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Structural analysis of commercial ceramides by gas chromatography–mass spectrometry

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Abstract

A simple method using gas chromatography–mass spectrometry was applied to analyse structures of ceramides. Identification of trimethylsilylated ceramides were obtained in short analysis times (derivatization of ceramides in 30 min at room temperature and 20 min gas chromatography mass spectrometry run) even for complex mixtures. For example in ceramide Type III, 18 peaks were observed which represent 27 various structures. The coeluted compounds were ceramides containing the same functional groups and the same carbon number but with a different distribution on the two alkyl chains of the molecule. They were accurately differentiated by mass spectrometry. Therefore, 83 structures of trimethylsilylated ceramides were identified in 11 different commercial mixtures. For 52 structures of these, mass spectral data were not described in the literature, neither full mass spectra nor characteristic fragments.

Keywords: Ceramides; Fatty acids; Amides

1. Introduction

Our laboratories are interested in the analysis of ceramides from the stratum corneum [1,2]. The number of different ceramide structures in the stratum corneum is higher than 1000 [3]. Consequently we have envisaged an analytical strategy in order to develop identification methods based on retention behaviour studies [1,2]. However, reference structures were necessary for this development. Less than ten structures are commercially available as pure standards. Commercial mixtures of ceramides are also available but with little information on their compositions. The aim of this work was to constitute an important set of structurally well defined ceramide molecules for our studies. Therefore, standards and commercial mixtures were investigated in order to identify the maximum of ceramide structures.

We were first interested in a gas chromatography–mass spectrometry (GC–MS) method after hydrolysis of the ceramides [2], which has been currently used [4–7]. The difficulty was to reconstitute the

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original composition of ceramides analysed in a mixture. GC–MS methods have also been successfully used for analysing intact ceramides after trimethylsilylation, first on packed columns in the early 1970s [8–13] and more recently on capillary columns [14,15].

From previous work, we tried to select the elements to establish the simplest but yet efficient GC–MS method in order to reach our objective. As commercial samples were specified as pure or containing ceramides with the same sphingoid base, we decided to use a non-polar high-temperature capillary column since the separation should be based principally upon chain length variations. This paper presents a summary of ceramide identification in 11 commercial samples (including Ceramide types III and IV from Sigma). Eighty-three different structures were found and they are given with two characteristic ions. From these results original full mass spectra of trimethylsilyl (TMS)-derivatized ceramides were extracted.

2. Experimental

2.1. Chemicals

Ceramide Type III, ceramide Type IV, *N*-palmitoyl-D-sphingosine $C_{16:0S}$, *N*-stearoyl-D-sphingosine $C_{18:0S}$, *N*-palmitoyl-DL-dihydrosphingosine $C_{16:0D}$, *N*-oleoyl-D-sphingosine $C_{18:1S}$, *N*-lignoceroyl-DL-dihydrosphingosine $C_{24:0D}$. *N*-nervonoyl-D-sphingosine $C_{24:1S}$ were all purchased from Sigma (St. Quentin Fallavier, France), ceramide III (CIII), ceramide VI (CVI) and ceramide IIIB (CIIIB) were a generous gift of Cosmoferm (Delft, Netherlands).

2.2. Structural abbreviations

Cn:xDn′ corresponds to ceramides with a Dihydrosphingosine base, Cn:xPn′ to ceramides with a Phytosphingosine base and Cn:xSn′ to ceramides with a Sphingosine base. n and x are, respectively, the number of carbons and the number of insaturations of the fatty acid chain, and n′ the number of carbons of the base chain. Ceramides with an α-hydroxylated fatty acid were symbolised as the following Cn:xα-OHSn′.

2.3. Silylation

The trimethylsilylation reagent, Sylon HTP, consisting of pyridine–hexamethyldisilazane–trimethylchlorosilane (9:3:1, v/v/v) was purchased from Supelco (Bellefonte, PA, USA). Trimethylsilylation was conducted at room temperature for 30 min with 100 μl of Sylon for 100 μg of ceramide. After the excess reagent was removed using rotary evaporation, the residue was dissolved in 100 μl of hexane and splitless injected (1 μl).

2.4. Apparatus

The GC–MS system consisted of a Series 3400 gas chromatograph (Varian, Walnut Creek, CA, USA) interfaced by direct coupling to an INCOS 50 quadrupole mass spectrometer (Finnigan, San Jose, CA, USA). The gas chromatograph was equipped with a 25 m×0.32 mm I.D. fused-silica column coated with a 0.1 μm film of HT5 5% phenylpolycarboranesiloxane (SGE, Australia). The carrier gas was helium at a pressure of 71 kPa. The injector and transfer line temperatures were set to 320 and 350°C, respectively. The chromatographic conditions were performed by the oven temperature programme 250–350°C at 5°C/min. Electron impact ionization (EI) mass spectra were measured in the total ion monitoring mode. The operating conditions for EI-MS were source temperature 180°C, filament emission current 750 μA, ionising voltage 70 eV and scan range from m/z 45 to 950 with a period of 1 s.

3. Results and discussion

Ceramides of the stratum corneum are molecules containing sphingosine, dihydrosphingosine or phytosphingosine base amide linked with a fatty acid which can be saturated, unsaturated or α-hydroxylated [3,16].

As ceramides contain between two and four hydroxyl groups, they should be derivatized. From the literature, we retained the trimethylsilylation performed at room temperature [8,9] which was transposed with a current commercial silylating reagent (Sylon HTP). Thereby only 30 min were necessary to obtain trimethylsilylated ceramides even

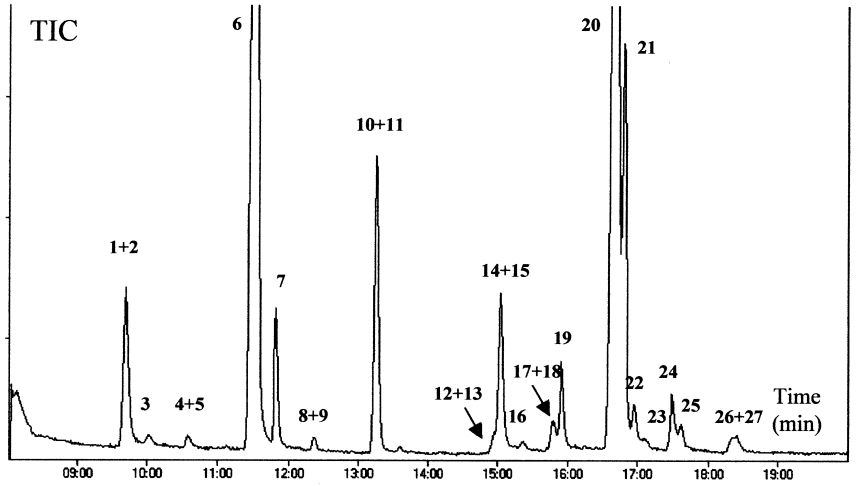

Fig. 1. TIC: total ion chromatogram of Ceramide Type III (Sigma); (1) C16:0S18, (2) C18:0S16, (3) C16:0D18, (4) C18:0S17, (5) C17:0S18, (6) C18:0S18, (7) C18:0D18, (8) C18:0S19, (9) C19:0S18, (10) C18:0S20, (11) C20:0S18, (12) C22:1S18, (13) C24:1S16, (14) C22:0S18, (15) C24:0S16, (16) C22:0D18, (17) C23:1S18, (18) C24:1S17, (19) C23:0S18, (20) C24:1S18, (21) C24:0S18, (22) C24:1D18, (23) C24:0D18, (24) C25:1S18, (25) C25:0S18, (26) C26:1S18, (27) C26:0S18.

for those possessing four hydroxyl groups (e.g. ceramides containing phytosphingosine and α-hydroxylated acid).

The gas pressure and the temperature conditions (gradient) were selected in order to be able to reach the best separation for the more complex samples. Figs. 1 and 2 correspond to the separations obtained with ceramides type III and IV, respectively, from Sigma containing sphingosines or dihydrosphingosines. In less than 20 min, 18 peaks were observed

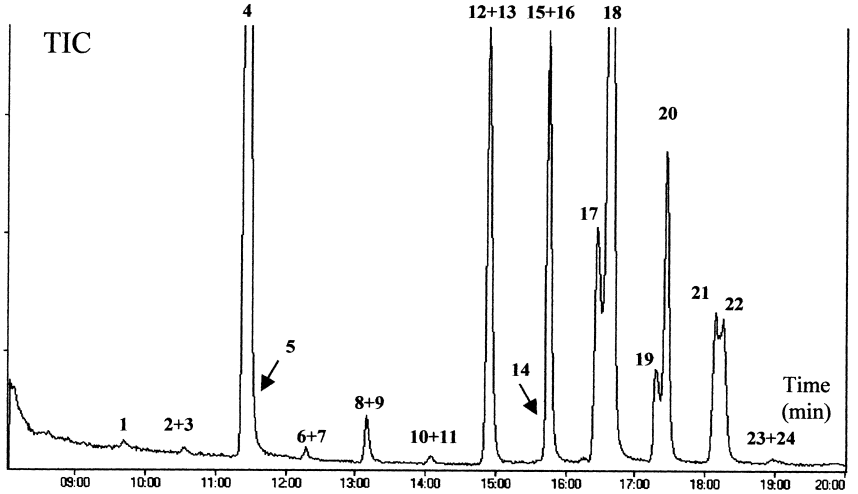

Fig. 2. TIC: total ion chromatogram of Ceramide Type IV (Sigma); (1) C18:0α-OHS16, (2) C17:0α-OHS18, (3) C18:0α-OHS17, (4) C18:0α-OHS18, (5) C18:0α-OHD18, (6) C18:0α-OHS19, (7) C19:0α-OHS18, (8) C18:0α-OHS20, (9) C20:0α-OHS18, (10) C23:0α-OHS16, (11) C22:0α-OHS17, (12) C22:0α-OHS18, (13) C24:0α-OHS16, (14) C23:1α-OHS18, (15) C23:0α-OHS18, (16) C25:0α-OHS16, (17) C24:1α-OHS18, (18) C24:0α-OHS18, (19) C25:1α-OHS18, (20) C25:0α-OHS18, (21) C26:1α-OHS18, (22) C26:0α-OHS18, (23) C27:1α-OHS18, (24) C27:0α-OHS18.

for type III and 15 for type IV which correspond to 27 and 24 various ceramide structures, respectively. It has been already reported that these samples contained an important structural heterogeneity of ceramides [2,17]. Fig. 3 presents the chromatograms obtained for less complex mixtures of ceramides from Cosmoferm containing phytosphingosines. For all the samples, the coeluted compounds were homologous ceramides, i.e. containing the same functional groups and the same carbon number but with a different distribution on the two alkyl chains. They were accurately differentiated by mass spectrometry. The separation is based on carbon number and degree of insaturation of either the fatty acid or the long chain base. Addition of one or two OTMS groups is of very little influence on retention times.

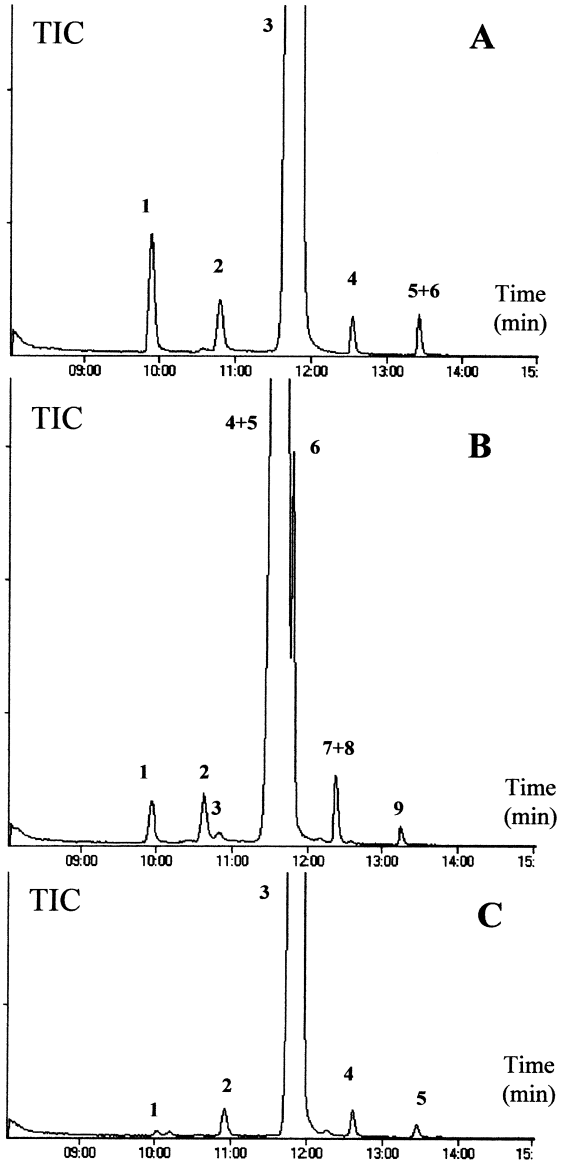

Fig. 3. TIC: total ion chromatograms of (**A**) Ceramide CIII (Cosmoferm); (1) C16:0P18, (2) C18:0P17, (3) C18:0P18, (4) C18:0P19, (5) C18:0P20, (6) C20:0P18; (**B**) Ceramide CIIIB (Cosmoferm); (1) C16:0P18, (2) C18:1P17, (3) C17:0P18, (4) C18:1P18, (5) C18:2P18*, (6) C18:0P18, (7) C18:1P19, (8) C18:2P19*, (9) C18:1P20; (**C**) Ceramide CVI (Cosmoferm); (1) C16:0α-OHP18, (2) C18:0α-OHP17, (3) C18:0α-OHP18, (4) C18:0α-OHP19, (5) C18:0α-OHP20. *Trace compounds.

The mass spectra obtained for the major compound of ceramide standards are in good agreement with published data [9]. The published mass spectra were generally recorded at a 22 eV ionising voltage. Consequently, they are qualitatively comparable to ours but with differences in relative intensities. They concern essentially ceramides constituted of sphingoid bases with 18 carbon atoms (most common chain length) and fatty acids with even numbers of carbon atoms. Therefore, for other ceramides, m/z values of specific ions have been calculated by methylene group incrementation. For minor compounds giving partial mass spectra, identification was made using fragmentometry with these specific ions.

The structural differences between the sphingoid

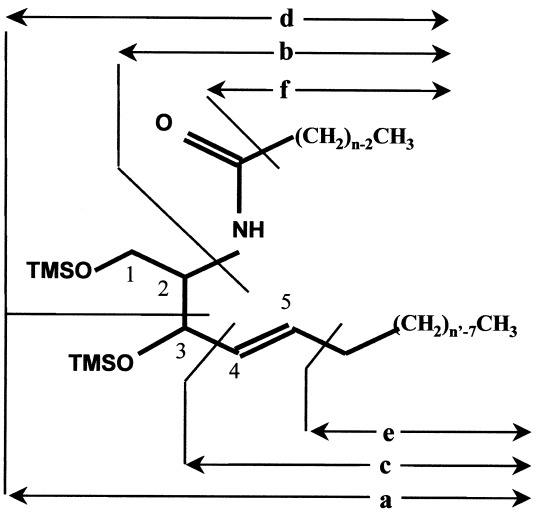

Fig. 4. Main cleavages of trimethylsilylated ceramides with sphingosine base.

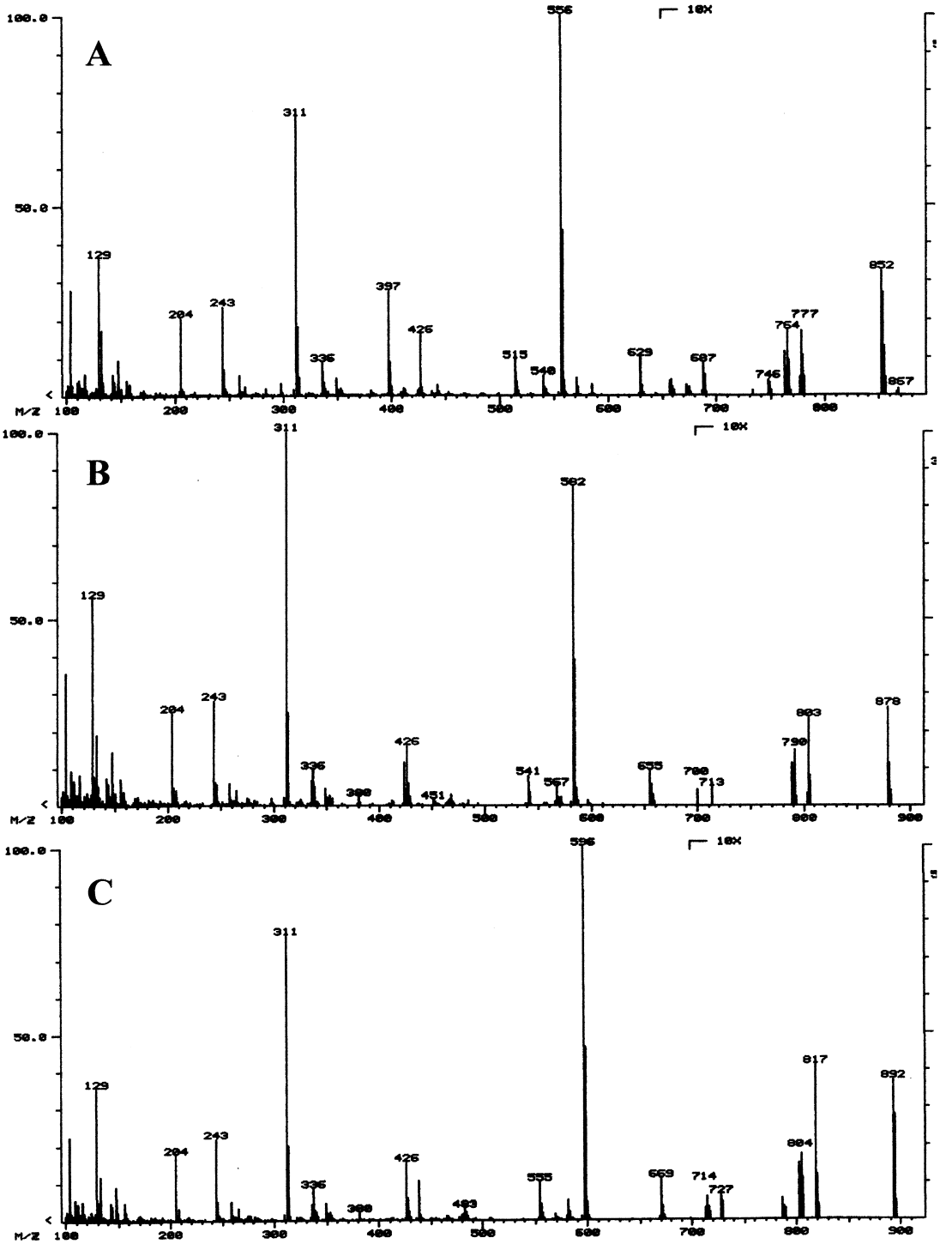

Fig. 5. Spectra of TMS ceramides with sphingosine base. (A) C23:0α-OHS18; (B) C25:1-αOHS18; (C)26:1-αOHS18.

bases are located on carbons 4 and 5, $-^{4}CH_2-^{5}CH_2$ pattern being observed for dihydrosphingosine base, and $-^{4}CH(OH)_2-^{5}CH_2$ for phytosphingosine base.

The structural identification of TMS ceramides requires at least two types of ions: characteristic ions of the base moiety and characteristic ions of the fatty acid moiety which give the nature and the carbon content of the base and acid moieties. Fig. 4 describes the main cleavages in TMS ceramides. The molecular peak was generally very weak or absent. However, the molecular mass can be indicated by fragments $[M-15]$ (loss of CH_3 from a TMS group), $[M-90]$ (loss of one trimethylsilanol), or $[M-103]$ (loss of CH_2OTMS after cleavage between carbon 1 and 2 of the base moiety). However, when ceramides were present in trace amounts, these ions were generally not observed.

The identification of the base moiety when ceramides contain sphingosine, can be established from the ions: $[M-d]$ (cleavage between C_2 and C_3 with charge retention on C_3), $[M-(b+1)]$ (loss of acylamide), $[M-(b+1+e)]$ (loss of acylamide and a

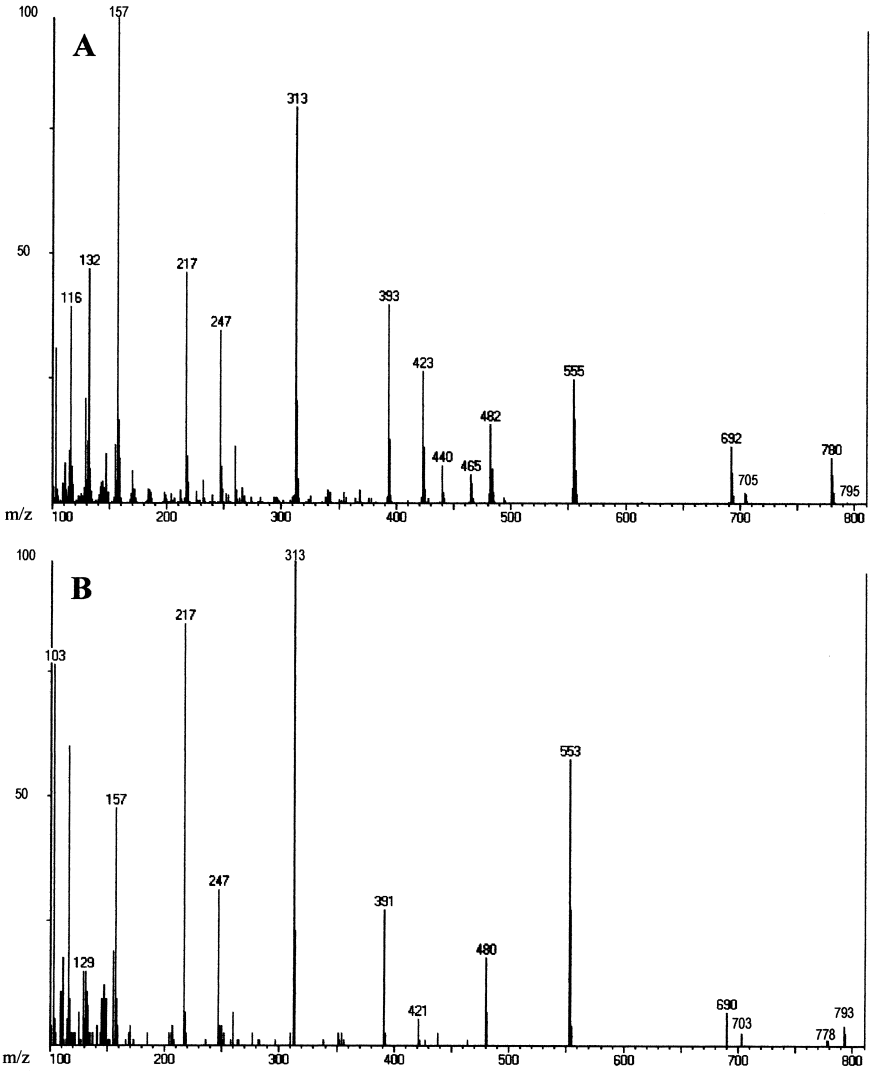

Fig. 6. Spectra of TMS ceramides with dihydrosphingosine. (A) C24:0D18 (B) C24:1D18.

part of the alkyl chain beyond carbon 5). [$M-$d] and [$M-$(b+ 1)] values depend of the chain length of the base. They are, respectively, at m/z 311 and m/z 426 for a sphingosine with 18 carbon atoms. The third ion is always observed at m/z 243.

For ceramide with dihydrosphingosine base, an intense [$M-$d] ion was also observed. However, the ion [$M-$(b+1)] was still observed, but weak. They were found, respectively, at m/z 313 and m/z 428 for a dihydrosphingosine with 18 carbon atoms. The nature of the base was confirmed by the ion [$M-$(b+1+c)] (loss of acylamide and a part of the alkyl chain beyond carbon 3) always found at m/z 217.

The fatty acid moiety of ceramides with sphing-

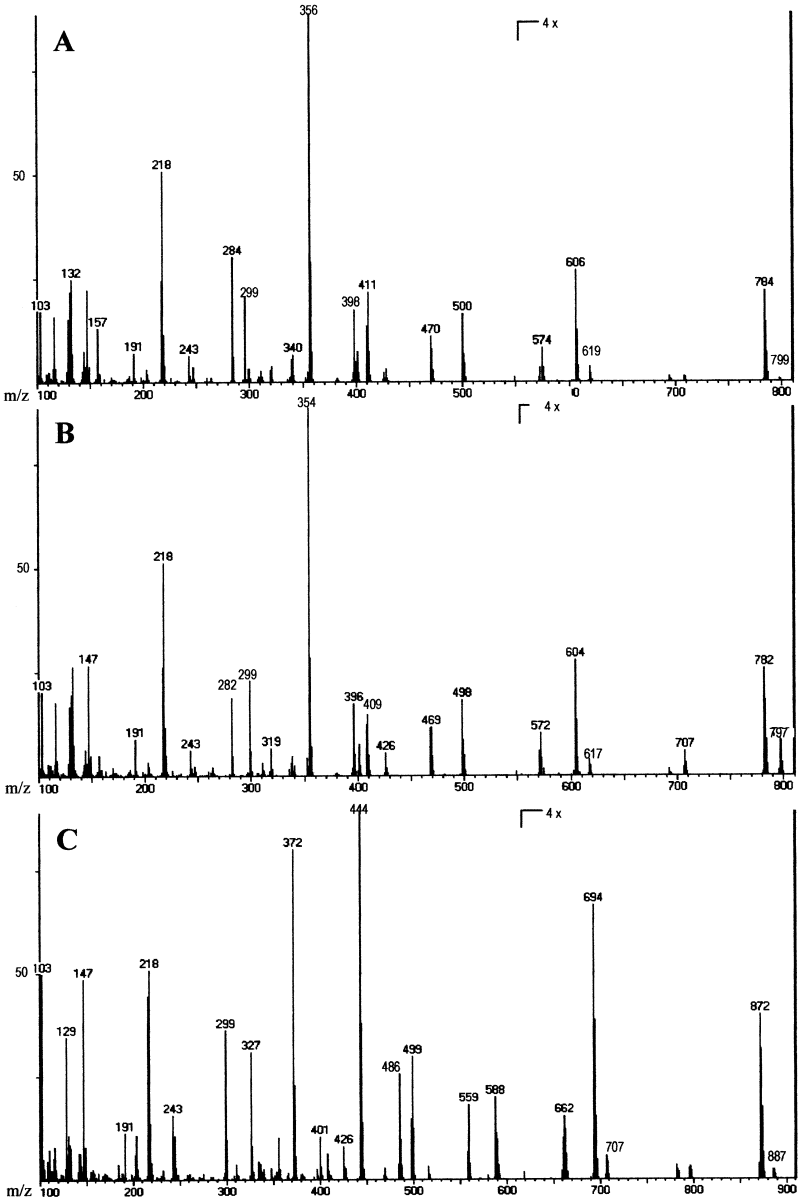

Fig. 7. Spectra of TMS ceramides with phytosphingosine. (A) C18:0P18; (B) C18:1P18; (C) C18:0-αOHP18.

osine and dihydrosphingosine, was characterised by the ions [$M-$a] (cleavage between C_2 and C_3 with charge retention on C_2) and [$M-$(a−73)] (same cleavage with transfer of a TMS group). These ions were observed whatever the nature of the fatty acid (saturated, unsaturated or α-hydroxylated).

For TMS ceramides containing phytosphingosine, the base moiety was characterised by the ions [$M-$d], [c] (cleavage between C_3 and C_4 with charge retention on C_4) and [$M-$(b+c)]. The values of [$M-$d] and [c] depend on the base chain length. For a ceramide with P18 phytosphingosine, they were found, respectively, at m/z 401 and m/z 299. The third ion was always observed at m/z 218.

For the characterisation of the acid moiety, ion [$M-$a] was still remaining, but with a weaker intensity. Conversely, the ion [(b+1+73] coming from [$M-$(c−73)] after complex rearrangement gave generally the base peak [12]. The fatty acid moiety was also characterised by ion [b+2].

For ceramides containing a sphingosine or dihydrosphingosine base, published mass spectra are abundant and major variations in the acid chains are illustrated. The originality of compounds encountered in commercial ceramides lies only in particular acid or base chain length. For example, Figs. 5 and 6 present original mass spectra of ceramides with sphingosine and dihydrosphingosine base linked with fatty acid of uncommon chain length. Partial data are also given in Table 2 for all the ceramides containing acid or base of uncommon chain length.

In the literature, the mass spectra of TMS ceramides containing a phytosphingosine base are scarce and concern always bases associated with saturated fatty acids [12]. Fig. 7 presents, for comparison, full mass spectra obtained for P18 phytosphingosines linked, respectively, with saturated, unsaturated and hydroxylated fatty acids. In addition, Table 1 presents the main characteristic fragments for most of the ceramides observed in the three studied samples from Cosmoferm.

Table 2 details the structures find in each sample and gives m/z values of the two ions required for identification when the spectrum was not described in the literature.

4. Conclusion

GC–MS analysis after trimethylsilylation is an efficient method in order to determine the composition of complex ceramide samples, also well adapted to the detection of minor compounds. Eighty-three different structures have been identified in the whole commercial products. For 52 of them,

Table 1
Characteristic fragments of TMS derivatives of ceramides with phytosphingosine base and various fatty acids

Ceramides	m/z (relative intensity, %)												
	$M-$(b+1+c)	$M-$(b+c)	b+2	c	f[a]	$M-$d	$M-$(b+1+90)	b+1+73	$M-$a	$M-$(c+89)	$M-$((a+1)−73)	$M-$c	$M-$103−90	$M-$15
C16:0P18	217(24)	218(47)	256(25)	299(18)		401(6)	426(2)	328(100)	370(16)	383(22)	422(11)	472(15)	578(5)	756(4)
C18:1P17	217(37)	218(64)	282(19)	285(25)		387(6)	412(2)	354(100)	396(16)	409(16)	468(10)	498(15)	590(5)	768(5)
C18:0P17	217(33)	218(56)	284(28)	285(30)		387(6)	412(11)	356(100)	398(17)	411(22)	470(10)	500(16)	592(7)	770(4)
C18:1P18	217(27)	218(51)	282(23)	299(24)		401(8)	426(6)	354(100)	396(18)	409(15)	468(12)	498(19)	604(7)	782(7)
C18:0P18	217(25)	218(51)	284(30)	299(21)		401(8)	426(3)	356(100)	398(18)	411(22)	470(11)	500(17)	606(7)	784(6)
C18:1P19	217(36)	218(64)	282(20)	313(21)		415(6)	440(1)	354(100)	396(17)	409(14)	468(11)	498(17)	618(6)	796(5)
C18:0P19	217(31)	218(54)	284(27)	313(18)		415(5)	440(1)	356(100)	398(15)	411(20)	470(8)	500(15)	620(4)	798(3)
C18:1P20	217(42)	218(66)	282(20)	327(20)		429(3)	454([b])	354(100)	396(12)	409(12)	468(9)	498(14)	632(3)	810(1)
C16:0α-OHP18	217(72)	218(55)	344(73)	299(x)	299(82−x)	401([b])	426([b])	416(100)	458(13)	471(17)	531(12)[c]	560(7)	666(7)	844(2)
C18:0α-OHP17	217(63)	218(66)	372(79)	285(45)	327(35)	387(11)	412(6)	444(100)	486(24)	499(30)	559(15)[c]	588(20)	608(14)	858(6)
C18:0α-OHP18	217(45)	218(51)	372(80)	299(36)	327(31)	401(11)	426(8)	444(100)	486(26)	499(30)	559(18)[c]	588(20)	694(17)	872(10)
C18:0α-OHP19	217(51)	218(56)	372(73)	313(27)	327(31)	415(5)	440(5)	444(100)	486(21)	499(28)	559(17)[c]	588(17)	708(12)	886(3)
C18:0α-OHP 20	217(65)	218(58)	372(69)	372(x)	327(55−x)	429(4)	454(2)	444(100)	486(24)	499(26)	559(13)[c]	588(5)	722(10)	900(2)

[a] Detected only with ceramides containing an α-hydroxy acid.
[b] Not detected.
[c] $M-$(a−73) for ceramides containing an α-hydroxy acid.

Table 2
Summary of the identified structures in each commercial sample

Sample name	Ceramide structures[a]
N-Stearoyl-D-sphingosine	C18:0S16 C16:0S18 C18:0S17 (398,297) C17:0S18 (384,311) C18:0S18 C18:0S19 (398,325) C18:0S20 (398,339)
N-Palmitoyl-D-sphingosine	C16:0S16 C14:0S18 C16:0S17 (370,297) C15:0S18(356,311) C16:0S18 C16:0S19 (370,325) C16:0S20 (370,339)
N-Oleoyl-D-sphingosine	C18:1S16 (396,283) C18:1S17 (396,297) C18:1S18 C18:1S19 (396,325) C18:1S20 (396,339)
N-Nervonoyl-D-sphingosine	C24:1S16 C24:1S17 (480,297) C24:1S18 C24:1S19 (480,325) C24:1S20 (480,339)
N-Palmitoyl-DL-dihydrosphingosine	C16:0D18
N-Lignoceroyl-DL-dihydrosphingosine	C24:0D18
Type III	C16:0S18 C18:0S16 C16:0D18 C18:0S17 (398,297) C17:0S18 (384,311) C18:0S18 C18:0D18 C18:0S19 (398,325) C19:0S18 (412,311) C18:0S20 (398,339) C20:0S18 C22:1S18 C24:1S16 C22:0S18 C24:0S16 C22:0D18 C23:1S18 (466,311) C24:1S17 (480,297) C23:0S18 C24:1S18 C24:0S18 C24:1D18 (553,313) C24:0D18 C25:1S18 C25:0S18 (496,311) C26:1S18 C26:0S18
Type IV	C18:0α-OHS16 (486,283) C17:0α-OHS18 (472,311) C18:0α-OHS17 (486,297) C18:0α-OHS18 C18:0α-OHD18 C18:0α-OHS19 (486,325) C19:0α-OHS18 (500,311) C18:0α-011S20 (486,339) C20:0α-OHS18 C23:0α-OHS16 (556,283) C22:0α-OHS17 (542,297) C22:0α-OHS18 C24:0α-OHS16 (570,283) C23:1α-OHS18 C23:0α-OHS18 C25:0α-OHS16 (584,283) C24:1α-OHS18 (568,311) C24:0α-OHS18 C25:1α-OHS18 (582,311) C25:0α-OHS18 (584,311) C26:1α-OHSI8 (596,311) C26:0α-OHS18 C27:1α-OHS18 (610,311) C27:0α-OHS18 (612,311)
Ceramide III	C16:0P18 C18:0P17 (356,285) C18:0P18 C18:0P19 (356,313) C18:0P20 (356,327) C20:0P18
Ceramide IIIB	C16:0P18 C18:1P17 (354,285) C17:0P18 (342,299) C18:2P18 (352,299) C18:1P18 C18:0P18 C18:2P19 (352,313) C18:1P19 (354,313) C18:1P20 (354,327)
Ceramide VI	C16:0α-OHP18 (416,299) C18:0α-OHP17 (444,285) C18:0α-OHP18 (444,299) C18:0α-OHP19 (444,313) C18:0α-OHP20 (444,327)

[a] When spectra were not described in the literature, the m/z values of characteristic ions, respectively, for the identification of acid and base moieties are indicated in brackets: [$M-$a] and [$M-$d] for sphingosine TMS ceramides, [$M-$(a$-$73)] and [$M-$d] for dihydrosphingosine TMS ceramides, [b+l+73] and [c] for phytosphingosine TMS ceramides.

this study provided the main fragments which are not available in the literature (e.g. ceramides with odd chain lengths) and also original mass spectra (e.g. Cn:1Pn′, Cn:0-αOHPn′).

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