with a PTFE lined screw cap, and evaporated to dryness under nitrogen to remove residual water and then dissolved in 1 ml of dry pyridine. After 1 min at 60°C, 400 μl of N-(trimethylsilyl)-imidazole was added after which the test-tube was left to react at 60°C for 5 min. The TMS derivatives were immediately injected into the GC–MS system after the reaction. For comparison non-acetylated LCBs were also submitted to the TMS reaction and subsequently analyzed by GC–MS.

2.4. HPLC

Evaluation of the different reaction performances, except the final TMS-derivatization, was done by HPLC according to a method used by Herslöf et al. [29], modified by Kroon [30]. The gradient HPLC system consisted of two LC-10AD HPLC pumps, a SCL-10A system controller, a SIL-10A auto-injector equipped with a 20 μl injection loop, a CTO-6AS column oven equipped with a pre-heater and a C-R3A electronic integrator, all from Shimadzu (Kyoto, Japan). Detection was done with an evaporative light-scattering detector, Cunow D.D.L. 11 (Cergy, St. Christophe, France), set at an air inlet pressure of 0.8 bar and a temperature of 50°C. The analytical column was a Diol (125×4.6 mm I.D.) from Merck, maintained at 55°C by the column oven. The mobile phases were composed of (A) *n*-hexane–2-propanol–acetic acid–triethylamine (81.5:17:1.5:0.08, v/v) and (B) 2-propanol–water–acetic acid–triethylamine (84.5:14:1.5:0.08). The gradient was from 7% B to 100% B over 15 min. The lipid samples were dissolved in *n*-hexane–2-propanol (4:1, v/v) before injection.

2.5. GC–MS

The GC–MS system was a Varian Saturn ion trap detector (Walnut Creek, CA, USA) connected to a Varian 3400 GC, equipped with a Varian 8100 auto-

sampler. Samples were injected onto the capillary column via a septum temperature programmable (SPI) on-column injector. The carrier gas was helium and the analytical column was a CP-Sil-5 CB (25 m×0.25 mm I.D., film thickness: 0.24 μm) purchased from Chrompack (Middelburg, Netherlands). The injector was temperature programmed as follows: 100°C (hold 30 s) to 250°C at 300°C/min, then hold for 30 s. 0.5 μl of the samples, dissolved in pyridine were injected. A multi-ramped temperature program over 17 min was applied for the GC separation: 100°C (hold 1 min) to 200°C at 50°C/min, then to 260°C at 5°C/min, hold at 260°C for 2 min. The transfer line was held at 260°C throughout the analyses. The mass spectrometer was operated in the electron impact mode (EI) with a manifold temperature of 260°C.

3. Results and discussion

Five units of sphingomyelinase was found to be able to hydrolyse two consecutive aliquots of substrate, i.e., 2×2 ml of the SPH suspension. All reaction steps were evaluated by HPLC with the method described above with the exception of the final TMS derivatives, which were evaluated by GC–MS. The total ion current (TIC) chromatograms from the GC–MS runs of the fully derivatized LCBs from bovine milk, egg yolk and bovine brain are shown in Figs. 1–3, respectively. In Table 1 a list of the identified peaks is given. The identification of the peaks in each of the chromatograms was based upon interpretation of their mass spectra. Judging from the fatty acid composition and the RP-HPLC separation [22,31] of intact molecular species of the three materials under investigation, a larger number of long-chain bases were expected in the SPHs from bovine milk than in those from bovine brain and chicken egg yolk. This was confirmed by the gas chromatographic analyses (Figs. 1–3). Bovine brain and egg yolk displayed similar LCB compositions (Table 1), containing mostly d18:0, whereas bovine milk contained two main long-chain bases, namely d16:1 and d18:1.

The interpretation of some mass spectra can be seen in Figs. 4–6. Several of the fragments have previously been identified by Krisnangkura and Sweeley [32]. A spectrum of N-acetyl-di-O-TMS-sphinganine (Fig. 6) is also published in that paper

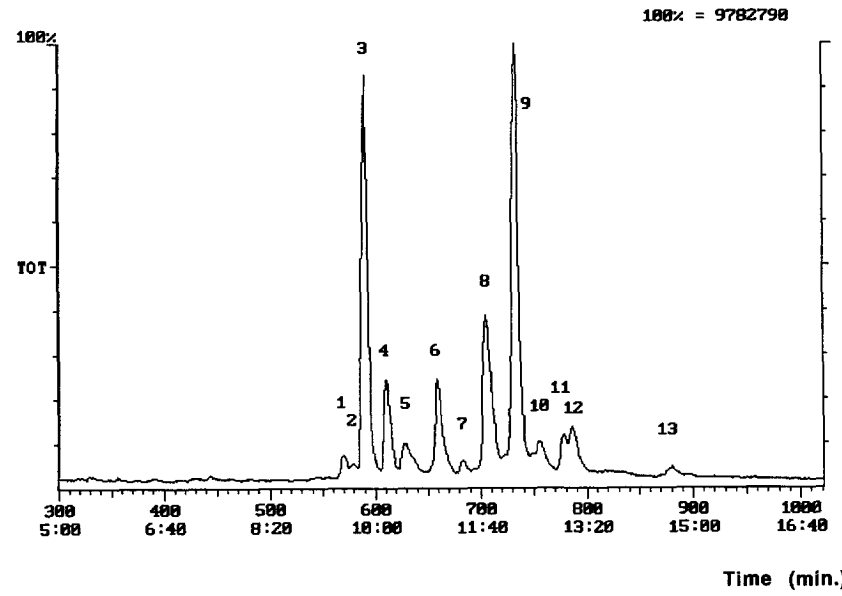


Fig. 1. Gas chromatogram of N-acetyl-O-TMS-long-chain bases of bovine milk SPH. GC temperature program: 100°C to 260°C, amount injected: 75 ng in pyridine. Peaks: see Table 1.

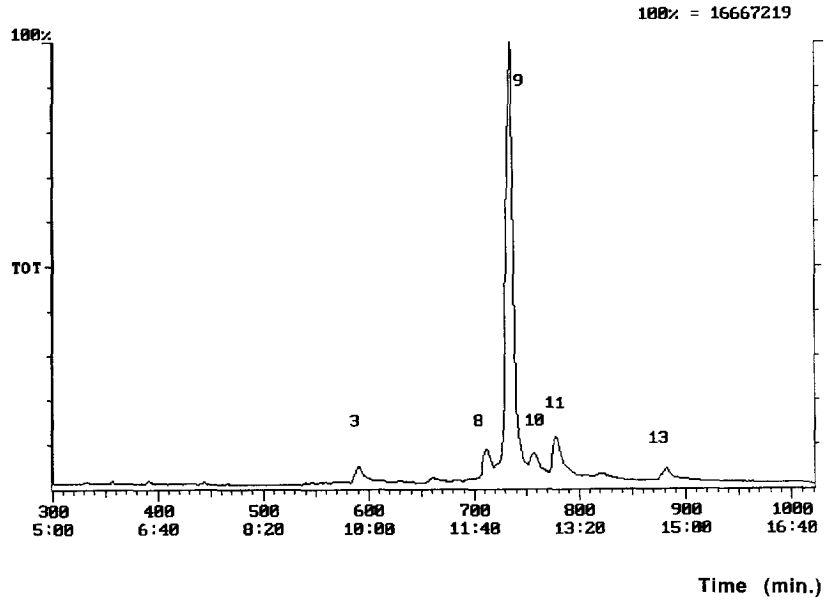


Fig. 2. Gas chromatogram of N-acetyl-O-TMS-long-chain bases of bovine brain SPH. GC temperature program: 100°C to 260°C, amount injected: 75 ng in pyridine. Peaks: see Table 1.

and may serve as a comparison. The presence of a peak at $(M+73)^+$ in each spectrum (Figs. 4–6) may be explained by either the presence of an N-acetyl-N-TMS substituent, created due to a preference of the secondary amide, formed after the acetylation reaction, to react with TMS-imidazole or the free

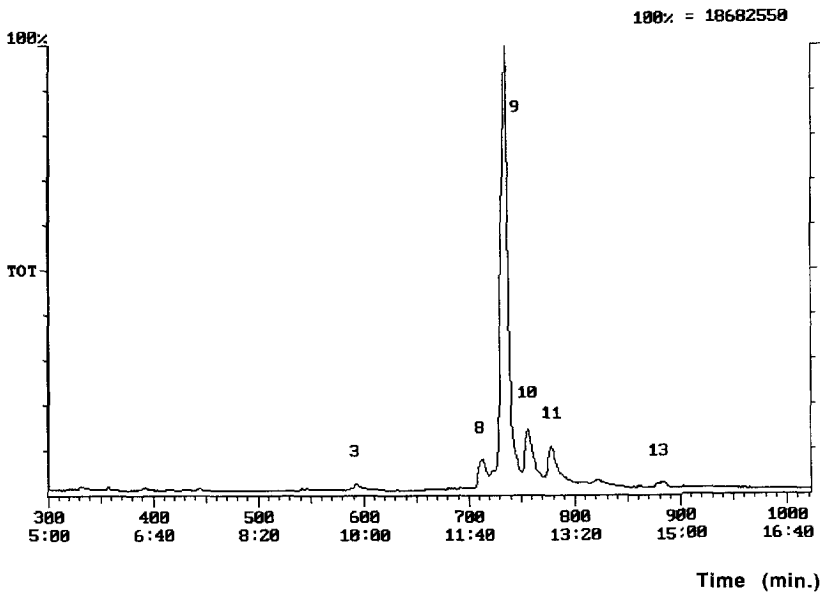


Fig. 3. Gas chromatogram of N-acetyl-O-TMS-long-chain bases of egg yolk SPH. GC temperature program: 100°C to 260°C, amount injected: 75 ng in pyridine. Peaks; see Table 1.

Table 1
Long-chain base composition of natural SPHs

Compound	Peak No.	Bovine milk		Bovine brain		Egg yolk	
		Straight chain (%)	Branched (%)	Straight chain (%)	Branched (%)	Straight chain (%)	Branched (%)
i d16:1	1		1.5				
ai d16:1	2		1.0				
d16:1	3	27.2		2.3		1.1	
br d16:0	4		7.1				
d16:0	5	3.6					
d17:1	6	8.1					
d17:0	7	0.7					
ai d18:1	8		14.2		5.4		5.1
d18:1	9	33.0		78.0		69.2	
d18:0	10	0.5		5.7		13.2	
i d19:1	11		2.3		8.6		11.4
ai d19:1	12		0.5				
Plasticizer	13						
Total		73.1	26.6	86.0	14.0	83.5	16.5

i=iso, ai=antesio, br=branched.

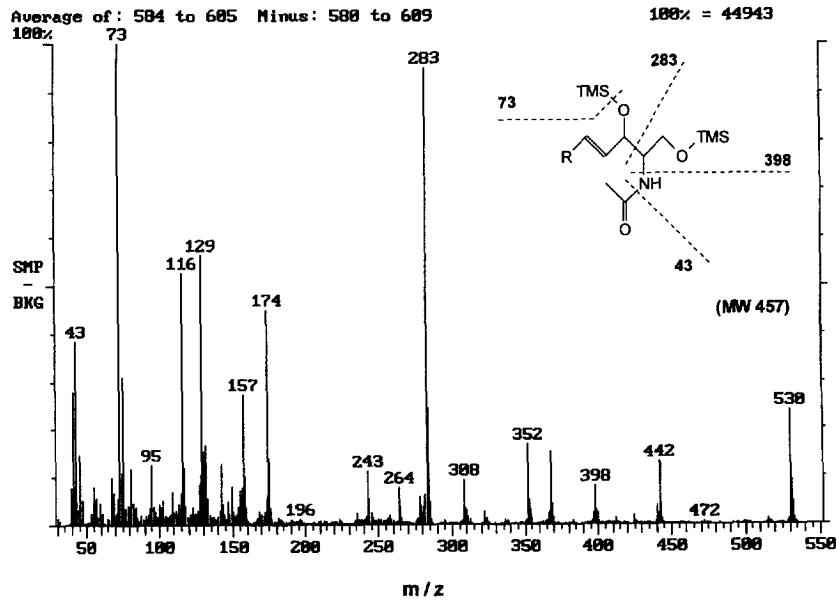


Fig. 4. EI mass spectrum of N-acetyl-di-O-TMS-d16:1. Manifold temperature 260°C.

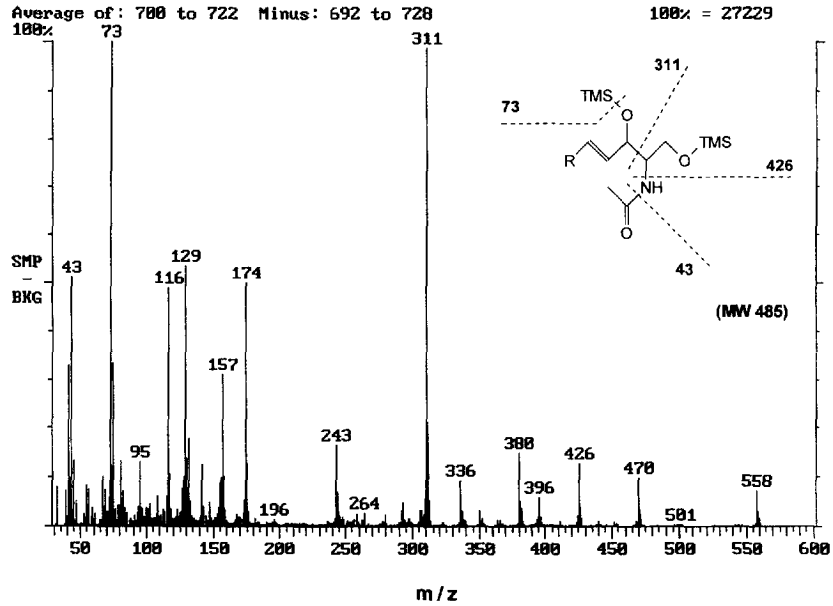


Fig. 5. EI mass spectrum of N-acetyl-di-O-TMS-d18:1. Manifold temperature 260°C.

hydroxyl group of the enol form (keto–enol tautomerism) of the acetylated amide to act likewise. Therefore, the $(M+73)^+$ was not regarded as the M^+ peak and the spectrum was interpreted accordingly. In this case an $(M-15)^+$ peak, indicating a loss of a methyl group from TMS, is visible in all the spectra. Peaks at m/z 174 (Figs. 4–6) indicate fragments of $(M-283)^+$, $(M-311)^+$ and $(M-313)^+$, respective-

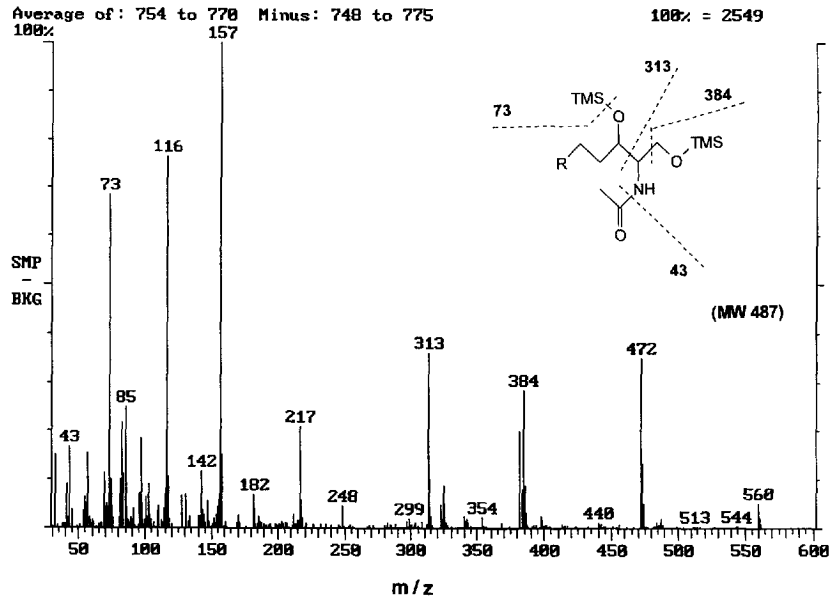


Fig. 6. EI mass spectrum of N-acetyl-di-O-TMS-d18:0. Manifold temperature 260°C.

ly. Peaks at m/z 116 attest the loss of NH-acetyl from the m/z 174 fragment. The ions at m/z 157 represent the fragment (N-acetyl-O-TMS-CH=CH_2)$^+$.

Exposing the underivatized LCBs to the TMS reaction alone did not result in the formation of $(M+73)^+$ peaks in the spectra. The non-acetylated TMS derivatives had a shorter retention time on the non-polar GC column than their fully derivatized analogs, due to a lower molecular mass, and the mass fragmentation revealed the presence of a free amine group, further supporting the structural determination. Also, the chromatographic performance was slightly worse for the non-acetylated sample which was also to be expected with the free amine group present.

In this study the complex reaction and derivatization sequence leading to the GC amenable long-chain base derivatives was improved. Chromatograms of derivatized long-chain base residues of bovine milk, bovine brain and chicken egg yolk SPH have been presented. The mass spectrum interpretation is straightforward and may serve as a confirmation of the LBC composition of SPHs from these and other sources and may be used in conjunction with RP-HPLC investigations of intact SPH molecular species.

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