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PREPARATIVE SEPARATION OF ALGAL POLAR LIPIDS AND OF INDIVIDUAL MOLECULAR SPECIES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND THEIR IDENTIFICATION BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A crude polar lipidic extract of the green fresh-water alga *Chlorella kessleri* cultivated under heterotrophic conditions was separated by high-performance liquid chromatography (HPLC) on a preparative silica gel column into a total of twelve lipid classes, in which the content of fatty acids was determined by means of gas chromatography–mass spectrometry (GC–MS). The separation was by gradient elution from hexane–isopropanol (6:8) to hexane–isopropanol–water (60:80:14) lasting 20 min and then isocratically for 30 min. A total of 17.5 mg of lipids were injected. Individual types of lipid classes were further separated into eighteen molecular species by isocratic HPLC on a reversed-phase C_{18} column using a mixture 20 mM choline hydrochloride in methanol–water–acetonitrile (90.5:7:2.5) in the preparative mode (5 mg injected). Phosphatidylcholine and phosphatidylethanolamine were hydrolyzed by phospholipase C and corresponding diglycerides were identified by GC–MS on a polar capillary column. In mono- and digalactosyldiglycerols, fatty acids in position 1 were identified after a specific hydrolysis by lipase. The recovery obtained with an UV detector in HPLC and a mass spectrometer in GC–MS is discussed. It was shown that the relative response of the UV detector decreases with increasing saturation of acids, whereas the relative response of the mass spectrometer increases.

INTRODUCTION

Many organisms, from bacteria to higher plants, contain complex mixtures of polar lipids, phospho- and glycolipids[1]. Hydrolysis of a mixture of total lipids or of individual lipid classes followed by derivatization of fatty acids and their identification and quantification by gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS) can yield information on the distribution of fatty acids in the mixture. Unfortunately, the method does not provide data concerning the distribution of fatty acids in individual molecular species and it is also impossible to identify them in the native form. For an accurate analysis it is first necessary to separate the total lipids into individual lipid classes, either by thin-layer chromatography (TLC)[2]

(one- and/or two-dimensional) or by high-performance liquid chromatography (HPLC)[3].

The present progress in instrumentation analysis makes it possible to replace the TLC methods with an HPLC method which is much more "friendly" with respect to the separated compounds and yields a direct detection of individual components in the effluent. However, the HPLC method has mostly been applied to animal lipids[4-6], rarely to plant lipids[7-10]. Polar lipids are separated most frequently on a silica column into individual classes of phospho- and glycolipids, and this is followed by the separation of each separated class into individual molecular species on a reversed-phase column. The method has certain limitations, e.g., it is necessary to detect individual compounds at 200–210 nm, when only the terminal absorption of the molecules in UV light is recorded. It is also impossible to use a refractive index detector as the desirable components are eluted with a gradient. In addition, several molecular species of lipids are eluted in a single peak. The identification of eluted compounds is also difficult unless LC–MS is used[11] and consists primarily in the collection of individual peaks, transesterification and subsequent analysis of the methyl esters by GC or GC–MS. It is also possible to identify individual molecular species by splitting polar moieties of phospholipids by phospholipase C (E.C. 3.1.4.3)[12-14] and after preparation of trimethylsilyl (TMS) or tert.-butyldimethylsilyl (t-BDMS) derivatives demonstrate individual diglycerides by means of GC[13,15], GC–MS[16] or LC–MS[12].

In plants, such studies have been concentrated mainly on polar lipids in classical experimental plants, such as spinach[9] or soya[17]. In green algae, particularly in those of the genus Chlorella, galactosyl diglycerides were separated by means of silver ion TLC[18,19], their sugar moieties were investigated[21], polar glyco- and phospholipids were separated[20,22] and the proportions of fatty acids in separated lipid classes were determined[18-20]. MGDGs (monogalactosyldiglycerols) were partially separated according to the number of double bonds and in some simple cases molecular species were demonstrated, e.g., 18:2-16:2 (ref. 19).

In the present work we studied individual lipid classes in the green alga Chlorella kessleri and determined the content of fatty acids in these classes. The main polar lipids were further separated into individual molecular species. This work thus extends our previous communications about triglycerides[23] and waxes[24]. However, the use of the given methods is not limited only to algae, they can also be applied to all photosynthesizing organisms containing glyco- and phospholipids.

MATERIALS AND METHODS

The green alga Chlorella kessleri was obtained from the Department of Autotrophic Microorganisms of the Institute of Microbiology in Třeboň in the form of centrifuged paste frozen at −25°C. A 10-g amount of fresh biomass (3.7 g dry mass) was extracted with 40 ml of chloroform–methanol (2:1) according to Blight and Dyer[25] and 290 mg of total lipids were obtained. The major part of neutral lipids and pigments was separated from glyco- and phospholipids on a silica gel column (20 g) washed with seven volumes of chloroform. Elution with methanol yielded complex lipids (175 mg) that were further separated by preparative HPLC.

Preparative HPLC was performed in the Gradient LC System G-I (Shimadzu, Japan) with two LC-6A pumps (5 ml/min), a SCL-6A system controller a SPD

ultraviolet detector (208 nm), a SIL-1A sample injector and a C-R3A data processor. Preparative columns 25 m × 21.2 mm I.D. packed with Zorbax SIL or ODS with 5-μm particles were used. After injection of 17.5 mg of polar lipids into the column with Zorbax SIL, it was eluted with a linear gradient from hexane–isopropanol (6:8) to hexane–isopropanol–water (60:80:14) for 20 min and then isocratically for 30 min[9,10]. Individual classes of polar lipids were collected manually and concentrated under reduced pressure and a nitrogen atmosphere. They were identified by comparison of their retention characteristics with those of standard mixtures (Serum lipid mixture; Supelco, Bellefonte, PA, U.S.A.) and by colour reactions after two-dimensional TLC [chloroform–methanol–water (65:25:4) or chloroform–methanol–28%ammonia (65:25:5) in the second direction]. Ten injectons, each of 17.5 mg in 200 μl chloroform, were necessary in order to obtain sufficient quantities of individual lipid classes.

Preparative HPLC of the molecular species of the individual lipids (injection of 5 mg in 100 μl) was performed in the same apparatus on a Zorbax ODS column in the isocratic mode using a mixture of 20 mM choline hydrochloride in methanol–water–acetonitrile (90.5:7:2.5)[4,9]. Individual fractions were again collected manually. All fractions were transesterified by sodium methoxide in methanol (except for fraction X_{v1}). The methylesters obtained were then identified and quantified by means of GC–MS.

Enzymatic degradation[14]

PC (phosphatidylcholine) and PE (phosphatidylethanolamine) (1–1.5 mg) were suspended in 3 ml of diethyl ether with 0.1 mg butylated hydroxytoluene (BHT) as an antioxidant and 3 ml of buffer [17.5 mM tris(hydroxymethyl)aminomethane (Tris) containing 1.0 mM calcium chloride, pH 7.2] with 35 units of phospholipase C (Type XIII from *Bacillus cereus*; Sigma, St. Louis, MO, U.S.A.) were added. After shaking for 3 h, diacylglycerols were extracted with 5 ml of diethylether and the extract was dried with sodium suphate. The enzymatic reaction proceeded with a yield of 98% (TLC; chloroform–methanol–water, 65:25:4). Groups of diacylglycerols from PC or PE were derivatized with TMS[13] for 30 min at room temperature and identified by GC–MS.

Pancreatic lipase (Type II, Sigma) was treated according to Safford and Nichols[19] and impurities were thus removed. The hydrolysis was performed with 1 mg of individual molecular species, *i.e.*, MGDG or DGDG (digalactosyldiglycerol) and with 1 mg of lipase in Tris buffer with calcium chloride at pH 7.6 and 40°C for 3 h under continuous shaking. After hydrolysis, the products were extracted with chloroform, the extract was separated by TLC (hexane–diethyl ether–acetic acid, 70:30:1) and free acids and monoacylgalactosyldiglycerides were methylated with a boiling mixture of methanol–benzene–sulphuric acid (20:10:1) for 1.5 h. Methyl esters were analyzed by GC–MS.

Amide hydrolysis

Fraction X_{v1} (2 mg) containing a lipid with a long base was hydrolyzed under mild conditions (for 1 h at room temperature, 0.6 M sodium hydroxide in methanol). After completion of the reaction the mixture was extracted three times with 5 ml of diethyl ether and the methyl esters were separated from the amide by TLC (choroform–methanol–28% ammonia, 65:25:5). The compound with a lower R_F (0.1) was hydrolyzed with boiling 1 M potassium hydroxide in methanol for 20 h, the

mixture was then cooled and extracted three times with diethyl ether (secondary aminoalcohol was obtained after evaporation). The acidified aqueous phase (3 M hydrochloric acid) was extracted three times with diethyl ether–hexane (1:1) (5 ml), the solvent was evaporated to dryness and methyl esters prepared by reaction with diazomethane were identified by GC–MS.

GC–MS

Methyl esters of fatty acids were separated in an HP 5995 B apparatus (Hewlett-Packard, Avondale, PA, U.S.A.) Chromatographic conditions: splitless injection; temperature, injector 230°C, column 100°C (1 min) then at 20°C/min to 160°C, than at 2°C/min to 270°C; column 30 m × 0.25 mm I.D. × 0.25 μm film, Supelcowax 10; carrier gas helium 30 cm/s; ionization voltage 70 eV; spectra scanned within m/z 50–500.

TMS-diglycerides were separated and identified on the same apparatus. Injection temperature: 100°C. Column (15 m × 0.25 mm I.D. × 0.25 μm film, Supelcowax 10) temperature: 100°C (1 min) then at 20°C/min to 230°C and at 2°C/min to 280°C; finally isothermal for 10 min. Carrier gas (helium), flow-rate: 70 cm/s. Spectra were scanned within m/z 150–750 at 70 eV.

RESULTS AND DISCUSSION

Lipids and fatty acids in Chlorella kessleri

Tables I–V summarize all the values determined. As far as the qualitative and quantitative occurrence of individual lipid classes in algae is concerned, only Nichols[20] separated but did not quantify individual lipid classes in the green alga *Chlorella vulgaris*. This author found practically all known lipid classes; only CL (cardiolipin) was not detected in our sample, however on the basis of analytical TLC and colour reactions it may be assumed that it is the fraction X_2. Khotimchenko[22] presented known phospholipid types in sea algae. He found common species, such as PG (phosphatidylglycerol), PC, PE and minority PI (phosphatidylinositol), PS (phosphatidylserine) and PA (phosphatidic acid). Brush and Percival[21] analyzed sugar moieties of galactosyllipids, identified MGDG, DGDG and TGDG (trigalactosyldiglyceride) and determined the quantitative order DGDG > MGDG > sulfolipid (SL) which is a characteristic feature of algae; a different order was found in higher plants.

TABLE I
POLAR LIPID COMPOSITION OF THE ALGA *C. KESSLERI* AS DETERMINED BY HPLC

Polar lipid fraction	%	Polar lipid fraction	%
MGDG	19.3	X_2	1.2
DGDG	10.9	PI	3.0
SL	2.3	PA	3.1
X_1	0.7	PS	2.8
PE	9.8	PC	37.2
PG	7.3	X_{v1}	2.4

TABLE II

FATTY ACIDS IDENTIFIED FROM POLAR LIPID CLASSES OF THE ALGA C. KESSLERI

Fatty acid[*]	Lipid class											
	MGDG	DGDG	SL	X_1	PE	PG	X_2	PI	PA	PS	PC	X_{v1}
12:0	—	—	—	1.4	0.7	1.0	1.1	0.8	1.4	1.0	0.6	—
i-14:0	—	—	—	2.1	—	—	2.5	—	—	—	—	—
14:0	1.5	1.3	2.1	11.4	3.1	1.7	0.7	3.1	3.1	2.2	2.7	—
9-14:1	—	—	—	1.1	0.2	0.8	5.1	0.1	0.3	0.3	0.3	—
ai-15:0	—	—	—	3.0	—	—	7.3	—	—	—	—	—
15:0	1.0	—	1.8	2.1	2.4	1.1	2.4	2.3	2.3	2.8	3.1	—
9-15:1	—	—	—	1.7	1.0	0.7	0.8	1.0	1.3	1.1	1.0	—
i-16:0	—	—	—	8.1	—	—	3.2	—	—	—	—	—
16:0	5.2	7.1	5.4	15.4	13.2	21.3	19.7	16.5	10.8	10.8	25.4	7.2
7-16:1	1.1	1.4	2.1	7.3	4.6	5.2	5.7	5.8	5.2	6.2	4.8	—
9-16:1	2.4	1.2	1.9	1.8	3.2	2.0	0.9	0.8	1.9	4.1	4.9	—
3t-16:1	—	—	—	—	—	7.1	1.3	—	—	—	—	—
7,10-16:2	14.2	18.7	12.7	—	1.4	2.3	0.6	3.4	2.2	3.1	2.8	—
7,10,13-16:3	23.1	14.8	15.7	—	3.1	0.7	—	3.1	0.7	2.5	1.9	—
17:0	1.0	1.0	1.1	7.9	2.5	1.8	4.3	0.9	1.6	0.3	0.5	—
br$_2$-17:0	—	—	—	5.3	—	—	1.2	—	—	—	—	—
9-17:1	0.5	0.6	1.3	2.3	0.7	2.1	2.7	2.3	1.7	1.1	1.4	—
18:0	2.5	4.5	3.6	13.5	3.4	5.8	16.0	7.2	4.3	22.5	3.8	1.0
9-18:1	5.3	8.1	4.9	14.8	19.8	14.9	22.1	13.1	14.2	17.1	17.4	2.1
9,12-18:2	24.8	25.4	21.4	—	14.1	18.3	1.7	19.6	20.0	14.4	17.1	—
6,9,12-18:3	10.4	5.6	17.8	—	12.2	7.9	—	7.8	2.7	2.7	1.4	—
9,12,15-18:3	7.0	10.3	8.2	—	13.8	5.3	0.7	12.4	15.6	2.1	10.9	—
9-19:1	—	—	—	0.7	—	—	—	—	—	—	—	0.7
20:0	—	—	—	0.1	—	—	—	—	—	0.6	—	3.4
11-20:1	—	—	—	—	0.6	—	—	0.4	3.1	3.1	—	5.1
22:0	—	—	—	—	—	—	—	—	—	—	—	4.8
13-22:1	—	—	—	—	—	—	—	—	—	—	—	7.0
23:0	—	—	—	—	—	—	—	—	—	—	—	2.1
15-24:1	—	—	—	—	—	—	—	—	—	2.0	—	18.3
26:0	—	—	—	—	—	—	—	—	—	—	—	14.2
17-26:1	—	—	—	—	—	—	—	—	—	—	—	17.0
28:0	—	—	—	—	—	—	—	—	—	—	—	3.2
28:1	—	—	—	—	—	—	—	—	—	—	—	4.5
30:0	—	—	—	—	—	—	—	—	—	—	—	4.2
30:1	—	—	—	—	—	—	—	—	—	—	—	5.1

* i = Isoacid; ai = anteisoacid; br$_2$ = methyl group on C$_2$; t = trans; numbers before the hyphen = position(s) of double bond(s); first number = number of carbon atoms in the chain, second number = number of double bonds.

In our case the results of quantification of galactosyl-lipids are not in agreement; they can be compared with the order found in higher plants (MGDG > DGDG > SL)[1].

Table II presents the occurrence of individual fatty acids in different phospholipid classes after their hydrolysis. The unidentified class X_{v1}, with very long acids up to C_{30} bound by an amide bond, has the most interesting content of fatty acids. On the basis of preliminary data it may be assumed that an amide with an unidentified aminoalcohol is involved. The content of longer fatty acids (above C_{18}) in PS is also quite interesting; it thus appears that the assumption of Murata et al.[26] that PS is a precursor of waxes is substantiated. Branched saturated acids were detected in fractions X_1 and X_2. Nichols[20] was again the only author who determined the qualitative and quantitative contents of individual fatty acids in phospholipid classes. His results are in good agreement with our data.

Table III shows the occurrence of individual molecular species of glycolipids. Fatty acids in positions 1 and 2 of MGDG were studied[19] again. The previous authors detected C_{16} acids in position 2. Our results are not in agreement with theirs. On the contrary, we found more saturated acids in position 1, irrespective of the chain length. The situation in lower and higher plants is quite complicated[1].

Tables IV and V present the percentage occurrence of the molecular species of individual phopholipids and/or of PC and PE. Unfortunately, to the best of our knowledge, literature data are practically absent, so that we have nothing with which to compare our results. Compared with higher plants, e.g., tobacco, potatoes, or soya, no pronounced differences in majority molecular species in PC and other phospholipids were detected, e.g., in tobacco leaves[9] most of the PCs were 18:3-18:3, 18:2-18:3, 16:0-18:3 and 16:0-18:2, whereas in the alga used in the present work most of the PCs were 18:3-18:3, 16:0-16:1 and 16:0-18:1.

This fact can be explained by a close taxonomic relationship between green fresh-water algae with higher plants, as the latter (Telomophyta) can be considered as a section of the tribe Chlorophyta (green algae) from which they are phylogenetically derived.

TABLE III

DISTRIBUTION (%) OF MOLECULAR SPECIES OF ALGAL GALACTOLIPIDS AS DETERMINED BY HPLC

Peak No.	Molecular species*	MGDG	% FA on C_1	DGDG	% FA on C_1	SL
1	16:3-18:3**	26.9	79.3	16.1	64.3	22.5
2	16:2-18:3**	10.6	94.5	12.1	86.9	12.2
3	18:3-18:3**	14.4	50.0	10.3	50.0	29.5
4	16:2-18:2	15.1	83.2	26.4	67.8	9.4
5	18:2-18:3**	20.1	47.1	16.4	44.9	20.2
6	16:0-18:3**	1.0	99.7	4.6	98.6	1.7
7	18:1-18:3**	4.3	90.1	5.3	95.4	1.1
8	16:0-18:2	1.4	96.7	7.4	99.0	2.1
9	18:1-18:2	6.2	72.6	1.4	84.3	1.3

    * First FA, it's FA on C_1 of glycerol.
   ** Positional isomer, e.g., 6,9,12-18:3 and 9,12,15-18:3.

TABLE IV

DISTRIBUTION (%) OF MOLECULAR SPECIES OF ALGAL PHOSPHOLIPIDS ACCORDING TO HPLC

Peak No.	Molecular species	Phospholipid					
		PE	PG	PI	PA	PS	PC
1	16:3-18:3	2.4	–	2.0	–	0.5	1.0
2	18:3-18:3	10.2	6.0	11.7	17.6	1.0	12.4
3	18:2-18:3	11.0	8.3	13.0	15.0	3.0	5.5
4	16:1-18:2	3.3	4.6	4.5	4.5	6.4	3.5
5	18:2-18:2	5.9	11.6	11.7	12.8	8.9	6.7
6	18:1-18:3	15.4	6.8	8.2	10.6	3.5	9.2
7	16:0-16:1	3.1	7.3	3.5	3.7	4.8	16.8
8	14:0-18:2	–	1.0	2.0	–	–	1.2
9	16:1-18:1	1.3	3.7	2.8	2.0	1.4	1.4
10	16:0-18:2	5.6	13.5	10.3	4.5	6.7	8.2
11	18:1-18:2	18.3	9.4	8.3	9.0	10.6	6.6
12	17:0-18:2	1.1	1.1	0.5	1.0	–	0.8
13	16:0-18:1	7.3	10.9	6.9	7.5	8.0	12.2
14	18:1-18:1	11.7	7.7	5.5	6.4	12.6	7.3
15	18:0-18:2	1.4	3.7	4.5	2.7	14.0	2.0
16	17:1-18:1	–	1.0	1.0	0.8	0.8	1.3
17	17:1-18:0	–	0.4	0.5	–	1.0	0.4
18	18:0-18:1	2.0	3.0	3.1	1.9	16.8	3.5

HPLC separation of polar lipids

In order to separate sufficient amounts for studies of individual classes of polar lipids or even molecular species of respective lipids, it is necessary to apply much larger samples for enzymatic degradation than those used for a bare determination of fatty acids. Therefore, on the basis of literature data, we performed preparative HPLC of polar lipids.

Fig. 1 shows a record of preparative HPLC of total lipids on a silica gel column. We were able to separate twelve lipid classes, of which MGDG, DGDG and PC were the main ones detected. Quantification was on the basis of the UV detector response; individual values are best seen in Table I. Individual lipid classes were separated practically down to the baseline even when 17.5 mg were injected. This amount is some orders of magnitude higher than that applied by Demandre et al.[9] (20–50 μg) and Rivnay[10] (2.5 mg). The order of elution was different in the two studies, in spite of the fact that the authors used the same mobile phase. The order of elution of the individual components of our sample is in agreement with that of Rivnay[10].

All fractions were thus completely separated, i.e., residual neutral lipids, triglycerides and residual photosynthetic pigments were separated from polar lipids, and all fractions were separated down to the baseline. In addition, it was possible to separate PC and the fraction X_{v1} (amide resembling sphingomyelin) and to use HPLC in the preparative mode and thus obtain milligram amounts of pure polar lipid classes after a single injection. These facts can also be utilized in further separations and reactions, thus, for instance, for PC it would not be necessary to repeat the HPLC separation as 6.4 mg of PC were obtained after a single injection which is sufficient for

TABLE V

MOLECULAR SPECIES OF ALGAL PC AND PE AS DETERMINED BY POLAR CAPILLARY GC–MS

Determined as TMS ethers after treatment by phospholipase C.

Peak No.	Molecular species	PC	PE	Peak No.	Molecular species	PC	PE
1	12:0-16:0	0.4	0.4	27	16:0-18:0	3.8	1.0
2	14:0-16:0 + 15:0-15:0	1.9 + 0.3	0.9	28	16:0-18:1	8.6	5.9
3	14:0-16:1*	0.4	0.3	29	16:1*-18:0	0.6	0.4
4	14:0-16:1	0.4	0.4	30	16:1**-18:0	0.6	0.5
5	15:0-16:0	2.2	0.7	31	16:1*-18:1	1.8	0.0
6	15:0-16:1*	0.4	0.5	32	16:1**-18:1	0.6	1.4
7	15:0-16:1**	0.4	0.3	33	16:0-18:2	4.9	4.2
8	16:0-16:0 + 14:0-18.0	6.5 + 0.3	3.8	34	16:1*-18:2	0.9	1.5
9	16:0-16:1*	8.3	1.4	35	16:1**-18:2	0.9	1.0
10	16:0-16:1**	8.4	0.0	36	16:0-18:3§	1.0	3.6
11	14:0-18:1	0.9	1.4	37	16:0-18:3***	3.8	4.1
12	16:1*-16:1*	0.4	0.5	38	16:1*,**-18:3***,§	1.4	3.3
13	16:1**-16:1*	0.3	0.3	39	17:1-18:1	0.5	0.3
14	16:1**-16:1**	0.4	0.5	40	18:0-18:0	0.4	0.3
15	16:0-16:2	1.9	0.4	41	18:0-18:1	2.3	2.5
16	14:0-18:2	0.6	1.0	42	18:1-18:1	5.4	8.9
17	16:1*-16:2	0.4	0.4	43	18:0-18:2	1.7	2.1
18	16:1**-16:2	0.4	0.5	44	18:1-18:2	3.4	6.3
19	16:0-16:3	1.3	0.9	45	18:0-18:3***	0.6	1.1
20	14:0-18:3***	0.4	1.0	46	18:2-18:2	2.4	6.1
21	16:1*-16:3	0.1	0.3	47	18:1-18:3§	1.3	5.2
22	16:1**-16:3	0.2	0.7	48	18:1-18:3***	3.8	4.0
23	15:0-18:0 + 16:0-17:0	0.3 + 0.3	1.1 + 0.2	49	18:2-18:3§	0.3	3.0
24	15:0-18:1 + 16:0-17:1	1.0 + 1.0	0.8 + 0.7	50	18:2-18:3***	2.0	4.3
25	15:0-18:2	0.6	0.6	51	18:3§-18:3§	1.8	3.2
26	15:0-18:3***	0.5	0.7	52	18:3**-18:3***	3.3	4.2

* 7-16:1.
** 9-16:1.
*** 9,12,15-18:3.
§ 6,9,12-18:3.

both reversed-phase HPLC and enzymatic hydrolysis and other determinations by GC–MS.

HPLC separation of polar lipids into molecular species

A successful separation of polar phospholipids and glycolipids into molecular species depends on the content of fatty acids and also on the polar head group. However, when using the given mobile phase their effect should not be pronounced. For instance, the difference in retention times of the molecular species 18:1-18:3 in PE, PG, PI, PA, PS and PC is negligible, not exceeding ± 7 min with respect to PS, which is in contradiction with Patton *et al.*[4] who reported much larger differences (PI about 40 min, PC and PE up to 100 min). On the contrary, the order of elution of the above molecular species 18:1-18:3 is retained in individual phospholipids, *i.e.*, PI has the shortest retention time (33.5 min), whereas PC, PE and PA have the longest retention
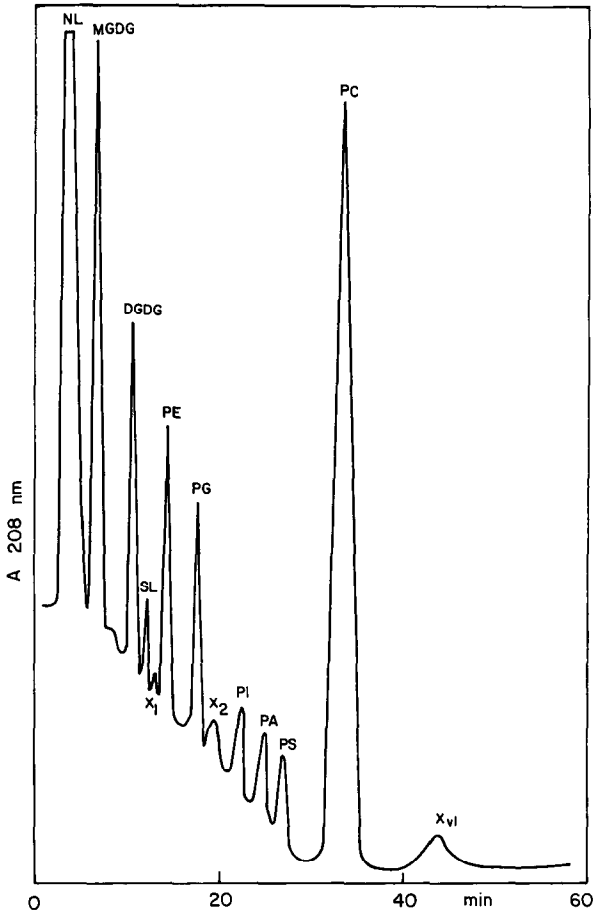
Fig. 1. Preparative HPLC of total polar lipids. For experimental conditions see Materials and Methods.

time (37.5 min). A question thus arises, whether the retention time is determined by the polarity of the given head group or by its polarizability. As a prolongation of the retention time is found in both PC (basic character) and PA (acidic character) it may be assumed that the polarizability plays a role here.

The separation was practically down to the baseline during 30 min when using the isocratic mode (Fig. 2). Thus a total of 9 molecular species of glycolipids were detected, whereas up to 18 molecular species were detected in phospholipids. As compared with Demandre et al.[9], we did not detect contamination of MGDG and DGDG by pigments, due probably to a previous separation by column chromatography (see Experimental). The optimum column load was approximately 5 mg. When increasing the injection volume the separation of individual components sharply decreased and the peaks of the molecular species 18:1-18:1 and 18:1-18:2 overlapped when injecting 10 mg of any phospholipid.
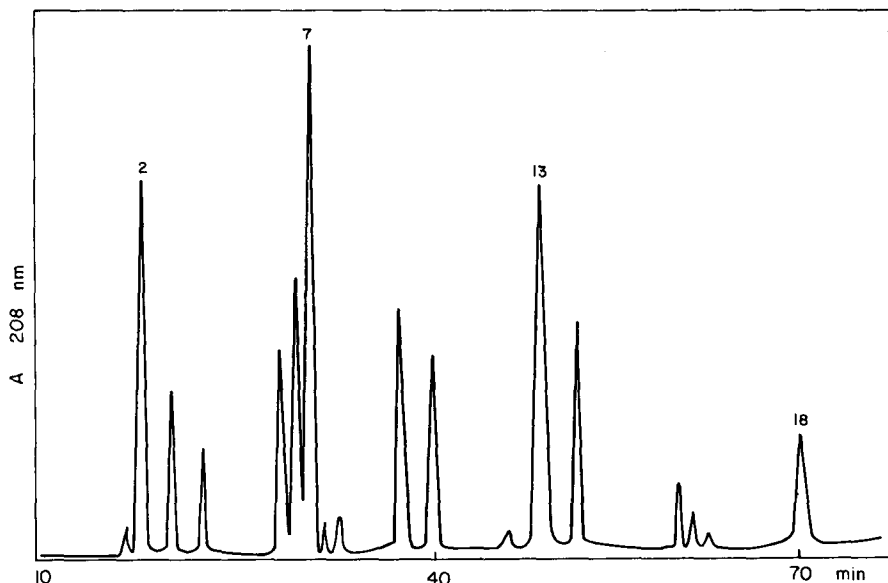
Fig. 2. Preparative reversed-phase HPLC of PCs. For experimental conditions see Materials and Methods; for peak numbers see Table IV. NL = Neutral lipids (mainly triacylglycerols, waxes, sterols, etc.).

GC–MS of TMS-diglycerides

The separation of individual molecular species of TMS-diglycerides on a 30-m capillary column packed with a non-polar phase was described[27]. However, the column efficiency did not permit the separation of even molecular species such as 16:0-18:1 and 16:0-18:2. Odd and even diglycerides including isoacyldiglycerides and diacyldiglycerides were separated on a packed column[16]. Myher and Kuksis[13,15] separated successfully individual molecular species, both TMS- and t-BDMS-diglycerides on a polar capillary column packed with SP-2330. Split and splitless injection and isothermal elution enabled the separation of even positional isomers, e.g., 11-18:1-18:2 from 9-18:1-18:2. Helium was reported to be unsuitable as a carrier gas, however when using this gas we were able to obtain a better separation than that obtained by the above authors. This was primarily due to the injection technique, i.e., on-column injection, and to the use of a less polar and particularly chemically bound and more thermally stable phase of the Supelcowax type which made it possible to use a temperature gradient and thus obtain a better separation (see Fig. 3). We thus separated even a mixture of position isomers, e.g., combination of all 16:1 (see peaks 12–14 in Fig. 3) or diglycerides with 18:3 in combination with other acids (see three peak pairs 47–48, 49–50 and 51–52 in Fig. 3).

Individual peaks in GC–MS can be identified[28] primarily on the basis of $M^+ - 15$ (molecular mass can be determined) and of the ions $M - R_1COO$ and $M - R_2COO$, where R_1COO and R_2COO are acyloxy groups bound to carbon 1 and carbon 2 of TMS-glycerol. In this way it is possible to discriminate even positional isomers, i.e., fatty acids in position 1 and/or 2, on the basis of the intensities of the above ions.
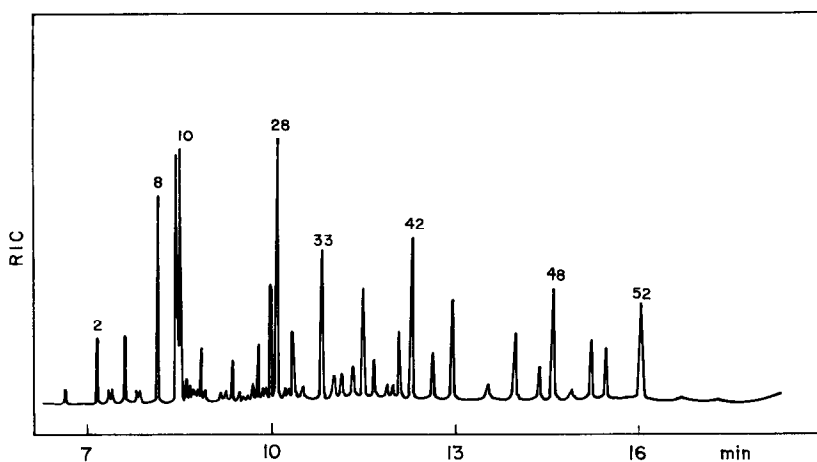
Fig. 3. GC–MS of TMS-diglycerides from PCs after hydrolysis by phospholipase C. For experimental conditions see Materials and Methods; for peak numbers see Table V.

Recovery in HPLC and GC–MS

As pure standards are not available, linearity of the GC–MS and HPLC (UV detector) is quite important for quantification of individual components. Table VI shows the recovery obtained with both detectors (MS an UV) for selected majority peaks in Tables IV and V, the values always being recalculated per 100%. This relationship can be explained by the fact that the terminal absorbancy of the double bond (bonds) is still detected at 208 nm and thus the response of the UV detector is higher than in the case of the saturated chain.

As far as we know, the problem has not yet been discussed in the literature, except in ref. 12. However, as those authors used LC–MS and GC (FID) in which no discrimination occurred, we cannot compare the data obtained. Baty et al.[29] when

TABLE VI

RELATIVE RESPONSES TO PCs AS THEIR TMS-DIGLYCERIDES OBTAINED BY HPLC WITH UV DETECTION AND GC–MS WITH TOTAL ION CURRENT DETECTION

Molecular species*	Number of C = C bonds	HPLC**	GC–MS**	Ratio LC/GC
18:3-18:3	6	14.0	9.1	1.54
18:2-18:3	5	6.2	4.1	1.51
18:2-18:2	4	7.6	6.3	1.21
18:1-18:3	4	10.4	9.0	1.16
18:1-18:2	3	7.5	6.1	1.23
16:0-18:2	2	9.3	8.7	1.07
18:1-18:1	2	8.3	9.6	0.86
16:0-16:1	1	18.9	27.7	0.70
16:0-18:1	1	13.8	15.3	0.90
18:0-18:1	1	4.0	4.1	0.98

* Classified according to the number of double bonds.
** The values were always recalculated per 100% from Tables IV and V.

studying the separation of fatty acid derivatives by HPLC (fluorescence detector) and GC (FID) also did not obtain unambiguous results. These discrepancies can be caused by the chromophore and by the detection method. Pind et al.[12] stated that "Theoretically, the polar capillary columns could be connected to the mass spectrometer, but no practical combinations of this type have yet been devised for work with diacylglycerols". On the contrary, we demonstrated that for the analysis of phospholipids the GC–MS method is suitable and more economic than the LC–MS method, due also to the fact that phospholipids are enzymatically hydrolyzed by phospholipase C also for LC–MS and thus the identical substrate can be prepared for both GC–MS and LC–MS.

Hence, it can be concluded that both methods, i.e., GC–MS and HPLC, are mutually complementary and facilitate a better and more efficient separation and quantification of natural mixtures of polar lipids.

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