methods

Gas-chromatography/mass-spectrometry analysis of human skin constituents as heptafluorobutyrate derivatives with special reference to long-chain bases

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Abstract The composition of the constituents (monosaccharides, long-chain bases, and fatty acids) found in an ethanol extract of the human skin could be determined, without time-consuming steps of purification, after acid-catalyzed anhydrous methanolysis, followed by the formation of volatile derivatives with heptafluorobutyric anhydride and gas-chromatography/mass-spectrometry analysis. Despite the extreme heterogeneity of such extracts, the electron impact analysis of the constituents allowed qualitative and quantitative determinations of monosaccharides, long-chain bases, fatty acids, and alkyl-glycerols. Throughout the different long-chain bases, sphingenines (Sphes), sphinganines, phytosphingosines, and 6-hydroxy-Sphes (6oh-Sphes) can be identified and quantified. Long-chain bases with a chain-length up to 28 carbon atoms can be identified through specific fragmentation patterns in the electron impact mode. Particular attention was drawn to the behavior of compounds of the family of 6oh-Sphes upon acid-catalyzed methanolysis.—Pons, A., P. Timmerman, Y. Leroy, and J-P. Zanetta. **GC/MS analysis of human skin constituents as heptafluorobutyrate derivatives with special reference to long-chain bases. *J. Lipid Res.* 2002.** 43: **794–804.**

Supplementary key words amino acids • monosaccharides • fatty acid • sphingenine • sphinganine • phytosphingosine • 6-hydroxy-sphingenine

In previous papers (1, 2), we demonstrated that the complete analysis of constituents of purified glycolipids could be performed by gas-chromatography/mass-spectrometry (GC/MS) analysis of heptafluorobutyrate derivatives of the different constituents liberated using acid-catalyzed methanolysis. Indeed, during methanolysis, the cleaved monosaccharides are transformed into their *O*-methyl glycosides and *O*-methyl glycosides of their methyl esters, fatty acids as their methyl esters (FAMEs), and long-chain bases (LCBs) as free compounds or perfectly identified degradation products. A high temperature acylation of the mixture with heptafluorobutyric anhydride allows the complete blockage of alcohol and amino groups as heptafluorobutyrate derivatives, a step after which all the constituents become volatile and consequently suitable for a subsequent analysis by GC. Based on the poor van der Waals interactions of heptafluorobutyrate (HFB) derivatives with the classical methyl-siloxane liquid phase of the capillary columns, the derivatives of monosaccharides are fully separated from FAMEs and from the derivatives of LCBs, allowing an easy GC and GC/MS identification of the different compounds. The attribution of correct relative molar responses to the different compounds (1, 2 and here for GC/MS) allowed to obtained reproducible molar compositions of glycosphingolipids, without steps of centrifugation, phase partition, etc., the sample remaining in the same test tube during all the steps of cleavage and derivatisation. The GC/MS analysis in the electron impact (EI) mode appeared especially convenient for the analysis of hydroxylated FAMEs and of LCBs, since the search of ions for HFB derivatives, then ions specific for each family of compounds, and finally of each compound allowed easy identification even in very complex mixtures.

This suggested that this method could be applied to very crude samples such as membrane preparations or total homogenates. Because human skin was shown to contain a large variety of long-chain bases [sphingenines (Sphes), sphinganines (Sphas), phytosphingosines (Phyts) and 6-hydroxy-Sphes (6oh-Sphes)] (3–5), we decided to test the capability of this method to provide a complete spectrum of its constituents in routine screening conditions. This manuscript reports that this is actually the case in total ethanol extract of human skin, in which most amino acids, monosaccharides, FAMEs, and long-chain bases can be identified and quantified. This manuscript also discusses the nature of the different compounds formed during acid-catalyzed anhydrous methanolysis of 6oh-Sphes.

Abbreviations: CI, chemical ionization; EI, electron impact; FAME, fatty acid methyl ester; Gal, galactose; GlcNAc, *N*-acetyl-glucosamine; GalNac, *N*-acetyl-galactosamine; GC, gas chromatography; HFB, heptafluorobutyrate; HFBAA, heptafluorobutyric anhydride; LCB, long-chain base; Man, mannose; MS, mass spectrometry; Phyt, phytosphingosine; Rt, retention time; Spha, sphinganine; Sphe, sphingenine; TIC, total ion counts.

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MATERIALS AND METHODS

Preparation of human skin extracts

Human hands were washed twice with 25 ml redistilled ethanol and the extract was concentrated with a rotary evaporator. The residue was suspended in 2 ml of chloroform-methanol, 1:1 (v/v), transferred to heavy walled Pyrex tubes, and centrifuged for 30 min at 3,000 RPM at 20°C. Aliquots of the clear supernatant (2–20 μl) were transferred to heavy walled Pyrex tubes with Teflon lined screw cap and dried under a light stream of nitrogen.

Methanolysis and acylation

Samples were supplemented with 0.5 ml of the methanolysis reagent and the closed vessels were left for 20 h at 80°C. The methanolysis reagent was obtained by dissolving anhydrous gaseous HCl (up to 0.5 M) at –50°C in anhydrous methanol previously redistilled on magnesium turnings (6). Gaseous HCl was prepared by the drop wise addition of concentrated sulfuric acid on crystallized sodium chloride.

After methanolysis, samples were evaporated to dryness under a light stream of nitrogen in a ventilated hood, followed by the addition of 200 μl acetonitrile and 25 μl of heptafluorobutyric anhydride (HFBAA) (Fluka AG, Buchs, Switzerland). The closed vessels were heated for 15 min at 150°C in a sand bath. After cooling at room temperature, the samples can be stored for months at room temperature in the closed vessel without need of re-acylation. When analysis had to be performed the samples were evaporated in a light stream of nitrogen in a ventilated hood in order to eliminate the excess of reagent and the heptafluorobutyric acid (HFBA) formed during the acylation, then taken up in the appropriate volume of acetonitrile; this acetonitrile was stored in a closed vessel in the presence of calcinated calcium chloride in order to eliminate traces of water. An aliquot of the acetonitrile solution of the HFB derivatives was introduced in the Ross injector of the GC/MS apparatus.

GC/MS

For GC/MS analysis, the GC separation was performed on a Carlo Erba GC 8,000 gas chromatograph equipped with a 25 m × 0.32 mm CP-Sil5 CB Low bleed/MS capillary column, 0.25 μm film phase (Chrompack France, Les Ullis, France). The temperature of the Ross injector was 280°C and the samples were analyzed using the following temperature program: 90°C for 3 min then 5°C/min until 260°C, followed by a plateau at 260°C for the cleaning of the column. The column was coupled to a Finnigan Automass II mass spectrometer (mass limit 1,000) for routine analysis. The analyses were performed either in the EI mode (ionization energy 70 eV; source temperature 150°C) or in the chemical ionization (CI) mode in the presence of ammonia (ionization energy 150 eV, source temperature of 100°C). The CI detection mode was performed for positive ions or for negative ions in separate experiments. The latter allowed the quite specific detection of heptafluorobutyrate derivatives with a higher sensitivity for compounds able to be derivatised by heptafluorobutyric anhydride (1). The qualitative and quantitative analysis of the GC/MS data were obtained with the Xcalibur software (Thermo-Finnigan; Les Ullis, France).

RESULTS AND DISCUSSION

As shown in **Fig. 1A** the total ion counts (TIC) profile of the compounds present in the ethanol extract of human skin appeared very complex. Indeed, such samples contained volatile derivatives formed from free amino acids (the carboxyl groups being methyl-esterified during methanolysis and alcohol, thiol, phenol, and amino groups being derivatised with HFBAA), FAMEs, vinyl-ethers derived from plasmalogens, alkyl-glycerol HFB derivatives derived from alkyl-acyl-glycerols, HFB derivatives of *O*-methyl glycosides (formed from free and glycolipid-bound mono- or oligosaccharides), and HFB derivatives of long chain bases and of hydroxylated FAMEs. Nevertheless, this complexity could be easily reduced through the search of ions specific to the various families of compounds. For example, saturated FAMEs (Fig. 1B) and FAMEs hydroxylated in position 2 (Fig. 1C) were revealed using the search of specific ions.

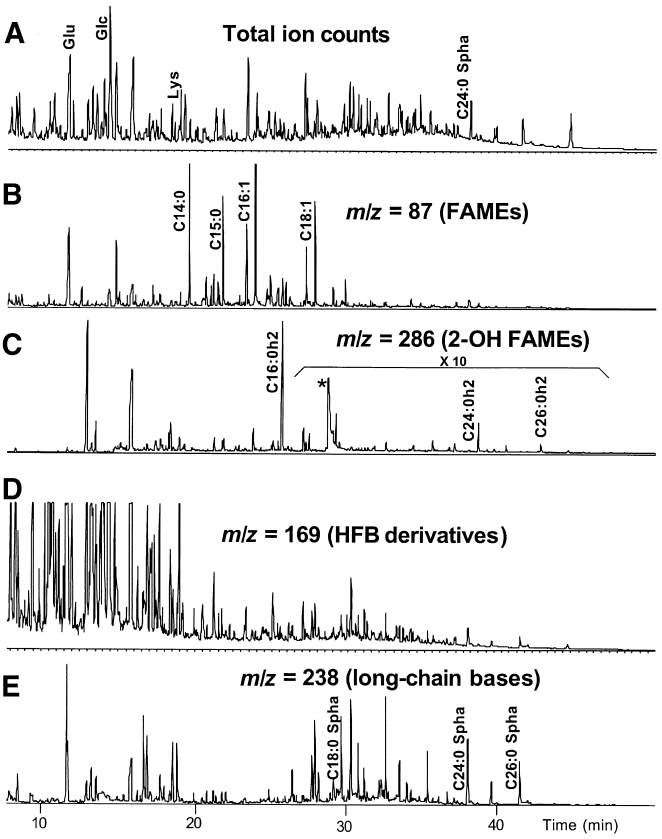

Fig. 1. Total ion counts and chromatogram reconstitution using ions specific of the different families of constituents. A: Total ion counts (this profile was used for all quantitative determinations); B: Search for the ion at $m/e = 87$, specific for the majority of saturated fatty acid methyl esters (FAMEs); C: Search for the ion at $m/e = 286$ $[H_3COC_{(1)}OH\text{-}C_{(2)}HOCOCF_2CF_2CF_3]^{+\circ}$ specific of 2oh-FAMEs (the compound labeled * was due to a contaminant at $m/e = 285$); D: Search for the ion at $m/e = 169$ characteristic of heptafluorobutyrate (HFB) derivatives; E: Search for the ion at $m/e = 238$ characteristic of most long-chain bases (LCBs). The positions of some major compounds found in the sample are indicated.

All compounds possessing one or several alcohol, thiol, amino, or phenol group(s) were specifically detected using the ion at $m/e = 169$ $[CF_3CF_2CF_2]^{+\circ}$ characteristic of HFB derivatives (Fig. 1D) (throughout these compounds, different families of compounds could be quite specifically identified: alkyl-glycerol revealed by an intense ion at $m/e = 467$ $[CH_2(CHOCOCF_2CF_2CF_3)CH_2OCOCF_2CF_2CF_3]^+$) (2), *O*-methyl glycosides (not shown) (2), and long chain bases (Fig. 1E) revealed through a specific ion of α-amino-alcohols at $m/e = 238$; $[CH_2{=}CNHCOCF_2CF_2CF_3]^+$ (2).

TABLE 1. Identification of the non long-chain base constituents found in the human skin extracts

AA	Rtr	Mass	El Reporter Fragment Ions
Ala*	0.1000	299	299-268-240-220-192-169-119-102
Arg2	0.6710	580	580-549-521-367-353-342-256-239-100
Arg3	0.7627	776	776-607-504-492-334-326-266-169-119
Asp	0.3432	357	357-326-298-266-256-236-128-113-100
(Cys)2	0.9424	660	660-601-447-330-298-270-238-169-118
Glu	0.4228	371	371-364-339-312-307-280-252-232-100
Gly*	0.0983	285	285-254-240-226-206-169-119-88
His	0.8174	365	365-334-320-306-163-137-109-91-79
Ile	0.2279	341	341-310-282-255-252-226-150-128-100
Leu	0.2233	341	341-310-282-253-240-226-150-128-100
Lys	0.6619	552	551-520-493-473-295-280-253-226-100
Met	0.7263	359	359-329-300-280-252-179-151-132-100
Phe	0.6349	375	375-316-169-163-162-119-103-77-69
Pro	0.3007	325	325-293-266-249-226-198-178-128-83
Ser	0.2164	511	511-480-452-432-285-282-253-240-100
Thr	0.2031	525	525-494-481-466-292-285-253-241-100
Tyr	0.6820	587	587-556-528-508-374-343-303-275-78
Trp1	0.8005	414	414-395-355-341-240-157-116-103-91
Trp2	0.8776	610	610-551-397-326-228-169-129-119
Val*	0.1628	327	327-296-268-253-226-169-119-88
Sugars	**Rtr**	**Mass**	**El Reporter Ions**
Gal	0.4818	978	947-765-704-551-519-491-337-277-169
GalNAc	0.6024	977	946-926-703-518-490-336-276-238-169
Glc	0.5171	978	947-765-704-551-519-509-491-337-277
GlcA	0.5816	810	597-537-505-478-383-323-283-267-169
GlcNAc	0.6082	977	946-926-703-518-508-490-336-276-238
Man	0.5074	978	947-765-704-551-519-509-491-337-277
Neu	0.7375	1275	944-789-756-602-542-322-300-169
FAMEs	**Rtr**	**Mass**	**El Reporter Ions**	**% of FAMEs**
C12:0	0.5340	214	214-87-74	1.26 ± 0.11
C14:0	0.7040	242	242-87-74	6.92 ± 0.08
C15:0	0.7836	256	256-87-74	13.00 ± 0.01
C16:0	0.8588	270	270-87-74	22.39 ± 0.16
C16:1	0.8387	268	268-87-74	21.29 ± 0.17
C16:0h2	0.9229	482	482-355-286-169	5.55 ± 0.04
C17:0	0.9426	284	284-87-74	0.82 ± 0.00
C17:0h2	0.9878	496	496-355-286-169	0.55 ± 0.00
C18:0	1.0000	298	298-87-74	5.06 ± 0.04
C18:1	0.9744	296	296-87-74	19.59 ± 0.12
C18:0h2	1.0497	510	510-355-286-169	1.17 ± 0.01
C22:0h2	1.2766	566	566-355-286-169	0.38 ± 0.00
C24:0h2	1.3842	594	594-355-286-169	1.29 ± 0.00
C25:0h2	1.4500	612	612-355-286-169	0.29 ± 0.00
C26:0h2	1.5304	626	626-355-286-169	0.42 ± 0.00

The derivative of His corresponded to the mono-HFB derivative, those of Arg to the di-HFB (Arg2) and tri-HFB (Arg3) derivatives and those of Trp to the mono- (Trp1) and di-HFB (Trp2) derivatives. The derivatives of the methyl esters of Gly*, Ala*, and Val* were too volatile to be quantitatively recovered. All derivatives of amino acids gave a weak molecular ion. Intense ions corresponded to M-31 and M-59. For the derivatives of the *O*-methyl glycosides, the data presented here corresponded to the major anomer of each compound. The FAME composition shown here does not include the extreme heterogeneity of FAMEs due to a very high number of ramified FAMEs of unknown origin. The presence of these compounds (unpublished data) is systematic and independent on the washing procedure applied to the hands before performing the ethanol extract. Rtr, relative retention time.

Because of the extremely poor interactions of HFB derivatives with siloxane liquid phases of the capillary columns, the mass of the compounds was not a determining factor for the GC separation, but the number of methyl and methylene groups in the chains. The first compounds to be eluted from the columns corresponded to amino acids (**Table 1**), although this method was not appropriate for the quantitative determination of free amino acids. As already described (7), the methyl esters of the HFB derivatives of Gly, Ala, and Val are too volatile to be recovered after elimination of the excess of HFBAA reagent. Nevertheless, all these compounds, including those (His, Arg, Trp) with a higher relative retention time (Rtr) could be identified without ambiguities.

The second family of compounds to be eluted included the HFB derivatives of *O*-methyl glycosides (Table 1) (2). These compounds were in large part separated from compounds endowed with a long aliphatic chain, i.e. FAMEs, plasmalogen derivatives, alkyl-glycerols, and long-chain bases. Consequently, most of the derivatives of fatty compounds were largely chromatographically separated from other constituents found in these complex extracts (Fig. 1).

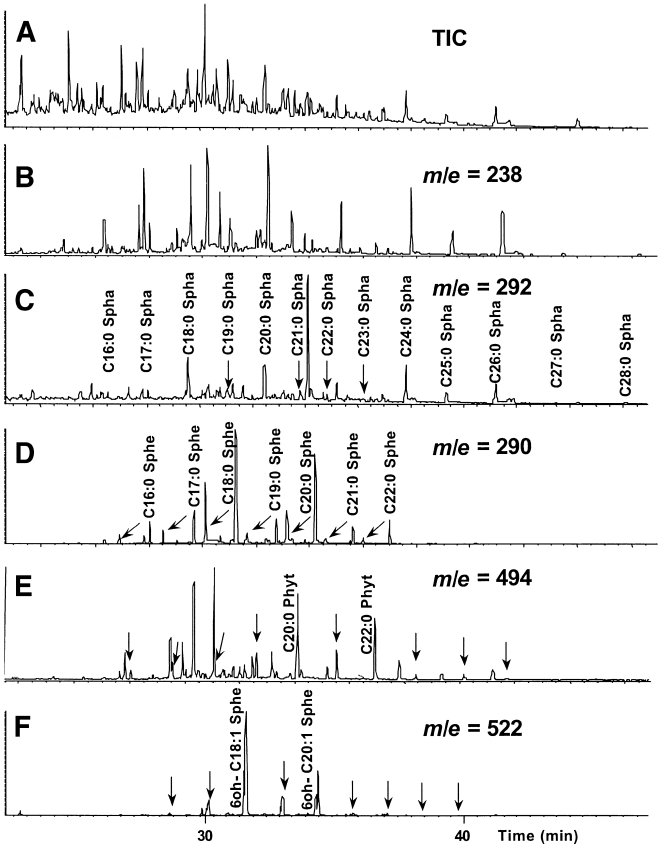

Fig. 2. Total ion counts and chromatogram reconstitution using ions specific of each family of long-chain bases. A: Total ion counts (TIC); B: Search for the ion at $m/e = 238$ characteristic of most LCBs; C: Search for the ion at $m/e = 292$ specific of compounds of the sphinganine (Spha) family; D: Search for the ion at $m/e = 290$ specific of compounds of the sphingenine (Sphe) family; E: Search for the ion at $m/e = 494$ specific of compounds of the furanic forms of the compounds of the phytosphingosine (Phyt) family (the derivatives of C16:0, C17:0, and C18:0 Phyt were actually detected but could not be quantified because they represented less than 0.1% of the C20:0 Phyt); F: Search for the ion at $m/e = 522$ specific of compounds of the 6-hydroxy-Sphe (6oh-Sphe) family. Some of the members of the different families of LCBs were labeled; the positions of other members are indicated by arrows. In C, the major peak at $m/e = 292$ was due to a leakage product from the column.

TABLE 2. Identification and relative abundance of the different LCBs found in the human skin extract

Compound	Rtr	Wt	% ± SD
C16:0 Spha	0.9490	861	1.47 ± 0.02
C17:0 Spha	1.0061	875	5.46 ± 0.04
C18:0 Spha	1.0622	889	18.52 ± 0.12
C19:0 Spha	1.1164	903	3.84 ± 0.05
C20:0 Spha	1.1664	917	16.60 ± 0.09
C21:0 Spha	1.2037	931	3.84 ± 0.04
C22:0 Spha	1.2645	945	8.35 ± 0.06
C23:0 Spha	1.3108	959	1.91 ± 0.03
C24:0 Spha	1.3586	973	16.50 ± 0.08
C25:0 Spha	1.4136	987	6.61 ± 0.07
C26:0 Spha	1.4822	1001	15.24 ± 0.14
C27:0 Spha	1.5613	1015	1.03 ± 0.02
C28:0 Spha	1.6626	1029	0.59 ± 0.00
C16:1 Sphe	1.0101	677	6.36 ± 0.09
C17:1 Sphe	1.0270	873	10.44 ± 0.19
C17:1 Sphe	1.0691	691	10.44 ± 0.19
C18:1 Sphe	1.0843	887	38.66 ± 0.42
C18:1 Sphe	1.1250	705	38.66 ± 0.42
C19:1 Sphe	1.1390	901	6.98 ± 0.07
C19:1 Sphe	1.1796	719	6.98 ± 0.07
C20:1 Sphe	1.1934	915	25.90 ± 0.28
C20:1 Sphe	1.2312	733	25.90 ± 0.28
C21:1 Sphe	1.2459	929	3.84 ± 0.06
C21:1 Sphe	1.2830	747	3.84 ± 0.06
C22:1 Sphe	1.2964	943	7.41 ± 0.08
C22:1 Sphe	1.3323	761	7.41 ± 0.08
C23:1 Sphe	1.3439	957	0.34 ± 0.00
C23:1 Sphe	1.3849	775	0.34 ± 0.00
C24:1 Sphe	1.3885	971	0.07 ± 0.00
C24:1 Sphe	1.4480	789	0.07 ± 0.00
C25:1 Sphe	n.d	985	Trace
C25:1 Sphe	1.5254	803	Trace
C26:1 Sphe	n.d	999	Trace
C26:1 Sphe	1.5981	817	Trace
C18:0Phyt	1.0227	1101	Trace
C18:0Phytf	1.1181	691	Trace
C19:0Phyt	1.0529	1115	11.74 ± 0.14
C19:0Phytf	1.1739	705	11.74 ± 0.14
C20:0Phyt	1.1026	1129	37.70 ± 0.31
C20:0Phytf	1.2272	719	37.70 ± 0.31
C21:0Phyt	1.1527	1143	12.38 ± 0.25
C21:0Phytf	1.2794	733	12.38 ± 0.25
C22:0Phyt	1.2000	1157	29.77 ± 0.31
C22:0Phytf	1.3295	747	29.77 ± 0.31
C23:0Phyt	1.2452	1171	2.04 ± 0.03
C23:0Phytf	1.3821	761	2.04 ± 0.03
C24:0Phyt	1.2902	1185	4.98 ± 0.04
C24:0Phytf	1.4458	775	4.98 ± 0.04
C25:0Phyt	1.3367	1199	1.40 ± 0.01
C25:0Phytf	1.5236	789	1.40 ± 0.01
6oh-C16:1 Sphe	1.0047	889	Trace
6oh-C16:1 Sphe	1.0371	889	Trace
6oh-C16:1 Sphe	1.0393	707	Trace
6oh-C17:1 Sphe	1.0573	903	8.04 ± 0.09
6oh-C17:1 Sphe	1.0645	903	8.04 ± 0.09
6oh-C17:1 Sphe	1.0833	903	8.04 ± 0.09
6oh-C17:1 Sphe	1.0915	721	8.04 ± 0.09
6oh-C18:1 Sphe	1.1114	917	51.60 ± 0.61
6oh-C18:1 Sphe	1.1192	917	51.60 ± 0.61
6oh-C18:1 Sphe	1.1404	917	51.60 ± 0.61
6oh-C18:1 Sphe	1.1429	735	51.60 ± 0.61
6oh-C19:1 Sphe	1.1685	931	9.92 ± 0.12
6oh-C19:1 Sphe	1.1739	931	9.92 ± 0.12
6oh-C19:1 Sphe	1.1898	931	9.92 ± 0.12
6oh-C19:1 Sphe	1.1930	749	9.92 ± 0.12
6oh-C20:1 Sphe	1.2215	945	23.08 ± 0.33
6oh-C20:1 Sphe	1.2272	945	23.08 ± 0.33
6oh-C20:1 Sphe	1.2384	945	23.08 ± 0.33
6oh-C20:1 Sphe	1.2416	763	23.08 ± 0.33
6oh-C21:1 Sphe	1.2733	959	2.80 ± 0.03
6oh-C21:1 Sphe	1.2787	959	2.80 ± 0.03
6oh-C21:1 Sphe	1.2856	959	2.80 ± 0.03
6oh-C21:1 Sphe	1.2895	777	2.80 ± 0.03
6oh-C22:1 Sphe	1.3233	973	2.80 ± 0.05
6oh-C22:1 Sphe	1.3288	973	2.80 ± 0.05
6oh-C22:1 Sphe	1.3295	973	2.80 ± 0.05
6oh-C22:1 Sphe	1.3356	791	2.80 ± 0.05
6oh-C23:1 Sphe	1.3810	987	0.26 ± 0.00
6oh-C23:1 Sphe	1.3842	987	0.26 ± 0.00
6oh-C23:1 Sphe	1.3871	987	0.26 ± 0.00
6oh-C23:1 Sphe	1.3932	805	0.26 ± 0.00
6oh-C24:1 Sphe	1.4368	1001	Trace
6oh-C24:1 Sphe	1.4436	1001	Trace
6oh-C24:1 Sphe	1.4429	1001	Trace
6oh-C24:1 Sphe	1.4490	819	Trace
6oh-C25:1 Sphe	1.5113	1015	Trace
6oh-C25:1 Sphe	1.5200	1015	Trace
6oh-C25:1 Sphe	1.5175	1015	Trace
6oh-C25:1 Sphe	1.5236	833	Trace
6oh-C26:1 Sphe	1.5960	1029	Trace
6oh-C26:1 Sphe	1.6039	1029	Trace
6oh-C26:1 Sphe	1.6021	1029	Trace
6oh-C26:1 Sphe	1.6082	847	Trace

Spha % LCBs ± SD	37.36 ± 0.35
Sphe % LCBs ± SD	19.76 ± 0.21
Phyt % LCBs ± SD	25.11 ± 0.41
6oh-Sphe % LCBs	17.77 ± 0.28

The relative abundance of each compound in a family was determined by integration of the peaks on the reconstituted chromatogram with the ions specific of each family. The relative proportion of each family was thereafter determined on the TIC chromatogram, by determining the relative areas of the major constituents of each family. For sphingenines (Sphe), only the two major peaks were indicated corresponding to the tri-HFB derivative and to the 3-*O*-methyl-di-HFB derivative. For compounds of the phytosphingosine family (Phyt), the Rt of the two peaks were indicated, corresponding to the tetra-HFB derivative (lower Rtr) and to the di-HFB derivative of the furanic dehydration product (Phytf). For the family of the 6-hydroxy-sphingenines (6oh-Sphe), the Rt of the four major peaks were indicated, corresponding to compounds II, III, IV and V, respectively.

Wt = molecular weight of the derivative. SD = Standard deviation obtained for 15 independent analyses of the same skin sample.

Analysis of FAMEs, plasmalogens, and alkyl-glycerols in human skin extracts

The composition of FAMEs showed a predominance of short chain FAMEs with linear C14:0, C15:0, C16:0, C16:1, C18:0, C18:1, and the 2-hydroxylated C16:0 (Fig. 1B) representing more than 85% of total FAMEs (Table 1). Nevertheless other FAMEs were present in low amounts. The linear very long-chain FAMEs were absent in contrast with the 2-hydroxylated FAMEs with 18, 22, 23, 24, 25, and 26 carbon atoms (Fig. 1C and Table 1). Alkyl-glycerols, compounds derived from glycero-phospholipids having a fatty alcohol instead of a fatty acid, were very minor constituents of the skin extract (not shown). Similarly, although identified, the derivatives of plasmalogens (dimethyl-acetals and vinyl-ethers) were extremely minor compounds

(not shown) (2). In fact, these compounds were totally and partially destroyed, respectively, during the HFBAA acylation procedure (2). Their quasi absence in human skin was verified analyzing a heptane extract of the methanolysis mixture without acylation with HFBAA.

Analysis of long-chain bases of human skin extracts

As shown in Fig. 1A and 1E, it was evident that LCBs (either bound to ceramides or free) were important fatty constituents of the skin extract. They could be easily selected through an ion at *m/e* = 238 characteristic of α-amino-alcohols (2). Throughout this very heterogeneous family, the constituents of each sub-family can be selectively detected through specific ions (**Fig. 2**).

Heterogeneity of Sphas

Throughout the different LCBs, Sphas (1,3-dihydroxy-2-amino-alkanes) were the major constituents (about 37% of total LCBs; **Table 2**). The HFB derivatives of these compounds were easily identified through a low but specific ion at *m/e* = 292; likely cyclic [-CH_2=CNH($COCF_2CF_2CF_3$)CH=$CHCH_2CH_2$-]$^{+\circ}$ (Fig. 2) (2), a very weak ion at M–F (M-19) and two relatively intense fragment ions at M-169 (minus $CF_3CF_2CF_2$) and M-213/214 (minus a heptafluorobutyric acid, or heptafluorobutyric amide, or heptafluorobutyrate group, respectively). This population of compounds was found to be extremely heterogeneous since the linear Sphas with a chain-length of 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, and 28 carbon atoms were present (**Fig. 3**). These data partially confirmed previous studies showing the abundance and heterogeneity of the Sphas in human skin (5), but provided additional information on the presence of shorter and longer chain Sphas. The quite specific detection of these compounds by their specific fragmentation patterns (Fig. 3) and, especially, the presence of the ion at *m/e* = 292, allowed us to ascertain the nature and the abundance of the minor compounds including those with 27 and 28 carbon atoms (Fig. 3, Table 2) without any time-consuming separation procedures involving the isolation of LCBs prior to GC/MS analysis.

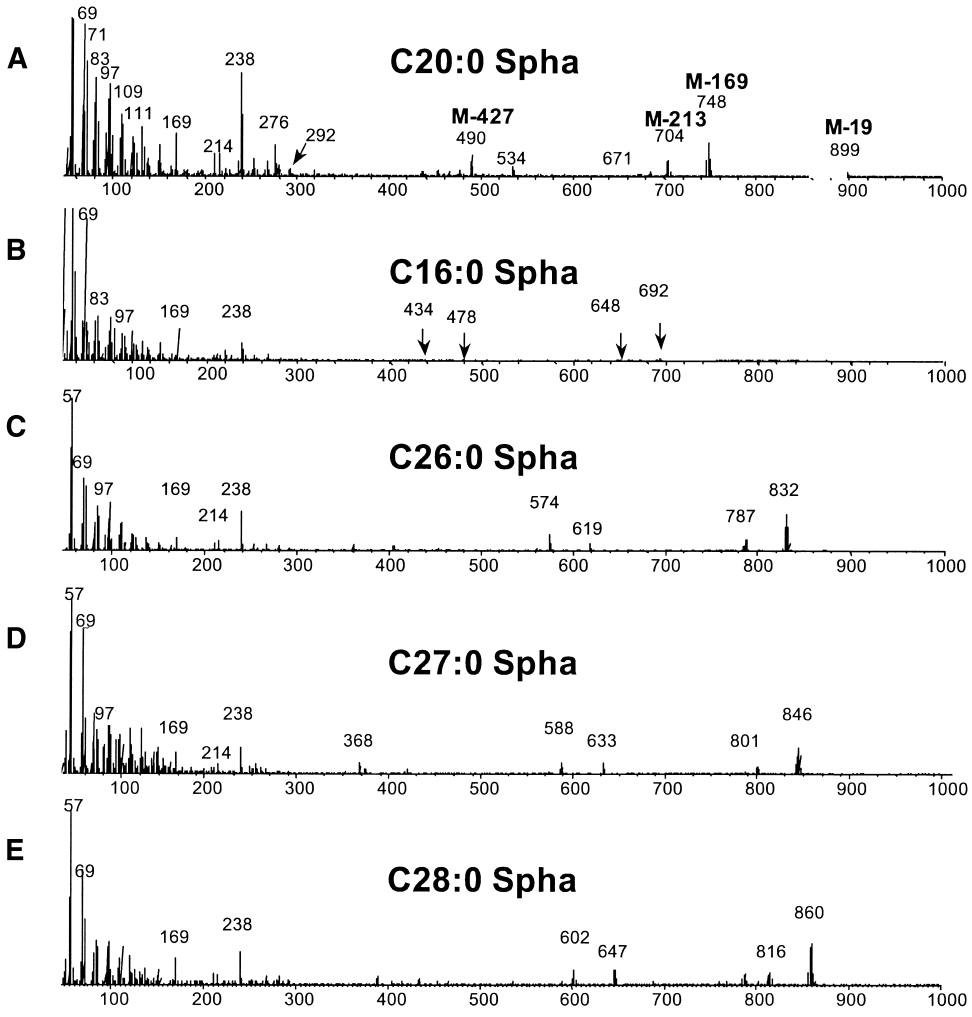

Fig. 3. Examples of mass spectra of different Sphas found in human skin. A: Note that these compounds were characterized by relatively intense ions at *m/e* = M-169 and M-213. Two other important reporter ions were derived from the former by a loss of one heptafluorobutyric acid unit (M-383 and M-427). Note that in short-chain Spha (like C16:0 Spha); B: The high mass ions were of lower intensities than in long-chain Spha; C: C26:0 Spha; D: C27:0 Spha; E: C28:0 Spha.

Heterogeneity of Sphes

The second family of compounds found in abundance in the extract was that of Sphes. As previously reported (1, 2, 8, 9), Sphes (1,3-dihydroxy-2-amino-4-ene-alkenes) gave three peaks upon acid-catalyzed anhydrous methanolysis. Indeed, these compounds form a carbonium ion in resonance between $C_{(3)}$ and $C_{(5)}$. A subsequent nucleophilic attack by CH_3O^- at the 3 or 5 position gave a mixture of derivatives, already observed with conventional methods (8). The compound of intermediate Rtr corresponded to the compound with all hydroxyl groups substituted with HFB (very weak molecular ion at m/e = 887 for sphingosine; C18:1 Sphe), whereas the two other peaks corresponded to compounds having a O-CH_3 group on $C_{(3)}$ or $C_{(5)}$ (very weak molecular ions at m/e = 705 and a doublet at M-31 and M-32). The fragmentation pattern indicated that the 3-*O*-methyl compound was eluted at a higher retention time (Rt), than the minor 5-*O*-methyl compound. Besides the ion at m/e = 238, these compounds were characterized by intense ions (although not absolutely specific) at m/e = 290 (2); likely cyclic $[\text{-}CH_2{=}CNH(COCF_2CF_2CF_3)CH{=}CHCH{=}CH_2\text{-}]^{+\circ}$, the equivalent of the ion at m/e = 292 of Spha, the intensity being due to its stabilization by the aromatic ring. The skin extracts showed the presence of Sphes with 18 and 20 carbon atom chain length as the major constituents (Fig. 2), but other members of the family were also unambiguously detected as minor compounds with chain length of 16, 17, 19, 21, 22, 23, 24, 25, and 26 carbon atoms (**Fig. 4** and Table 2).

Heterogeneity of Phyts

Compounds of the Phyt family (1,3,4-trihydroxy-2-amino-alkanes) were also present. As previously described (2, 8), these compounds gave two peaks upon acid-catalyzed anhydrous methanolysis corresponding to the intact form and to a furanic form due to a dehydration between the $C_{(1)}$ and $C_{(4)}$ carbon atoms. The furanic forms were characterized (besides the ion at m/e = 238) by three additional ions of equal intensities (Fig. 2 and **Fig. 5**) at m/e = 252 $[CH_2CH{=}COCOCF_2CF_2CF_3]^+$, m/e = 256 $[CHOH{=}CHOCOCF_2CF_2CF_3]^+$, and m/e = 494 (positively charged pyranic ring). The intact form was characterized by a triplet of ions at M-169, M-197, and M-213/214 (not shown) (2). Seven constituents of this family were detected with a chain length of 19, 20, 21, 22, 23, 24, and 25 carbon atoms (Table 2).

Heterogeneity of 6oh-Sphes

The evidence for the existence of 6oh-Sphes (1,3,6-trihydroxy-2-amino-4-ene-alkenes) was only provided relatively recently (3–5), these compounds being relatively abundant in human skin. In fact, analyzing by GC/MS the glycosphingolipid composition of several human samples, we observed a significant reduction of the quantitative ratio between LCBs and FAMEs, concomitant with the presence of additional peaks presenting the ion at m/e = 238 (suggesting the presence of LCBs) and showing fragmentation patterns compatible with the presence of such com-

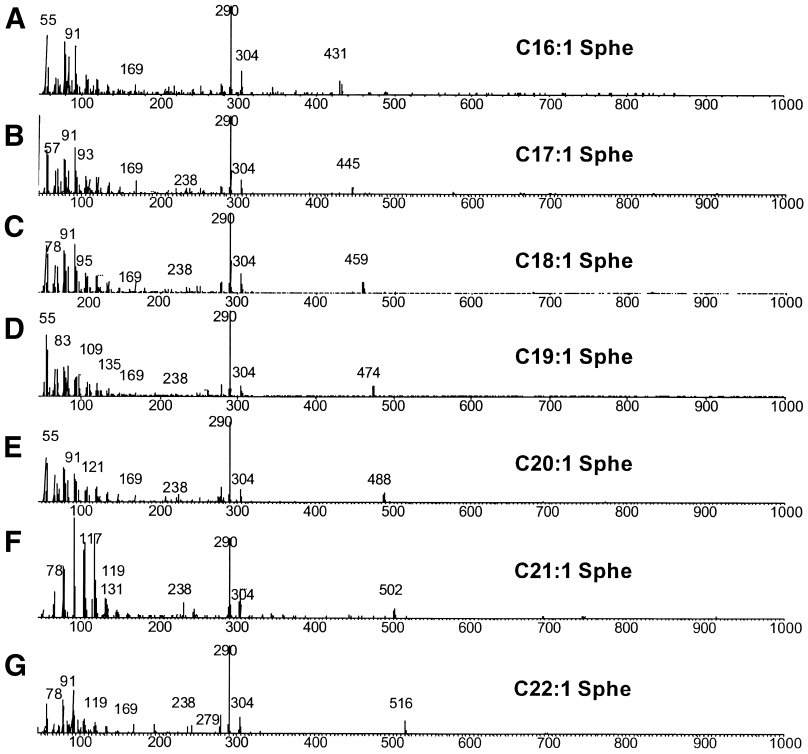

Fig. 4. Mass spectra of the derivatives of the Sphes found in the human skin extract. Note that all these compounds showed an intense ion at m/e = 290 characteristic of Sphes. The individual constituents were identified by intermediate intensity ions at m/e = M-214–32, corresponding to the loss of one methanol group and one heptafluorobutyric acid group for the 3-*O*-methyl-1,2-di-HFB derivatives (Table 2).

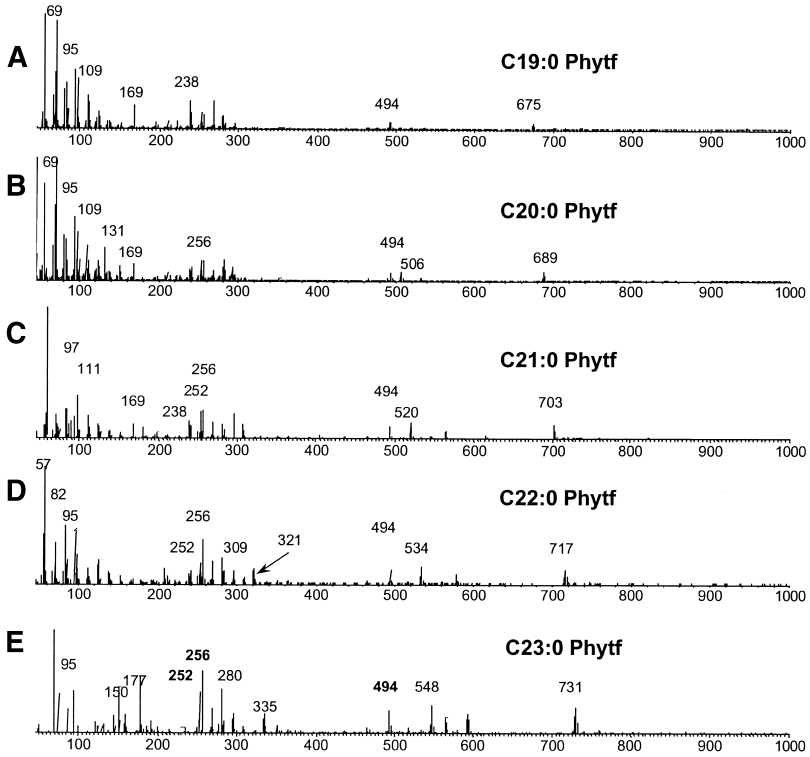

Fig. 5. Examples of mass spectra of the furanic dehydration products, Phytf, of compounds of the Phyt family. The furanic forms were characterized by three common ions at *m/e* = 252, 256, and 494. The higher mass ions of these compounds corresponded to M-30 (opening of the furanic ring and loss of $CH_2 = O$ from the $C_{(1)}$ carbon atom).

pounds. Therefore, it was of interest for us to analyze human skin extracts known to be enriched in 6oh-Sphe and to define their behavior using the present technique.

GC/MS identification of products related to 6oh-Sphes. The GC/MS profiles of human skin extracts showed additional compounds possessing both the ions characteristic of HFB derivatives (*m/e* = 69, 119, 169) and that characteristic of long-chain bases (*m/e* = 238). A series of compounds differing by a mass difference of 14 atomic mass units was detected, showing also a weak ion at *m/e* = 290 characteristic of Sphes (2), the remaining part of the mass spectra being essentially different from those of Sphes. In fact, the analysis of the GC/MS spectra suggested that five peaks were derived from each compound. Indeed, these peaks were always found in reproducible (within a 2% error for each value) proportions (0.8%, 13.93%, 32.31%, 14.32%, and 38.53%, respectively), independent of the chain length. This was essentially different from Sphes that always gave three peaks in reproducible proportions. These observations suggested that they could be the different members of the family of 6oh-Sphes (3–5). Indeed, these compounds had two hydroxyl groups in an α-position relative to a double bound, a symmetrical situation that, relative to classical Sphes, theoretically increased the number of products provoked by the resonance of the carbonium ion between $C_{(3)}$ and $C_{(5)}$ formed during anhydrous acid-catalyzed methanolysis of Sphes up to five different compounds (**Fig. 6**). Although the MS-derived interpretations of the structures of the different compounds was a priori difficult, the presence in human skin of compounds with different chain length allowed an easy discrimination between ions related to the chain lengths and those related to their common portions.

The identification of the different compounds was solved in several steps based on specific ions due to classical mechanisms of fragmentation. Intense ions were observed representative of the chain length (for example 253 and 281 for compounds with chain lengths of 18 and 20 carbon atoms, respectively). These ions were particularly intense for compounds II and III (Fig. 6), this ion being accompanied by an intense ion at *m/e* = 71, which was in part the result of a secondary fragmentation process involving an *O*-methyl group in a α-position to a double bond as already observed for vinyl ethers (2). One of the most important ions (of very weak intensity but fundamental for the identification of the compounds) was the ion corresponding to the cleavage in the α-position relative to the double bond. This gave specific weak ions (Fig. 6) at *m/e* = 704 for compound I, 748 for compound II, 478 for compound III, and a relatively strong ion at *m/e* = 522 for compounds IV and V.

These ions were accompanied by ions derived from the molecular ion by a loss of a heptafluorobutyric acid group (−214) and/or ketene/methanol group (−30/−32), the latter loss of mass indicating the presence of an *O*-methyl group. Based on these ions, and ions characteristic of the chain length, it was possible to identify the five different compounds formed from each 6oh-Sphe during acid-cata-

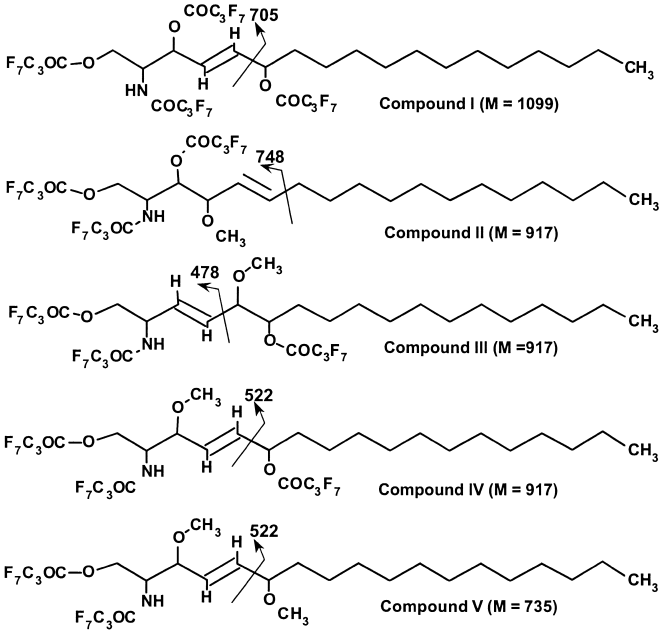

Fig. 6. Structures of the HFB derivatives of the five products formed from 6oh-Sphes during anhydrous acid-catalyzed methanolysis. The compounds were termed I–V according to their retention time. The very minor compound I corresponded to the unmodified molecule, all polar groups being blocked by HFB groups. Compounds IV and V were characterized by a positively charged ion at $m/e = 522$ corresponding to a cleavage adjacent to the double bond and by a very intense ion ($m/e = 308$) derived from the former by an additional loss of a heptafluorobutyric acid group.

lyzed methanolysis (**Fig. 7**). The very minor (0.8%) compound I, corresponding to the intact 6oh-Sphes, showed a fragmentation pattern very similar to that of Sphes with successive losses of heptafluorobutyric acid/amide groups (2). Compounds II and III gave similar fragmentation patterns with losses of a fluorine atom, associated with the loss of a heptafluorobutyric acid/amide group and of a methanol group. Compound IV differed from the two latter compounds because of the absence of loss of the fluorine atom. Finally, compound V was characterized by two successive losses of methanol/ketene groups.

These conclusions were reinforced by analyses of the different compounds in the chemical mode (CI) of ionization in the presence of ammonia and detection of positive or negative ions. For CI with detection of the positive ions, extremely weak pseudo-molecular ions $[M+H]^+$ were observed. The major ions corresponded to a loss of a HFBA group relative to the pseudo-molecular ion $[M+NH4]^+$ and to an ion derived from the $[M+H]^+$ ion by a loss of two HFBA groups. An ion of similar intensity corresponded to a positively charged chain depleted of all substituents except hydrogen atoms (Fig. 7). The analysis of these compounds in the CI mode with detection of negative ions gave an extremely weak pseudo-molecular ion $[M\text{-}F]^-$ and an intermediate intensity ion derived from the former by a loss of a HFBA group. For compounds I, II, III, and IV, the basic ion corresponded to the loss of the two first carbon atoms (−452) relative to the classical $[M\text{-}F]^-$ pseudo-molecular ion. Such a combined loss of a fluorine atom and of the HFB substituted $C_{(1)}$ and $C_{(2)}$ could not be obtained from compound V. Indeed, this compound only possessed two HFB groups (on $C_{(1)}$ and $C_{(2)}$), explaining the quasi absence of response of this compound using this detection mode.

Heterogeneity of the family of 6oh-Sphes. As mentioned above, four peaks corresponding to each 6oh-Sphes could be easily identified on a GC/MS chromatogram. The search of the ion at $m/e = 522$ (Fig. 6) and overall the very intense ion at $m/e = 308$ (derived from the former by the loss a heptafluorobutyric acid/heptafluorobutyrylamide group) specific of compounds IV and V allowed us to distinguish even very minor compounds in a very complex chromatogram. The presence of these compounds could be confirmed by the search for the intense ions related to the chain-length and corresponding to compounds II and III (225, 239, 253, 267, 281, 295, 309, 323, 337, 351, and 367 for the 6oh-Sphe with 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, and 26 carbon atoms). Eleven 6oh-Sphes were detected in the human skin extracts differing by their chain length (between 16 to 26 carbon atoms). As shown in Table 2, the 6oh-C18:1 Sphe was the major constituent (51.6%) followed by the 6oh-C20:1 Sphe. Very long-chain constituents were detected using this technique, but they could not be integrated for quantitative data since they represented less than 1/1,000 of the major compounds of this family.

Quantitative determinations of long-chain bases

The quantitation of a large variety of compounds in complex mixtures presented difficulties because of the frequent superimposition of constituents of different natures. Such determinations could not be performed using GC alone because the retention time is not a sufficient criterion for proving the purity of a compound. GC/MS allowed solving these ambiguities through the search of specific ions. Nevertheless, the specific ions of each family of compounds were not suitable for quantitation because they were not representative of the abundance of the different products. Only the TIC response could be used for such determinations. Consequently, the quantitative determinations were performed into two steps: *i*) integration of the areas of related peaks for a single family of compounds. For example, the integration could be performed on the Sphe peaks revealed by the specific ion at $m/e = 290$. Because the proportion of the three peaks resulting from methanolysis were constant, the integration could be performed on one of them, and the proportion between the different compounds of the family being deduced from such a simple calculation. For such determination on the specific ion chromatogram, the compounds need be free from other compounds producing this ion; and *ii*) calculation of the proportion between the different families of long-chain bases, based on the areas of the peaks obtained on the TIC chromatogram. This operation could be performed on a single member of each family, the chosen compound being not contaminated. At this stage, it was evident that a correction had to be performed

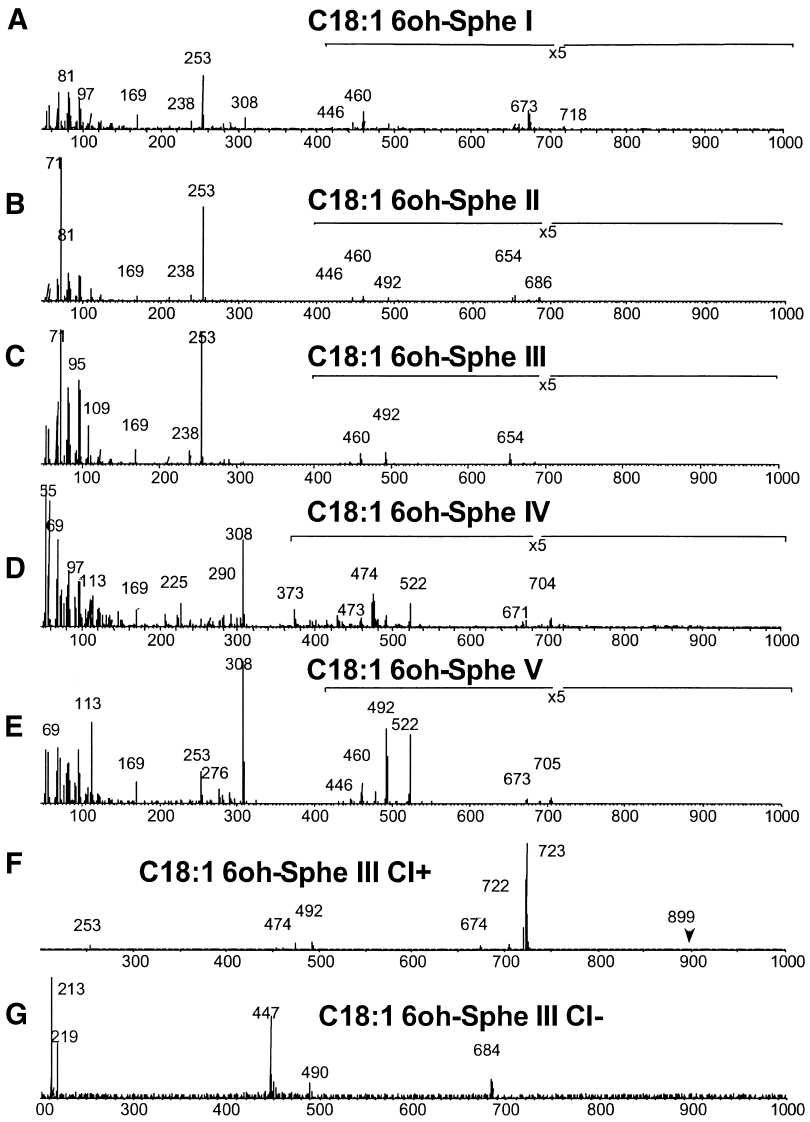

Fig. 7. Mass spectra of the different compounds produced from the 6oh-Sphe (C18:1 6ohSphe) during methanolysis. A–E: Corresponded to the electron impact (EI) spectra of compounds I–V, respectively. Note the intensity of the ions at m/e = 71 and 253 in compounds II and III and the intense ion at m/e = 308 for compounds IV and V. For compound I (A) the high mass ions at m/e = 718 and 673 corresponded to M-169–213 and M-213–214, respectively. For compound II (B) and III (C), the ions at m/e = 686 and 654 corresponded to M-19–213 and M-19, -213, -32, respectively. For compound IV (D), the ions at m/e = 704 and 671 corresponded to M-214 and M-214–32, respectively. For compound V (E), the ions at m/e = 705 and 673 corresponded to M-30 and M-30–32, respectively. F: Chemical ionization (NH3 gas and detection of positive ion) spectrum of compound III. The intense ion at m/e = 723 corresponded to the pseudo-molecular ion $[M+18]^+$ having lost the heptafluorobutyramide group. This ion was also the major ion obtained from compounds II, IV, and V. G: Chemical ionization spectrum (NH3 gas and detection of negative ions) of compound III. The compound underwent an intense fragmentation. The classical pseudo-molecular ion $[M\text{-}F]^-$ (m/e = 899) was at the limit of detection. The ion at m/e = 447 was due to the loss of the substituted $C_{(1)}$-$C_{(2)}$ carbon atoms relative to the pseudo-molecular ion. The ion at m/e = 684 corresponded to the loss of the heptafluorobutyric amide from the pseudo-molecular ion.

taking into account the fact that some compounds were present as multiple peaks. When the analyses were performed in routine conditions with controlled reagents, the proportions of the different peaks were reproducible from one experiment to the other in such a way that this correction factor could be calculated once on standard compounds and applied thereafter to mixtures.

The TIC response could differ dramatically for one compound to the other depending on the fine regulation of the apparatus (for example, a choice between mass precision and intensity of high mass ions). In our hand, such difficulties could be overcome using a frequent contaminant (or endogenous compound) in all samples so far analyzed, i.e., the *O*-methyl glycosides of glucose. The best compromise was to calibrate the apparatus in order to obtain a ratio of 0.60 between the intensities of the ions at

704 and 277 of the HFB derivative of the α-anomer of the *O*-methyl-glycoside of Glc (evidently choosing a non-saturating portion of the peak).

Based on analysis of multiple purified ceramides and glycosphingolipids of different origins (2, 10), the following relative molar TIC response factors were: 1,000 for derivatives of hexoses, FAMEs, LCBs, sialic acid, and Kdn, 1,300 for derivatives of hexosamines and pentoses, 1,280 for derivatives of deoxy-hexoses. In these conditions, quantitative molar composition could be obtained within less than 2% error, independent on the nature of monosaccharides, FAMEs, and fatty acids present in the samples. The data on human skin were actually reproducible within the same range of error for all constituents when separate initial aliquots of the same sample (15 analyses) were methanolyzed, derivatized, and analyzed separately at different periods. However, the composition could be extremely different between different skin extracts, especially due the conditions of skin washing before performing the ethanol extract. Nevertheless, this method allowed an easy determination of the long-chain base and hydroxylated FAME composition of the human skin as shown in **Table 3**. Indeed, analyses of ethanol extracts from six different individuals showed a relatively homogeneous composition with the presence of the same compounds (hydroxylated FAMEs, Sphes, Sphas, Phyts, and 6oh-Sphes) in relatively close molar compositions. Although this pattern could vary dramatically in pathological cases, the overall profiles remained the same, suggesting that the major compounds were not of microorganism origin. Thus, the presence of the four classes of long-chain bases in human skin, and especially that of 6oh-Sphes and Phyts, appeared as a common feature, asking the question of the biosynthesis of these compounds in the human skin.

TABLE 3. Composition of long-chain bases and hydroxylated FAMEs of ethanol extracts of the skin of six different individuals (results are given as percentage of each compound in the different families)

	Sample					
Compound	1	2	3	4	5	6
Sphe						
C16:1	6.21	5.05	6.03	7.05	8.25	2.25
C17:1	21.43	12.13	12.12	11.87	14.39	13.81
C18:1	46.42	53.62	50.32	52.41	53.79	48.78
C19:1	7.41	6.81	5.47	6.98	5.57	7.82
C20:1	12.81	15.82	14.60	15.14	13.49	19.13
C21:1	2.23	2.33	2.13	2.39	1.63	3.60
C22:1	1.98	2.57	2.77	2.92	1.84	4.34
C23:1	1.08	0.88	3.62	0.73	0.42	0.27
C24:1	0.42	0.79	2.95	0.50	0.61	0.00
Spha						
C16:0	2.54	2.73	2.61	3.38	3.75	3.19
C17:0	12.91	11.33	12.78	13.06	14.67	13.53
C18:0	26.76	26.80	28.48	27.31	28.76	22.61
C19:0	11.79	9.54	10.11	11.83	9.79	9.32
C20:0	28.32	23.23	23.75	23.56	22.60	22.11
C21:0	4.40	5.23	4.40	5.31	4.79	6.46
C22:0	6.35	7.51	8.64	8.55	6.72	6.62
C23:0	1.44	2.41	1.32	1.36	0.94	2.39
C24:0	3.19	5.68	4.70	3.05	4.59	7.47
C25:0	1.35	1.84	1.38	1.32	1.55	2.32
C26:0	0.94	3.69	1.83	1.27	1.84	3.98
2oh-F						
C14:0	1.80	1.12	0.52	0.35	1.52	2.01
C15:0	3.67	2.07	5.05	2.60	2.81	3.26
C16:0	38.78	32.55	41.40	25.05	34.04	32.72
C17:0	3.48	2.72	2.19	2.67	2.65	2.97
C18:0	6.86	5.43	4.82	4.67	5.72	5.24
C19:0	0.89	0.73	0.89	0.67	0.75	0.36
C20:0	2.51	2.46	2.27	2.06	2.24	2.65
C21:0	0.56	0.66	0.61	0.61	1.13	0.71
C22:0	4.11	4.28	3.83	3.61	3.96	3.57
C23:0	2.82	3.50	3.95	3.48	4.94	3.63
C24:0	19.03	22.18	17.97	17.55	20.71	21.15
C25:0	6.23	6.97	6.42	6.69	6.86	7.89
C26:0	9.26	15.34	10.08	11.35	12.67	13.84
Phyto						
C16:0	3.22	0.00	0.83	0.27	1.06	1.66
C17:0	5.37	3.18	1.57	1.18	3.69	2.36
C18:0	41.94	40.95	43.89	40.35	44.15	36.44
C19:0	7.00	8.02	6.82	9.22	5.22	7.87
C20:0	24.09	23.35	24.62	26.74	20.73	22.80
C21:0	6.75	8.09	8.15	7.89	5.33	8.92
C22:0	10.01	11.15	10.98	13.35	11.85	15.41
C23:0	1.62	5.26	3.13	0.99	6.67	4.54
C24:0	Trace	Trace	Trace	Trace	1.29	Trace
6oh						
C16:1	5.66	5.77	5.99	4.27	5.77	4.69
C17:1	12.27	10.58	11.52	9.29	9.69	6.94
C18:1	59.97	58.12	55.75	59.96	50.79	60.32
C19:1	6.23	6.53	6.94	7.73	2.34	9.23
C20:1	12.49	14.14	13.55	13.44	28.14	11.56
C21:1	1.29	1.54	2.33	1.85	0.54	2.60
C22:1	1.66	2.50	3.08	2.64	1.90	3.72
C23:1	0.24	0.38	0.41	0.39	0.55	0.52
C24:1	0.19	0.43	0.43	0.43	0.29	0.41
C25:1	Trace	Trace	Trace	Trace	Trace	Trace
C26:1	Trace	Trace	Trace	Trace	Trace	Trace
	1	2	3	4	5	6
Sphe	33.98	39.78	37.55	37.64	37.68	39.09
Spha	20.34	18.17	16.95	20.16	18.58	19.03
Phyt	27.58	25.51	26.79	25.97	26.26	24.24
6oh	18.10	16.53	18.71	16.23	17.47	17.65

CONCLUSION AND PERSPECTIVES

This work demonstrates the suitability of combining acid-catalyzed methanolysis and GC/MS of the heptafluorobutyrate derivatives for the analysis of LCBs in very complex mixtures, even on total homogenates (unpublished results). Mixtures containing amino-acids, oligosaccharides, fatty acids, and long-chain bases can be safely analyzed in a single experiment without time-consuming purification steps. This is due in large part to the insensitivity of HFB derivatives to impurities (including salts), their stability with time, and their poor retention on methylsiloxane column. In contrast, fatty constituents form strong van der Waals interactions with these liquid phases and are eluted at retention times much greater that the abundant contaminants. Furthermore, the HFB derivatives can be easily detected using specific ions that allow discrimination os these compounds within a very high background of non-HFB derivatized compounds. Finally, the specificity of detection of the different LCBs using the chromatogram reconstitution with specific ions described here allows reaching an extremely high level of sensitivity of detection of the different compounds. The optimal chromatographic

conditions and TIC responses for integration are in the range of the nanogram of each compound. But constituents 1,000- to 10,000-fold less abundant than the major ones can be easily detected and, with a single subtraction of the random background of liquid phase leakage, can be unambiguously identified through clear EI spectra. Even quantitative determinations can be performed at the picogram level when a LCB constituent is present in these complex mixtures.

Consequently, besides the analysis of purified compounds (1, 2, 10), this method allows the easy and routine identification of many compounds, starting from very crude extracts. It could provide a methodological breakthrough in many fields of biological analyses, i.e., quality controls in industry, fundamental research and, likely, forensic analyses. Furthermore, this method provides a very easy identification of LCBs, especially 6oh-Sphes, compounds found as major LCBs only in human skin (3–5).

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