Japanese Sardine Oil as a Source of 16:3(n-4) and 16:4(n-1) Fatty Acids

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Open tubular gas liquid chromatographic (GLC) analysis of fatty acids in Japanese sardine oil showed the contents of 1.57% hexadecatrienoic acid (16:3) and 2.55% hexadecatetraenoic acid (16:4). Concentrations of 16:3 and 16:4 were carried out through the urea adduct method, argentation thin layer chromatography (TLC) and reversed phase TLC, with the purities of 16:3 and 16:4 samples obtained being both higher than 92%. They were recognized as 16:3(n-4) and 16:4(n-1) by nuclear magnetic resonance analyses of the methyl esters and GLC analyses of the partially hydrazine hydrogenated products and their oxidative ozonolysis products. The structures were supported by the results of gas chromatographic–mass spectrometric analyses of the pyrrolidides. Other isomers of 16:3 and 16:4 have not been detected in the GLC analysis of the methyl esters. These results indicate that sardine oil is useful as a source of these fatty acids.

The largest production of fish oil in Japan is sardine oil (430,000 tons in 1986). Fatty acids abundant in polyenoic acids are used for the production of 20:5(n-3) (EPA) and 22:6(n-3) (DHA). The latter are used in biological tests and medical areas. In the separation process for EPA and DHA, hexadecatrienoic acid (16:3) and hexadecatetraenoic acid (16:4) are concentrated in some stages, such as early eluates of reversed phase (RP) column chromatography and early extracts of supercritical fluid carbon dioxide extraction. These fatty acids in marine lipids have often been neglected on account of their overlaps with methyl stearate and oleate peaks in gas liquid chromatography (GLC) on columns having polar phases such as DEGS, Silar 10C, Silar 7CP, SP 2340, and SP 2330 (1).

In this study, the peaks of 16:3 and 16:4 were separated with GLC from those of other fatty acids. The column of low polarity gave the percentages of the fatty acids in Japanese sardine oil and structural analyses showed that they were 16:3(n-4) and 16:4(n-1).

MATERIALS AND METHODS

Materials. Japanese sardines caught in Chiba (A) and Miyagi (B) prefectures were obtained at food markets in Hakodate in mid-April of 1987 and 1988, respectively. The samples were an average of 22.2 (A) and 22.3 (B) cm in length and 86 (A) and 83 (B) g in weight per head.

Preparation of fatty acid methyl esters. All the lipids were extracted from Japanese sardines with the method used by Bligh and Dyer (2). The lipids were saponified by refluxing them with 1 M KOH-EtOH for 1 hr, and the unsaponifiable part was extracted with ether. Following acidification of the mixture with dilute HCl, fatty acids were recovered by ether extraction and converted to methyl esters by refluxing them with 7% BF_3-MeOH at 70°C for 14 min. Methyl esters for GLC were purified by thin layer chromatography (TLC) with Kiesel Gel 60 G (Merck, Darmstadt, Federal Republic of Germany) plates of 0.5 mm thickness by developing them with ether/n-hexane (15:85, v/v).

Open tubular GLC of methyl esters. Open tubular GLC of methyl esters was done with a Shimadzu GC 6AM instrument (Shimadzu Seisakusho Co., Kyoto, Japan) equipped with a dual FID detector and a glass capillary WCOT column (50 m × 0.28 mm i.d.) coated with Silar 5CP. The carrier gas was H_2. The column temperature was 185°C, and the injector and detector were 230°C. Peak area percentages were obtained with a Shimadzu integrator C-R6A. The component of each peak on the gas chromatogram was identified on the basis of an agreement of the retention data with the components of methyl esters of sea urchin fatty acids (3). The log plot procedure was used concurrently for identification.

Urea adduct method. The concentration of methyl polyenoates was carried out by urea adduct methods using 9 g of urea and 50 ml of methanol per gram of fatty acid methyl esters. The methyl polyenoates were recovered from the nonadduct fraction by ether extraction.

Argentation TLC ($AgNO_3$-TLC). Methyl esters were fractionated according to their degree of unsaturation on silver nitrate impregnated layers of Kiesel Gel 60 G by developing the plates with ethyl acetate/n-hexane (5:95 or 35:65, v/v).

RP-TLC. Methyl esters were fractionated according to their carbon number on Whatman $KC_{18}F$ RP-TLC plates by developing them with ethanol/water (90:10, v/v).

Preparation of fatty acid pyrrolidides. Fatty acid pyrrolidides were prepared by heating 20 mg of methyl esters in 2 ml of pyrrolidine, and 0.2 ml of acetic acid at 100°C for 30 min in a sealed tube (4). Pyrrolidides were purified by TLC with Kiesel Gel 60 G plates of 0.5 mm thickness by development with n-hexane/ether (1:1, v/v).

Gas chromatography-mass spectrometry (GC-MS). GC-MS analyses of the methyl esters and the pyrrolidides were carried out with a JEOL D-300 GC-MS system (Nippon Denshi Co., Tokyo, Japan) equipped with a glass column (2 m × 2 mm i.d.) packed with 5% XF-1150 on 60–80 mesh Shimalite W using He as a carrier gas. All spectra were obtained at 23 eV ionizing electron energy, and at a source temperature of 150°C for the methyl esters and 200°C for the pyrrolidides. The methyl esters were analyzed under high resolution conditions.

^{13}C and ^{1}H-nuclear magnetic resonance (NMR). A JEOL FX-90Q spectrometer (Nippon Denshi Co., Tokyo, Japan) in the Fourier transform mode at 22.53 MHz for ^{13}C and 89.60 MHz for ^{1}H was used to obtain the NMR spectra of methyl esters in $CDCl_3$.

Partial hydrogenation with hydrazine. Partial hydrogenation of unsaturated fatty acids was carried out by the method previously described (5). A mixture of 10 ml of 10% (v/v) hydrazine hydrate in methanol and 10 mg of fatty acids was stirred in the presence of air at 50°C for 4 hr by a magnetic stirrer. The products extracted with n-hexane were converted to methyl esters with 7% BF_3-MeOH. Methyl esters obtained were analyzed by

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GLC under the conditions described above with the exception that the column temperature was 160°C.

Oxidative ozonolysis. Oxidative ozonolyses of 2 mg methyl esters were carried out in BF_3-MeOH medium at −70°C using the procedure of Sebedio and Ackman (6,7). Ozone that had been generated by a Nippon Ozone Model 0-10-2 at 75 V was bubbled through the solution at a total gas flow of 250 ml/min. The products were extracted into $CHCl_3$, and these extracts were concentrated to about 10% (w/w). The products were analyzed by GLC under the same conditions as above with the exception that the column temperature was 80°C and 160°C.

RESULTS AND DISCUSSION

In this study, two peaks, X (relative retention time [RRT] 0.762 to the peak of 18:0) and Y (RRT 0.892) appeared in GLC on a Silar 5CP open tubular column. The contents of X and Y in Japanese sardine oil from sample (A) were 1.57 and 2.55%, respectively, of the total fatty acids, and 4.0 and 6.5% of the total polyenoic acids. The components of X and Y were confirmed as 16:3 and 16:4, respectively, since the mass numbers of the molecular ion peaks obtained for them under the high resolution conditions with a D-300 instrument agreed with those calculated for 16:3 and 16:4 methyl esters. Those calculations were: found m/e 264.20910 for X (calcd. $C_{17}H_{28}O_2$ 264.20893 for 16:3 methyl ester) and found m/e 262.19326 for Y (calcd. $C_{17}H_{26}O_2$ 262.19328 for 16:4 methyl ester).

The separation of 16:3 and 16:4 from fatty acid methyl esters in sample (A) was conducted using procedures I to IV shown in Table 1. The fractions of 16:3 and 16:4 obtained were found to contain 92.1% of 16:3 and 96.2% of 16:4.

The ^{13}C-NMR of 16:3 and 16:4 methyl esters from the sardine oil showed the peaks listed in Table 2. The ^{13}C-NMR spectra for 16:3 and 16:4 were close in agreement with those for 16:3(n-4) and 16:4(n-1), calculated by the set of additive substituent parameters (8–10), except for C_{12} and C_{13} in 16:4(n-1). The latter could not be calculated through the data from previous papers. The chemical shifts for C_{15} and C_{16} in 16:4 from the sardine oil revealed the occurrence of a terminal olefinic bond in 16:4. These results established that 16:3 and 16:4 from the sardine oil were the n-4 and n-1 structures, respectively. Their ^{1}H-NMR spectra characterized also these structures. The spectrum of 16:4(n-1) showed the multiplets at 4.92–4.96 and 5.62–5.88 ppm for the three protons of terminal olefinic bond (11), while that of 16:3(n-4) showed the triplet at 0.83–0.98 ppm for the three protons of the terminal methyl group which is in the gamma position to the olefinic bonds (11).

GLC of the oxidative ozonolysis products produced from partially hydrogenated products of 16:3 and 16:4 showed seven large peaks of methyl butanoate, methyl heptanoate, methyl decanoate, methyl palmitate, dimethyl hexanedioate, dimethyl nonanedioate, and dimethyl dodecanedioate, respectively, as shown in Table 3. Each peak component was identified on the basis of retention time agreement with those of the authentic reference esters. These results showed the locations of three olefinic bonds as 6, 9, and 12 in both 16:3 and 16:4. The peak of

TABLE 1

Concentration of 16:3(n-4) and 16:4(n-1) (wt%)

Order	I	II	III	IV	
Separation	Urea method	$AgNO_3$-TLC[a]	RP-TLC	$AgNO_3$-TLC[b]	
Fraction	Non-adduct	Polyenoate	Shorter chain fr.	16:3 (n-4)	16:4 (n-1)
Yields %	38.6	74.8	15.0	26.9	36.2
16:3(n-4)	3.95	5.03	28.75	92.15	—
16:4(n-1)	6.42	9.69	50.86	—	96.25
18:4(n-3)	5.16	6.69	11.24	—	1.17
20:5(n-3)	35.02	41.30	1.38	—	1.74
22:5(n-3)	3.64	4.46	—	—	—
22:6(n-3)	21.77	17.20	—	—	—
Others	24.04	15.63	7.77	7.85[c]	0.84

Developed with ethyl acetate/n-hexane [a](5:95, v/v) and [b](35:65, v/v).
[c]Containing 18:3(n-6): 3.97% and 18:3(n-3): 1.39%.

TABLE 2

^{13}C Chemical Shifts of Methyl Esters of 16:3(n-4) and 16:4(n-1)

Location of C atom	16:3(n-4)		16:4(n-1)	
	Found	Calcd[a]	Found	Calcd[a]
1	174.062	174.25	174.062	174.25
2	33.967	34.05	34.021	34.05
3	24.595	24.65	24.649	24.65
4	29.091	29.15	29.145	29.15
5	26.870	26.90	26.924	26.90
6	129.585	129.63	129.693	129.74
7	128.231	128.32	128.176	128.20
8	25.624	25.75	25.624	25.75
9	128.068	128.10	128.393	128.42
10	128.393	128.45	128.014	128.08
11	25.624	25.75	25.624	25.75
12	127.797	127.82	127.093	—[b]
13	130.127	130.13	128.939	—[b]
14	29.308	29.35	31.529	31.45
15	22.753	22.90	136.736	136.90
16	13.760	13.80	114.741	114.75
OCH_3	51.411	51.31[c]	51.411	51.31[c]

[a]Calculated shifts for each carbon were obtained with the method reported by Bus *et al.* and Gunstone *et al.* (8–10).
[b]Chemical shifts for these carbons could not be calculated by the previous data (8–10).
[c]Chemical shifts for these carbons were taken from the data of 18:3(n-6) (10).

TABLE 3

Compositions of Oxidative Ozonolysis Products Produced from Partially Hydrogenated Products (wt%)

Product	From 16:3(n-4)	From 16:4(n-1)
Methyl butanoate	7.38	3.85
Methyl heptanoate	9.78	9.44
Methyl decanoate	10.96	10.82
Methyl palmitate	20.75	20.16
Dimethyl hexanedioate	24.33	25.82
Dimethyl nonanedioate	14.83	16.78
Dimethyl dodecanedioate	11.97	13.13

TABLE 4

Compositions of Partially Hydrazine Hydrogenated Products of 16:3(n-4) and 16:4(n-1) (wt%)

Product[a]	Retention time (min)	From 16:3(n-4)	From 16:4(n-1)
16:0	21.34	16.13	15.31
6-16:1	22.91	13.50	13.12
9-16:1	23.55	13.57	14.17
12-16:1	24.96	14.97	15.23
6,9-16:2	25.98	9.46	9.88
6,12-16:2	27.05	13.97	14.13
9,12-16:2	28.20	10.43	10.77
6,9,12-16:3	30.85	7.97	7.39

[a]For example, 6-16:1 and 6,9-16:2 show *cis*-6-hexadecenoic and *cis,cis*-6,9-hexadecadienoic acids, respectively.

dimethyl propanedioate expected from the structure of divinylmethylene did not appear as was described in the previous paper (6). The compositions of the partially hydrogenated products of 16:3 and 16:4 are shown in Table 4. Six peaks appeared between the peaks of 16:0 and 16:3(n-4) in GLC. They were assigned to *cis*-6-hexadecenoic acid (6-16:1), 9-16:1, 12-16:1, *cis,cis*-6,9-hexadecadienoic acid (6,9-16:2), 6,12-16:2, and 9,12-16:2 on the basis of the results of the oxidative ozonolyses. The retention times and peak area percentages of the components in the products from 16:4 agreed with those from 16:3. This result also supported the similarity in the structures of 16:3 and 16:4 with the exception of the terminal olefinic bond in 16:4(n-1). In the gas chromatogram of the products from 16:4, the peaks of products having the terminal olefinic bond were not found. The hydrogenation of the terminal olefinic bond of fatty acid with hydrazine proceeds much faster than that of the internal olefinic bond, because of less steric hindrance (12). This result indirectly showed the occurrence of the terminal olefinic bond in 16:4 from the sardine oil. Therefore, GLC of the partially hydrogenated products and their oxidative ozonolysis products also supported the structures of 16:3(n-4) and 16:4(n-1).

The mass spectra of the fatty acid pyrrolidides of 16:3 and 16:4 from the sardine oil showed the irregular intervals of m/e 12 between the maxima in the fragment ion peaks for each carbon atom as follows: C_5 (m/e 154, intensity 2.6% to the parent ion peak)-C_6 (m/e 166, 2.6%); C_8 (m/e 194, 4.5%)-C_9 (m/e 206, 2.2%) and C_{11} (m/e 234, 2.1%)-C_{12} (m/e 246, 3.0%) for 16:3; and C_5 (m/e 154, 2.9%)-C_6 (m/e 166, 2.7%) and C_8 (m/e 194, 5.0%)-C_9 (m/e 206, 2.0%) for 16:4. These fragments showed that 16:3 was the n-4 structure and that two olefinic bonds occurred between carbon 6 and 7, and also between 9 and 10 in 16:4. A series of fragments m/e 260 (M-41), 272 (M-29), and 286 (M-15) obtained in the mass spectrum of the pyrrolidide of 16:4 characterized the terminal olefinic bond in 16:4(n-1) (4). The irregular interval between C_{11}-C_{12} expected to appear in the spectrum of 16:4(n-1) pyrrolidide was not observed. Ther terminal olefinic bond may influence the interval between C_{11}-C_{12}.

In this study, 16:3 and 16:4 separated from Japanese sardine oil were confirmed as the n-4 and n-1 structures, respectively, while other isomers of 16:3 and 16:4 were not found. In menhaden oil, the main fish oil produced in the USA, the occurrences of 16:3 and 16:4 were also frequently reported (13,14). However, according to reported data, these fatty acids were the mixtures of 16:3(n-4) and (n-3) or 16:4(n-1) and (n-3) (13). On the other hand, 16:3 (1.57%) and 16:4 (2.55%) in Japanese sardine oil consisted almost solely of 16:3(n-4) and 16:4(n-1), respectively. In this respect, Japanese sardine oil is useful as a source of 16:3(n-4) and 16:4(n-1) for the dietary materials for biological tests and medical uses. The ratios of the contents of 16:3(n-4) and 16:4(n-1) to that of EPA were 9.23 and 14.99%, respectively, and the sardine oil contained more 16:4(n-1) than 18:4(n-3) (2.06% to the total fatty acids).

The retention times of the peaks of major 16:1 and 16:2 isomers in Japanese sardine oil agreed with those of 16:1(n-7) and 16:2(n-4) in the partially hydrogenated products of 16:3(n-4) in GLC, respectively. A series of major components of C16 unsaturated fatty acids in Japanese sardine oil is shown as follows: 16:1(n-7) (RRT 0.564, 8.64%)—16:2(n-4) (RRT 0.686, 1.23%)—16:3(n-4) (RRT 0.762)—16:4(n-1) (RRT 0.892). Fish oils, which are usually used as the source of n-3 polyenoic acids, EPA and DHA, may be worth using as the source of n-1 and n-4 polyenoic acids. The unknown biological and dietary effects of the n-1 and n-4 fatty acids would be interesting subjects.

In the oil extracted from Japanese sardine sample (B), a sample of oil from flesh with skin contained a larger amount of 16:3(n-4) and 16:4(n-1) than the viscera oil. The contents of 16:3(n-4) and 16:4(n-1) were 1.27 and 2.35%, respectively, in the flesh oil (light brownish yellow color), and 0.78 and 1.91%, respectively, in the viscera oil (dark green color). Therefore, the flesh oil would be of more value when used for preparation of 16:3(n-4) and 16:4(n-1).

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