The composition of marine-oil triglycerides as determined by silver ion-thin-layer chromatography

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ABSTRACT The fractionation of marine-oil triglycerides according to their degree of unsaturation was achieved on silica gel thin layers with 8% silver nitrate (w/w). Under the optimum conditions, cod-liver and whale oils were reproducibly separated into seven and six fractions, respectively. The fatty acid compositions of the fractions obtained from cod-liver and whale oils were further studied by gas–liquid chromatography. The following was found in both oils: Saturated and monoenoic acids were not only abundant in their corresponding fractions, but also comprised about two-thirds of the fatty acids in the more unsaturated fractions. Instead, polyenoic fatty acids of similar degrees of unsaturation predominated only in the particular fraction which corresponded to their number of double bonds. Thus, the distribution of fatty acids of varying degrees of unsaturation among marine triglycerides is not random.

SUPPLEMENTARY KEY WORDS highly unsaturated triglycerides · polyunsaturated fatty acids

Since its introduction in 1962 by Barrett, Dallas, and Padley (1), de Vries (2), and Morris (3), silver ion-TLC has been widely used in the fractionation of triglycerides according to their degrees of unsaturation. However, its application has been restricted to triglycerides containing acids with no more than two or three double bonds. This, of course, excluded the separation of marine triglycerides which contain fatty acids with up to five or six double bonds. To our knowledge, only reversed-phase TLC in the hands of Kaufmann and Khoe (4) has been shown to be effective in the fractionation of cod-liver oil.

In recent years, the concept has developed that different substances are separated at different optimum concentrations of silver nitrate. Morris (5) has discussed this problem and its possible relation to the formation of disilver compounds. In our experiments several levels of silver nitrate impregnation of silica gel layers were tested in the fractionation of marine triglycerides. A concentration of 8% silver nitrate in silica gel was found most suitable. The procedure was then applied, in conjunction with GLC, to the study of two marine oils of similar fatty acid compositions but different intramolecular distributions.

EXPERIMENTAL

Commercial cod-liver and whale-blubber triglycerides were purified from the original oils by preparative TLC on silica gel, using petroleum ether (bp 30–60°C)–ethyl ether–acetic acid 60:40:1.6 (v/v/v) as the developing solvent system.

Silver ion-TLC layers were prepared by a slight modification of the procedure of Barrett, Dallas, and Padley (6). Layers of 20 × 20 cm × 0.5 mm were prepared by dissolving various amounts of silver nitrate in 100 ml of water and adding to the solution 60 g of silica gel with 10% calcium sulfate binder (Adsorbosil-1, Applied Science Laboratories Inc., State College, Pa.). The layers were dried at room temperature for about 12 hr in the dark and then activated at 110°C for 1 hr. Between 20 and 25 mg of sample was applied under a nitrogen blanket in the device of Cruess and Seguin (7). Chromatograms were developed in the dark in lined

Abbreviations: TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

tanks with a mixture of chloroform–absolute ethanol 94:6 (v/v) at room temperature. After development the plates were sprayed with a solution of dichlorofluorescein and the fractions were visualized under ultraviolet light. For fatty acid analysis, the fractions were scraped off the plates immediately after completion of the chromatography, and the triglycerides were extracted with ethyl ether, 5% methanol in ethyl ether (8), or 10% methanol in ethyl ether. The silica gel was acidified with 10% HCl and then reextracted with ethyl ether (9). Fatty acid methyl esters were formed by refluxing the triglycerides with a 2% solution of sulfuric acid in methanol, under nitrogen. The proportion of each fraction was determined quantitatively by adding to each fraction isolated by TLC a known amount of methyl arachidate and analyzing the methyl ester mixtures by GLC (8).

Pancreatic lipase hydrolyses were performed according to the procedure of Luddy, Barford, Herb, Magidman, and Riemenschneider (10). The fatty acids of all four products of lipolysis were analyzed.

GLC was performed in a Beckman GC-5 gas chromatograph equipped with a dual flame ionization detector. Stainless steel columns, 6 ft × $^1/_8$ in. I.D., packed with 10% siliconized ethylene glycol succinate polyester on Chromosorb P, 100–120 mesh (EGSS-X, Applied Science Laboratories Inc.), were used. Quantitative results with National Heart Institute Fatty Acid Standard D agreed with the stated composition data with a relative error of less than 5% for major components (>10% of total mixture) and less than 33% for minor components (<10% of total mixture).

RESULTS AND DISCUSSION

Conditions for the Fractionation

Several parameters were tested to obtain conditions which would allow suitable separations. Levels of silver nitrate were tried at 2, 5, 8, 10, and 17% (silver nitrate in silica gel, w/w). Only the 5% and 8% layers provided acceptable resolution, the 8% layers being slightly better. Both above and below the 5–8% range poor resolution resulted, with marked streaking and trailing, especially in the region close to the origin. The 8% level of impregnation was, therefore, chosen for further experimentation. Examples of the type of results that can be obtained are presented in Figs. 1 and 2, which are chromatograms of cod-liver oil and whale oil fractionated into 7 and 6 bands, respectively. Developing the plates at low temperature (about 4°C) did not improve the general characteristics of the separations. Poor chromatograms—streaking and low resolution—were obtained when the sandwich technique of Brenner and Niederwieser (11) was attempted on 20 × 40 cm plates of silica gel impregnated with 8% silver nitrate and developed in benzene containing 0–2% water.

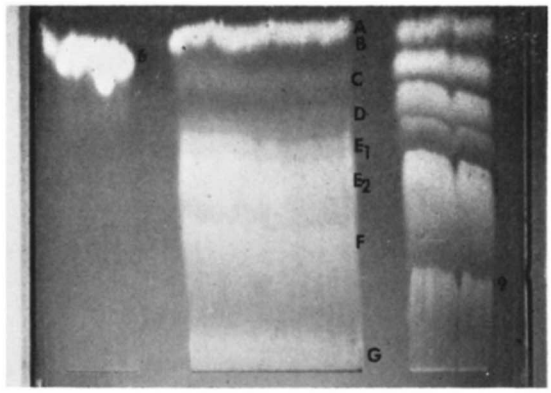

FIG. 1. Fractionation of cod-liver-oil triglycerides (center row) by silver ion-TLC (8% $AgNO_3$ in silica gel, w/w). The layer is slightly overloaded to show minor components clearly. At left, trilinolein standard (*6*); at right, linseed oil standard (trilinolenin, *9*). Bands *A*, E_2, and *F* are designated as major bands in the text and in (*A* and *F*) Table 2. A manual streaker (Applied Science Laboratories Inc.) was used for the chromatograms that were photographed and for those used in quantitative analyses.

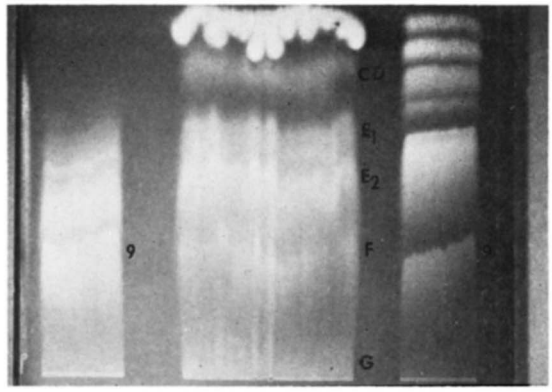

FIG. 2. Fractionation of whale-oil triglycerides (center row) by silver ion-TLC (8% $AgNO_3$ in silica gel, w/w). At left, trilinolenin standard showing *cis-trans* isomerization; at right, linseed oil. Other comments as in Figure 1.

Reproducibility

The reproducibility of the method was tested as follows: Cod-liver-oil triglycerides were separated on six TLC plates under the optimum conditions. The silica gel fractions were scraped off the plates and collected separately. Since the minor triglyceride fractions from only one plate did not provide enough material for GLC analysis, corresponding fractions from two plates were pooled. The triglycerides were then converted into fatty acid methyl esters which were analyzed by GLC. The results are presented in Table 1. Three separate determinations of the fatty acid compositions of two major fractions, arbitrarily designated in Fig. 1 as *A* and *F*, are shown in Table 2. The reproducibility

TABLE 1 FATTY ACID COMPOSITION OF COD-LIVER-OIL TRIGLYCERIDES

Fatty Acid	Fractions after Ag^+-TLC*							Total Triglycerides	
	A	B	C	D	$E_1 + E_2$	F	G	Determined	Recalc.†
	% of total fatty acids								
12:0‡	0.7	0.2	—	0.1	tr	tr	tr	tr	
13:0	0.7	0.2	0.2	0.4	tr	tr	tr	—	
14:0	5.8	2.9	3.3	3.0	2.9	2.5	2.5	4.3	4.5
15:0	0.4	0.6	0.3	0.6	0.3	0.1	0.3	0.2	
16:0	14.9	7.8	12.2	12.9	11.6	7.8	10.1	9.8	12.9
18:0	2.0	1.3	3.5	4.4	3.0	2.0	3.1	2.0	2.2
14:1?	tr	0.5	—	—	tr	0.1	—	—	
15:1?	—	0.1	0.1	—	0.2	0.1	—	0.1	
16:1 (n−7)§	23.5	19.2	10.5	17.4	11.2	11.2	9.3	17.2	18.7
17:1?	—	0.9	—	0.1	—	—	—	—	
18:1 (n−9)§	27.2	22.5	14.5	17.3	15.6	18.4	13.2	19.8	22.6
20:1 (n−9)	13.4	11.7	11.1	17.5	12.4	13.3	9.8	12.4	12.9
22:1 (n−9)	9.0	5.9	5.8	7.2	7.7	6.5	5.3	6.5	8.2
16:2 (n−4)	0.3	6.7	0.9	0.5	0.2	0.5	0.8	1.0	0.3
18:2 (n−6)	0.6	12.3	3.7	1.6	1.3	1.9	2.5	2.0	1.3
20:2 (n−9)	—	1.2	0.2	—	—	0.3	0.8	0.3	
20:2 (n−6)	—	—	—	0.5	—	—	—	tr	
16:3 (n−6)	—	—	4.9	5.7	—	—	1.0	—	
18:3 (n−6)	0.1	—	2.7	1.7	0.2	tr	0.8	0.1	
18:3 (n−3)	—	—	—	—	—	—	—	—	
20:3 (n−6)	tr	0.1	0.9	0.6	tr	0.2	0.9	tr	
22:3?	0.1	1.1	1.3	0.3	0.3	—	3.1	—	
18:4 (n−3)	—	0.3	tr	0.6	4.9	0.5	3.8	1.8	1.2
20:4 (n−6)	tr	—	—	—	—	—	—	—	
20:4 (n−3)	0.1	0.4	0.3	0.9	1.9	0.3	2.2	0.6	
22:4 (n−6)	0.5	0.1	2.7	0.1	0.9	0.9	0.9	0.7	
20:5 (n−3)	tr	0.3	0.1	0.3	22.1	9.5	14.5	11.9	6.0
22:5 (n−3)	—	tr	0.2	—	1.8	1.3	1.9	1.1	0.6
22:6 (n−3)	0.1	0.2	7.3	0.8	0.3	21.4	10.4	7.2	2.7
Sum of unknowns	0.6	3.5	13.3	5.5	1.2	1.2	2.8	1.0	

* Fractions were extracted from silica gel with ethyl ether.

† Recalculation based on fatty acid compositions of the fractions and the triglyceride composition of the oil (Table 5). Major fatty acids only.

‡ Number of carbon atoms: number of double bonds.

§ May include other positional isomers.

was quite good. The variations between samples were not too far from the variations found in consecutive GLC analyses of the same sample. For major components (>10% of sample) there was less than 12% departure from the average. Similar data for two groups of minor bands are also given in Table 2.

Minor fractions were not as reproducible and contamination became significant in some cases, e.g., the high levels of components with the retention times of 22:6 and higher in fraction C of Table 1.

Table 2 also compares two solvents used for extracting triglycerides from the silica gel after TLC. The fatty acid compositions do not differ significantly whether one uses ethyl ether only or 5% methanol in ethyl ether. Similar agreement was obtained when fractions of whale oil were extracted with ethyl ether alone or with 10% methanol in ethyl ether followed by acidification and reextraction with ethyl ether (results not shown in Table 2).

Factors in Glyceride Fractionation

Gunstone and Padley (12) found that their silver ion-TLC layers retained a triglyceride containing one linolenic and two saturated acid chains (300)[1] to a greater degree than one with two linoleic acid residues and one saturated acid (220). These authors did not take into consideration glycerol positional isomers. More recently, Wessels and Rajagopal (13) have confirmed that triglycerides containing fatty acids such as linolenic may have lower R_F values than might be expected from the number of double bonds. Figs. 1 and 2 show a similar effect in that the degree of migration of trilinolein (222) is greater than that of fraction E_2 of the marine oils, which is essentially (015) according to

[1] In the notation (300) the numbers indicate the number of double bonds in each of the three acyl residues in a triglyceride. The parentheses denote that glycerol positional isomerism is not taken into consideration.

Table 1. It is obvious then that positions of the double bonds in the fatty acid chains play a role in the separation of highly unsaturated triglycerides by silver nitrate-TLC. The affinity of the silver ions for the double bonds seems to be greater when they are clustered in one acid than when they are in different acids. Perhaps the formation of the disilver compounds mentioned above is somehow related to this effect.

Glycerol positional isomerism also seems to play a role in the fractionation of triglycerides by silver nitrate-TLC. Barrett et al. (6) have shown that 2-oleodistearin can be made to migrate faster than 1-oleodistearin, and Wessels and Rajagopal (13) have shown that 2-oleodipalmitin moves ahead of 1-oleodipalmitin. In the present case we can compare the migration of fraction F of cod-liver oil, which has the 22:6 fatty acid mainly in the middle position of its triglycerides (Table 3), with the migration of fraction F of whale oil, which has the 22:6 mainly in the 1- and 3-positions. The reference can be the trilinolenin standard (Figs. 1 and 2). One can see that, as expected, fraction F of cod-liver oil is less strongly retained than fraction F of whale oil. This may be due to less exposure of the polyenoic fatty acid in the fish oil to the silver ions.

The present results with highly unsaturated triglycerides thus confirm previous reports on studies of less unsaturated triglycerides, i.e., separations of triglycerides by silver nitrate-TLC depend on the degree of unsaturation, on the position of the double bonds along the fatty acid chains, and on the positions of the fatty acids on the glycerol backbone. Chain length does not seem to be a significant factor in these separations (13).

TABLE 2 Reproducibility in the Fractionation of Cod-Liver-Oil Triglycerides. Major Fatty Acid Composition of Some Fractions

	Major Fractions (>10%)								Minor Fractions (<10%)				
	A				F				C		D		C–D
Fatty Acid	Extraction with Ether			Extraction with 5% Methanol in Ether	Extraction with Ether			Extraction with 5% Methanol in Ether	Extraction with Ether				Extraction with 5% Methanol in Ether
	1	2	3		1	2	3		1	2	1	2	
	% of total fatty acids												
14:0	5.8	3.2	4.7	5.6	2.5	2.9	2.3	2.8	3.3	0.7	3.0	1.7	4.7
16:0	14.9	13.1	13.6	14.5	7.8	11.0	7.8	6.8	12.2	10.0	12.9	21.9	11.6
18:0	2.0	2.1	1.8	1.5	2.0	2.9	2.1	1.9	3.5	6.2	11.4	9.1	1.7
16:1	23.5	20.4	22.2	23.3	11.2	12.2	13.7	12.3	10.5	7.3	17.4	11.5	17.9
18:1	27.2	26.4	25.1	23.7	18.4	20.4	17.9	17.9	14.1	20.0	17.3	22.8	19.4
20:1	13.4	18.3	17.5	15.3	13.3	13.1	12.9	12.9	11.1	7.6	17.5	6.8	18.2
22:1	9.0	10.6	9.7	9.0	6.5	7.8	6.8	7.4	5.8	4.9	7.2	2.9	7.8
20:5	tr	tr	0.1	—	9.5	6.2	9.7	7.1	0.1	4.7	0.3	0.3	—
22:6	0.1	tr	tr	—	21.4	15.9	20.6	24.1	7.3	tr	0.8	—	0.1

TABLE 3 Major Fatty Acid Distribution in Cod-Liver and Whale Oils as Determined by Pancreatic Lipase Hydrolysis*

	Cod-liver Oil			Whale Oil		
Fatty Acid	Original TG†	2-MG†	% in 2-Position‡	Original TG	2-MG	% in 2-Position
	% of total fatty acids			*% of total fatty acids*		
14:0	4.7	5.4	38	5.2	11.4	73
16:0	11.0	14.6	44	12.5	8.7	23
16:1	21.6	14.1	22	12.5	22.7	60
18:0	1.8	0.2	4	2.1	0.6	10
18:1	22.3	8.0	12	30.9	39.8	43
20:1	11.9	9.0	25	10.2	4.3	14
20:5	11.0	14.4	44	3.7	1.1	10
22:1	5.2	5.2	33	7.1	0.7	3
22:6	4.3	17.2	100?	4.5	0.5	4

* When located at the 1,3-positions of a triglyceride, the 20:5 and 22:6 fatty acids are resistant to pancreatic lipase hydrolysis (16). However, the presence of the resistant acids in the 2-position has little effect on the hydrolysis of the acids at positions 1 and 3.

† TG, triglycerides; MG, monoglycerides.

‡ % in 2-position, $\frac{\text{MG}}{3 \times \text{TG}} \times 100$.

Composition of Marine Oils

Silver nitrate-TLC allows a new insight into the composition of marine triglycerides.

The fatty acid compositions of the fractions obtained from cod-liver oil are presented in Table 1. The acids have been grouped by number of double bonds to facilitate the discussion of the data. There are two distinct patterns in the distribution of the acids among fractions, based essentially on the degrees of unsaturation of the fatty acid components. The first pattern is that of the saturated and monoenoic acids, which are most abundant in fraction A but are also present in significant levels in all other fractions. In most fractions the sum of the percentages of saturated and monoenoic fatty acids is about two-thirds of the total acids. This confirms (14) that two of the positions of fish-oil triglycerides are esterified by saturated and monoenoic acids whereas the third position carries acids with higher unsaturation. This general trend does not apply to fraction G, which may possibly contain triglycerides with more than one polyunsaturated fatty acid per molecule (15). Alternatively, fraction G could be a composite of unresolved fractions.

The second pattern of distribution is that of the highly unsaturated fatty acids. They appear at relatively high levels in one particular fraction without expanding into the others (except fraction G) to any significant extent. For example, the 20:5 acid predominates in fraction $E_1 + E_2$ and 22:6 is predominant in fraction F. This pattern is more apparent in the composition of whale-oil triglycerides presented in Table 4 and discussed below.

The two patterns of distribution discussed above applied not only to groups of fatty acids of equal degrees of unsaturation but to the individual acids as well.

Kaufmann and Khoe (4) fractionated cod-liver-oil triglycerides on silica gel and on paper impregnated with paraffin oil, and performed a qualitative analysis of the fatty acids on the resulting fractions. They found

TABLE 4 FATTY ACID COMPOSITION OF WHALE-OIL TRIGLYCERIDES AND OF THEIR PRODUCTS OF FRACTIONATION BY SILVER ION-TLC

Fatty Acid	Fractions after Ag^+-TLC*								Total Triglycerides	
	A_1	A_2	A_3	C–D	E_1	E_2	F	G	Determined	Recalc.†
	% of total fatty acids									
12:0	—	0.1	tr	—	—	—	—	tr	—	
13:0	tr	0.1	0.1	—	0.1	—	tr	tr	tr	
14:0	4.4	14.8	7.7	4.4	4.9	4.9	4.3	2.8	5.2	5.7
15:0	0.3	1.0	0.6	0.8	0.5	0.7	0.6	0.4	0.5	
16:0	90.9	32.9	14.7	12.0	13.4	15.2	11.6	10.0	12.5	14.1
18:0	1.7	6.4	2.5	1.5	2.8	3.9	2.4	2.2	2.1	2.4
14:1?	—	tr	0.8	0.8	0.5	0.7	0.6	0.4	0.5	
15:1?	0.1	tr	0.1	tr	tr	0.2	tr	0.1	0.1	
16:1 (n−7)‡	tr	7.3	15.0	11.3	6.8	9.3	10.4	7.8	12.5	13.7
17:1?	0.4	1.4	tr	—	—	tr	—	—	—	
18:1 (n−9)‡	0.4	23.3	29.7	28.2	19.5	22.7	24.5	16.4	30.9	32.4
20:1 (n−9)	0.3	5.3	15.3	21.1	10.6	6.7	7.7	6.9	10.2	11.0
22:1 (n−9)	0.1	4.1	11.0	9.8	12.1	4.8	5.6	5.1	7.1	8.0
16:2 (n−4)	0.1	0.4	0.9	0.3	0.2	0.5	0.9	0.6	0.7	
18:2 (n−6)	0.1	—	0.5	1.8	0.9	0.5	2.1	1.7	1.0	1.6
20:2 (n−9)	—	0.1	tr	—	0.2	2.2	0.4	0.4	0.1	
20:2 (n−6)	tr	0.1	tr	—	—	0.2	0.1	0.1	tr	
16:3 (n−6)	—	—	—	1.5	—	—	—	—	—	
18:3 (n−6)	0.1	0.4	0.1	0.2	0.2	0.3	0.1	0.4	0.6	
18:3 (n−3)	—	—	—	—	—	—	—	2.3	—	
20:3 (n−6)	—	0.1	—	0.4	0.1	0.2	0.2	0.5	0.4	
22:3?	—	—	—	—	—	—	—	0.1	—	
16:4?	—	—	—	—	2.3	—	—	0.5	—	
18:4 (n−3)	0.1	—	—	1.5	10.5	1.1	0.5	4.1	1.8	0.6
20:4 (n−6)	0.7	—	—	—	6.7	—	0.4	2.8	1.3	0.6
20:4 (n−3)	—	—	—	—	—	1.2	—	—	—	
22:4 (n−6)	—	0.5	0.4	3.2	3.5	1.0	0.9	4.5	0.4	
20:5 (n−3)	tr	tr	0.1	0.2	1.5	13.6	5.1	10.4	3.7	1.1
22:5 (n−3)	—	—	—	tr	0.1	7.0	4.8	6.3	2.1	0.6
22:6 (n−3)	—	—	—	tr	0.1	tr	14.6	10.3	4.5	1.1
Sum of unknowns	0.3	1.7	0.5	0.1	2.5	3.1	2.2	2.9	1.8	

* Fractions were extracted from silica gel with ethyl ether.

† Recalculation based on fatty acid compositions of the fractions and the triglyceride composition of the oil (Table 5). Major fatty acids only.

‡ May include other positional isomers.

TABLE 5 TRIGLYCERIDE COMPOSITION OF COD-LIVER AND WHALE OILS*

Fraction	Cod-Liver Oil†	Whale Oil‡
	wt %	
A–B	61.2	76.7
C–D	2.7	2.8
E_1	22.0	3.4
E_2		9.2
F	12.1	5.6
G	2.0	2.3

* Determined by GLC using methyl arachidate as internal standard (8).

† Fractions extracted from silica gel with 5% methanol in ethyl ether (8).

‡ Fractions extracted from silica gel with 10% methanol in ethyl ether followed by acidification with HCl and reextraction with ethyl ether (9).

C_{16} and C_{18} fatty acids in the fractions containing C_{20} and C_{22} acids with 5 and 6 double bonds.

It is well known (14) that in fish oils the highly unsaturated fatty acids are esterified mostly in the 2-position of the glycerol molecules but that in whale-oil triglycerides those acids occupy predominantly the 3-position. Therefore, it was of interest to fractionate a whale oil which had a fatty acid composition similar to that of the cod-liver oil. In this case, the fractionation on 8% silver nitrate layers was supplemented by a further fractionation of fraction A by the sandwich technique on 20 × 40 cm layers containing 17% silver nitrate. Three subfractions, A_1, A_2, and A_3, which essentially correspond to trisaturated, monounsaturated, and diun saturated triglyceride species, respectively, were obtained. The fatty acid compositions of the fractions are shown in Table 4. As in cod-liver oil, a high degree of organization based on the degrees of unsaturation of the fatty acids is apparent. As before, two types of distributions, that of saturated and monoenoic fatty acids and that of polyenoic fatty acids, are seen. In both fish and whale triglycerides the distribution of fatty acids among similar triglyceride fractions is similar, although the positional distribution of at least two-thirds of the fatty acids differ in the two oils, as indicated by the data in Table 3 in confirmation of previous stereospecific analysis by others (14).

Studies in progress in this laboratory show that similar distribution patterns of saturated + monoenoic versus polyenoic acids are observed in the triglycerides of land mammals which are fed marine triglycerides. Thus, the rules of distribution of polyenoic acids among triglyceride molecules seem to have quite a general character.

Triglyceride Composition

The proportions of the fractions that compose the cod-liver and whale oils are presented in Table 5. These data and the fatty acid compositions of the fractions were used to recalculate the fatty acid compositions of the original oils. The results are shown in the right-hand column of Tables 1 and 4. There is good agreement between the recalculated and the original data concerning the less unsaturated fatty acids. The agreement is poor for the polyunsaturated fatty acid data. It is difficult to say which of the steps involved in the TLC–GLC analysis used here may be responsible for the apparent losses of polyunsaturated fatty acids. It is obvious, however, that extreme care must be exercised in handling easily alterable glycerides such as those of marine origin.

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