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RELATIVE GAS–LIQUID CHROMATOGRAPHIC RETENTION FACTORS OF TRIMETHYLSILYL ETHERS OF DIRADYLGLYCEROLS ON POLAR CAPILLARY COLUMNS

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SUMMARY

Gas–liquid chromatography (GLC) on polar capillary columns provides a highly reproducible resolution and quantitation of molecular species of diradylglycerols when analyzed as the trimethylsilyl (TMS) ethers. In the absence of peak collection and determination of fatty acids or mass spectrometry, peak identification is obtained on the basis of relative retention times of reference standards or of relative retention times calculated from the additive contributions of component fatty chains. Unlike simple esters, complex mixtures of diradylglycerols present special problems in GLC peak identification, which must be attended to by auxiliary separations prior to GLC analysis. In the present study the positional *sn*-1,2(2,3)- and X-1,3-isomers were resolved by borate thin-layer chromatography (TLC) while the alkenylacyl-, alkylacyl- and diacylglycerols were separated as their TMS ethers by normal-phase high-performance liquid chromatography. The diradylglycerol nature of the sample was further verified by GLC determination of the carbon number distribution, which must be consistent with the composition of the fatty chains of the sample. Under these conditions the identification and quantitation of the molecular species on the polar capillary columns was always consistent with the total fatty acid composition of the sample, as well as with the fatty acid composition of any argentation TLC fractions isolated from some of the samples prior to the polar capillary GLC. Due to the great complexity of the natural diradylglycerol mixtures some peak overlaps occurred, which were reflected in their relative retention times. Nevertheless, a determination of diradylglycerol peak identity from relative retention times proved very satisfactory provided the above described procedures were employed.

INTRODUCTION

The availability of polar capillary columns of high temperature stability now permits the resolution of molecular species of diradylglycerols with the ease of fatty acids[1–4]. In comparison to the packed columns containing polyester[5,6] and cyano-alkylpolysiloxane[7] liquid phases, the polar capillary columns give shorter retention times at comparable oven temperatures and higher recoveries, while retaining the same overall order of elution of molecular species. The polar capillary columns have been

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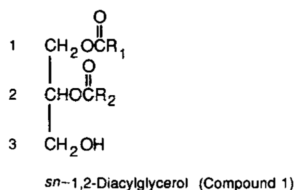
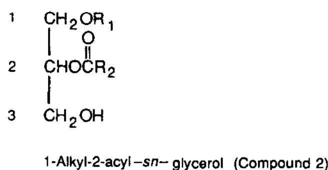
further improved by cross-bonding of the liquid phase, which has reduced the column bleed and improved overall performance. The polar capillary columns described by Geeraert and Sandra[8] for the resolution of natural triacylglycerols on the basis of degree of unsaturation, and which have proved adequate for gas chromatography–mass spectrometry (GC–MS)[9], however, are not suitable for the separation of polyunsaturated diradylglycerols in the form of trimethylsilyl (TMS) or *tert.*-butyldimethylsilyl (TBDMS) ethers, because of the adverse effects of the high temperature needed to maintain column polarity.

The high-resolution capillary columns yield a large number of major and minor peaks, which in the case of natural diacylglycerols require knowledge of fatty acid composition and in the case of the alkylacyl and alkenylacylglycerols also the knowledge of the alkylglycerol and dimethylacetal composition. Since peak collection is impractical in capillary gas–liquid chromatography (GLC) and MS not available in most laboratories, the peak identities must be established by comparison of the relative retention times of the unknowns to those of the reference compounds, which are difficult to prepare and impossible to purchase. The present report describes a variety of retention factors, which we have determined by reference to the few standard diacylglycerols, that are commercially available, or to secondary reference standards easily prepared in the laboratory. We have found them useful for the identification of the TMS ethers of natural diacyl, alkylacyl and alkenylacylglycerols.

EXPERIMENTAL

The structures of the compounds to be resolved are shown in Fig. 1. Compound **1** represents an *sn*-1,2-diacylglycerol, which is the major diradylglycerol moiety of





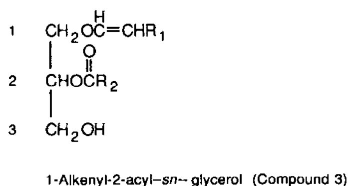


Fig. 1. General structures of diacyl-, alkylacyl- and alkenylacylglycerols.

natural glycerophospholipids. It usually contains a saturated fatty acid in the *sn*-1-position, while the 2-position is occupied by an unsaturated fatty acid. However, isomers, where the positional placement of the fatty acids is reversed, are also known. Compound **2** represents an *sn*-1-alkyl 2-acylglycerol, which is a minor diradylglycerol moiety in natural glycerophospholipids. The alkyl group is usually saturated, while the fatty acyl group is unsaturated. Compound **3** represents an *sn*-1-alkenyl 2-acylglycerol, which may also be a minor diradylglycerol moiety of natural glycerophospholipids, although in special instances it can represent the bulk of a natural glycerophospholipid class (*e.g.* ethanolamine phosphatide). The alkenyl groups may be saturated or oligounsaturated, while the fatty acid chains are oligo- and polyunsaturated. The alkyl and alkenyl groups are not known to occur in the 2-position of the diradylglycerol moieties of natural glycerophospholipids.

Sources of diradylglycerols

The natural diradylglycerols used in the compilation of the retention data were obtained as described in recent studies on the glycerophospholipids in human erythrocytes[10] and plasma[11], rat intestinal mucosa[12] and soybean phosphatidylinositols[4]. Reference *sn*-1,2-diacylglycerols, *rac*-1,2-diacylglycerols and X-1,3-diacylglycerols containing the more common fatty acids were purchased from Serdary Research Labs. (London, Canada; Supelco, Bellefonte, PA, U.S.A.; and Sigma, St. Louis, MO, U.S.A.). Other *sn*-1,2- and *sn*-2,3-diacylglycerols of known composition were synthesized in the laboratory[13]. Secondary reference *sn*-1,2(2,3)-diacylglycerol standards were obtained by Grignard degradation of randomized mixtures of synthetic and natural triacylglycerols[1].

Preparation of derivatives

It is important to avoid even a minimal conversion of the *sn*-1,2-diradylglycerols to 1,3-isomers during trimethylsilylation because these compounds may overlap and interdigitate during GLC separation of the TMS ethers. We have found that the formation of 1,3-isomers is avoided if the *sn*-1,2-diradylglycerols are silylated with trimethylchlorosilane in the presence of hexamethyldisilazane and pyridine as described[2]. Alkylglycerol diacetates are prepared with acetic anhydride–pyridine (1:1, v/v) after transmethylation of the alkylacylglycerol TMS ether with 1 *M* sodium methoxide in methanol–toluene (3:2, v/v) for 15 min at 20°C[10]. The identity of the reference compounds and of the major diradylglycerols in the natural mixtures was confirmed by MS[2,13,14].

Resolution of diradylglycerol subclasses

It is important to avoid even a minimal cross-contamination of the *sn*-1,2-diacylglycerols with the *sn*-1,2-alkylacyl- and *sn*-1,2-alkenylacylglycerols because some of the members of these classes of compounds tend to overlap and interdigitate during the GLC separation of the TMS ethers. It was found that the natural diradylglycerols are readily resolved into the alkenylacyl-, alkylacyl- and diacylglycerols, when run as the TMS ethers using high-performance liquid chromatography (HPLC) on a Supelco sil LC-Si-15 (5 μm) column (250 mm × 4.6 mm I.D.) with 0.3% isopropanol in hexane (1 ml/min)[10–12]. Total running time was 10 to 15 min at 30°C. The peaks were detected at 214 nm.

GLC analyses

Diradylglycerol TMS ethers and alkylglycerol diacetates were resolved principally on the basis of carbon number using a fused-silica capillary column (8 m × 0.32 mm I.D.) coated with bonded SE-54 liquid phase. Samples were injected on-column and temperature was programmed in four ramps from 40 to 350°C as described[14]. The molecular species were separated according to carbon number and degree of unsaturation on a fused-silica capillary column (15 m × 0.32 mm I.D.) coated with cross-bonded RTx 2330 (Restek, Port Matilda, PA, U.S.A.). Diradylglycerol TMS ethers were separated isothermally at 250 or 260°C using a split injector (split ratio, 7:1). Some analyses were made using a 15 m × 0.32 mm I.D. fused-silica column coated with stabilized SP 2380 (Supelco). All the samples were analyzed on a Hewlett-Packard Model 5880 gas chromatograph equipped with a hydrogen flame ionization detector and a Level IV microprocessor. The carrier gas was hydrogen at 2 p.s.i. or 3 p.s.i. head pressure.

Calculation of relative retention times

The calculated relative retention times for any combination of fatty chains in a diradylglycerol molecule were obtained by computing retention factors, as described under Results and discussion. These could be used as multiplication factors for the estimation of theoretical retention times for any diradylglycerol containing the corresponding fatty chains.

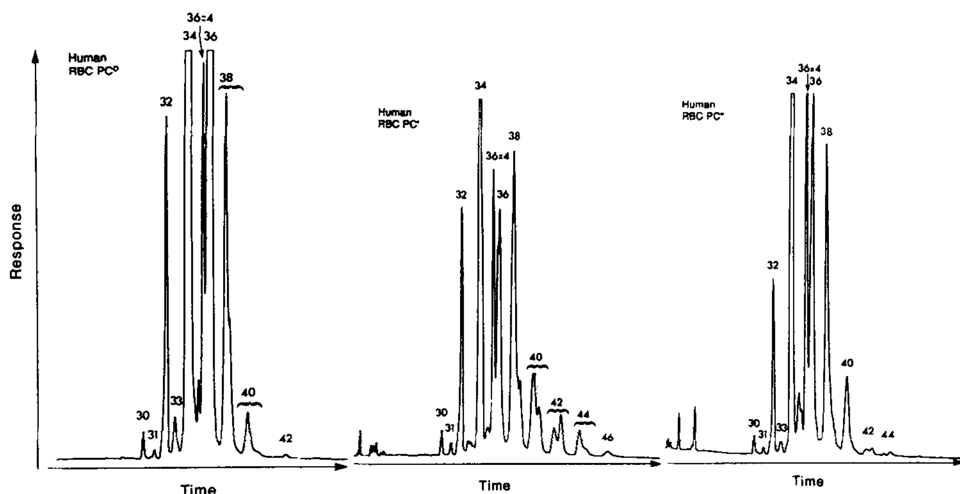


Fig. 2. Carbon number resolution of diacyl (PC⁰), alkylacyl (PC') and alkenylacyl (PC") glycerol moieties of human red cell diradylglycerophosphocholine. GLC conditions: column, 8 m × 0.32 mm I.D. fused-silica capillary coated with cross-linked 5% phenylmethylsilicone (HP-5), 0.17 μm film thickness; carrier gas, hydrogen, 6 p.s.i.; instrument and other operating conditions as given in text. Sample: diradylglycerol TMS ethers. Peaks are identified by the number of carbons in the two radyl moieties. The peak containing the species 16:0–20:4ω6 is indicated as 36:4.

RESULTS AND DISCUSSION

Retention on non-polar columns

Fig. 2 shows the separation obtained on a non-polar capillary column for the TMS ethers of the diacyl-, alkylacyl- and alkenylacylglycerol moieties of the choline phosphatides of human red cells. Although each of the subclasses of diradylglycerols yields excellent peak resolution with full baseline separation, there is considerable overlapping and shouldering when the diradylglycerols are run as a mixture. Extensive peak overlapping occurs also between the *sn*-1,2- and X-1,3-diradylglycerols in a mixture. Table I gives the absolute retention times (in minutes) for the *sn*-1,2-diradylglycerols in the 30–46 carbon range. The presence of polyunsaturated species within a carbon number resulted in a doublet with the unsaturated species being eluted ahead of the saturated ones. It is seen that the diacylglycerol species are retained longer than the alkylacylglycerol species, which are retained longer than the alkenylacylglycerol species of the same carbon number. Although the principle of elution is different, the order is similar to that noted on normal-phase HPLC, where the alkenylacylglycerol species also are eluted first and diacylglycerol species last.

Retention on polar columns

Fig. 3 compares the elution patterns obtained on two polar capillary columns for the TMS ethers of the diacylglycerol moieties of total phosphatidylcholine of rat liver. Both columns, which are presently commercially available, give about the same resolution and elution order of the components, indicating closely similar polarities. Similar polarities were also evident from the elution order of standard and natural (menhaden oil) mixtures of fatty acid methyl esters, although the absolute retention times were slightly different on the two columns. Table II gives the mean relative retention times and standard deviations (S.D.) as determined for the *sn*-1,2-diacylglycerol species from a variety of sources along with the calculated relative retention

TABLE I

ABSOLUTE RETENTION TIMES OF SELECTED *sn*-1,2-DIRADYLGLYCEROLS ON A NON-POLAR CAPILLARY COLUMN (SE-54)

Carbon number	*Diradylglycerols*		
	Diacyl	*Alkylacyl*	*Alkenylacyl*
30	9.57	9.30	9.1
32	10.34	10.1	9.82
33	10.72		
34	11.16	10.82	10.60
36:4	11.78	11.41	11.21
36:(1–3)	11.94	11.60	11.41
38	12.56	12.19	12.04
40	13.36	13.00	12.84
42[a]		13.9	13.61
44[a]		14.95	14.57
46[a]		16.0	15.65

[a] Unsaturated part of doublet.

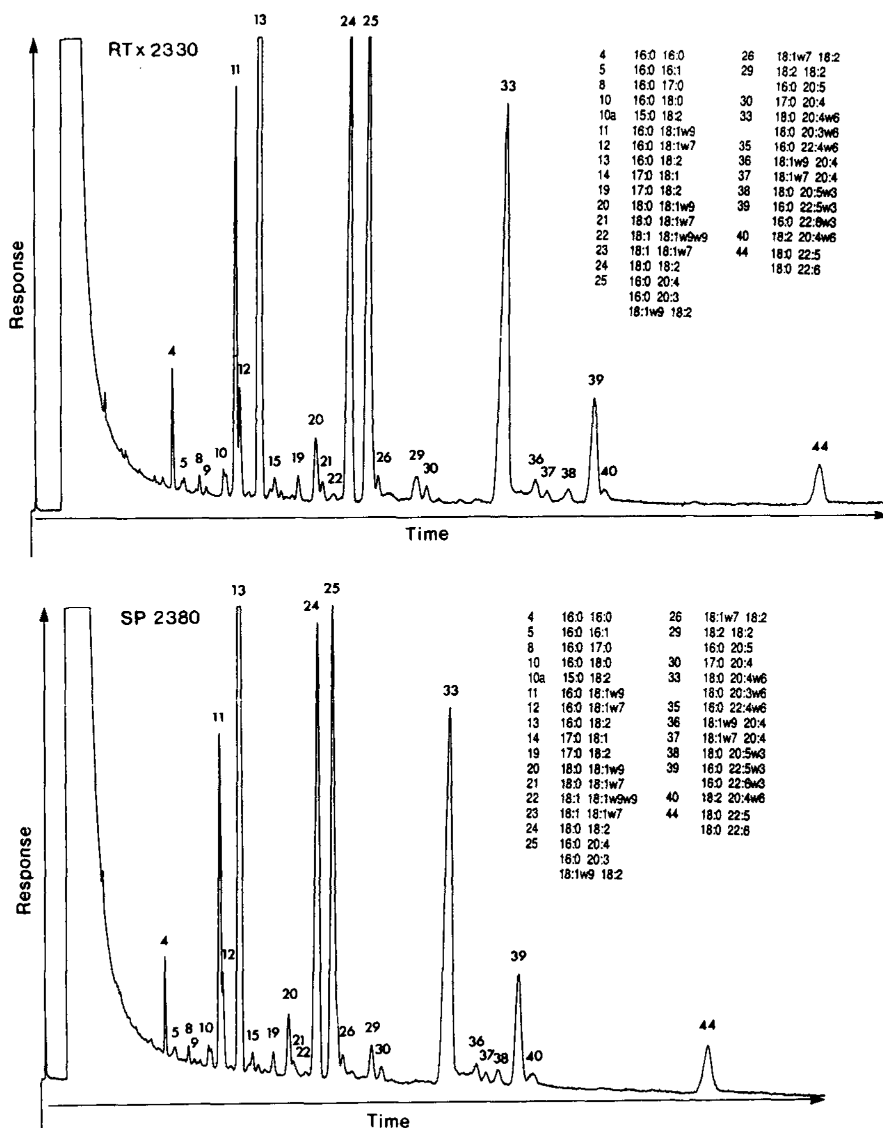


Fig. 3. Polar capillary GLC profiles of the diacylglycerol moieties of rat liver phosphatidylcholine on RTx-2330 and SP 2380 columns. GLC conditions: first column, 15 m × 0.32 mm I.D. fused-silica capillary coated with cross-bonded RTx-2330 (Restek); second column, 15 m × 0.32 mm I.D. fused-silica capillary coated with cross-bonded SP 2380 (Supelco); carrier gas, hydrogen, 3 p.s.i.; temperature, 250°C, isothermal; instrument and other operating conditions as given in text. Sample: 1 μl of 0.1% diacylglycerol TMS ethers in hexane. Split ratio: 7:1.

times for the RTx-2330 column. The standard deviations are very small and range from 0.001 to 0.005, attesting to a high reproducibility of the runs at least over short periods of time. There is a close agreement between the determined and the calculated

TABLE II

RELATIVE RETENTION TIMES OF SELECTED MOLECULAR SPECIES OF *sn*-1,2-DIACYLGLYCEROLS ON A POLAR CAPILLARY COLUMN (RTx-2330)

Peak No.	*Molecular species*	*Relative retention time*		
		Mean	*S.D.*	*Calculated*[a]
1	14:0–16:0	0.445	0.01	0.445
	15:0–16:0			
4	16:0–16:0	0.636	0.01	0.635
5	16:0–16:1ω7	0.710	0.01	0.710
8	14:0–18:2ω6	0.781	0.01	0.768
9	15:0–18:1	0.825	0.01	0.789
10	16:0–18:0	0.931	0.003	0.928
10a	15:0–18:2	0.948	0.003	0.943
11	16:0–18:1ω9	1.000	0.000	0.997
12	16:0–18:1ω7	1.025	0.002	1.021
13	16:0–18:2ω6	1.135	0.003	1.126
14	17:0–18:1	1.204	0.005	1.205
15	16:1–18:2	1.234	0.004	1.259
18	16:0–18:3	1.338	0.004	1.328
	18:0–18:0	1.332	0.006	1.357
19	17:0–18:2	1.358	0.004	1.362
20	18:0–18:1ω9	1.452	0.003	1.457
21	18:0–18:1ω7	1.490	0.005	1.492
22	18:1ω9–18:1ω9	1.557	0.005	1.564
24	18:0–18:2	1.633	0.006	1.646
25	16:0–20:4	1.748	0.008	1.723
	18:1ω9–18:2	1.763	0.009	1.767
26	18:1ω7–18:2	1.807	0.007	1.810
	18:0–18:3	1.889	0.007	1.941
28		1.89	0.008	
29	16:0–20:5	2.018	0.008	1.963
	18:2–18:2	2.018	0.008	1.997
30	17:0–20:4	2.074	0.009	2.083
32		2.37	0.008	
	18:2–18:3	2.398	0.009	2.355
33	18:0–20:4ω6	2.487	0.012	2.519
34		2.540	0.007	
35	16:0–22:4ω6	2.604	0.013	2.570
36	18:1ω9–20:4ω6	2.698	0.010	2.704
37	18:1ω7–20:4ω6	2.761	0.012	2.770
38	18:0–20:5ω3	2.878	0.012	2.869
39	16:0–22:5ω3	3.017	0.016	2.967
	16:0–22:6ω3	3.017	0.016	
40	18:2–20:4ω6	3.093	0.015	3.080
	18:1ω9–20:5ω3	3.131	0.017	3.080
41a		3.578	0.014	
41	18:0–22:4ω6	3.726	0.016	3.757
	18:0–22:5ω6	3.825	0.019	
	18:1ω9–22:4ω6	4.017	0.018	4.033
	18:0–22:5ω3	4.273	0.020	4.337
	18:0–22:6ω3	4.273	0.020	4.337
	18:1ω9–22:5	4.646	0.020	4.656
	18:1ω9–22:6	4.646	0.020	4.656
	20:4ω6–20:4ω6			4.674
	18:2ω6–22:6ω3			5.262
	20:5ω3–20:5ω3			6.066

[a] Calculated relative retention, $R_{AB} = F_A \cdot F_B$, where F_A and F_B are retention factors characteristic of fatty chains in *sn*-1- and *sn*-2-positions of the diradylglycerol molecule, respectively.

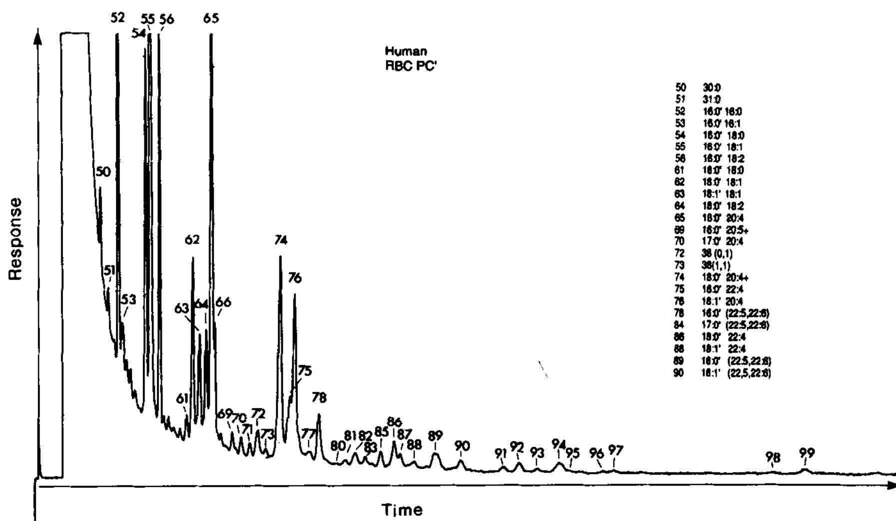


Fig. 4. Polar capillary GLC profile of the alkylacylglycerol moieties of human red cell alkylacylglycerophosphocholine. GLC conditions as given in Fig. 3 first column. Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane.

values, which indicates that the contributions to the free energy of interaction of the various acyl groups are independent and additive.

Fig. 4 shows the elution pattern obtained for the alkylacylglycerol moieties of the choline phosphatide of human erythrocytes.Table III gives the means and standard deviations determined for the RRT of the alkylacylglycerols from a variety of sources, along with the calculated relative retention times for the RTx-2330 column. Again the runs are readily reproduced as indicated by the small standard deviations (0.001–0.005). The agreement between the determined and calculated values was also close, which suggests that the alkyl groups contribute independently and additively to the total retention time of an alkylacylglycerol molecule.

Fig. 5 shows the elution pattern obtained for the alkenylacylglycerol moieties of the ethanolamine phosphatides from human erythrocytes. Table IV gives the means and standard deviations for the RRT of the alkenylacylglycerols from a variety of sources, along with the calculated values. There is good reproducibility of the retention times with the standard deviation ranging from 0.003 to 0.009, as well as a good agreement between the determined and calculated relative retention time values. Obviously, the alkenyl group also makes an independent and additive contribution to the total retention time of the alkenylacylglycerol molecule.

Fig. 6 shows the GLC separation on a polar capillary column of an isomerized sample of diacylglycerols containing approximately equal amounts of *sn*-1,2(2,3)- and X-1,3-isomers. We have calculated that the X-1,3-isomers are eluted later than the corresponding *sn*-1,2(2,3)-isomers by a factor ranging from 1.070 for the monoenes and dienes to 1.085 for the tetraenes and hexaenes.

The performance of the 15-m RTx-2330 columns was determined by calculating the Trennzahl from the measured retention times and peak widths of various homologous pairs of fatty acid methyl esters and diacylglycerols. The average

TABLE III

RELATIVE RETENTION TIMES OF SELECTED MOLECULAR SPECIES OF *sn*-1-ALKYL-2-ACYLGLYCEROLS ON A POLAR CAPILLARY COLUMN (RTx-2330)

Peak No.	*Molecular species*	*Relative retention time*		
		Mean	*S.D.*	*Calculated*[a]
50	30:0	0.245	0.001	0.254
51	31:0	0.300	0.001	0.304
52	32:0	0.366	0.002	0.363
53				
54	34:0	0.542	0.002	0.531
55	16:0–18:1ω9	0.576	0.001	0.570
56	16:0–18:2ω6	0.634	0.002	0.644
57		0.665	0.002	
58	35:1	0.697	0.002	0.689
59		0.728	0.003	
60	35:2	0.769	0.003	0.779
61	36:0	0.813	0.004	0.776
62	18:0–18:1ω9	0.849	0.004	0.833
63	18:1ω9–18:1ω9	0.894	0.003	0.888
64	18:0–18:2ω6	0.938	0.004	0.941
65	16:0–20:4ω6	0.976	0.005	0.986
66		0.996	0.005	
69	16:0–20:5ω3	1.116	0.005	1.123
70	17:0–20:4ω6	1.174	0.005	1.191
71		1.228	0.004	
72	20:0–18:1	1.278	0.003	1.210
73	20:1–18:1	1.329	0.002	1.277
74	18:0–20:4ω6	1.429	0.003	1.440
75	16:0–22:4ω6	1.500	0.003	1.471
76	18:1ω9–20:4ω6	1.525	0.002	1.535
		1.551		
77	18:0–20:5ω3	1.619	0.003	1.640
78	16:0–22:5ω3	1.695	0.005	1.698
78	16:0–22:6ω3	1.695	0.005	1.698
79		1.732	0.004	
80		1.818		
81		1.863	0.004	
82		1.932	0.004	
83		1.992	0.003	
84		2.022	0.004	
85	20:0–20:4ω6	2.088	0.004	2.093
86	18:0–22:4ω6	2.176	0.005	2.148
87	20:1–20:4ω6	2.217	0.004	2.207
88	18:1–22:4ω6	2.307	0.005	2.290
89	18:0–22:5ω3	2.444	0.005	2.480
89	18:0–22:6ω3	2.444	0.005	2.480
		2.461	0.006	
90		2.614	0.004	
91		2.901	0.005	
92		2.990	0.006	
93	22:0–20:4ω6	3.109	0.006	3.092
94	22:1–20:4ω6	3.266	0.006	3.249
95	22:1–20:4ω6	3.327	0.005	
96	22:2–20:4ω6	3.547	0.007	3.528

(Continued on p. 196)

TABLE III *(continued)*

Peak No.	*Molecular species*	*Relative retention time*		
		Mean	*S.D.*	*Calculated*[a]
97	20:0–22:5ω3	3.607	0.008	3.604
97	20:0–22:6ω3	3.607	0.008	3.604
97a	20:1–22:5ω3	3.787	0.006	3.801
97a	20:1–22:6ω3	3.787	0.006	3.801
98	24:0–20:4ω6	4.618	0.008	4.627
99	24:1–20:4ω6	4.865	0.008	4.839
100	24:1–20:4ω6	4.960	0.009	
100a	24:2–20:4ω6	5.321	0.008	5.293
100b	22:1–22:5ω3	5.564	0.009	5.596
100b	22:1–22:6ω3	5.564	0.009	5.596

[a] See footnote to Table II.

Trennzahl value (number of peaks resolved between homologues differing by one methylene unit) of 5.9 found for the diacylglycerols was significantly lower than the average value of 8.2 obtained for the fatty acid methyl esters. In spite of this slight loss in column efficiency the achieved separations are very good.

Columns may vary slightly in properties from batch to batch even if supplied by the same manufacturer, and it is important to test them with a few known mixtures. Columns also age. In the short term, columns may initially show some effective increase in polarity following use at 250–260°C. In long term, effective column polarity gradually decreases due to loss of liquid phase.

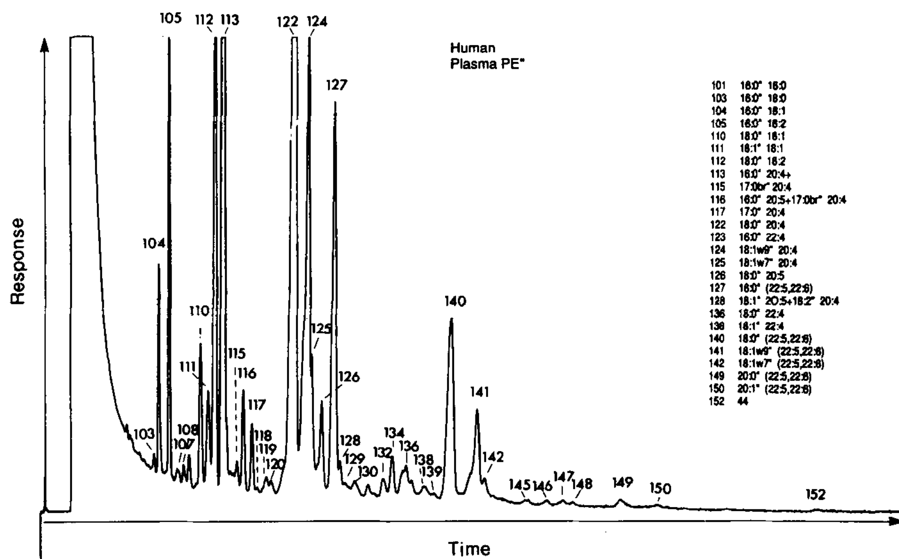


Fig. 5. Polar capillary GLC profile of the alkenylacylglycerol moieties of human red cell diradylglycerophosphoethanolamine. GLC conditions as in Fig. 4. Sample: 1 μl of 0.1% diradylglycerol TMS ethers in hexane.

TABLE IV

RELATIVE RETENTION TIMES OF SELECTED MOLECULAR SPECIES OF *sn*-1-ALKENYL-2-ACYLGLYCEROLS ON A POLAR CAPILLARY COLUMN

Peak No.	*Molecular species*	*Relative retention time*		
		Mean	*S.D.*	*Calculated*[a]
	30:0	0.225	0.003	
101	16:0–16:0	0.335	0.002	0.340
102a	16:0–17:0	0.412	0.003	0.410
103	16:0–18:0	0.501	0.004	0.496
104	16:0–18:1ω9	0.534	0.003	0.538
105	16:0–18:2ω6	0.598	0.002	0.602
107		0.648	0.003	
108		0.683	0.004	
109		0.716	0.003	
110	18:0–18:1ω9	0.787	0.003	0.777
111	18:1ω9–18:1ω9	0.833	0.004	0.833
112	18:0–18:2ω6	0.874	0.003	0.878
113	16:0–20:4ω6	0.922	0.004	0.921
115		1.013	0.003	
116	16:0–20:5ω3	1.055	0.003	1.049
116	17:0br–20:4ω6	1.055	0.003	1.049
117	17:0–20:4ω6	1.105	0.004	1.109
118		1.130	0.003	
119		1.191	0.005	
120		1.218	0.004	
121				
122	18:0–20:4ω6	1.340	0.004	1.343
123	16:0–22:4ω6	1.411	0.006	1.374
124	18:1ω9–20:4ω6	1.437	0.005	1.440
125	18:1ω7–20:4ω6	1.464	0.006	1.457
126	18:0–20:5ω3	1.521	0.005	1.530
127	16:0–22:5ω3	1.597	0.005	1.586
127	16:0–22:6ω3	1.597	0.005	1.586
128		.1.636	0.005	
129		1.675	0.006	
130		1.725	0.004	
131		1.814	0.006	
132		1.882	0.005	
133	17:0–22:5ω3	1.927	0.004	1.910
133	17:0–22:6ω3	1.927	0.004	1.910
134	20:0–20:4ω6	1.956	0.006	1.939
136	18:0–22:4ω6	2.037	0.006	2.003
137				
138	18:1–22:4ω6	2.157	0.005	2.148
139		2.203	0.007	
140	18:0–22:5ω3	2.295	0.007	2.312
140	18:0–22:6ω3	2.295	0.007	2.312
141	18:1ω9–22:5ω3	2.455	0.008	2.480
141	18:1ω9–22:6ω3	2.455	0.008	2.480
142	18:1ω7–22:5ω3	2.508	0.008	2.509
142	18:1ω7–22:6ω3	2.508	0.008	2.509
145		2.759	0.007	
146		2.883	0.009	
147		2.979	0.007	

(Continued on p. 198)

TABLE IV *(continued)*

Peak No.	*Molecular species*	*Relative retention time*		
		Mean	*S.D.*	*Calculated*[a]
148		3.036	0.008	
149	20:0–22:5ω3	3.328	0.009	3.339
149	20:0–22:6ω3	3.328	0.009	
150	20:1–22:5ω3	3.544	0.011	3.555
150	20:1–22:6ω3	3.544	0.011	

[a] See footnote to Table II.

Calculation of retention factors

The relative retention time, R_{AB}, of any species AB, were A and B are alkyl, alkenyl or acyl moieties, is given by the expression

$$R_{AB} = F_A \cdot F_B$$

or

$$\log R_{AB} = \log F_A + \log F_B$$

where F_A and F_B are appropriate retention factors for the A and B portions of the molecular species. This relationship is in accordance with Martin's equation[16] and assumes that each ester or ether moiety contributes independently to the retention time of the whole molecule. This assumption has been shown previously to apply to the GLC retention factors of diacylglycerols[1,7] and HPLC retention factors of triacylglycerols[17]. In the present case contributions from the rest of the molecule are assumed

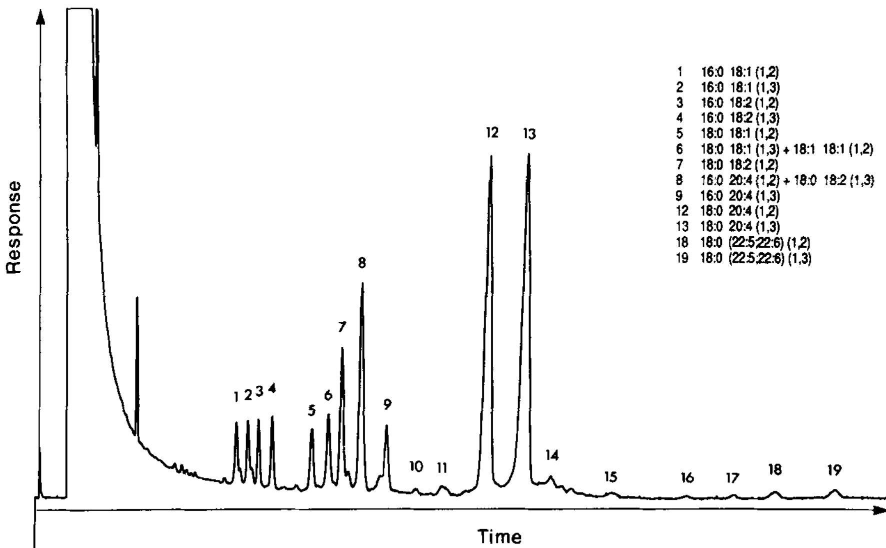


Fig. 6. Polar capillary GLC profile of isomerized diacylglycerol moieties of human plasma phosphatidylinositol. GLC conditions as given in Fig. 4. Sample: 1 μl of 0.1% diacylglycerol TMS ethers.

to be constant and are incorporated into the individual factors. Each factor has been determined in several ways by an iterative procedure and then averaged. The retention time of a single acid species, such as 16:0–16:0 and 18:1–18:1, are used to initiate the process. Thus,

$$F_{16:0} = \sqrt{R_{16:0-16:0}} \text{ and } F_{18:1} = \sqrt{R_{18:1-18:1}}.$$

Other determinations of $F_{18:1}$ can be made from the retention data of other species, such as 16:0–18:1 and 18:0–18:1, using the factors for 16:0 and 18:0. In the end the factors represent averages from as many combinations of fatty chains as the experimental data permit.

It is assumed that the factors are independent of the positional placement of the fatty chains in the diradylglycerol molecules. This is consistent with the observation that reverse isomers, such as 16:0–18:1 and 18:1–16:0, are not resolved. GLC runs of random mixtures containing equal proportions of reverse isomers show no resolution or peak broadening due to the presence of both isomers in a peak.

TABLE V

RETENTION FACTORS USED TO CALCULATE THEORETICAL RETENTION TIMES

Fatty chain	*Retention factors (Fx)*		
	Acyl[a]	*Alkyl*[b]	*Alkenyl*[c]
14:0	0.558		
15:0	0.667		
16:0	0.797	0.456	0.426
16:1ω7	0.891		
17:0	0.964	0.551	0.513
18:0	1.165	0.666	0.621
18:1ω9	1.251	0.710	0.666
18:1ω7	1.281		0.674
18:2ω6	1.413		
18:3ω3	1.666		
20:0	1.70	0.968	0.897
20:1		1.021	0.955
20:4ω6	2.162		
20:5ω3	2.463		
22:0		1.430	
22:1		1.503	
22:2		1.632	
22:4ω6	3.225		
22:5ω6	3.284		
22:5ω3	3.723		
22:6ω3	3.723		
24:0		2.140	
24:1		2.238	
24:2		2.448	

[a] $F_{acyl} = R_{diacyl}/F_{acyl}$

[b] $F_{alkyl} = R_{alkylacyl}/F_{acyl}$

[c] $F_{alkenyl} = R_{alkenylacyl}/F_{acyl}$

In the case of the alkylacyl- and alkenylacylglycerol species, the acyl factors were set equal to those found for the diacyl species. The factors for the alkyl and alkenyl moieties were found by dividing the retention times of the molecular species by the appropriate acyl factors. For example,

$$F_{16:0} = R_{16:0-18:1}/F_{18:1}$$

Again, the final factors are averages.

Table V gives the retention factors calculated for the acyl, alkyl and alkenyl groups of the diradylglycerols. There are marked differences in the retention factors among these different radyl moieties. For corresponding carbon numbers, the acyl retention factor is larger than the alkyl retention factor, which is larger than the alkenyl retention factor. Since each diradylglycerol class has the secondary acyl group in common, these factors represent the elution order of equivalent diradylglycerol species from the polar capillary column.

If one plots the carbon number, C, *versus* log Fx for the saturated acyl, alkyl and alkenyl factor (Fx) three parallel lines corresponding to the equation

$$C = m \log Fx + b$$

are obtained. The (m, b) values obtained by linear regression are (12.16, 17.20), (12.24, 20.17) and (12.36, 20.58) for the acyl, alkyl and alkenyl factors, respectively. By substitution of the Fx values for the three types of 16:0, 18:0, 18:1 and 20:0 moieties into the regression line for the acyl moieties, it is possible to show that equivalent alkylacyl and alkenylacyl species elute 2.97 and 3.34 carbon number units earlier than the corresponding diacyl species. In terms of relative retention times equivalent alkylacyl- and alkenylacylglycerol species elute earlier than diacylglycerol species by factors of 0.570 and 0.532, respectively. On a glass capillary column coated with SP-2330 we found[2] a value of 0.525 for the ratio of the relative retention times of the TMS ethers of corresponding alkenylacyl/diacylglycerol species.

Identification of diradylglycerols containing isomeric fatty chains

The identification of diradylglycerol species containing isomeric fatty acids, such as 16:0–18:1ω9 and 16:0–18:1ω7 is based on a number of different experimental lines of evidence. Thus, the observed splitting for species containing ω9 and ω7 monoenoic fatty acids is consistent with the known behavior of these acids on this and similar liquid phases[1,18]. Although other octadecenoic acids can be present especially in samples from subjects consuming partly hydrogenated fats, the principal isomers present in mammalian tissues are the ω9 and ω7 isomers[19]. Therefore, peaks coeluting with reference samples of 18:1ω9 and 18:1ω7 can be justifiably assumed to be identical with the standards. In some cases, monoenoic fractions isolated by argentation thin-layer chromatography (TLC) have been characterized by GC–MS[13]. The GC–MS data shows that no unknown species are present that can be responsible for the observed splitting.

Effect of temperature and flow-rate

As expected, a change in flow-rate changes all the retention times by the same factor. In contrast a temperature shift affects the various molecular species differently

TABLE VI

EFFECT OF TEMPERATURE AND FLOW-RATE ON THE RELATIVE RETENTION TIME (RRT) OF SELECTED DIACYLGLYCEROLS ON POLAR CAPILLARY GLC COLUMNS

Molecular species	*260 °C*			*250 °C*	
	Adjusted retention time		*RRT*[a]	*Adjusted retention time*	*RRT*
	2 p.s.i.	*3 p.s.i.*		*(3 p.s.i.)*	
16:0–16:0	3.30	2.09	0.653	3.37	0.639
16:0–18:0	4.72	3.00	0.936	4.91	0.932
16:0–18:1	5.04	3.205	1.000	5.27	1.000
16:0–18:2	5.61	3.59	1.117	5.88	1.116
18:0–18:1	7.19	4.56	1.425	7.69	1.459
18:0–18:2	8.00	5.08	1.585	8.52	1.617
16:0–20:4	8.59	5.39	1.693	8.99	1.706
16:0–20:5	9.66	6.14	1.916	10.20	1.935
17:0–20:4	9.99	6.34	1.980	10.67	2.025
18:0–20:4	11.92	7.61	2.370	12.92	2.452
16:0–22:6	14.08	8.98	2.798	15.21	2.886
18:0–22:6	19.70	12.54	3.911	21.77	4.131

[a] Average of retention times at 2 and 3 p.s.i.

due to an increase in column polarity at higher temperatures[20]. In general increasing the oven temperature decreases the separation between homologous species and increases the separation between species differing in unsaturation. A relatively sensitive indicator of the temperature effect due to differing degrees of unsaturation is provided by the 16:0–20:4/18:0–18:2 diacylglycerol retention time ratio, which increases with increasing temperature. Tables VI and VII gives the shifts in the retention times obtained for various diacylglycerol species by increasing the column temperature from 250 to 260°C or by increasing the head pressure of the hydrogen carrier gas from 2 to 3 p.s.i. In general, the diradylglycerol species containing 20:4 elute earlier than anticipated from the retention time of the fatty acid methyl esters on the same polar phase. This is due to two opposing effects. It is known that on non-polar phases the 16:0–20:4 elutes earlier than either 18:0–18:2, 18:1–18:2 or 16:0–20:3

TABLE VII

EFFECT OF TEMPERATURE ON THE SEPARATION FACTORS OF SELECTED PAIRS OF DIACYLGLYCEROLS

Ratio of Molecular species	*Oven temperature*		*Ratio*[a]
	260 °C	*250 °C*	
18:0–18:1/16:0–18:1	1.425	1.459	0.977
18:0–18:2/18:0–18:1	1.113	1.108	1.005
16:0–20:4/18:0–18:2	1.067	1.1055	1.011
16:0–20:4/18:0–18:1	1.188	1.169	1.016
16:0–22:6/18:0–20:4	1.181	1.177	1.003
17:0–20:4/16:0–20:5	1.033	1.047	0.987

[a] Ratio of relative retention times at 260 and 250°C.

TABLE VIII

RELATIVE RETENTION TIMES (RRT) AND EQUIVALENT CHAIN LENGTH (ECL) VALUES OF ALKYLGLYCEROL DIACETATES

Alkyl glycerol	*Retention time (min)*	*RRT*[a]	ECL_1[a]	ECL_2[b]
14:0	8.66	0.748	14.00	
15:0	9.35	0.808	15.00	
16:0 (iso)	9.66	0.835	15.42	
16:0	10.08	0.871	16.00	16.00
17:0 (iso)	10.39	0.898	16.42	16.46
17:0 (ai)	10.58	0.914	16.67	16.69
17:0	10.81	0.934	17.00	17.00
18:0	11.57	1.000	18.00	18.00
18:1t[c]	11.97	1.035	18.53	18.52
18:1c (ω9)	12.10	1.046	18.70	18.71
18:1c (ω7)	12.21	1.055	18.84	18.83
18:2	12.92	1.117	19.78	19.75
20:0	13.09	1.131	20.00	20.00
20:1t[c]	13.48	1.165	20.51	20.47
20:1c[c]	13.64	1.179	20.72	20.67
20:1c[c]	13.75	1.188	20.87	
20:2	14.45	1.249	21.79	21.61
22:0	14.62	1.264	22.00	22.00
22:1t[c]	15.02	1.298	22.52	22.41
22:1c[c]	15.18	1.312	22.72	22.59
22:1c[c]	15.27	1.320	22.84	22.70
22:2	15.97	1.380	23.74	23.48
24:0	16.17	1.398	24.00	24.00
24:1	16.68	1.442		24.53
24:2	17.52	1.514		25.35

[a] Temperature programmed, from 100°C to 180°C at 20°C/min, then to 240°C at 5°C/min.
[b] Isothermal, 210°C.
[c] Unidentified positional isomers.

species. As the polarity of the liquid phase increases, 16:0–20:4 tends to elute later. For SP-2380 or RTx-2330, 16:0–20:4 elutes later than 18:0–18:2, but slightly earlier than 18:1–18:2. Similarly, the species pairs, such as 16:0–22:5ω3 and 16:0–22:6ω3 elute either together or with 16:0–22:5ω3 slightly delayed.

Effect of column age

As the column ages, the effective polarity of the liquid phase decreases. This is apparently due to loss of liquid phase, although changes in the properties of the phase also could have taken place. Again a good indication of the gradual loss of polarity is the ratio of the retention times of the species 16:0–20:4/18:0–18:2, which decreases with increasing column usage. Eventually these species begin to overlap and it is time to replace the column. It is not possible to reverse the loss in separation sufficiently by increasing the column temperature.

Resolution of enantiomers and reverse isomers

The enantiomeric *sn*-1,2- and *sn*-2,3-diradylglycerols are not resolved on either the non-polar or polar capillary columns. It is possible that such resolution could be

obtained following preparation of diastereoisomeric derivatives, which can be resolved on non-chiral liquid phases. A chiral HPLC column has been reported to effect a complete resolution of the enantiomeric diradylglycerols in the form of dinitrophenylurethane derivatives[21].

There was no resolution between reverse isomers, such as 16:0–18:2 and 18:2–16:0. The interaction with the liquid phase is probably different for primary and secondary functions, but any positional effect must be largely independent of the exact identity of the acyl chains. Thus, interchanging the two acyl chains makes no net difference in the interaction. However, it is possible that reverse isomers differing significantly in two fatty chains could be resolved on longer capillary columns containing either a non-polar or polar liquid phase.

Resolution of alkylglycerol diacetates

The alkylglycerol diacetates elute much later than the corresponding fatty acid methyl esters. Whereas methyl stearate elutes at 4.77 min, the corresponding stearylglycerol diacetate elutes at 11.57 min. In fact, palmitylglycerol diacetate elutes after methyl docosahexaenoate. Only myristylglycerol diacetate emerges in the elution range of the common mammalian fatty acid methyl esters. The alkylglycerols recovered from mammalian diradylglycerophospholipids are predominantly of 16 and 18 carbons, but 20–26 carbon species are also known to occur[10,11]. Table VIII gives the retention times of the alkylglycerol diacetates, prepared from the glycerophospholipids of human plasma and erythrocytes, as obtained on the RTx-2330 column. The equivalent chain length (ECL) values are based on isothermal runs made at 210°C. Retention times and relative retention times are also given for temperature programmed runs, which are better from an analytical standpoint. As expected, there is good resolution between the saturated and unsaturated glyceryl ethers. There is also some resolution of isomeric monoenes. Although these isomers have not been completely characterized, each monoenoic species from human blood has at least three components. An early component tentatively identified as *trans*-isomers and two *cis*-isomers. This identification of these components is consistent with their behavior on argentation TLC. The peak identified as *cis*-18:1ω9 coelutes with authentic *cis*-18:1ω9 from dogfish[22]. The peak identified as *cis*-18:1ω7 coelutes with the minor 18:1 satellite of the salachyl alcohol from dogfish. These assignments are also consistent with the major isomers of 18:1 fatty acids found in human body fats, which are known to be precursors of the alkylglycerol moieties[23]. The longer chain length homologues, C_{20-24}, may be chain elongation products. Similarly, the alkenyl chains from mammalian lipids are predominantly ω9 and ω7 isomers[24].

Comparison with other methods

Molecular species of glycerophospholipids have been resolved using reversed-phase HPLC[25]. This method has the advantage of eliminating the need for chemical derivatization and possible distortion of species profile. It permits metabolic studies with radiolabeled bases, which are lost using other methods. The direct method, however, has the major disadvantage of not being able to resolve the three diradylglycerophospholipid classes occurring in natural mixtures. There is no convenient method of resolving intact glycerophospholipids into the diacyl, alkylacyl and alkenylacyl subclasses, although the plasmalogens can be selectively destroyed[26].

Furthermore, the direct method is difficult to reproduce and the resolution of species is not as good as that after dephosphorylation and derivatization.

After dephosphorylation with phospholipase C, the diradylglycerols can be converted into UV absorbing[27] or fluorescent[28] derivatives, and the molecular species resolved by reversed-phase HPLC. This method has the advantage that peaks can be collected for further analysis. The principles of analysis by retention data are the same for isothermal GLC and isocratic HPLC. Reversed-phase HPLC also permits the resolution of diradylglycerols. The main disadvantages of this method are lower sensitivity and poorer resolution, when compared to capillary GLC. The HPLC peaks can be admitted to a mass spectrometer only via special interfaces, while the effluent of capillary GLC columns can be admitted directly to the mass spectrometer for peak identification. Thus, polar capillary GLC holds a clear advantage over other methods of analysis of the molecular species of glycerophospholipids.

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