Preparative separation of sphingolipids and of individual molecular species by high-performance liquid chromatography and their identification by gas chromatography–mass spectrometry

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ABSTRACT

Six fractions containing tri- to pentaglycosylceramides were isolated from the green, fresh water alga *Chlorella kessleri*, grown heterotrophically, by using preparative high-performance liquid chromatography (HPLC). Up to twelve fractions were obtained by further reversed-phase HPLC of each glycosylceramide. The use of a polar capillary column with Supelcowax 10 as the stationary phase allowed an excellent separation of the individual molecular species of ceramides, even though the separation did not occur when the ceramides differed only in the position of the amide bond. The individual molecular species (even if present in mixtures) were identified by gas chromatography–chemical ionization mass spectrometry. The evidence for a complete structure was obtained by enzyme splitting with α- and β-galactosidases (the sequence of monosaccharides) and by negative ionization fast atom bombardment mass spectrometry. More than 400 molecular species of glycosylceramides were identified.

INTRODUCTION

A very interesting group of lipids, so far studied only in mammals, are sphingolipids. Several review papers and monographs have covered their isolation and occurrence[1-3]. The identification of these compounds always includes their separation according to the number of saccharides followed by separation according to the number of carbon atoms in the ceramide part of the molecule. Recently, thin-layer chromatography (TLC) has been superseded by high-performance liquid chromatography (HPLC), which is used either in the normal-phase mode (separation according to the number of monosaccharides) or a modification of reversed-phase (RP) HPLC

(separation according to the number of carbon atoms in ceramide)[4–10]. The problem of a complete separation into the individual molecular species of glycosylceramides has not yet been satisfactorily tackled, even though the peaks after HPLC were collected and identified by using mass spectrometry (MS)[9] or HPLC couples with MS (LC–MS)[11]. The variability of long-chain bases (LCB) was very small in mammals; in most instances sphingosine prevailed.

In contrast to mammals, the knowledge of sphingolipids in plants and fungi is modest. In a chapter concerning the occurrence of sphingolipids, Hakomori[2] devoted only two pages to plant sphingolipids, in comparison with several tens of pages to animal sphingolipids. In addition, only phosphosphingolipids were included. There are only two reports on glycosylceramides; one of them was discovered in wheat flour and the other in the fungus *Neurospora crassa*[12]. Mono-, di- and trihexosylceramide were isolated from the flour[13], but only trigalactosylglucosylceramide from the fungus. 2-Hydroxy acids and the bases sphingosine and sphinganine represented major components in both the flour and the fungus; phytosphinganine and its C_{20} homologue were another two major compounds identified in the latter material.

In view of the lack of studies on the occurrence of sphingolipids in plants, we attempted to carry out a detailed analysis of these compounds in algae, in which, to our knowledge, sphingolipids had not previously been detected. A knowledge of the occurrence and structure of the sphingolipids molecules can help in understanding phylogenesis of the lower plants to which algae belong.

Working with the green, freshwater alga *Chlorella kessleri*, we previously isolated and identified triacylglycerols[14], wax esters[15] and polar lipids[16]; in this paper, we made a detailed study of sphingolipids present under the conditions of heterotrophic growth.

EXPERIMENTAL

Chemicals

The enzymes α- and β-galactosidase, both from *E. coli*, benzoic anhydride, cellobiose and gluco- and lactocerebrosides were obtained from Sigma ((St. Louis, MO, U.S.A.). Other chemicals were purchased from Lachema (Brno, Czechoslovakia).

Isolation

An amount of 1 kg of the disintegrated, lyophilized biomass of *Chlorella kessleri* obtained from the Department of Autotrophic Microorganisms, Institute of Microbiology, Třeboň, Czechoslovakia, was used for isolation. The extraction procedure according to Bergelson[17] yielded a total of 630 mg of crude sphingolipids.

Derivatization, cleavage and hydrolysis

The total sphingolipids were hydrolysed under alkaline or acidic conditions and, after preparing the methyl esters, were determined by gas chromatography (GC)–MS[2,18,19]. The preparation of the dinitrophenyl (DNP) derivatives of amino alcohols and of FAME (fatty acid methyl esters) from DNP-alcohols was described previously[2]. The preparation of per-O-benzoylglycosphingolipids and their debenzoylation was described by Gross and McLuer[7].

Alditol acetates and partially methylated alditol acetates[2,18], free ceramides (from glycosylceramides) and their trimethylsilyl (TMS) derivatives were prepared by methods described in the literature[2,18,19]. Enzymatic hydrolysis has been described previously[10].

GC–MS

All GC–MS separations were performed by using a Finnigan MAT (San Jose, CA, U.S.A.) 1020 B apparatus with electron impact (EI) or chemical ionization (CI).

Per-TMS-bases. An HP-1, cross-linked methylsilicone (Hewlett-Packard, Palo Alto, CA, U.S.A.) fused-silica capillary column (25 m × 0.2 mm I.D., 0.11 μm film thickness) was used. The injection temperature was 260°C (splitless) and the temperature was programmed from 150 to 300°C at 5°C/min. The linear velocity of the carrier gas (helium) was 45 cm/s and the ionization energy was 70 eV (EI mode).

FAME. A fused-silica capillary column (Supelcowax 10; Supelco, Bellefonte, PA, U.S.A.) (30 m × 0.25 mm I.D., 0.25 μm film thickness) was used. The temperatures were as follows: splitless injection, 240°C; column, 100°C for 1 min, then increased at 20°C/min to 160°C and at 2°C/min to 280°C. The carrier gas was helium at 36 cm/s and the ionization energy was 70 eV (EI mode).

Alditols. Alditol acetates and partially methylated alditol acetates were separated on the HP-1 fused-silica capillary column under the above conditions. For alditol acetates EI was used, and for partially methylated alditol acetates both EI and CI (isobutane; 1.0 Torr) were used.

TMS-ceramides. An injection temperature of 100°C and a Supelcowax 10 column (15 m × 0.25 mm I.D., 0.25 μm film thickness) were used. The temperature programme was as follows: 100°C for 1 min, then increased at 20°C/min to 230°C and at 2°C/min to 280°C, which was maintained for 10 min. The carrier gas was hydrogen at a flow-rate of 120 cm/s. Ammonia (0.6 Torr) was used as the CI reagent gas. The spectra were scanned within the range m/z 250–900.

HPLC

Preparative HPLC was performed with a G-I gradient LC system (Shimadzu, Kyoto, Japan) with two LC-6A pumps (5 ml/min), an SCL-6A system controller, an SPD ultraviolet detector (230 nm), an SIL-1A sample injector and a C-R3A data processor. Preparative columns (25 cm × 21.1 mm I.D.) packed with Zorbax-SIL or ODS (Shimadzu) with 5-μm particles were used.

Per-O-benzoylglycoceramides. These were separated on a preparative column in the normal-phase mode (injection of 27 mg). A linear gradient from 89:10:1 to 50:10:40 hexane–tetrahydrofuran–dioxane in 39 min was used, followed by a 15-min isocratic step. When the analysis was finished, the column was washed with pure dioxane for 30 min and conditioned to the original state by washing with the mobile phase for 1 h. The chromatographic step was repeated 40 times. The individual peaks were collected manually and the solvent evaporated before further use.

RP-HPLC. The individual peaks (A–F) were separated on a preparative column with ODS (see above). The amount injected was 20 mg. A linear gradient from 49:1:50 to 1:49:50 methanol–isopropanol–acetonitrile in 48 min was used, followed by a 15-min isocratic step. When the preparation was finished, the column was washed with pure isopropanol for 30 min and then conditioned with the initial elution

mixture. The individual peaks were collected manually and the solvent evaporated to dryness before further use.

Negative ionization fast atom bombardment MS (NI-FAB-MS)

A 1–5-μg amount of glycosylceramide after debenzoylation [peaks A-4 and (B–F)-6 after RP-HPLC] was dissolved in 10 μl of chloroform–methanol mixture (1:1) and 5 μl of triethanolamine were added. The sample was analysed by using a Hewlett-Packard HP 5988 A apparatus with a FAB source and a high-mass range. Xenon was used as the reaction gas with an energy of 5 keV.

RESULTS AND DISCUSSION

Isolation

Our previous results indicated that the fraction X_{v1} would represent about 0.1% of the total[16], which was consistent with other reports (sum of glycosylcera-mides in flour = 0.043 ± 0.015% of the total)[13], and consequently a modified proce-dure of Bergelson[17] was employed to obtain a greater amount of these compounds. To simplify the extraction and reduce the handling of large volumes, a several-fold extraction of the lyophilized biomass was performed (see Experimental). The result-ing mass of 630 mg, representing 0.063% of the initial biomass, is in keeping with the above-mentioned data. During the extraction, a partial loss could take place, counter balanced by the subsequent rapid reduction of the volumes handled. If the classical method had been used, volumes of the solvent of up to several tens of litres would have had to be handled, requiring the use of pilot-plant equipment. The mild alkaline hydrolysis is a very suitable step that enabled us to remove most contaminating lipids.

HPLC

Neutral glycosylceramides were mostly isolated by TLC, sometimes after acety-lation. As a result of advanced technology, the HPLC of both free sphingolipids and their derivatives, mostly strongly absorbing UV light, has recently been intro-duced[4–8,10]. As the ceramides had to be in a free form for further analyses, benzoic anhydride, which reacts only with the hydroxy and not the amido groups, was em-ployed for derivatization. The absorption of the benzoyl group at 230 nm is compara-ble to that of the *p*-nitrobenzoyl groups at 254 nm (ε = 10 000), which exceeds by several orders of magnitude the value for free sphingolipids. Therefore, a wavelength of 210 nm (or preferably even lower) should be used for detection when these com-pounds are chromatographed.

The analysis of benzoyl derivatives has been reported several times[7,10], but unfortunately not in the preparative or, at least, semi-preparative mode. Therefore, we adapted the classical method of Gross and McCluer[7] by using tetrahydrofuran to prepare a ternary mixture. The addition of tetrahydrofuran resulted in an enhance-ment of the solubility of benzoyl derivatives, better miscibility of dioxane with hexane and a decrease in the viscosity of the mobile phase. Even though benzoylation was repeated, the expected mass increase was not obtained. Therefore, we assume that other additional lipids are present in crude sphingolipids (alkyl- and alkenylglycerols, sterol glycosides, etc.). The total mass of the peaks A–F (Table I) was 473 mg, which represented a yield of 43.8% (relative to the crude benzoyl derivatives). To obtain this
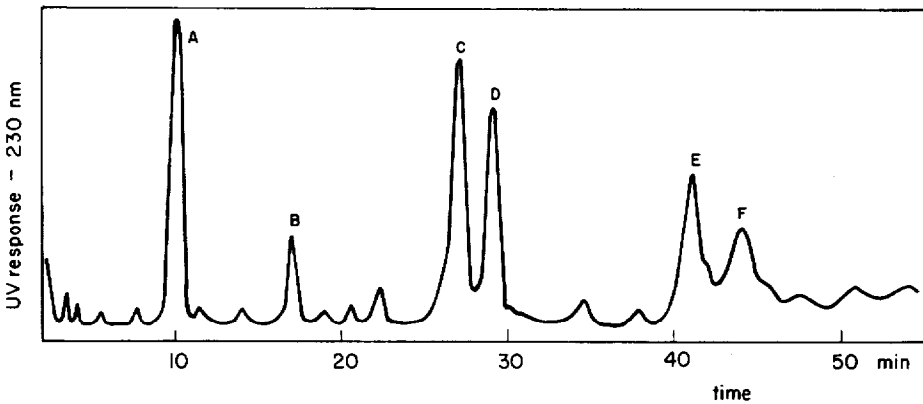
Fig. 1. Preparative HPLC of crude per-O-benzoylglycosylceramides. Peaks A–F are per-O-benzoylglyco-ceramides. For structures see Table I and for experimental conditions see text.

TABLE I

STRUCTURES AND AMOUNTS OF GLYCOSYLCERAMIDES FROM *C. KESSLERI*

Peak	Structure of glycosylceramide	Proportion (%)	Monosaccharides (molar ratio)	Structure of partially methylated alditol acetates
A	Gal–α-1–4-Gal–β-1–4-Glc–1–1-Cer	26.4	Gal,Glc (2:1)	2,3,4,6-Tetra-O-Me-galactitol[a] 2,3,6-Tri-O-Me-galactitol[b] 2,3,6-Tri-O-Me-glucitol[a]
B	Gal–1–4-Gal–1–4-Glc–1–1-Cer 3 1 Rha	7.2	Gal,Glc,Rha (2:1:1)	2,3,4,6-Tetra-O-Me-galactitol[a] 2,3,4-Tri-O-Me-rhamnitol[a] 2,6-Di-O-Me-galactitol[b] 2,3,6-Tri-O-Me-glucitol[a]
C	Rha–1–3-Gal–1–4-Gal–1–4-Glc–1–1-Cer	23.2	Gal,Glc,Rha (2:1:1)	2,3,4-Tri-O-Me-rhamnitol[a] 2,3,4,6-Tetra-O-Me-galactitol[a] 2,3,6-Tri-O-Me-glucitol[b] 2,6-Di-O-Me-galactitol[b]
D	Fuc–1–4-Gal–1–4-Gal–1–4-Glc–1–1-Cer	17.8	Gal,Glc,Fuc (2:1:1)	2,3,4-Tri-O-Me-fucitol[a] 2,3,6-Tri-O-Me-galactitol[b] 2,3,6-Tri-O-Me-glucitol[a]
E	Fuc–1–4-Gal–1–4-Gal–1–4-Glc–1–1-Cer 3 1 Rha	14.2	Gal,Glc,Fuc,Rha (2:1:1:1)	2,3,4-Tri-O-Me-rhamnitol[a] 2,3,4-Tri-O-Me-fucitol[a] 2,3,6-Tri-O-Me-galactitol[b] 2,3,6-Tri-O-Me-glucitol[a] 2,6-Di-O-Me-galactitol[b]
F	Fuc–1–4-Gal–1–4-Gal–1–4-Glc–1–1-Cer 3 1 Rha	11.2	Gal,Glc,Fuc,Rha (2:1:1:1)	2,3,4-Tri-O-Me-rhamnitol[a] 2,3,4-Tri-O-Me-fucitol[a] 2,3,6-Tri-O-Me-galactitol[b] 2,3,6-Tri-O-Me-glucitol[a] 2,6-Di-O-Me-galactitol[b]

[a] Identified by standards and GC–MS; Me = methyl.
[b] Identified by GC–MS only.

TABLE II

COMPOSITION OF ALGAL GLYCOSYLCERAMIDES AFTER RP-HPLC

Peak No.	ECN	Glycosylceramides (%)						Content of LCB	Content of FA
		A	B	C	D	E	F		
1	28	5.9	1.0	0.8	0.7	0.9	1.0	15:1 + 16:1 + 18:2	16:1
2	30	10.2	2.4	2.3	1.9	2.5	2.7	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1	18:1 + 16:1
3	32	23.1	3.9	3.5	4.1	4.3	3.9	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1	20:1 + 18:1 + 16:1
4	34	31.5	11.4	14.6	14.1	9.9	10.3	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1 + 22:1	22:1 + 20:0 + 20:1 + 18:1 + 16:1
5	36	22.3	20.3	19.8	22.3	20.1	18.7	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1	24:1 + 22:0 + 22:1 + 20:0 + 20:1
6	38	3.9	22.2	19.4	20.7	25.1	19.9	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1 + 22:1	26:1 + 24:0 + 24:1 + 22:0 + 22:1 + 20:0 + 20:1
7	40	2.0	18.1	18.6	17.4	18.2	19.1	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1 + 22:1 + 24:1	28:1 + 26:0 + 26:1 + 24:0 + 24:1 + 22:0 + 22:1 + 20:0 + 20:1
8	42	0.9	12.4	10.8	12.1	11.7	12.9	15:1 + 16:1 + 18:2 + 16:0 + 18:1 + i-18:1 + 18:0 + 20:1 + 22:1 + 24:1	30:1 + 28:0 + 28:1 + 26:0 + 26:1 + 24:0 + 24:1 + 22:0 + 22:1 + 20:0
9	44	0	6.2	6.8	5.4	6.1	7.3	16:0 + 18:1 + i-18:1 + 18:0 + 20:1 + 24:1	30:1 + 28:0 + 28:1 + 26:0 + 26:1 + 24:0 + 24:1 + 22:0
10	46	0	1.5	2.1	0.9	1.1	1.9	20:1 + 18:0 + 22:1 + 24:1	30:1 + 28:0 + 28:1 + 26:0 + 26:1 + 24:0
11	48	0	0.5	1.0	0.3	0.1	1.4	22:1 + 24:1	30:1 + 28:0 + 28:1 + 16:0
12	50	0	0.1	0.3	0.1	0	0.9	24:1	30:1 + 28:0

amount, the HPLC had to be repeated as many as 40 times (for a chromatogram, see Fig. 1). We tried to increase the mass of the injected mixture, but if the injection exceeded 30 mg of the crude derivatized sphingolipids peaks C–D and E–F were not separated. In contrast, baseline separation was observed on the analytical scale, but the amount of sample injected was about 5 mg. Using this amount for injection would have caused the number of repeated injections to be too high.

Structure of oligosaccharides bound to ceramides

The suggested structures obtained by four independent methods are shown in Table I. First, the proportion of oligosaccharides (*e.g.*, alditol acetate in peaks A–F after normal-phase HPLC; Fig. 1) was determined. Second, partially methylated alditol acetates that were further characterized after capillary GC–MS were prepared by methylation of glycosylceramides followed by hydrolysis, reduction and acetylation of the free hydroxy groups (Table I). The structures of hexitols and 6-deoxyhexitols were determined by using the mass spectra, and those of tetra-O-methyl-Gal, tri-O-methyl-Glc, fucitol and rhamnitol also by comparing their retention times with those of standards. The identification of the inner saccharides (*i.e.*, partially methylated Gal and Glc) was difficult. The tabulated retention data of many tens of derivatives were used for this purpose[20–24].

It was necessary to distinguish only 2,6-di-O-methyl-Gal and 2,3,6-tri-O-methyl-Gal from 2,3,6-tri-O-methyl-Glc, the last of them being available as a standard prepared from cellobiose. Owing to the use of capillary GC–MS, the identification of 2,6-di-O-methyl and 2,3,6-tri-O-methyl alditols was easy. The only problem was to distinguish the 4-linked Gal from Glc. By using the results of McNeil and Albersheim[22], we were able to distinguish these two alditols, employing GC–MS with CI (isobutane). With the Gal derivatives, the proportions of $M+1$, $M+1-32$ and $M+1-60$ ions (M = molecular ion) were 3:1:1, whereas with the Glc derivatives they were 10:1:30. These intensity ratios clearly indicate the suggested structure and are fully consistent with literature data[22].

To obtain independent evidence for the structure of oligoglycosylceramides, we employed NI-FAB-MS[25–27]. During this type of soft ionization, not only fragments were detected (*i.e.*, splitting off the individual saccharides from the molecule) but also a pseudo-molecular ion. Unfortunately, owing to the wide variation of the chain lengths of LCB and FA (fatty acids), peaks A–F could not be used directly for the study and they had to be separated by RP-HPLC [peaks A-4 and (B–F)-6; Table II]. The M peaks overlapped the M − saccharide peaks that had longer LCB and FA. In this way, saccharides having different molecular weights can be distinguished (*e.g.*, Fuc and Glc), but not, for example, Gal and Glc. In spite of this the method yielded excellent results that enabled us to discern peaks E and F. Fig. 2 shows the difference between the spectra of E-6 and F-6, which confirmed the structures given in Table I. In the region below m/z 400, the structure of the spectrum is very complex, resulting from the presence of many ions formed by splitting the saccharide and ceramide part of the molecule. These ions are not essential for the structure determination, so this region was not examined. In this way, the ions representing ceramides became the base peak, in contrast with some other studies[25–28]. Our values determined for the individual ions differ by as many as 2 a.m.u. owing to the isotopic increments. It should be realized that, for a molecule having a molecular weight of about 1400
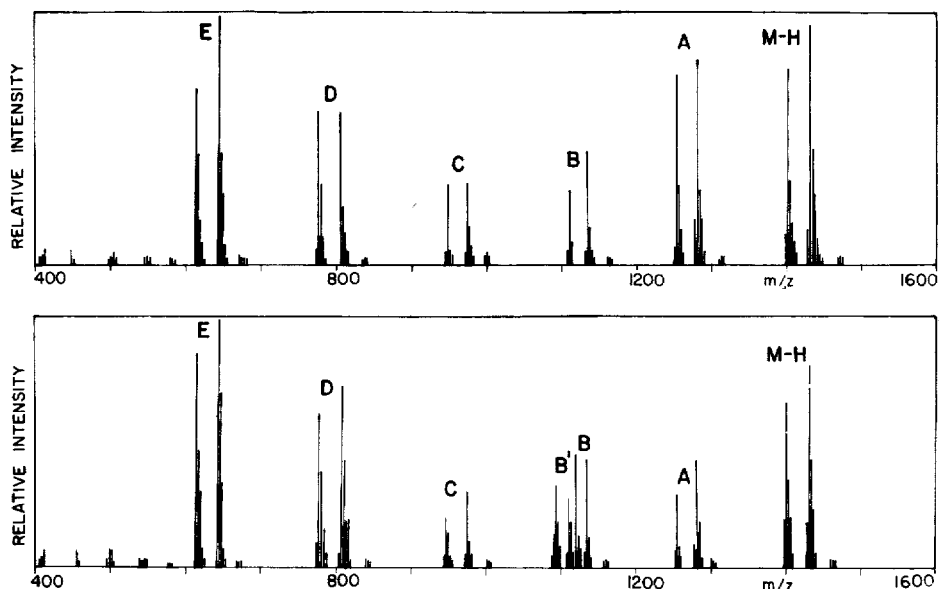
Fig. 2. NI-FAB-MS of peaks F-6 (top) and E-6 (bottom). A = M − H − Fuc or Rha; B = M − H − Fuc − Rha; B' = M − H − Fuc − Gal; C = M − H − Fuc − Rha − Gal; D = M − H − Fuc − Rha − Gal − Gal; E = M − H − pentasaccharide.

a.m.u., the isotopic increment can be as high as 2 a.m.u. The molecular formula for peaks E-6 and F-6 having LCB 18:1 and FA 24:1 is $C_{72}H_{131}NO_{26}$, *i.e.*, M.W. 1425. $M^{+} - H$ has M.W. 1424 whereas, if calculated with the isotopic increment, it is 1426.83.

Another source of information concerning the structure of the oligosaccharides was the enzyme splitting by α- and β-galactosidase. Peak A-4 was found to be suitable for this treatment as the material after the preparation using the normal- and reversed-phase modes amounted to about 39 mg. By using the two enzymes, Gal–Glc–Cer and Glc–Cer were successively obtained; these two compounds had similar chromatographic (HPLC after benzoylation) and mass spectrometric (NI-FAB-MS) properties to the commercial standards. They differed only in the lengths of the LCB and FA chains in ceramide. In our sample, a greater variation in the chain length was observed.

The structures shown in Table I were proposed on the basis of all four methods employed, suggesting that, in all six glycosylceramides, a repeating Gal–Gal–Glc–Cer unit was present, substituted by one or two 6-deoxyhexoses. Our further work will be focused on the complete determination of this structure, including the configuration of the glycosidic bonds and the type (L or D) of the saccharide present.

RP-HPLC

Fig. 3 shows a chromatogram of pentaglycosylceramide (peak F). It clearly indicates that compounds differing in their ECN (equivalent chain number; *e.g.*, chain length minus twice the number of double bonds) were easily separated. Un-
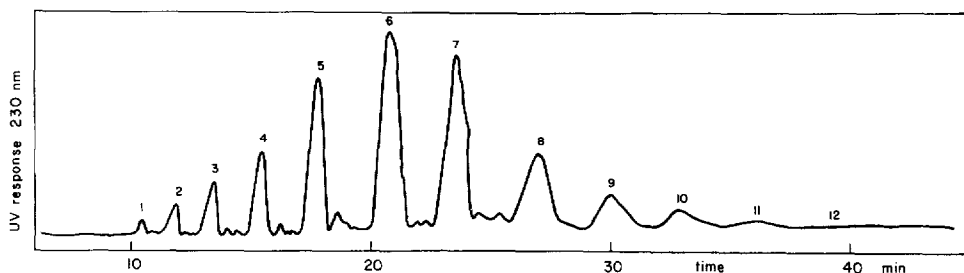
Fig. 3. Semi-preparative RP-HPLC of peak F from Fig. 1. Peaks 1–12 are the molecular species of per-O-benzoylglycoceramides. For structures see Table II and for experimental conditions see text.

fortunately, the great variability in the length of the FA and LCB chains present resulted in the formation of so-called critical pairs when several molecular species were present in one peak, which was indicative of their partial separation. According to the literature, this behaviour can be overcome by changing the temperature[29], by addition of acetonitrile to the mixture[10,30] or by argentation HPLC[31].

In spite of the use of the second method, we were not able to separate the compounds on the semi-preparative scale, which can be explained by the presence of even more than ten molecular species in one peak. The previously described increase in the retention time (tens of minutes)[10], related to the increased number of benzoyl groups in the saccharide, was also observed, but by employing a gradient of the methanol–acetonitrile–isopropanol ternary mixture we were able to eliminate this unfavourable effect almost completely. As a result, e.g., peaks (B–F)-6 had a retention time of 21.3 ± 2.5 min. After collecting all the peaks, acid hydrolysis of the compounds was carried out and the LCB and FA present were identified in each peak. To give a clear arrangement of the results, Table II shows only those LCB and FA whose amounts exceeded 3% of the sum of components of the chromatogram.

Concerning the amount injected, the value of 400 μg indicated in the literature could be exceeded as much as 50-fold, which enabled us to perform a separation on a semi-preparative scale. On using an even greater amount of the compounds separated, overlapping of the peaks was observed[9]. The use of the relative retention time (RRT) in relation to the ECN of the FA and LCB separated, however, did not improve the identification of the individual glycosylceramides because of the great variability in the chain length. Therefore, in addition to the above-mentioned hydrolysis, the collected peaks were also debenzoylated and the free glycosylceramides oxidatively split to obtain ceramides. Periodic acid was chosen as the most suitable reagent for this splitting[18,19]. As other degradations took place during this step, only the peaks isolated by RP-HPLC representing more than 10% of the total amount were identified by GC–MS (Tables III and IV).

GC–MS

The GC of ceramides in the form of their derivatives [most often TMS or tert.-butyldimethylsilyl (t-BDMS)] has been performed on packed columns[32,33]. Unfortunately, by using these columns, the compounds were separated only in relation to the number of carbon atoms and not the number of double bonds in the molecule. A similar result was obtained when capillary columns with a non-polar stationary

TABLE III

MAJOR CERAMIDES AFTER DEGRADATION IDENTIFIED BY POLAR CAPILLARY GC–MS

Glycosylceramide after HPLC and RP-HPLC		Proportion (%)	LCB–FA Composition[b]	
A-2	32:1[a]	1.9	16:0–16:1	
	32:2	3:2	15:1–18:1	
	i-34:2	6.7	i-18:1–16:1	
	34:2	85.2	16:1–18:1/18:1–16:1	M/L
	36:3	3.0	18:2–18:1	
A-3	34:1	3.7	16:0–18:1/18:0–16:1	S/M
	35:2	7.4	15:1–20:1	
	i-36:2	3.6	i-18:1–18:1	
	36:2	78.3	16:1–20:1/18:1–18:1/20:1–16:1	L/L/S
	38:3	7.0	18:2–20:1	
A-4	35:1	2.0	15:1–20:0	
	36:1	10.6	16:1–20:0	
	36:1	1.8	16:0–20:1/18:0/18:1	M/S
	37:2	5.3	15:1–22:1	
	i-38:2	4.0	i-18:1–20:1	
	38:2	1.9	18:2–20:0	
	38:2	69.5	16:1–22:1/18:1–20:1/20:1–18:1/22:1–16:1	L/L/S/S
A-5	36:0	0.5	16:0–20:0	
	37:1	1.1	15:1–22:0	
	i-38:1	1.6	i-18:1–20:0	
	38:1	22.1	16:1–22:0/18:1–20:0	L/L
	38:1	2.3	18:0–20:1/16:0–22:1	L/L
	39:2	3.1	15:1–14:1	
	i-40:2	4.3	i-18:1–22:1	
	40:2	1.0	18:2–22:0	
	40:2	61.1	16:1–24:1/18:1–22:1/20:1–20:1	L/L/S
	42:3	2.9	18:2–24:1	

[a] First number, number of carbon atoms in the chains; second number, number of double bond(s); i = isoacid.

[b] S = Small, M = medium, L = large (intensity according to ions $M - a_x$, see text).

phase were employed[34–36]. Much better separation was achieved on polarizable or polar capillary columns, using both the high-temperature phase RSL-300[34] (methyl-phenylsilicone) or RTX 2330 (cyanopropylsilicone)[37,38]. Unfortunately, no chromatogram of the separation of a mixture of di-TMS-ceramides was published in either paper by Myher et al.[37,38]. Thus, only the chromatogram from an RSL-300 column remains[33].

Ceramides were baseline separated, even 16:1–24:1 and 18:2–22:0, e.g., the compounds having identical ECN (36). As can be seen in Fig. 4, we were able to separate on the baseline even the molecular species differing only in the presence of a double bond in LCB or FA, e.g., the peaks 40:1 (16:1–24:0 and 16:0–24:1). The peaks containing branched LCB were also easily separated from those of compounds having a straight chain in LCB. Very similar molecular species may thus be discerned by chromatography on a polar capillary column.

The mass spectra of ceramides, mainly their splitting by electron-impact ionization (EI-MS), were studied in detail[32,33]. Splitting in the region of the molecular

TABLE IV

MAJOR CERAMIDES (FROM PENTAGLYCOSYLCERAMIDE = F) AFTER DEGRADATION, IDENTIFIED BY POLAR CAPILLARY GC–MS

| Glycosylceramide after HPLC and RP-HPLC		Proportion (%)	LCB-FA composition[b]	
F-4	35:1[a]	2.0	15:1–20:0	
	36:1	10.6	16:1–20:0	
	36:1	1.8	16:0–20:1/18:0–18:1	L/M
	37:2	5.3	15:1–22:1	
	i-38:2	4.0	i-18:1–20:1	
	38:2	69.5	16:1–22:1–20:1/20:1–18:1/22:1–16:1	L/L/S/S
	40:3	4.9	18:2–22:1	
F-5	36:0	0.5	16:0–20:0	
	37:1	1.0	15:1–22:0	
	38:1	5.3	16:1–22:0	
	38:1	2.2	16:0–22:1	
	i-38:2	3.5	i-18:1–20:1	
	38:2	15.9	18:1–20:1	
	39:2	3.8	15:1–24:1	
	40:1	2.6	18:0–20:1	
	i-40:2	4.9	i-18:1 22:1	
	40:2	0.9	18:2–22:0	
	40:2	55.7	16:1–24:1/18:1–22:1/20:1–20:1	M/L/S
	42:3	3.7	18:2–24:1	
F-6	38:0	0.7	16:0–22:0/18:0–22:0	L/L
	.39:1	2.1	15:1–24:0	
	i-40:1	1.5	i-18:1–22:0	
	40:1	27.9	16:1–24:0/18:1–22:0/20:1–20:0	L/L/S
	40:1	3.2	16:0–24:1/18:0–22:1	L/M
	41:2	1.7	15:1–26:1	
	i-42:2	4.4	i-18:1–24:1	
	42:2	2.0	18:2–24:0	
	42:2	54.9	16:1–26:1/18:1–24:1/20:1–22:1/22:1–20:1	M/L/S/S
	44:3	1.6	18:2–26:1	
F-7	40:0	1.2	16:0–24:0/18:0–22:0	L/L
	41:1	1.6	15:1–26:0	
	i-42:1	4.4	i-18:1–24:0	
	42:1	54.6	16:1–26:0/18:1–24:0/20:1–22:0/22:1–20:0	M/L/S/S
	42:1	3.6	16:0–26:1/18:0–24:1	M/L
	43:2	0.9	15:1–28:1	
	i-44:2	2.1	i-18:1–26:1	
	44:2	1.5	18:2–26:0	
	44:2	29.3	16:1–28:1/18:1–26:1/20:1–24:1/22:1–22:1/24:1–20:1	M/L/M/M/S
	46:3	0.8	18:2–28:1	
F-8	42:0	1.3	16:0–26:0	
	43:1	1.7	15:1–28:0	
	i-44:1	4.5	i-18:1–26:0	
	44:1	49.6	16:1–28:0/18:1–26:0/20:1–24:0/22:1–22:0/24:1–20:0	S/L/M/S/S
	44:1	3.8	16:0–28:1/18:0–26:1	M/L
	45:2	0.8	15:1–30:1	
	i-46:2	2.5	i-18:1–28:1	
	46:2	1.6	18:2–28:0	
	46:2	33.5	16:1–30:1/18:1–28:1/20:1–26:1/22:1–24:1/24:1–22:1	M/L/S/M/S
	48:3	0.7	18:2–30:1	

[a,b] See Table III.
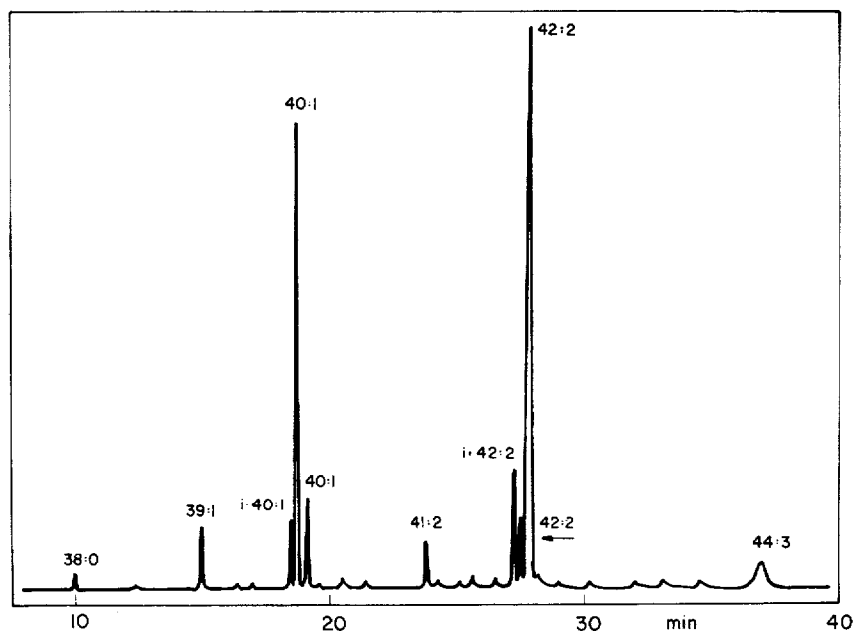
Fig. 4. GC–MS of di-O-TMS-ceramides from glycosylceramides (peak F-6, Fig. 3) after degradation, on a polar column. For conditions see text.

ion (M) resulting in the formation of the ions $M-15$, $M-90$ and $M-103$ was observed, M being mostly undetectable. Another very important group of the ions are those formed through cleavage of the molecule, *e.g.*, the splitting of a C–C bond next to the carbon atoms bearing heteroatoms[32,33].

Being fully aware of the limitations of EI-MS, Oshima *et al.*[39] used GC–CI–MS for the detection of various molecular species as early as 1977. Using this type of ionization, M or, more exactly, the quasi-molecular ion (QM) is always detectable and its intensity depends on the kind of ionizing gas employed. In our case, ammonia was used, QM becoming the base peak. The structure of the other ions is in keeping with the literature data[32,33,39]. The $M-a_x$ ions (see Fig. 5), their structures in the literature[31,32,38] enabled us to determine the structure of the amide-bound FA, whereas the ions $M-d_x$ (see Fig. 5) indicated the numbers of carbon atoms and double bonds present in the molecule of LCB. The interpretation of the data was much hindered by the presence of the isotopic increase. Fig. 5 shows the mass spectrum of the peak 42:2 from Fig. 4. The corresponding molecular weight was 791. Taking into account the isotopic increase, the value was, however, 792.5, which affected the QM value, giving 810.5 a.m.u. On the basis of splitting in MS, we sought not only to identify the individual peaks but also, as far as the peaks containing several molecular species were concerned, to assess their quality and quantity. As far as the qualitative determination is concerned, the length and the degree of unsaturation of the chains of LCB and FA could have been determined on the basis of the ions $M-a$ and $M-d$. However, to our knowledge, no one has been able to separate ceramides of the same molecular weight, whose LCB and FA would have different
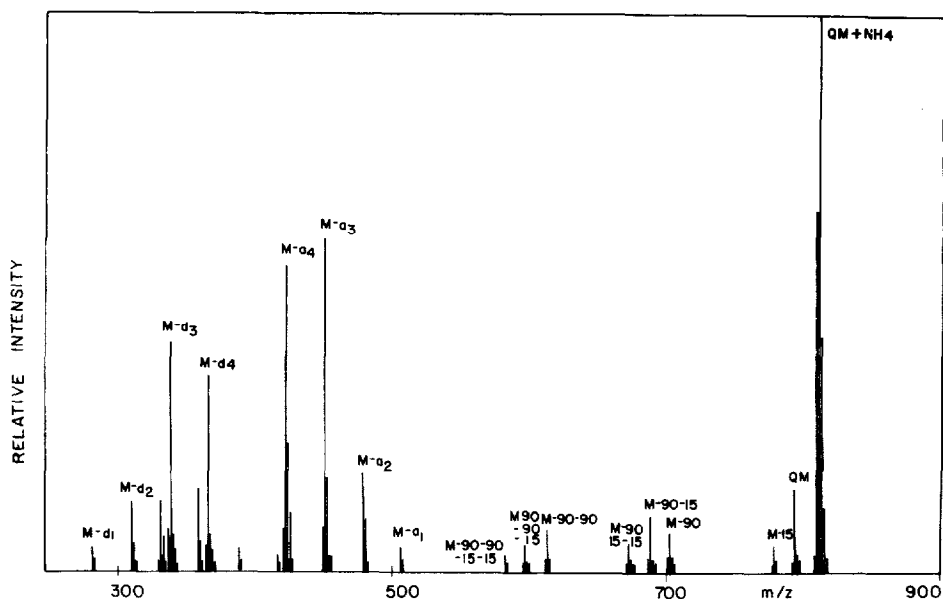
Fig. 5. Mass spectrum of peak 42:2 (major) from Fig. 4. For explanation see text.

chain lengths. We presume that the problem in the separation of these critical pairs is similar to that of wax esters, where some progress has recently been made. Under special conditions, esters differing from each other in the length of the alcohol and acid moieties by more than two carbon atoms can now be separated[15,40].

Irrespective of the fact that we sought to apply previous experience, we were not able to separate the critical pairs in the natural sample. We assume that we could be successful with a synthetic mixture, such as LCB-FA 16:0–20:0 or 20:0–16:0. As far as the proportion of the molecular species is concerned, we attempted to quantify approximately the species present (small, medium, large); however, for a precise determination of the proportion of the individual molecular species, tens of standards would have been necessary for the calibration. In this respect, we do not fully agree with the attempt of Myher et al.[35] to assess the amounts of the molecular species on the basis of GC–MS without an adequate calibration.

The combined use of HPLC, RP-HPLC and the polar capillary column GC–MS together with enzyme splitting, TLC and NI-FAB-MS enabled us to identify more than 400 molecular species of sphingolipids, or more precisely glycosylceramides. It is clear that they represent only major molecular species. We conclude that such a combination of modern analytical methods will help to give a better understanding of the structure of lipids and that this complex approach can be also applied to other types of biological material.

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