METHOD

Stereospecific Analysis of Triacylglycerols Rich in Long-Chain Polyunsaturated Fatty Acids

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ABSTRACT: Six oils of marine, algal, and microbial origin were analyzed for stereospecific distribution of component fatty acids. The general procedure involved preparation of *sn*-1,2-(2,3)-diacylglycerols by partial deacylation with ethylmagnesium bromide or pancreatic lipase, separation of X-1,3- and *sn*-1,2(2,3)-diacylglycerols by borate thin-layer chromatography, resolution of the *sn*-1,2- and *sn*-2,3-enantiomers by chiral phase high-performance liquid chromatography following preparation of dinitrophenylurethane derivatives, and determination of the fatty acid composition by gas chromatography. Unexpected complications arose during a stereospecific analysis of triacylglycerols containing over 33% of either 20:4 or 22:6 fatty acids. The *sn*-1,2(2,3)-diacylglycerols made up of two long-chain polyunsaturated acids migrated with the X-1,3-diacylglycerols and required separate chiral phase resolution. Furthermore, the enzymatic method yielded *sn*-1,2(2,3)-diacylglycerols, overrepresenting the polyenoic species due to their relative resistance to lipolysis, but prolonged digestion yielded correct composition for the 2-monoacylglycerols. The final positional distribution of the fatty acids was established by pooling and normalizing the data from subfractions obtained by normal- and chiral-phase separation of diacylglycerols. The molecular species of X-1,3-, *sn*-1,2- and *sn*-2,3-diacylglycerol dinitrophenylurethanes were identified by chiral-phase liquid chromatography/mass spectrometry with electrospray ionization, which demonstrated a preferential association of the paired long-chain acids with the *sn*-1,2- and *sn*-2,3-diacylglycerol isomers.

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Knowledge of the stereospecific distribution of fatty acids in natural fats is of interest because of the positional specificity of the lipolytic enzymes (1–3), the intestinal absorption, and reesterification of intact 2-monoacylglycerols (MAG) (4,5), and the desirability to reproduce the structure of certain scarce natural fats and oils (6,7) by blends of natural or synthetic fats and oils. Recently specialty or designer oils rich in n-6 and/or n-3 polyunsaturated C_{20} and C_{22} fatty acids have been introduced for dietary supplementation (8), but the triacylglycerol (TAG) structure of these oils has not been determined. In fact, application of conventional methods of stereospecific analyses to such oils has proved difficult due to isolation of nonrepresentative diacylglycerol (DAG) intermediates (Myher, J.J., Kuksis, A., and Park, P.W., 1994, unpublished results). We have traced this problem to the overlap of long-chain *sn*-1,2(2,3)-DAG with short- and medium-chain X-1,3-DAG during the boric acid thin-layer chromatography (TLC) used for the preliminary resolution of the *sn*-1,2(2,3)- and X-1,3-DAG. Usually natural oils contain only one long-chain polyunsaturated fatty acid per TAG molecule and borate TLC yields representative *sn*-1,2(2,3)-DAG from Grignard degradation (5,9). The present study demonstrates that valid stereospecific analyses of the oils enriched in polyunsaturated fatty acids can be obtained provided special care is taken in the isolation and identification of the enantiomeric DAG intermediates.

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Abbreviations: DAG, diacylglycerol; DNPU, dinitrophenylurethane; ECN, equivalent carbon number; ELSD, evaporative light-scattering detection; GC, gas chromatography; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; MAG, monoacylglycerols; PN, partition number; TAG, triacylglycerol; TLC, thin-layer chromatography.

EXPERIMENTAL PROCEDURES

Materials. Six polyunsaturated oils of marine (tuna) (B), microalgal (*Chrypthecodinium cohnii*) (C and D) and microbial micelia (*Mortierella alpina*) (A, E, and F) origin were available in the laboratory. Three were rich in arachidonic acid (A, E, and F), and three were rich in docosahexaenoic acid (B, C, and D). Reference *sn*-1,2- and *sn*-2,3-DAG dinitrophenylurethane (DNPU) derivatives prepared from standard TAG were available in the laboratory from previous studies (10,11). The 3,5-dinitrophenylisocyanate was purchased from Sumitomo Chemical Co. (Osaka, Japan). Ethylmagnesium bromide and boric acid were obtained from Aldrich Chemical Co. (Milwaukee, WI). Pancreatic lipase was obtained from Sigma Chemical Co. (St. Louis, MO).

Purification of TAG. Five drops of each oil were dissolved in 1 mL of chloroform, and 250 μL of the solution was applied to a 20 × 20 cm TLC plate coated with a 250-μm thin-layer of Silica Gel H (Merck, Darmstadt, Germany). The plate was developed with hexane/ethyl acetate (88:11, vol/vol) (12). The TLC bands corresponding to standard steryl ester, TAG, and phosphatidylcholine were scraped off and eluted

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with chloroform/methanol (2:1, vol/vol) and the solvents evaporated under nitrogen. Care was taken in collecting the TAG fraction to avoid loss of short- and long-chain TAG that might have trailed or preceded the bulk of species. The TAG fraction was subjected to immediate stereospecific analysis, while the other fractions were saved for later analyses. Aliquots were taken for nonpolar capillary gas chromatography (GC) of fatty acids and reversed-phase high-performance liquid chromatography (HPLC) of the intact lipid esters (see below).

Partial deacylation of TAG. Ten mg of the purified TAG were dissolved in dry diethyl ether (1 mL), freshly diluted 0.5 M ethyl magnesium bromide in dry ethyl ether (250 μL) was added, and the mixture was shaken for 1 min before glacial acetic acid (6 μL) in hexane (5 mL) and water (2 mL) were added to stop the reaction (5). The organic layer was washed twice with water (2 mL) and dried over anhydrous sodium sulfate. After evaporating the solvent in a stream of nitrogen at room temperature, the mixture of deacylation products was separated by borate TLC (13) using chloroform/acetone (88:12, vol/vol). The *sn*-1,2(2,3)- and X-1,3-DAG were separately recovered. Alternatively, the TAG were partially deacylated by digestion with pancreatic lipase using another established procedure (14). The digestion was performed in presence of gum arabic for 30 s and the digestion products extracted with diethyl ether. The *sn*-1,2(2,3)- and X-1,3-DAG and 2-MAG, were resolved by boric acid TLC and separately recovered as described above. For preparation of representative 2-MAG the digestion was prolonged to 2–3 min. After taking aliquots of the DAG fractions for fatty acid analyses, the remainder of the X-1,3- and *sn*-1,2(2,3)-DAG were converted into the DNPU derivatives for chiral phase HPLC resolution (see below).

Preparation of 3,5-DNPU. The X-1,3- and the *sn*-1,2(2,3)-DAG (1 mg) were dissolved in dry toluene (400 μL) containing 40 μL of dry pyridine and about 2 mg of 3,5-dinitrophenylisocyanate and allowed to stand for 1 h at room temperature (5). The partly overlapping long-chain *sn*-1,2(2,3)-DAG and the short-chain X-1,3-DAG were converted to the DNPU derivatives together, while the short-chain *sn*-1,2(2,3)-DAG and the long-chain X-1,3-DAG were derivatized separately. The resulting 3,5-DNPU derivatives were purified by TLC on a silica gel plate (Kodak, Rochester, NY). Prior to use, the plate was activated at 110–120°C for 1 h. The reaction mixture, dissolved in 400 μL of chloroform, was spotted on the plate and developed up to 15 cm using petroleum ether/1,2-dichloroethane/ethanol (40:10:3, by vol) as the developing solvent. Bands were located under ultraviolet and the 3,5-DNPU fractions (R_f = 0.5–0.6) were scraped off the plate. The pure 3,5-DNPU were recovered from the adsorbent by extraction with diethyl ether.

Chiral phase HPLC. The enantiomeric *sn*-1,2- and *sn*-2,3-DAG in the *sn*-1,2(2,3)- and in the X-1,3-DAG fractions were resolved using the chiral (R)-(+)-1-(1-naphthyl)ethyl-amine phase bonded to silica (YMC Pack A-KO_3; YMC Inc., Kyoto, Japan) and installed in a Hewlett-Packard (Palo Alto, CA) Model 1090 liquid chromatograph, as previously described (10,11). The analysis was performed isocratically at 23°C using hexane/1,2-dichloroethane/ethanol (40:10:1, by vol) as the mobile phase at a constant flow rate of 0.5 mL/min. Peaks were monitored at 254 nm using a variable wavelength detector. The corresponding *sn*-1,2- and *sn*-2,3-DAG from the two separations were collected and combined for fatty acid analysis.

GC of fatty acids. The fatty acid composition of the purified TAG and their deacylation products was determined following transmethylation with 6% H_2SO_4-MeOH as described (12). The methyl esters were resolved by polar capillary GC and the component acids were quantitated by flame-ionization detection (12).

HPLC of TAG. Intact TAG were resolved by reversed-phase HPLC as previously described (15). The eluted peaks were monitored and quantitated by an evaporative light-scattering detector (ELSD).

Liquid chromatography/mass spectrometry (LC/MS). LC/MS with on-line electrospray of the DAG DNPU was performed using the YMC Pack A-KO_3 column installed in a Hewlett-Packard Model 1090 liquid chromatograph. The separations of X-1,3-, *sn*-1,2- and *sn*-2,3-DAG were obtained with hexane/dichloromethane/ethanol (40:10:1). After post-column mixing with 0.3 mL/min isopropanol/1% aqueous ammonia, mass spectra of eluting peaks were acquired by negative mode nebulizer assisted electrospray interfaced with a Hewlett-Packard MS Engine Quadrupole mass spectrometer (16).

Calculations. The mole % fatty acid composition of the *sn*-1, *sn*-2 and *sn*-3 positions of the TAG molecules were calculated by subtraction of the mole % fatty acid compositions as follows (5):

$$sn\text{-}2 = (\text{2-MAG}), \text{ from pancreatic lipase deacylation} \quad [1]$$

$$sn\text{-}1 = 2 \times (sn\text{-}1,2\text{-DAG}) - (sn\text{-}2\text{-MAG}) \quad [2]$$

$$sn\text{-}3 = 2 \times (sn\text{-}2,3\text{-DAG}) - (sn\text{-}2\text{-MAG}) \quad [3]$$

Alternatively, the positional fatty acid distribution was calculated as follows:

$$sn\text{-}1 = 3 \times (\text{total TAG}) - 2 \times (sn\text{-}2,3\text{-DAG}) \quad [4]$$

$$sn\text{-}3 = 3 \times (\text{total TAG}) - 2 \times (sn\text{-}1,2\text{-DAG}) \quad [5]$$

The final composition of the *sn*-1 and *sn*-3 positions was obtained by averaging the results of the two calculations.

RESULTS AND DISCUSSION

Preliminary characterization. TLC of the oil samples in a neutral solvent system revealed the presence of a large TAG band preceded by a faint band corresponding to steryl esters and small amounts of polar material left at the origin of the plate. There was evidence for the presence of trace amounts of free sterols or free DAG in the E and F, and C and D oil samples. Table 1 gives the fatty acid composition of the purified TAG

TABLE 1
Fatty Acid Composition of Purified Triacylglycerols (mole %)

Fatty acids	Triacylglycerols					
	A	B	C	D	E	F
10:0			0.18	0.75		
12:0	0.00	0.04	3.42	6.47		
14:0	0.36	3.70	14.79	19.76	0.38	0.35
15:0	0.17	1.29	0.06	0.01	0.12	0.14
16:0	13.93	22.29	15.31	17.08	8.01	9.31
16:1n-7	0.21	5.04	1.59	1.76	0.08	0.08
17:0	0.36	2.31	0.08	0.01	0.46	0.54
18:0	6.43	5.77	1.68	0.24	11.18	10.91
18:1n-9	15.53	15.48	14.63	9.01	14.80	8.13
18:2n-6	21.52	1.58	1.41	0.41	6.88	5.68
18:3n-6	3.17	0.14	0.41		2.93	3.25
20:0	0.65	0.43	0.18	0.03	1.06	1.12
18:3n-3	2.13	0.47	0.13	0.14	0.23	0.03
20:1	0.53	1.07	0.14	0.06	0.42	0.50
20:2	0.44	0.30	0.15	0.02	0.61	0.74
20:3n-6	3.10	0.13	0.31	0.01	2.11	2.68
22:0	1.84	0.25	0.32	0.02	0.00	0.00
20:4n-6	24.75	2.12	6.45	0.08	47.87	52.98
22:1n-9	0.09	0.13	0.00			
20:5n-3	0.00	7.10	0.06	0.05	0.22	0.26
24:0	2.89		0.19	0.01	1.03	1.48
24:1		0.42	0.08			
22:6n-3	0.02	29.89	37.21	43.51	0.05	0.04
Other	1.88	0.02	1.22	0.57	1.56	1.78

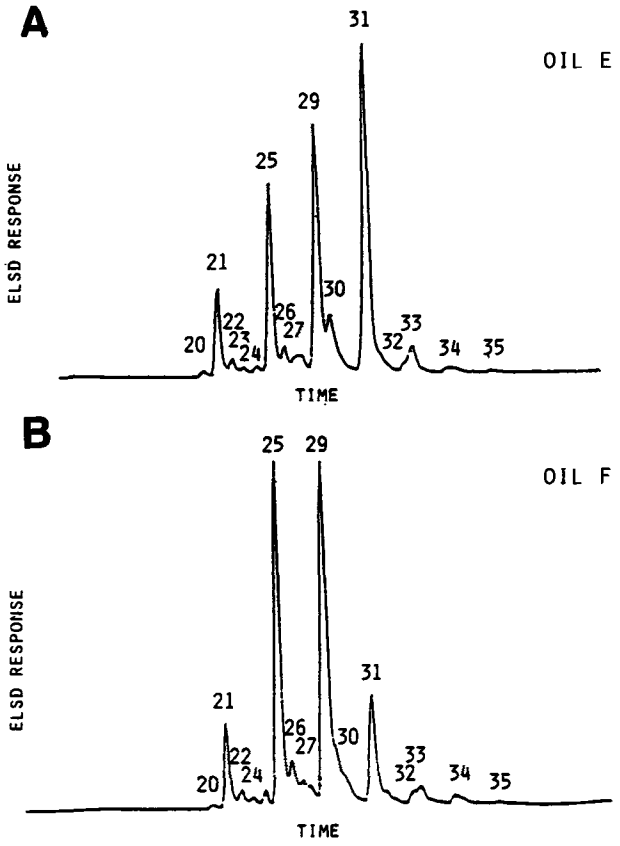

FIG. 1. Reversed-phase high-performance liquid chromatography (HPLC) profiles of E and F polyunsaturated oils as obtained with light-scattering detector. HPLC conditions: Hewlett-Packard Model 1050 liquid chromatograph (Palo Alto, CA) equipped with a Supelcosil LC-18 column (25 × 0.46 cm. i.d.) coupled to a Varex evaporative light-scattering detection (ELSD) II light scattering detector. Solvent: linear gradient of 10–90% isopropanol in acetonitrile at 25°C over a period of 70 min (1 mL/min). Peak identification by fractional equivalent chain-length number is given in Table 2.

fractions. It is seen that the TAG samples can be arranged in three groups on the basis of the major fatty acid content. Thus, samples E and F are characterized by a high content of 20:4, which averages about 50% of total. In contrast, samples C and D are characterized by a high content of 22:6, which averages about 40%. The C sample also contained a significant amount of 20:4 (6.5%). Finally, samples A and B contain mixed polyunsaturates as major components. Thus, sample A contains both 18:2 (21%) and 20:4 (25%) as polyunsaturates, while sample B contained 20:5 (7.1%) and 22:6 (29.9%) as the polyunsaturates. Sample B also contained 16:1 (5.0%) and was by far the most complicated of all the oil samples.

Figures 1–3 show the reversed-phase HPLC elution profiles of the purified TAG as monitored by the ELSD. Sample C oil gives excellent separation with almost complete baseline resolution (often complete resolution), despite the high content of 22:6 in these samples. The resolution of the E sample is poorer, and poor resolution appears to be due mainly to the higher content and greater variety of the unsaturated acids. Less than perfect separation was obtained also for the other 20:4-rich TAG oil, sample A. There was an abundance of double peaks. Likewise, double peaking was present in the B sample, although extensive resolution was still obtained.

Tables 2 and 3 give the quantitative composition of the TAG peaks by equivalent carbon number (ECN) as estimated by HPLC with ELSD. The reversed-phase HPLC resolves TAG by partition or ECN, where one double bond decreases the retention time equivalent to shortening the carbon chain by two methylene units. However, fractional ECN numbers also may be calculated to accommodate slight differences in retention time due to differences in location of the double bond in the fatty chain and of the fatty acid in the TAG molecule. In the present instance, the ECN values range from 38 to 54. Although a tentative identification of the major TAG species can be calculated from statistical consideration of the composition and positional distribution of the fatty acids, exact identification requires MS (see below). Thus, sample D would be expected to contain 14:0-22:6-22:6 (58:12 = PN 34), 16:0-22:6-22:6 (60:12 = PN 36) and 14:0-16:0-22:6 (52:6 = PN 40), where PN is the partition number (the acyl carbon number minus two times the number of double bonds), and major peaks can be found corresponding to these structures in the elution pattern. The identity of the major species can be confirmed and the identity of others established by on-line MS, which, however, was not completed on all samples as it was not considered necessary for stereospecific analysis of the positional distribution of the fatty acids.

Deacylation of TAG. Accurate stereospecific analysis depends on the precise execution of each one of the multistep

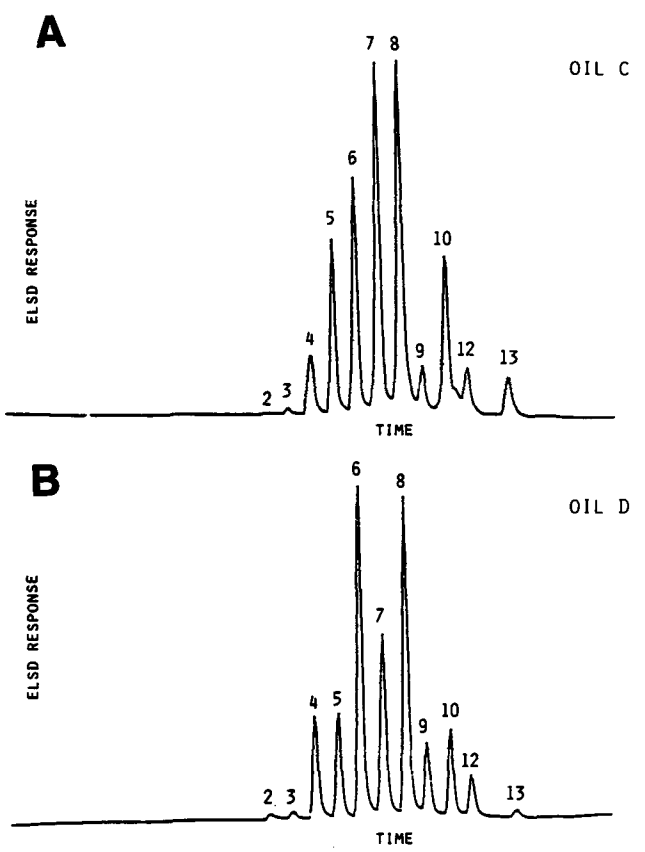

FIG. 2. Reversed-phase HPLC profiles of C and D polyunsaturated oils as obtained by light-scattering detector. HPLC conditions as given in Figure 1. Peak identification as given in Table 2. See Figure 1 for abbreviations.

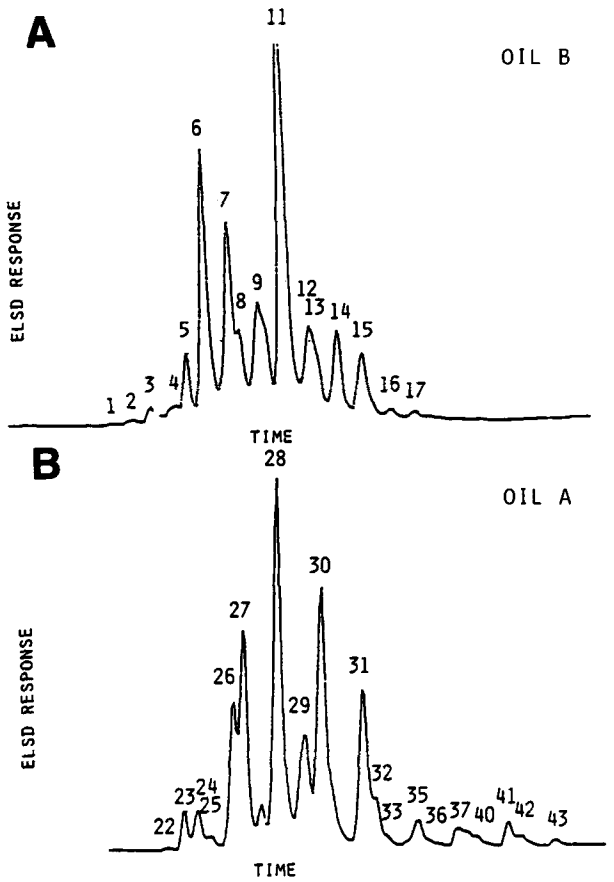

FIG. 3. Reversed-phase HPLC profiles of B and A polyunsaturated oils as obtained with light-scattering detector. HPLC conditions and abbreviations as given in Figure 1. Peak identification as given in Table 3.

procedure and frequent reconstitutions must be made to ascertain that each step has been adequately completed. Of critical importance is the formation of representative (random) *sn*-1,2(2,3)-DAG. In this study, neither the Grignard reaction nor pancreatic lipase yielded representative *sn*-1,2(2,3)-DAG, as isolated by borate TLC. This was due to the faster TLC migration of the long-chain polyunsaturated *sn*-1,2(2,3)-DAG, which led to an overlap with the X-1,3-DAG. Only after chiral-phase HPLC of the X-1,3-DAG was it recognized that the long-chain polyunsaturates in this fraction were due to contamination with *sn*-1,2(2,3)-DAG (see below). Because of the chain-length segregation and background contamination, it was also difficult to obtain representative samples from the small 2-MAG fraction produced during the Grignard reaction. However, representative 2-MAG could be recovered by prolonged pancreatic lipolysis, which yields 2-MAG as the final reaction products. In this case the TLC band was so strong that a representative 2-MAG fraction was readily obtained without contamination and undesirable segregation based on chain length. Pancreatic lipase is known to discriminate against the long-chain polyunsaturated fatty acids during brief digestion periods leading to nonrepresentative formation of the DAG intermediates. Table 4 shows the fatty acid composition of the original and reconstituted TAG for the D oil, along with corrected and calculated composition of the *sn*-1,2(2,3)-DAG obtained by Grignard deacylation, and of 2-MAG recovered from pancreatic and Grignard deacylation. There is reasonably good agreement between the corrected experimental *sn*-1,2(2,3)-DAG and the *sn*-1,2(2,3)-DAG calculated from the original TAG and the 2-MAG derived from pancreatic digestion. Thus, it became necessary to correct the *sn*-1,2- and *sn*-2,3-DAG compositions for loss of the polyunsaturated species in the X-1,3-DAG fraction in all the oils enriched in polyunsaturated long-chain acids. The need for such corrections was minimal in case of the A and B oils, the *sn*-1,2(2,3)-DAG of which showed only limited overlap with the X-1,3-DAG.

Stereospecific analyses. Figure 4 shows the chiral-phase HPLC separation of the *sn*-1,2- and *sn*-2,3-DAG derived from two of the TAG samples, one rich in 20:4 (F) and the other rich in 22:6 (D) fatty acid. Both show excellent resolution of the *sn*-1,2- and *sn*-2,3-enantiomers, with the latter emerging last. The X-1,3-isomers not removed by borate TLC emerge just ahead of the *sn*-1,2-enantiomers. There is considerable resolution of molecular species within the *sn*-1,2- and *sn*-2,3-enantiomers. The species were identified by chiral-phase LC/MS with electrospray. As noted above, during borate TLC, the long-chain *sn*-1,2(2,3)-DAG were observed to mi-

TABLE 2
Relative Proportions of Triacylglycerol ECN Values in the Test Oils as Estimated by High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection (area %)[a]

Peak no.	Fractional ECN	Triacylglycerol oils C	D	E	F
1	35.76	trace	trace		
2	37.02	1.18	1.56		
3	37.97	1.50	1.96		
4	38.88	6.03	9.18		
5	39.86	10.34	9.32		
6	40.79	13.37	19.93		
7	41.77	17.64	14.50		
8	42.69	18.95	19.59		
9	43.59	5.44	8.06		
10	44.61	11.01	8.67		
11	44.99	3.4	0.0		
12	45.47	5.27	5.51		
13	47.37	4.49	1.72		
14	50.40	1.37			
20	40.232			1.32	1.01
21	40.854			7.47	7.33
22	41.408			2.74	2.84
23	41.848			1.93	2.21
24	42.437			1.72	2.57
25	42.986			13.25	20.25
26	43.545			3.92	5.38
27	44.018			2.65	3.35
28	44.231			3.14	3.26
29	44.942			18.97	23.87
30	45.523			8.48	8.62
31	47.098			22.90	10.64
31a	47.615			3.07	
32	48.646			2.45	2.95
33	49.020				
34	50.549			4.77	2.63
35	52.450			1.90	
Other		0.01		0.68	3.09

[a]Abbreviation: ECN, equivalent carbon number.

TABLE 3
Relative Proportions of Triacylglycerol Species by ECN in the Test Oils as Estimated by High-Performance Liquid Chromatography with Evaporative Light-Scattering Detection (area %)[a]

Peak no.	Fractional ECN	Triacylglycerol oils A	B
1	38.388		
2	39.175		1.56
3	39.810		2.65
4	40.619		2.87
5	41.072		5.17
6	41.690		13.59
7	42.548		11.74
8	43.392		5.62
9	43.566		11.30
10	43.778		
11	44.447		19.27
12	45.366		9.75
13	45.592		
14	46.334		7.13
15	47.197		5.10
16	48.167		2.39
17	49.060		1.98
22	40.221	0.5	
23	40.851	2.1	
24	41.331	2.9	
25	41.820	1.5	
26	43.273	5.7	
27	42.938	8.4	
27a	43.576	2.6	
28	44.097	12.7	
29	45.066	6.8	
30	45.633	35.6	
31	47.093	7.4	
32	47.521	2.6	
33	47.951	1.2	
34	48.615	0.0	
35	49.032	3.0	
36	49.465		
37	50.422	2.8	
38	50.787		
39	51.195		
40	51.604		
41	52.349	2.0	
42	52.835	1.2	
43	54.148	1.0	

[a]See Table 2 for abbreviation.

grate with the short-chain X-1,3-DAG. Figure 5 shows the chiral-phase LC/MS elution patterns for the selected ions representing the mass of the 22:6-22:6 species. Peaks of comparable intensity are seen to be eluted in the *sn*-1,2- and *sn*-2,3-enantiomer regions of the chiral chromatogram. Similar separations were necessary for the oils rich in 20:4 acid. In this instance, the *sn*-1,2-enantiomers would be expected to show higher intensity than the *sn*-2,3-enantiomers.

Tables 5–7 give the corrected fatty acid composition of the *sn*-1, *sn*-2, and *sn*-3 positions of the six oils. It is seen that the 20:4 in E and F (Table 5) is found in the *sn*-1 position in double the concentration in the *sn*-3 position, with an intermediate value in the *sn*-2 position. The *sn*-3 position contains the bulk of 16:0 and 18:0, while the 18:1 and 18:2 are largely found in the *sn*-2 position. In contrast the 22:6 in C and D (Table 6) was found mainly in the *sn*-2 position, with the *sn*-1 position containing about half as much and the *sn*-3 position an intermediate value of this acid (especially in D). While 18:1 again was preferred for the *sn*-2 position, the saturated fatty acids 14:0 and 16:0 were about equally divided between the *sn*-1 and *sn*-3 positions with much less found in the *sn*-2 position. The 22:6 distribution in B (Table 7) again was confined largely to the *sn*-2 and *sn*-3 positions, with about one quarter of each in the *sn*-1 position. Interestingly, in this instance the 18:1 was divided nearly equally between the *sn*-1 and *sn*-3 positions, with much smaller amounts in the *sn*-2 position. The saturated acids, 14:0 and 16:0, were found primarily in the *sn*-1 and *sn*-2 positions, while 18:0 was found primarily in the *sn*-1 position. The 20:4 distribution in A (Table 7) was characterized by nearly equal proportions of the acid in all three positions of the TG, while the other major unsaturated acids, 18:1 and 18:2, were found in the *sn*-2 position in approximately twice the proportions found in *sn*-1 and *sn*-3 positions, which were about equal. The 16:0 was found in high proportions in *sn*-1 and *sn*-3 positions, while 18:0 was

TABLE 4
Fatty Acid Composition of Original and Reconstituted TAG[a] of Sample D, the Corrected and Calculated[b] Grignard *sn*-1,2(2,3)-DAG, and Pancreatic Lipase 2-MAG[c]

Fatty acids	TAG		2-MAG		1,2(2,3-DAG)	
	Original	Reconstituted (G)	Grignard	Lipase	Grignard	Calculated
10:0	0.75	0.75	0.00	0.00	0.00	0.56
12:0	6.47	6.47	0.53	0.44	1.78	4.97
14:0	19.76	19.76	9.14	4.75	16.85	16.00
16:0	17.08	17.08	9.39	2.72	14.22	13.49
16:1	1.76	1.76	2.25	2.03	2.06	1.83
18:0	0.24	0.24	1.17	0.44	0.61	0.29
18:1	9.01	9.01	20.93	22.86	14.58	12.47
18:2	0.41	0.41	0.00	0.72	0.58	0.49
18:3n-3	0.14	0.14	0.13	0.09	0.05	0.13
20:4n-6	0.08	0.08	0.00	0.09	0.00	0.08
20:5n-3	0.05	0.05	0.07	0.00	0.12	0.04
22:6	45.31	45.31	53.37	64.62	47.38	48.79
Other			3.31	1.42	1.89	0.99

[a]From data in Table 6, reconstituted triacylglycerols (TAG) = (position 1 + position 2 + position 3)/3.
[b]Calc. 1,2(2,3)diacylglycerols (DAG) = (3 × TAG + *sn*-2-MAG)/4.
[c]*sn*-2-Monoacylglycerols (MAG) from pancreatic lipolysis.

preferentially associated with the *sn*-3 position, with smaller amounts in *sn*-1 position. In contrast, the modest amounts of 24:0 were divided nearly equally between the *sn*-1 and *sn*-3 positions, with very little in the *sn*-2 position. Although the B and A oils are more complex in the composition and positional distribution of the fatty acids, it appears that they conform to the distribution of the polyunsaturated and saturated fatty acids established for the simpler oils (E and F, and C and D series).

Significance. It is difficult to compare the results of this work with published literature because natural oils containing over 33% of long-chain polyunsaturated fatty acids have not been previously subjected to stereospecific analyses. Analyses of TAG of human milk have shown that the 20:4n-6 and

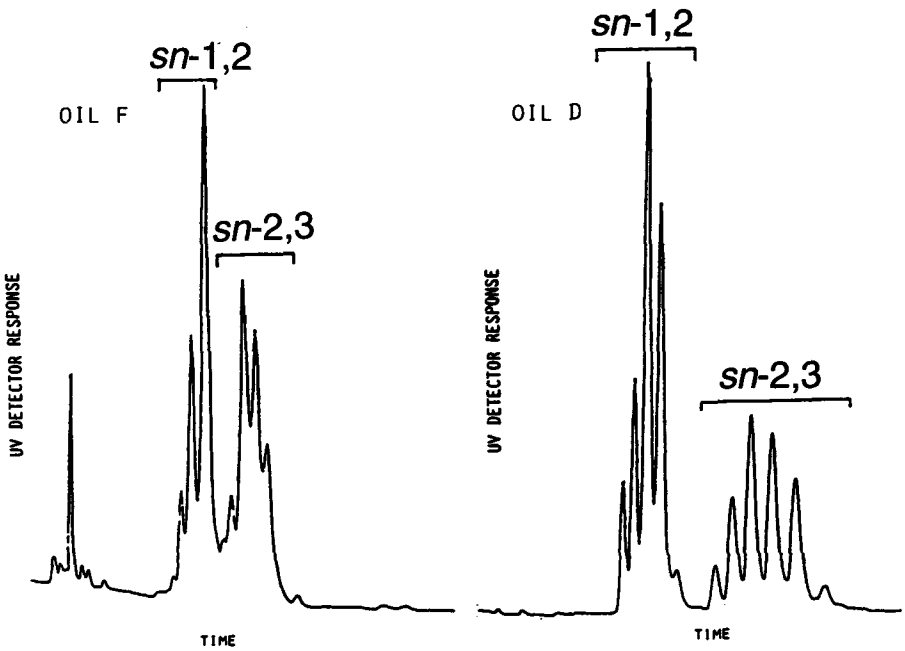

FIG. 4. Chiral-phase HPLC of the diacylglycerol dinitrophenyl urethane derivatives of F and D polyunsaturated triacylglycerols. *sn*-1,2-, *sn*-1,2-diacylglycerols (DAG); *sn*-2,3-, *sn*-2,3-DAG. Chiral-phase HPLC conditions: Hewlett-Packard Model 1090 liquid chromatograph equipped with a chiral column (25 cm × 4.6 mm i.d.) containing R-(+)-1-(1-napthtyl)ethylamine polymeric phase chemically bonded to 300 Å wide-pore spherical silica (YMC-Pack A-KO_3; YMC Inc., Kyoto, Japan). Solvent system: isocratic system of hexane/dichloromethane/ethanol 40:10:1, by vol, at 0.5 mL/min at an oven temperature of 23°C. Ultraviolet detector at 254 nm. See Figure 1 for abbreviations and company source address.

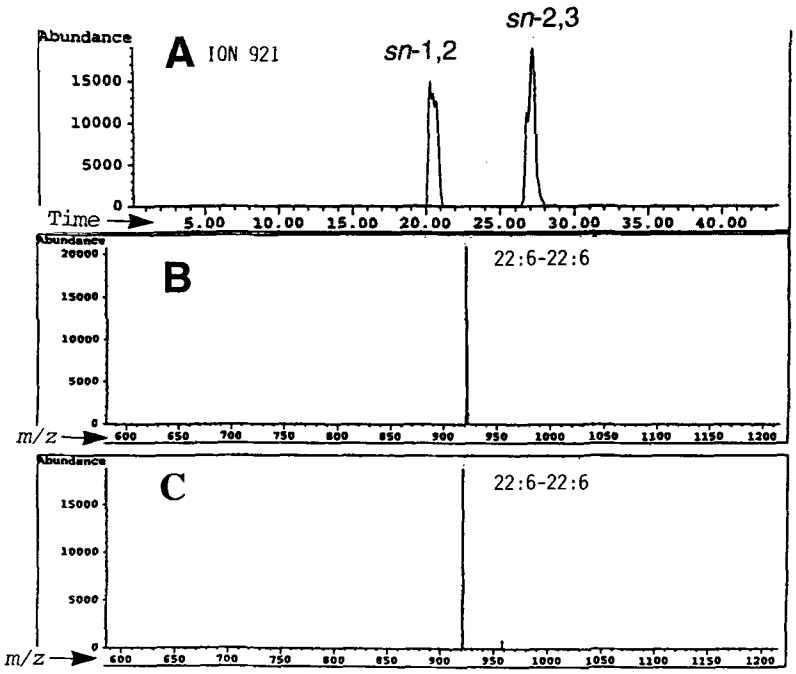

FIG. 5. Chiral-phase liquid chromatography/mass spectrometry (LC/MS) of the dinitrophenylurethane derivatives of oil C polyunsaturated *sn*-1,2- and *sn*-2,3-DAG recovered in the X-1,3-DAG fraction during borate thin-layer chromatography. Chiral-phase HPLC conditions as in Figure 4. LC/MS conditions as given in Experimental Procedures section. A, selected ion [*m/z* 921] profile corresponding to 22:6-22:6 species of DAG; B, total ion spectrum of peak corresponding to *sn*-1,2-enantiomers; C, total ion spectrum of peak corresponding *sn*-2,3-enantiomers. See Figures 1 and 4 for other abbreviations.

22:6n-3 acids are located mainly in the *sn*-2 and *sn*-3 positions (17,18), with the *sn*-1 position being relatively deficient in these acids (17). These findings have led to the observation that the fish oil and mother's milk on digestion would provide polyunsaturated 2-MAG, which would promote the recovery of these acids in the chylomicron TAG (19). On this basis, the oils rich in 20:4 (E and F) and 22:6 (C and D) would also yield high proportions of n-3 and n-6 polyunsaturated 2-MAG. In contrast to mother's milk, the present oils contain also high proportions of polyunsaturated fatty acids in the *sn*-1 position of the glycerol molecule. The metabolic significance of the location of the polyunsaturated fatty acids in the *sn*-1 position of the glycerol molecule is not known. Sample A oil is unusual in the content of comparable proportions of 20:4 in all three positions of the glycerol molecule. It would probably resemble the E and F in its metabolic fate.

These oils enriched in 20:4 and 22:6 acids also possess the unusual feature of containing significant amounts of TAG with two polyunsaturated long-chain fatty acids per molecule. In short-term incubations, pancreatic lipase discriminates against polyunsaturated long-chain fatty acid esters (20,21), but it is not known if this discrimination is more serious when two or three long-chain acids are present in a TAG molecule. During the present stereospecific analyses, there was a clear evidence for discrimination against the polyunsaturated TAG at early times, while at later times 2-MAG of representative composition were obtained.

The positional location and molecular association of long-chain polyunsaturated fatty acids may be pertinent to the resistance of these oils to peroxidation. Recent work has suggested (22) that a polyunsaturated fatty acid in the 2-position is more vulnerable to peroxidation than a similar fatty acid in the primary position. Assuming a fork shape for the TAG molecule, polyunsaturated fatty acids in the primary positions were thought to interact for mutual protection against oxidation. These observations are contradictory to earlier findings, showing that polyunsaturated acids in the primary positions were more vulnerable to peroxidation (23,24). Perhaps the protective interaction between the polyunsaturates in the primary positions is observed only when both are present in the same TAG molecule. The effect of the structural features of these unusual oils upon intestinal digestion and absorption, and upon peroxidation are subject to experimental determination and correlation with the positional distribution of the fatty acids.

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TABLE 5
Averaged Positional Distribution of Fatty Acids in the Purified Triacylglycerols of E and F Oils (mole %)

Fatty acids	E			F		
	sn-1[a]	*sn*-2	*sn*-3[a]	*sn*-1[a]	*sn*-2	*sn*-3[a]
14:0	0.45	0.23	0.45	0.31	0.30	0.44
15:0	0.11	0.06	0.20	0.16	0.06	0.19
16:0	7.15	1.25	15.62	6.09	1.36	20.47
16:1n-7	−0.09	0.40	−0.07	−0.04	0.30	−0.03
17:0	0.25	0.04	1.08	0.21	0.06	1.35
18:0	5.28	1.05	27.20	4.65	1.12	26.96
18:1n-9	8.60	29.52	5.38	3.66	17.97	2.40
18:1n-7	0.48	0.16	0.26	0.41	0.16	0.31
18:2n-6	1.87	16.70	2.07	0.56	15.11	1.38
18:3n-6	2.15	4.82	1.83	2.02	6.08	1.63
20:0	0.30	0.07	2.81	0.34	0.08	2.93
18:3n-3	0.02	0.60	0.09			
20:1n-9	0.42	0.13	0.71	0.38	0.17	0.94
20:2n-6	0.30	0.71	0.84	0.40	0.78	1.04
20:3n-6	1.28	3.03	2.03	1.43	4.33	2.27
22:0	1.71	0.15	3.83	2.03	0.11	3.74
20:4n-6	66.60	39.14	32.16	72.37	49.95	30.74
20:5n-3	0.26	0.12	0.29	0.37	0.04	0.36
24:0	1.20	0.09	1.81	3.19	0.10	1.15
24:1	0.28	0.00	0.28			
22:6	0.03	0.08	0.03	0.06	0.00	0.06
Other	1.26	1.65	1.03	1.40	1.97	1.65

[a]Average of the two calculations given in text for *sn*-1 and *sn*-3 positions, respectively; composition of *sn*-2 position was not averaged.

TABLE 6
Averaged Positional Distribution of Fatty Acids in the Purified Triacylglycerols of C and D Oils (mole %)

Fatty acids	C			D		
	sn-1[a]	*sn*-2	*sn*-3[a]	*sn*-1[a]	*sn*-2	*sn*-3[a]
10:0	0.27	0.00	0.27	1.12	0.00	1.12
12:0	1.50	0.94	7.82	8.76	0.44	10.22
14:0	24.18	5.44	14.77	30.53	4.75	23.99
15:0	−0.05	0.22	0.00	−0.29	0.18	0.15
16:0	29.68	2.91	13.34	29.81	2.72	18.70
16:1n-7	1.23	2.33	1.20	1.83	2.03	1.42
17:0	−0.01	0.04	0.22	-0.03	0.00	0.06
18:0	−0.01	0.53	4.53	0.84	0.44	−0.57
18:1n-9	3.02	31.54	9.35	3.90	22.86	0.26
18:2n-6	0.36	2.51	1.37	−0.35	0.72	0.86
20:0	0.03	0.00	0.52	−0.18	0.00	0.25
18:3n-3	0.08	0.14	0.16	0.22	0.09	0.11
20:1n-9	−0.04	0.12	0.34	0.09	0.00	0.09
20:3n-6	0.12	0.33	0.48			
22:0	0.20	0.12	0.64	0.03	0.00	0.03
20:4n-6	8.10	4.27	6.97	0.07	0.09	0.07
24:0	0.33	0.07	0.16			
24:1	0.12	0.00	0.12			
22:6	29.84	47.09	34.68	23.58	64.62	42.34
Other	0.89	1.47	3.06	0.78	1.06	0.33

[a]Average of the two calculations given in text for *sn*-1 and *sn*-3 positions, respectively; composition of *sn*-2 position was not averaged.

TABLE 7
Averaged Positional Distribution of Fatty Acids in the Purified Triacylglycerols of B and A Oils (mole %)

Fatty acids	B			A		
	sn-1[a]	*sn*-2	*sn*-3[a]	*sn*-1[a]	*sn*-2	*sn*-3[a]
12:0	0.13	0.13	−0.12			
14:0	3.86	5.17	2.20	0.68	0.16	0.24
15:0	1.71	1.62	0.60	0.74	0.06	−0.27
16:0	35.83	21.31	10.44	23.16	3.15	15.49
16:1n-7	7.33	3.89	4.11	1.68	0.63	−1.68
17:0	4.08	2.30	0.50	0.42	0.09	0.56
18:0	12.18	2.50	2.75	5.72	1.59	11.97
18:1n-9	16.48	6.17	18.47	9.55	20.94	13.19
18:2n-6	1.63	1.33	1.87	12.15	35.67	16.75
18:3n-6	0.02	0.22	0.18	1.96	3.32	4.23
20:0	0.40	0.14	0.79	0.16	0.16	1.64
18:3n-3	0.32	0.35	0.79	2.29	2.28	1.83
20:1n-9	0.80	0.61	1.84	0.10	0.14	1.36
20:2n-6	0.22	0.25	0.43	0.57	0.55	0.20
20:3n-6	0.15	0.09	0.15	2.15	4.79	2.35
22:0	0.26	0.10	0.42	1.93	0.50	3.10
20:4n-6	0.84	2.87	2.79	25.02	22.70	26.54
20:3n-3				−0.45	0.58	1.51
20:5n-3	3.34	6.81	11.69			
24:0				4.83	0.68	3.17
24:1n-9	0.40	0.11	0.76			
22:6	9.07	43.57	39.26	−0.02	0.09	−0.02
Other	1.10	0.20	0.03	2.57	1.92	0.13

[a]Average of the two calculations given in text for *sn*-1 and *sn*-3 positions, respectively; composition of *sn*-2 position was not averaged.

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