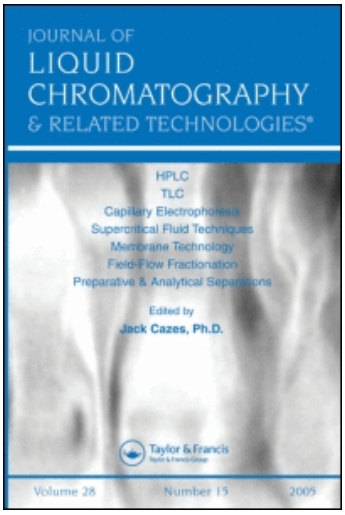

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CONTRIBUTION TO LIQUID CHROMATOGRAPHIC ANALYSIS OF CUTANEOUS CERAMIDES

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ABSTRACT

Ceramides constitute the major group of lipids in the Stratum Corneum where they play a crucial role in maintaining the water permeability barrier of the mammalian epidermis. Cutaneous ceramides are highly heterogeneous in structure. Consequently commercial standards of all these molecules are not available and no simple analytical system supports this magnitude at this time. Our work suggests a strategy, which offers the possibility of having the first simple tool for ceramide structural identification in the absence of a ceramide standard. The ceramide separation is done in NARP (non-aqueous reverse phase) liquid chromatography and the ceramide structural identification in GC/MS (gas chromatography / mass spectrometry). The correlation between these two methods establishes the retention behaviour of ceramides. This retention has been shaped into abacuses.

This approach is based on various theories: the linear progression of t'_R in a linear gradient, methylene selectivity and the incrementation on homologous series. This approach by modelisation has the advantage of being completed by iterations each time a new structure is available and without having to carry out the optimisation of the system again.

INTRODUCTION

Ceramides constitute the major group of lipids in the Stratum Corneum where they play a crucial role in maintaining the water permeability barrier of the mammalian epidermis.[1-6] Structural studies indicate that cutaneous ceramides are constituted by a sphingoide base (sphingosine, phytosphingosine, dihydrosphingosine, or 6-hydroxy-sphingosine) linked with a fatty acid (non OH fatty acids, α-OH fatty acids or ω-OH fatty acids) with amide-linked.[7-8] Furthermore the hydrocarbon chain of both of these moieties changes in length. That constitutes the basis of a highly heterogeneous family of compounds.

Up to now considerable effort has been undertaken to determine the different classes of the total lipid content of the Stratum Corneum. Although HPTLC[9] is the most used, other analytical methods have been used, like HPLC,[10-11] GC/MS,[12] FTIR,[13] NMR.[8] However, at this time there is no simple analytical system able to provide an efficient separation of ceramides based on the various functional groups per molecule as well as, the length of the hydrophobic chains.

Our strategy is to build a model, which would link together the structures of ceramides with their chromatographic retention. This allows us to impute an unknown peak to a possible structure and calculate the retention of a new standard without redoing the optimisation of the chromatographic system. This approach is justified by the lack of well defined reference substances for all cutaneous ceramides.

The large hydrocarbon volume of ceramides leads us to investigate the potentiality of NARP chromatography[14] for their separation. This technique uses C_{18} or C_8 grafted silica phases with organic solvents whereas in conventional RP-HPLC, hydro-organic solvents are used.

The use of an organic solvent mixture enables us to make the ceramide soluble. NARP has already shown successful separation for highly hydrophobic compounds. The structural study was carried out in GC/MS.

EXPERIMENTAL

Samples

Ceramide type III, Sphingosine (S), N-Palmitoyl-D-sphingosine (C16:0S), N-Stearoyl-D-sphingosine (C18:0S), N-Palmitoyl-DL-dihydrosphingosine (C16:0D), N-Oleoyl-D-sphingosine (C18:1S), N-lignoceroyl-DL-dihydro-sphingosine (C24:0D), N-Nervonoyl-D-sphingosine (C24:1S) were all purchased from Sigma (St. Quentin Fallavier, France). Phytosphingosine (P), ceramide III (CIII) and ceramide IIIB (CIIIB) were a generous gift of Cosmoferm (Delft, Netherlands).

High Performance Liquid Chromatography

Standards were prepared in methanol at 0.03 $mg.mL^{-1}$ or 0.3 $mg.mL^{-1}$ except for ceramide type III which was prepared at 1 $mg.mL^{-1}$. Measurements were carried out with a Thermo Separation Products P1000 XR gradient pump with a TSP SCM1000 (Thermo Separation Products, San Jose, California, USA), vacuum membrane degasser connected with a Kontron autosampler 360 (Kontron Instruments, Milan, Italy) equipped with a 3 μL sample loop injection valve. Detection was performed with a Cunow DDL 11 evaporative light-scattering detector (Eurosep, Cergy, France). The chromatograms were recorded with a Kontron PC-integration pack Rev. 3.90 (Kontron Instruments, Milan, Italy). The flow-rate was set at 0.4 $mL.min^{-1}$. The column (125 x 2.0 mm I.D.) was Kromasil C_{18} (Eka Nobel, Bohus, Sweden) with a particle size of 5 μm supplied by Macherey-Nagel (Hoechst, Düren, Germany). The column dead time was measured by injection of a 0.5 $mg.L^{-1}$ solution of internal standard of urea diluted in methanol. The gradient delay time was measured with a Kratos Spectroflow 773 detector (ABI, PE, Norwalk, Connecticut, USA) at 230 nm by observing the rise in the baseline while running a gradient from 100% methanol to a methanol-0.2% acetone mobile phase. The experimental value of the gradient delay time was 1.25 min for the flow-rate set at 0.4 $mL.min^{-1}$. Every mobile phase contains a constant amount of triethylamine (0.15%) and equimolecular formic acid.

Gas Chromatography and Mass Spectrometry

Materials and reagents were all of analytical grade. The trimethylsilylation reagent, REGISI1 + 1% TMCS, consisting of bis(trimethylsilyl)tri-fluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) was purchased from REGIS Technologies, Inc, Morton Grove, I1 60053, U.S.A.

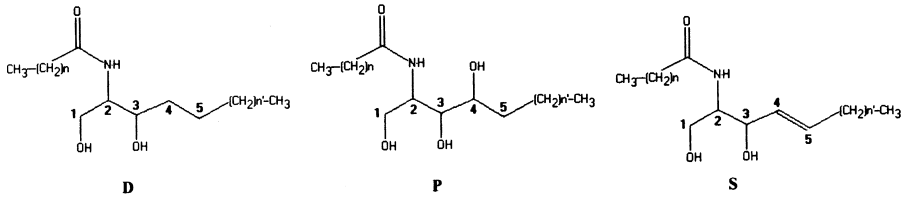

Figure 1. General structures of ceramides. n=12 to 30 and n'=10 to 20 for the phytosphingosine base or n'=10 to 16 for the dihydrosphingosine and sphingosine bases. the acidic part could be purely hydrocarbon, α=hydroxylated or ω=hydroxylated. (The two last kinds of structures are not shown here.)

Methanolysis was carried out as previously reported.[23] Samples (typically 1 mg) were taken up in 0.5 mL of methanolic HCl solution prepared by adding acetyl chloride (0.4 mL) to 15 mL of methanol. Methanolysis was conducted at 80°C for 24 hrs.

Thereafter, HCl was neutralized by adding pyridine and methanol was removed using a nitrogen stream. BSTFA/TMCS 1% (0.25 mL) and CH_3CN (0.25 mL) were added to the dried material. The solutions were then heated to 80°C for 2hrs. GC-MS analysis was performed directly with 1 μL of this solution.

The GC-MS system consisted of a SHIMADZU GC-17A gas chromatograph interfaced by direct coupling to a SHIMADZU QP 5000 quadrupole mass spectrometer (SHIMADZU CORPORATION, Kyoto, Japan, supplied by Touzart et Matignon, Courtaboeuf, France). The gas chromatograph was equipped with a 30 m x 0.25 mm I.D. fused silica column coated with a 0.25 μm film of DB-5 poly (5% phenyl, 95% methylsiloxane) (J. & W. Scientific, Folsom, CA, USA).

The carrier gas was helium at a flow rate of 1mL/min (measured at 50°C). Injector and transfer line temperatures were set to 300°C and 250°C, respectively. A splitless mode injection (splitless time 30 sec.) was followed by the oven temperature program:50°C-150°C at 30°C/min, 150°C-290°C at 10°C/min and 290°C for 10 mins.

Electron impact (EI) mass spectra were collected in the total ion monitoring mode. Operating conditions for EI-MS were: source temperature 160°C, filament emission current 750 μA, ionizing voltage 70 eV, scan range from m/z 45 to m/z 700 with a period of 2 sec. The other operating parameters were those set by the instrument's automatic calibration routine.

Table 1

Ceramide Identification*

	Major Acid n + 2	Major Base n' + 6	Minor Acid n + 2	Minor Base n' + 6
Ceramide				
CIIIB (a)	C18:1	P18	C18:0	P17
			C16:0	P19
			C18:2	P20
CIII (a)	C18:0	P18	C16:0	P17
			C20:0	Cy18
C16 :0S (b)	C16:0	S18	C18:0	S20
			C17:0	
			C15:0	
			C14:0	
			C12:0	
C16 :0D (b)	C16:0	D18	C18:0	D20
C18 :0S	C18:0	S18	C16:0	S16
			C20:0	S20
C18 :1S (b)	C18:1	S18	C18:0	S16
			C16:0	S20
				S19
C24 :1S (b)	C24:1	S18		
C24 :0D (b)	C24:0	D18		
Amine Base				
Phytosphingosine (a)		P18		
Sphingosine (b)		S18		S20

* (a) ceramides from Cosmoferm, (b) Ceramides from Sigma,
P: Phytoshingosine, S: Sphingosine, D: Dihydrosphingosine,
Cy: Cycloanhydrophytosphingosine. n and n' refer to the structures described in Figure 1.

RESULTS AND DISCUSSION

Two commercial sources of ceramides were considered for our experiments: Sigma and Cosmoferm.[15] There were two determining factors in this choice. First they provide structurally well known ceramides like those found in the skin. Secondly each sample is generally composed of one major product with traces of homologues. This last point enables us to get a maximum of variety in the chemical structures of the ceramides. The general structures of ceramides are shown on Fig. 1.

Mass Spectrometry

Gas chromatography-mass spectrometry (GC/MS) was used to determine ceramide structures after cleavage by acidic methanolysis and conversion to volatile trimethylsilyl derivatives. This method was widely applied on triglycerides, waxes,[16] and sugars[17] to achieve an efficient structural determination of these compounds. The methanolysis of ceramides leads to the formation of fatty acid methyl esters and long chain base. The silylation was performed by BSTFA which is described as an efficient sylilating agent for the amino compounds.[18-19]

Table 1 summarises all of the different fatty acids and amine bases found for each sample.

Identification of the fatty acid moiety

Three different types of acids were encountered : saturated fatty acid, unsaturated fatty acid, and saturated hydroxy fatty acid. Fatty acid methyl esters are very stable. Their mass spectra are well documented in the literature, and many homologues are represented in commercially available libraries.

Identification of the long chain base moiety

The long-chain bases encountered in this study were mainly of three types: sphingosine, dihydrosphingosine, and phytosphingosine. With the silylation procedure employed, they were converted into N,O-trimethylsilyl (TMS) derivatives. Mass spectral data concerning exactly this kind of derivatives are scarce.[20-21] They are more abundant concerning O-TMS derivatives,[22-24] their N-acetylated forms[25] or O-TMS ceramides.[26-27]

The mass spectra of N,O-TMS derivatives were analysed based on these results together with ours, obtained with authentic samples in the cases of C_{18} sphingosine and C_{18} phytosphingosine. (See Figure 2.)

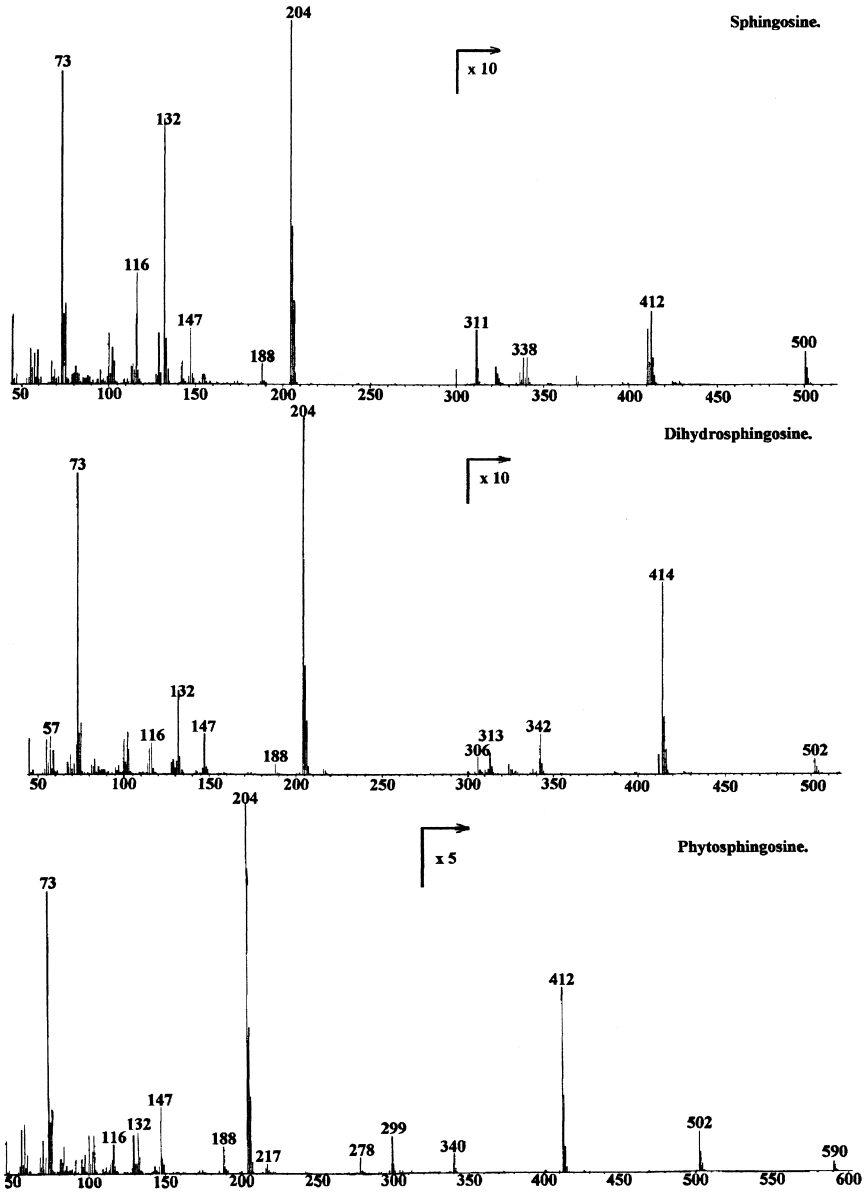

Figure 2. Mass spectra of the three N,O-TMS base derivatives.

Table 2

Characteristic Ions for N, O-TMS Long Chain Bases*

	m/z Values					
LCB	**M-15**	**M-15-90**	**M-103**	**M-103-90**	**M-204**	**M-306**
S16	472	382	385	W	283	A
S18	500	410	412	W	311	A
S20	528	438	440	W	339	A
D18	502	W	414	W	313	A
P17	576	A	488	398	A	285
P18	590	A	502	412	A	299
P19	604	A	516	426	A	313
P20	618	A	530	440	A1	327

* Abbreviations: S = sphingosine; D = dihydrosphingosine; P = phytosphingosine; A = absent; W = weak.

The molecular peak is generally absent but the molecular weight is indicated by fragments at (M-15) and (M-103) formed by elimination of CH_3 from one of the TMS groups and the terminal CH_2OTMS respectively (Table 2). The consecutive loss of one molecule of trimethylsilanol yields the ion at (M-15-90) in the spectra of sphingosines and the ion at (M-103-90) in the spectra of phytosphingosines. Cleavage between C-2 and C-3 after initial charge localization on the nitrogen at C-2 results in the most abundant ion at m/z 204 $[(CH_3)_3SiOCH_2CH=N^+HSi(CH_3)_3]$. In the spectra of sphingosine and dihydrosphingosine, the same cleavage after initial charge localization on the oxygen at C-3 results to ions (M-204). For the phytosphingosine, the presence of the additional trimethylsilyloxy group at C-4 leads to the cleavage between C-3 and C-4 with a charge retention on the oxygen at C-4, to form ions (M-306).

In the mass spectra of N,O-TMS long chain bases, characteristic ions are observed which are also detected in the mass spectra of the corresponding non N-silylated bases. The most intense is observed at m/z 132 $[(CH_3)_3SiOCH_2CH=N^+H_2]$. This is the equivalent of the ion at m/z 204 for the non N-silylated bases. Additional ions are also present in the mass spectrum of the sphingosine (m/z 338: M-105 and m/z 340: M-103) and in the mass spectrum of the dihydrosphingosine (m/z 342: M-103). They are also present with different relative intensities in published mass spectra[20,21,26] but their formation has never been explained.

During this work, we have noticed that the relative intensity of these ions strongly decreased with the fall of the ion source temperature. Thus, we can suppose that their formation involves a partial thermal decomposition of the N-silylated derivative leading to the non N-silylated compound.

The mass spectral behaviour of N,O-TMS long chain bases, including cyclo-anhydrophytosphingosine will be detailed in a future paper.

Liquid Chromatography

Development of the mobile phase

Both, selectivity and detection were considered. The evaporative light scattering detector (ELSD) is considered as it is a nearly universal[27] and sensitive detector for lipids.[28] As the response of this detector is connected with the size of the droplets generated by the nebulizer, it is also influenced by the nature of the mobile phase. This criterion has to be taken in consideration during the mobile phase optimisation. According to the results obtained on triglycerides in NARP[14] acetonitrile (ACN) and methanol were considered for the selection of the weak solvent. No elution occurred with a 100 % of ACN mobile phase. Pure methanol appeared to be a stronger solvent, leading to the elution of ceramides (with chain lengths equal to 18 carbons) with factors of retention below 5.

Since for more hydrophobic ceramides (with fatty acids at 24 carbons), the elution strength of methanol would be insufficient, propanol (PrOH) was used in order to obtain the suitable elution strength. As the scale of polarity for all of the studied ceramides is very large, the use of gradient became necessary to allow the elution of all ceramides without a loss in separation of the early eluting solutes.

Stronger solvents (tetrahydrofuran, dichloromethane, chloroform) improve the response of the detector for the identification of minor peaks. Tetrahydrofuran (THF) leads to a better sensitivity. The chromatographic measurements have been realised with the following gradient mode from ACN / THF (95 :5) to ACN / THF / PrOH (65 :35 :5) in 30 min.

The mobile phase was modified by adding 0.15% of triethylamine in order to minimise silanol interactions. An equimolar amount of formic acid is also added to neutralise the pH. For each sample, both concentrations were prepared; the weaker one to measure the retention time of the major peak, and the other, more concentrated, to measure the retention time of the traces.

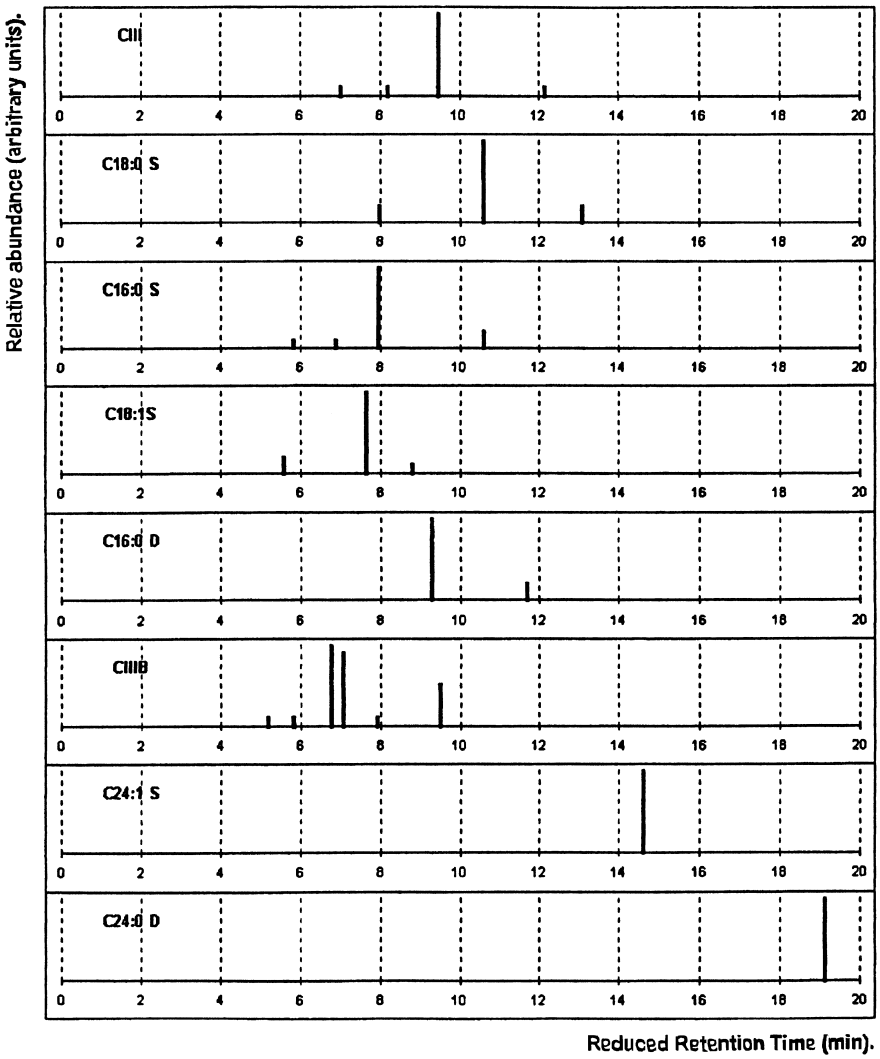

Figure 3. Schematic chromatographic profiles represented with the reduced retention times obtained with a gradient THF/acetonitrile [5:95] to THF/propanol/acetonitrile [5:60:35] in 30 min.

In the latter cases, this provokes a detector overload for the major peaks. Chromatographic profiles are schematised with bar graphs on Figure 3 using the average of the reduced retention times (t_R') obtained from four injections done at different days.

Table 3

Calculated Values of Increments from Reduced Retention Time of Major Peaks

Structural Variations	Increments t_R' (min)	Standard Deviation	Relative Standard Deviation(%)	Number of Data (n_d)
ΔCH_2	1.25	0.08	6	3
$\Delta C = C$	-2.82	0.12	4	2
$\Delta S/P$	-1.01	0.12	11	2
$\Delta S/D$	1.36			1

The heights of the peaks are not proportional to those observed on the chromatograms. It gives information about the abundance of the different components of each sample, if they have to be considered as a trace or as a major product.

Structural information from HPLC measurements

As we used linear gradients and homologous series of ceramides, we have constant time intervals between the same kind of variations in structure (i.e. methylene group, unsaturations, or hydroxyl group). Increments are calculated between these differences in structure by considering only the average of reduced retention time of the major peaks (Table 3) of our ceramide samples.

The values of these increments have to be understood as a variation of the reduced retention time values of the ceramide equal to:

- 1.25 min when the fatty acid chain increases of one CH_2.
- -2.82 min when an unsaturated fatty acid is present instead of a saturated one with the same chain length.
- -1.01 min when a phytosphingosine base is present instead of sphingosine one.
- 1.36 min when a dihydrosphingosine base is present instead of sphingosine one.

As shown in Reference 31, the value of the ΔCH_2 may vary (about 5%) with the polar head nature of homologous series. The lack of samples prevented us to taking into account this feature to calculate ΔCH_2 due to hydrocarboned chain on the different bases. So the recorded values in Table 3 for ΔCH_2 and $\Delta C=C$ are average values from ceramides with different bases. The CH_2 and

C=C are considered on the fatty acid chain length. ΔCH_2 was calculated between two fatty acid chain lengths for each base (n_d =3). $\Delta C{=}C$ was computed from differences in retention between ceramides with saturated and unsaturated C_{18} fatty acids containing either sphingosine or phytosphingosine (n_d =2). $\Delta S/P$ was calculated from ceramides containing saturated and unsaturated C_{18} fatty acids (n_d =2). $\Delta S/D$ was obtained from ceramides with C_{16} fatty acids (n_d =1).

Correlation between mass spectrometry and HPLC data

The reduced retention times are related to three major structural differences: the base structure, the fatty acid chain length, and the unsaturation of the fatty acid.

With these increments and the mass spectrometric data, we plotted a graph which represents a prediction of the chromatographic profiles. In Figure 4, the number of peaks for each sample comes from all of the possible statistical combinations between base and acid entities found in mass spectrometry. For each sample, the estimated reduced retention times for each expected structure were calculated by adding the reduced retention time of the major peak, to the value of increment(s) corresponding to the variation(s) in the structure of the minor peaks.

The comparison between the Figures 3 and 4, provides the first step of the trace amount identification with the following remarks:

Gas chromatography is a more sensitive technique than liquid chromatography, so we had expected a greater number of peaks than we actually observed in liquid chromatography. The same phenomenon was observed considering the observed methyl esters of carboxylic acids by GC and NARP chromatography of triacylglycerols.[32]

The intensity of the peaks obtained in GC/MS indicates the relative abundance of the recombined structures.

There is a slight variability of the experimental t_R' due in particular to some variation in temperature, (even small, 0.5°C). This leads to intervals of retention which are not easily presentable on the graph for reasons of clarity.

For CIII, C18 :0S, C16 :0S, C18 :1S and C16 :0D, all the minor peaks present on the chromatograms have been assigned to a predicted structure. In these cases, the real data fit well enough to give unambiguous identification. Furthermore, our identifications have been confirmed in sample C16:0S by spiking this sample with the C18:0 S18 standard. The comparison of the

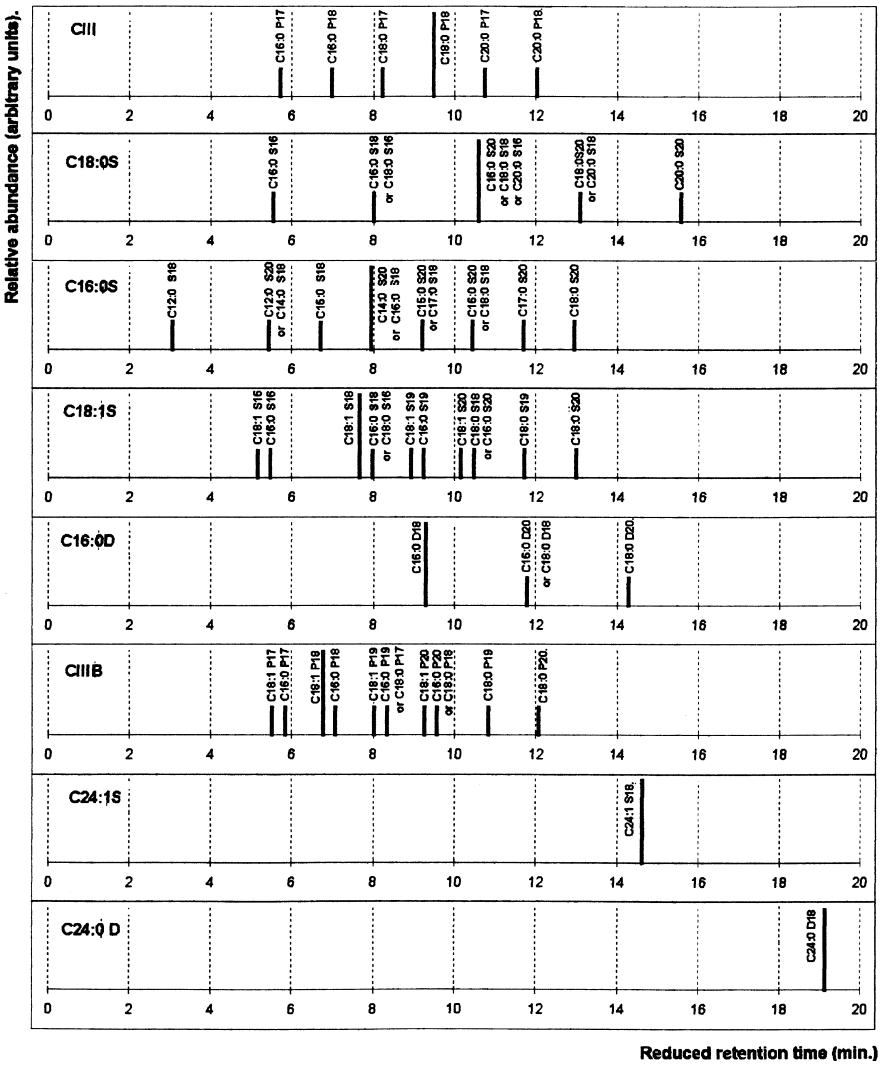

Figure 4. Predicted chromatographic Profiles. t_R' of the major peaks are experimental values. t_R' of the minor peaks are calculated from the increments.

respective heights of each peak of the enriched C16 :0S chromatogram with those of the pure non enriched C16 :0S chromatogram allowed the confirmation of the peak assignment. By using this spiking technique, the assignment of the C16 :0 S18 in the sample C18 :0S has been confirmed too.

CIIIB is a particular case, because in mass spectrometry the presence of another base (called cycloanhydrophytosphingosine) and a fatty acid C18:2 were found. As this fatty acid is contained in only one sample, it is impossible to estimate the influence of this structure on ceramide retention. Furthermore, this new base is not a relevant structure for our model, since the presence of such a base has never been observed in the skin. So, we cannot determine, without ambiguity, any peak structure from this chromatogram. For this sample, every hypothesis must be treated with caution. There is only one exception, the presence of C18:0 P18 was confirmed by injecting its authentic standard into the sample and observing the increase of a peak higher than a trace.

In conclusion, the new structures of ceramides found are: C16:0 P18, C20:0 P18, C18:0 P17, C20:0 S18 (or C18:0 S20), C14:0 S18 (or C12:0 S20), C15:0 S18, C18:0 D18 (or C16:0 D20), C18:1 S19, C16:0 S16.

Building a global diagram

The provisional diagram is built on the basis of linear variation of the t_R' obtained by linear gradient and the equivalent chain length (ECL) of the fatty acid in each ceramide. The integer values on the abscissa axis represent the saturated fatty acid chain length (one unit = one CH_2). The abscissa for an insaturation on the fatty acid chain is calculated using Equation 1:[30]

$$ECL = n + 2\frac{\left(t_R{}'_{U(n)} - t_R{}'_{S(n)}\right)}{t_R{}'_{S(n+2)} - t_R{}'_{S(n)}} \qquad (1)$$

where;

- n is the saturated fatty acid chain length,
- $t_R'_{U(n)}$ is the reduced retention time of the unsaturated fatty acid with a chain length of n CH_2 units,
- $t_R'_{S(n)}$ is the reduced retention time of the saturated fatty acid with a chain length of n CH_2 units,
- $t_R'_{S(n+2)}$ is the reduced retention time of the saturated fatty acid with a chain length of n+2 CH_2 units.

This equation can be rewritten using the values presented in Table 3 as :

$$ECL = n + \frac{\Delta C = C}{\Delta CH\,2} \qquad (2)$$

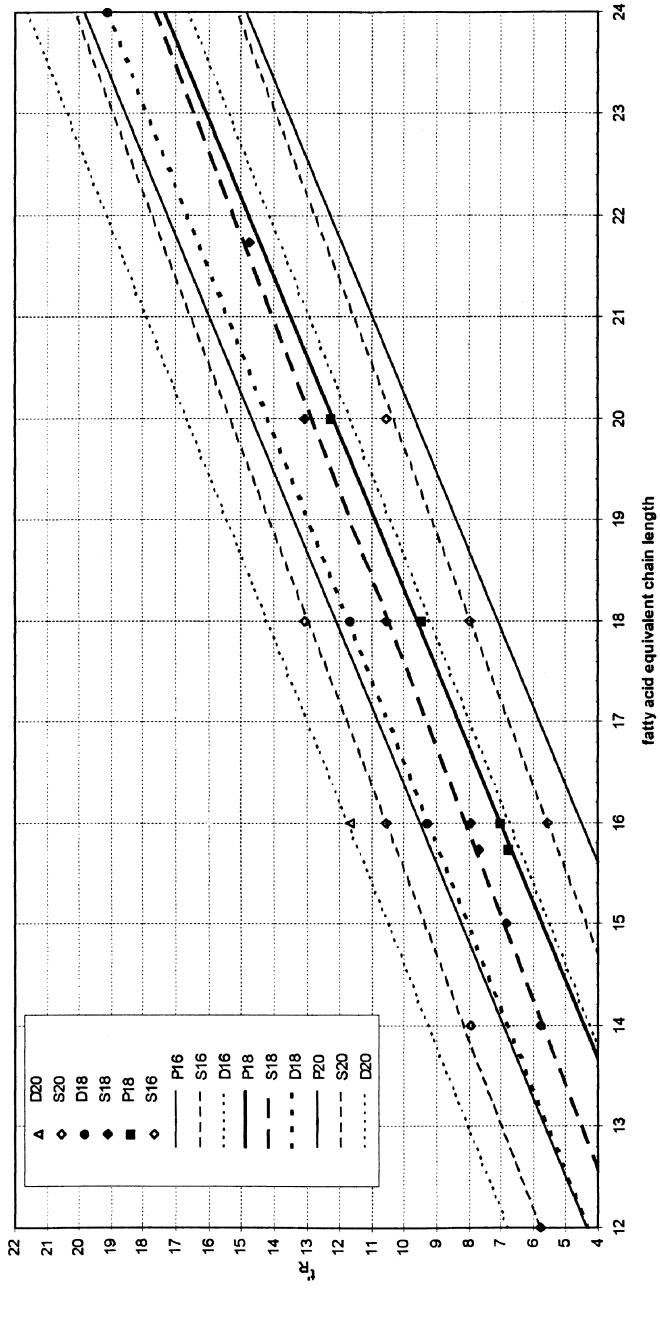

Figure 5. Plot of t_R' as a function of the equivalent chain length of the fatty acid moiety for ceramides containing various bases of different chain length. Thick lines: linear regression from experimental data; ■ base: phytosphingosine (C18); ▲ base: dihydrosphingosine (C18); ◆ base: sphingosine (c18); thin lines: base sphingosine, dashed line; phytosphingosine, solid line; dihydrosphingosine, dotted line.

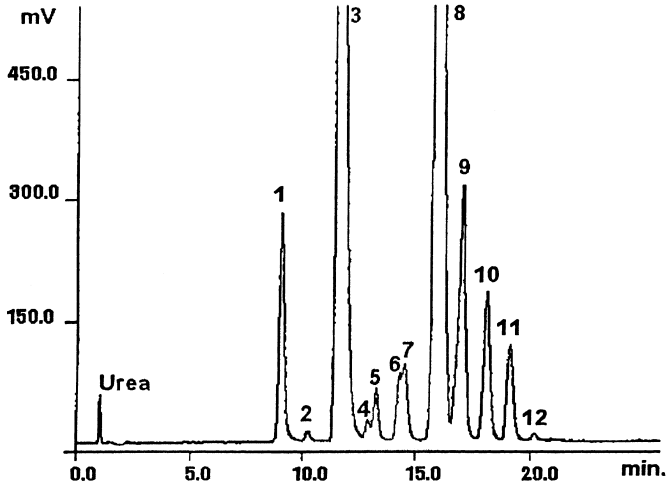

Figure 6. HPLC chromatogram of ceramide Sigma type III.

The value of $\frac{\Delta C = C}{\Delta CH2} = -2.26$ represents the difference in abscissa between two ceramides with the same fatty acid chain length saturated and unsaturated.

With all of these ceramide structures (standards and identified traces), we built abacuses (Figure 5) where each straight line represented the reduced retention times of an amine base linked with all of the possibilities in chain length fatty acids.

The thick lines represent each amine base with a C_{18} chain which are obtained by linear regression from the experimental values of t_R' represented as solid black symbols. The thin lines are extrapolated by translating the corresponding regression line with a shift corresponding to the number of ΔCH_2. These extrapolations have been confirmed in some cases by experimental values (white symbols). The extrapolation has been done for ceramides with bases ranging from 16-20 carbons in length which represents the chain base length encountered in our samples. In the skin, the major chain lengths for the bases encountered are 18, 20, 22 carbons long.[7]

So the validation of these abacuses was established by injecting in our chromatographic system a mixture of ceramides from Sigma: ceramide Type III. The obtained chromatograms in liquid and gas chromatography are shown in Figures 6 and 7.

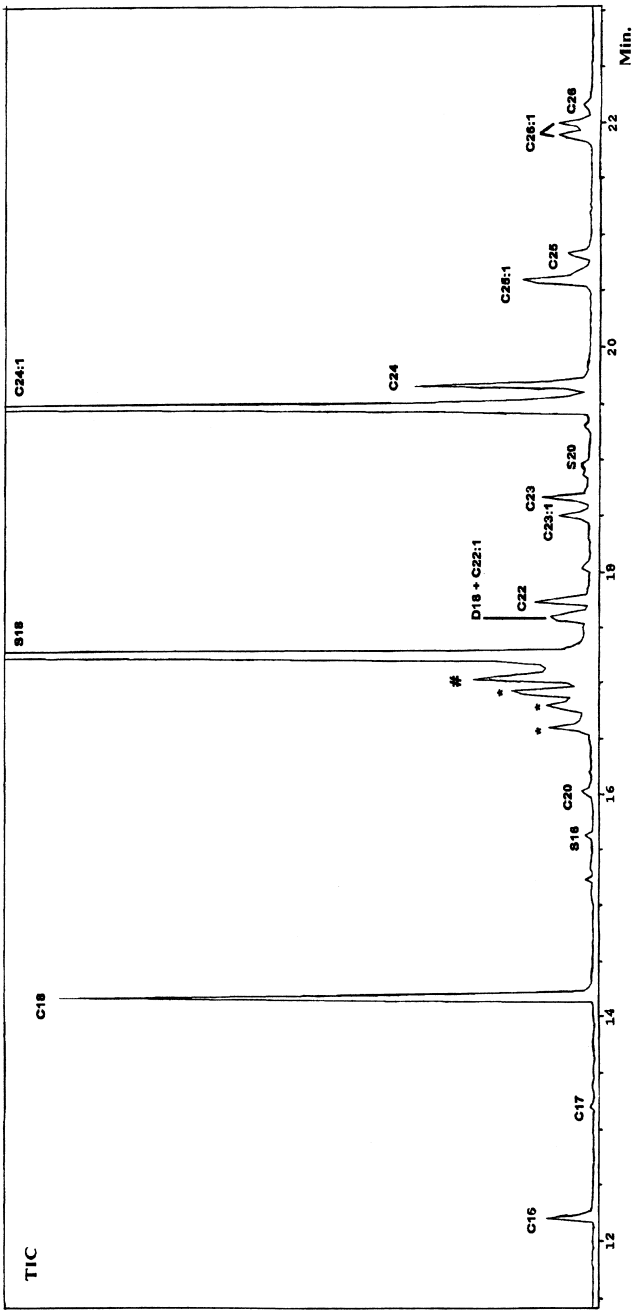

Figure 7. GC chromatogram for ceramide Sigma type III. Total ion current chromatogram (recorded in E1 mode) of reaction mixture obtained from methanolysis and trimethylsilylation. * secondary products from sphingosine formed during methanolysis. = O ~ TMS S18.

Table 4

Results for the Ceramide Type III

Peak N°	Average t_R'	Standard Deviaiton	Peak Intensity in LC	Possible Structures from Abacuses	GC/MS Confirmation Acid & Base	
1	8.04	0.04	High	C13:0D20 / C15:0D18 / 17:0D16C14:0S20 / C**16:0S18** / C18:0S16C18:1 P19 / C19:1P18 / C20:1P17	Minor C16:0	Intense S18
2	9.25	0.04	Trace	C14:0D20 / **C16:0D18** / C18:0D16C19:0S16 / C17:0S18C21:1P17 / C20:1P18	Minor C16:0	High D18
3	10.57	0.05	Intense	C19:0P18 / C21:1P18 C16:0S20 / **C18:0S18** / C20:0S16C13:0D22 / C15:0D20 / C17:0D18	Intense C18:0	Intense S18
4	11.87	0.06	Trace	C20:0P18 / C22:1P18 / C24:1P16C14:0D22 / C16:0D20 / **C18:0 D18** C15:0S22 / C17:0S20	Intense C18:0	High D18
5	12.23	0.05	Minor	C18:0P21 / C20:0P18 **C24:1S16**	Intense C24:1	Minor S16
6	13.28	0.06	Minor	C17:0P22 / C19:0P20 C24:1P17 / C25:1P16		
7	13.52	0.05	Minor	C21:0P18 / C22:0P17 / C23:0 P16**C23:1S18** / C25:1 S16	Minor C23:1	Intense S18
8	14.90	0.09	Intense	C23:1D18 / C25:1D16C22:1 S20 / **C24:1S18** / C26:1S16 C24:0P16	Intense C24:1	Intense S18
9	15.98	0.09	High	C21:0P20 / C23:0P18 / C25:0 P16**C25:1S18** / C27:1 S16	High C25:1	Intense S18
10	17.07	0.09	High	C20:0P22 / C22:0P20 **C26:1S18** / C28:1S16	High C26:1	Intense S18
11	18.07	0.1	Minor	C23:1P22 / C25:1P20 / C27:1 P18C19:0D22 / C21:0D20		
12	19.2	0.08	Trace	C21:0S22C20:0D22 / C22:0D20 / **C24:0D18**	High C24:0	High D18

Table 4 shows all the attributes found with the abacuses for each t_R' of this sample. For each peak, an important number of possibilities is found: there is more ambiguity about the number of methylene group than the base nature. The abacuses give information about the general number of methylene groups present in the molecule. That explains why a great number of possible structures is assigned to each peak. Since the diagram is built on the basis of an identical methylene selectivity for the amine and acid chain, this does not allow us to fix the chain length of the acid and base moieties.

The choice of the preferential structure (printed in bold in Table 4) has been done on the basis of:

-the knowledge of the origin of this sample (bovine brain) which is mainly composed of sphingosine.
-the alkyl chain of the base is generally about 18 carbons.
-the data in literature specify that the fatty acids contained in ceramides are often composed of chains with an even number of carbon atoms.

In order to confirm the structures deduced from HPLC data, a GC/MS analysis of the sample was performed. Although the response coefficient may be slightly different for each ceramide entity, we have considered that these coefficients would be approximately the same in GC/MS analysis. The same statement may be said for the ELSD, so the two chromatographic profiles should be comparable regarding peak intensity. High peak observed with the ELSD should be confirmed by at least equally high peak for the base and the acid in GC/MS. So the relative abundance of each component has been roughly estimated in both the chromatographic systems and classified in Table 4 as intense, high, minor, and trace. Furthermore in gas chromatography, the abundance of each entity is not directly related with the abundance of the initial ceramide, since an acid or a base could belong to several ceramides.

The comparison between the abundance in the both methods provides the confirmation of almost all peak identifications. Two peaks (6 and 11) are not identified. The low resolution between peaks 6 and 7 alters the reduced retention time. And peak 11 occurs in a domain of retention which is one of the boundaries of our model. For ceramides with a fatty acid chain length greater than 20 carbons, the reliability of the abacuses is ensured by only 2 experimental values. Furthermore, the existence of breaks[31] has been underscored in the linear curves of methylene selectivity of homologous series.

Although these abacuses are not self-sufficient at this time for the identification of the ceramide structures, most of the constituents of the ceramide type III (from Sigma) have been identified without proceeding to the isolation of each component. This confirms that our theoretical approach is suitable to establish structure-chromatographic behaviour correlation for

identifying ceramides with only a reduced set of available standard substances. From a practical point of view, the flaws of this model comes principally from the fact of the small number of standards which are mainly constituted with only one chain length for the amine base and a reduced domain for the fatty acid chain length. So the more the structures of the base and the acid moieties of a ceramide differ from those of the model, the more their identification becomes inaccurate.

These abacuses have emphasised the importance of having a chromatographic system which ensures a good discrimination face with the polar head of the ceramide. However the construction of these abacuses has shown an original and efficient approach for the analysis of ceramides. The interest of this approach is the possibility of improving the reliability of the model by iterations each time a new ceramide structure would be found. In this way, the extension of this model to a new ceramide structure would be possible (i.e. ceramides with α-OH fatty acid).

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