Occurrence of All-*cis*-5,8,11,14,17,20,23-Hexacosaheptaenoic Acid (26:7n-3) in the Roughscale Sole *Clidoderma asperrimum* Flesh Lipids

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Abstract

Fatty acid analysis of roughscale sole *Clidoderma asperrimum* flesh lipids was carried out by gas chromatography. An unidentified peak appeared in the chromatogram in the elution region of ≥C24 fatty acids. After enrichment by solvent partitioning, reversed-phase TLC, and argentation TLC, the peak component was subjected to structural analyses. The partially hydrogenated products after reaction with hydrazine hydrate gave seven isomers of *cis*-hexacosenoic acid (26:1). GC-MS analysis of their dimethyl disulfide (DMDS) adducts identified the monounsaturates as 5-, 8-, 11-, 14-, 17-, 20-, and 23-26:1. The peak component was assigned to all-*cis*-5,8,11,14,17,20,23-hexacosaheptaenoic acid (26:7n-3). GC-MS analyses of the 4,4-dimethyloxazoline (DMOX) derivative and methyl ester confirmed this structure. This fatty acid is a rare, very long chain polyunsaturated fatty acid (VLCPUFA). The concentrations of the acid found in roughscale sole were 0.69 ± 0.34 % (*N*=5) of the total fatty acids in the flesh lipids. Roughscale sole appears to be characterized by the occurrence of 26:7n-3, which is lacking in popular sources of methylene-interrupted VLCPUFA, such as vertebrate retina, spermatozoa, and herring.

Keywords

Hexacosaheptaenoic acid, Very long chain polyunsaturated fatty acid, Fatty acid, Roughscale sole, *Clidoderma asperrimum*, GC, GC-MS

Introduction

Very long chain polyunsaturated fatty acids (VLCPUFA) occur in many species of animals, plants, and lower organisms [1–4]. Mammalian retinas [5,6], brains [7–9], testes [10], and spermatozoa [11–12] include methylene-interrupted VLCPUFA of the n-3 and n-6 series up to C34–C40. Aquatic organisms also contain VLCPUFA [13]. Baltic herring include those up to C28 [14–16], some of which are represented in the fatty acids of the predatory ringed seal [17]. Bottom-living flathead flounder is rich in 24:6n-3 [18,19], which presumably originated from their diet of brittle star [20–22]. Additionally, dinoflagellates and some species of microalgae include 28:8n-3 and 28:7n-6 [4], and even- and odd-numbered VLCPUFA up to C36 were observed in a species of dinoflagellate [23,24].

All-*cis*-5,8,11,14,17,20,23-hexacosaheptaenoic acid (26:7n-3) is also a VLCPUFA. However, this fatty acid is a very rare one among the VLCPUFA of n-3 series. To the best of our knowledge, 26:7n-3 has been found in only 6 species of microalgae (Criptophyceae, Prymnesiophyceae and Dinophyceae) at less than 0.3% of the total fatty acid content [23–25]. This fatty acid also cannot be prepared from popular sources of n-3 and n-6 VLCPUFA standards such as retinas, sperm, and herring [1]. A detailed assignment of the structure has also not been reported.

In the present study, the fatty acids of roughscale sole *Clidoderma asperrimum*, i.e., ‘samegarei’ in Japanese, which is one of the edible fish in Japan, were investigated for VLCPUFA. The fatty acid analysis of the sole revealed the occurrence of 0.3%–1.3% of 26:7 in their flesh lipids. This paper reports the structural assignment of this fatty acid as 26:7n-3 along with their enrichment process and the fatty acid composition of the roughscale sole.

Materials and methods

Materials

Five individuals of roughscale sole caught in the Pacific waters off Hidaka, Hokkaido, Japan, and in the Sea of Okhotsk off Shari, Hokkaido, Japan, were purchased in stores in May 2009. Two of the Pacific samples were obtained as whole fish (females; body lengths, 39.8 and 41.2 cm; body weights, 2.4 and 2.8 kg) and another in frozen form without skin and viscera (body length, 34.8 cm). The two Okhotsk samples were obtained in frozen form without the head, skin, or viscera (lengths of trunk and tail, 24.0 and 25.8 cm). The flesh was removed, minced, and stored at −30°C before lipid extraction.

Fatty acid methyl esters

Total lipids were extracted from 150 g of the flesh by the method reported by Bligh and Dyer [26]. Fatty acid methyl esters were prepared from the lipids by transesterification with 7% BF_3-methanol for 1 h at 100°C under a nitrogen atmosphere. Methyl esters were purified by thin-layer chromatography (TLC) on silica gel G plates (10 × 10 cm, 0.25 mm thickness; Analteck, Newark, USA) with hexane/diethyl ether (85:15, v/v) for development.

For quantity preparation, the total lipids were saponified by refluxing them with 1 M KOH in ethanol for 1 h; the unsaponifiable portion was extracted with diethyl ether. Following acidification of the aquatic phase using dilute HCl, the fatty acids were recovered by ether extraction. The fatty acids were converted to methyl esters by refluxing them with 7% BF_3-methanol at 70°C for 14 min.

Fractionation of fatty acids

The fatty acid methyl esters were divided into two fractions obtained through a solvent partition method using a solvent system of 2,2,4-trimethylpentane and ethanol/water (1:1, v/v) containing 0.25 g/ml of silver nitrate [27,28].

The methyl esters were fractionated according to their partition number by reversed-phase

TLC (RP-TLC) on Partisil KC18F plates (20 × 20 cm, 0.2 mm thickness; Whatman, Maidstone, England) with acetonitrile/water (95:5, v/v) for double developments [29].

The methyl esters were fractionated according to the degree of unsaturation by argentation TLC (Ag-TLC) on 10% silver nitrate-impregnated layers of silica gel 60G (20 × 20 cm, 0.5 mm thickness; Merck, Darmstadt, Germany) with hexane/acetone (70:30, v/v) for double developments [29].

Derivatization for structural analysis

Partial hydrogenation of the polyunsaturated fatty acids was carried out using hydrazine hydrate [30,31]. A mixture of 5 mg of free fatty acids and 10% (v/v) hydrazine hydrate in methanol (5 ml) was stirred at 50°C for 7 h with aeration. The products, which were extracted with diethyl ether, were converted to methyl esters using 7% BF_3-methanol. The monounsaturated fatty acids were isolated from the products by Ag-TLC on 5% silver nitrate-impregnated silica gel 60G with hexane/acetone (95:5, v/v), and then fractionated according to the olefinic bond position by Ag-TLC on 15% silver nitrate-impregnated silica gel 60G with hexane/toluene (50:50, v/v) [32,33].

Dimethyl disulfide (DMDS) adducts of monounsaturated fatty acids were prepared following the procedure of Shibahara *et al.* [34,35]. The methyl esters were reacted with DMDS (1 ml) in the presence of catalytic I_2 (13 mg) for 1 h at 35°C before adding 30% aqueous $NaHSO_3$. The resulting adducts that were extracted by hexane/diethyl ether (50:50, v/v) were purified by TLC on a silica gel G plate with hexane/diethyl ether/acetic acid (80:20:1, v/v/v) for development.

4,4-Dimethyloxazoline (DMOX) derivatives of fatty acids were prepared by the procedure reported by Christie [36]. Acid chlorides formed from the free fatty acids by a reaction with oxalyl chloride were reacted with a 10 mg/ml solution of 2-amino-2-methyl-1-

propanol in dichloromethane (0.5 ml) for 1 h at room temperature. After the solvent was evaporated, trifluoroacetic anhydride was added to the residue and the mixture was left at 40°C for 1 h. The DMOX derivatives were purified by TLC on a silica gel G plate with hexane/diethyl ether/acetic acid (50:50:1, v/v/v) for development.

Instrumental analysis

The fatty acid methyl esters were analyzed by GC using a Shimadzu GC-18A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a Restek FAMEWAX column (30 m × 0.32 mm i.d., 0.25 μm film thickness; Restek, Bellefonte, USA). The column temperature was programmed to either increase from 170 to 240°C at a rate of 4°C/min, or to remain isothermal at 240°C. The injector and detector temperatures were 240°C, and the carrier gas was helium (85 kPa). Peak area percentages were obtained using a Shimadzu C-R6A integrator.

The monounsaturated fatty acids isolated from the hydrazine hydrogenation products were analyzed using a Shimadzu GC-17A gas chromatograph equipped with a flame ionization detector and a SLB-IL100 column (60 m × 0.32 mm i.d., 0.26 μm film thickness; Supelco, Bellefonte, USA) [37]. The column temperature was isothermal at 200°C, the injector and detector temperatures were 240°C, and the carrier gas was helium (117.5 kPa). The peaks were monitored using a Shimadzu C-R3A integrator.

GC-MS analysis was carried out using an HP 6890 series gas chromatograph (Hewlett-Packard, Palo Alto, USA) linked to a JEOL JMS-700TZ mass spectrometer (JEOL, Tokyo, Japan). The latter was used in the electron impact mode at 70 eV with source temperatures of 240°C for methyl esters, 270°C for DMOX derivatives and 280°C for DMDS adducts. The GC was fitted with split/splitless injection. For the analyses of the methyl esters and DMOX derivatives, a DB-23 column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent, Santa

Clara, USA) was used. The column temperatures were programmed from 40°C to 230°C and from 40°C to 270°C at 20°C/min for the methyl esters and DMOX, respectively. For the analysis of the DMDS adducts, a Zebron ZB-1ms column (30 × 0.25 mm i.d., 0.25 μm film thickness; Phenomenex, Torrance, USA) was used. The column temperature was held at 40°C for 1 min then raised to 175°C at 40°C/min and then to either 265°C at 5°C/min or to 280°C at 20°C/min. Helium was used as the carrier gas.

Fourier transform infrared spectra were measured in CCl_4 solutions using a JASCO FT-IR 5300 spectrometer (JASCO, Tokyo, Japan).

Fatty acid composition determined by GC of methyl esters was represented by mean ± standard deviation of the five individuals of roughscale sole.

Results

GC and enrichment of 26:7

The GC analysis of the fatty acid methyl esters derived from roughscale sole flesh lipids showed two remarkable peaks, A and B, after elution of 24:1n-9 (Fig. 1). Their equivalent chain lengths (ECLs) on FAMEWAX at 240°C were 26.14 and 28.30, for peak A and B, respectively. The ECL of peak A was in fair agreement with that of 24:6n-3, which was previously found in flathead flounder [18,19] and brittle star [20,21]. The component of peak B was confirmed to be hexacosaheptaenoic acid (26:7) since the mass number of the molecular ion peak obtained under high resolution conditions agreed with that calculated for a 26:7 methyl ester; the *m/z* found was 396.30280 and the value calculated was 396.30283 for $C_{27}H_{40}O_2$.

For the structural analysis, the enrichment of 26:7 was conducted by the solvent partition procedure, RP-TLC, and 10% Ag-TLC in this order (Table 1). The concentration of 26:7, which started at 1.3% of the total fatty acids, was boosted to 10.4% by the solvent partition, 27.7% by RP-TLC, and finally 83.0% by Ag-TLC. A coexistent minor component of the final fraction was

24:6n-3 (17.0%).

Structure of 26:7

The infrared spectrum of the 26:7 concentrate showed absorptions at 1650 cm^{-1} and 710 cm^{-1}, but not that near 970 cm^{-1} which is characteristic of a *trans*-olefinic bond. Hydrazine hydrogenation yielded *n*-26:0 as a saturated fatty acid. These results indicate that the fatty acid is normal-chain 26:7 with all *cis*-geometry.

The monounsaturated fatty acids, which were produced by the partial hydrogenation of 26:7, separated into four fractions on the 15% Ag-TLC plate. The GC analyses of these fractions showed a total of seven peaks corresponding to 26:1 isomers with ECLs in the range of 26.41–27.23 on SLB-IL100 at 200°C. The mass spectra of the DMDS adducts of the 26:1 isomers gave a molecular ion at *m/z* 502 corresponding to the DMDS adduct of 26:1 methyl ester and a series of key fragment ions showing the olefinic bond position in 26:1 (Fig. 2). In Fig. 2a, the fragment ions at *m/z* 161 and 341 indicate cleavage between the methylthio-substituted carbons of C5 and C6. The fragment ion at *m/z* 129 was due to the loss of methanol (*m/z* 32) from the ion at *m/z* 161. A set of the fragment ions indicated the structure of 5-26:1. In the same manner, the other isomers were identified as 8-, 11-, 14-, 17-, 20- and 23-26:1, as shown in Figs. 2b-2g. Hydrazine reduces olefinic bonds without positional and geometrical isomerization of the remaining olefinic bonds [30]. The structure of 26:7 was assigned as all-*cis*-5,8,11,14,17,21,23-hexacosaheptaenoic acid (26:7n-3).

The mass spectrum of the DMOX derivative of 26:7 showed irregular intervals of *m/z* 12 between the maxima in the fragment ion peaks for each carbon atom as follows: C7 (*m/z* 180, intensity 19.0%)–C8 (*m/z* 192, 9.7%); C10 (*m/z* 220, 23.2%)–C11 (*m/z* 232, 15.0%); C13 (*m/z* 260, 16.5%)–C14 (*m/z* 272, 8.6%); C16 (*m/z* 300, 13.7%)–C17 (*m/z* 312, 7.3%); and C19 (*m/z* 340, 15.6%)–C20 (*m/z* 352, 9.8%) (Fig. 3a). These fragments indicate the occurrence of olefinic

bonds at the Δ8, 11, 14, 17, and 20 positions in 26:7 [29]. An olefinic bond at the Δ5 position was shown by the fragment ion due to cleavage at this position (*m/z* 152, 17.6%) accompanied by an intense odd-numbered peak at *m/z* 153 (32.3%) [38]. GC-MS analysis of the 26:7 methyl ester revealed fragment ions characteristic of n-3 and Δ5 series polyunsaturated fatty acids at *m/z* 108 (33.4%) and 180 (14.1%), respectively [29], confirming the structure of 26:7n-3 (Fig. 3b).

Fatty acids of the roughscale sole flesh lipids

The fatty acid composition of the roughscale sole flesh lipids is shown in Table 2. The major fatty acids (>5% of the total lipids) were 14:0, 16:0, 16:1n-7, 18:1n-9+18:1n-11, 20:1n-11+20:1n-13, and 20:5n-3. The proportions of these fatty acids were not very different from those previously reported for deep-sea flounders [39], in which the monounsaturated fatty acids were rich in the liver and flesh neutral lipids. The major highly unsaturated fatty acids of the roughscale sole were 20:5n-3, 22:5n-3, 22:6n-3, 24:6n-3, and 26:7n-3. The content of 26:7n-3 was 0.69 ± 0.34% of the total lipids, ranging from 0.33% to 1.26% among the five individuals. The lipid content of the flesh was 30.5 ± 4.6% on wet-weight base, much higher than the previous datum (5.6%) observed for smaller-sized roughscale sole (mean body length, 22.3 cm; mean body weight, 199.8 g) [39].

Discussion

In the present study, 26:7n-3 and 24:6n-3 were found in the roughscale sole flesh lipids as their VLCPUFA. Methylene-interrupted VLCPUFA of n-3 series are typical of vertebrate retinas (up to 36:6n-3) [5,6], spermatozoa (up to 34:6n-3 in chain length and 32:7n-3 in unsaturation) [11,12], and Baltic herring (generally up to 28:7n-3) [14–16]. These tissues or fish have been recognized as convenient sources of VLCPUFA standards for the n-3 and n-6 series [1]. One of

the shorter-chain VLCPUFA, 24:6n-3, was rich in flathead flounder [18,19] and brittle stars [20–22], and was also found in sea lilies [20], coelenterates [40,41], gorgonians [42], jellyfish [43], and gastropods [44]. Freshwater crustacea of the order Bathnellacea were reported to contain more than 50 VLCPUFA up to 40:8n-3 [45,46]. Marine dinoflagellates contain 28:8n-3 together with 28:7n-6 [23–25,47–53]. Fatty acids of the dinoflagellate *Amphidinium carterae* included even- and odd-numbered chain VLCPUFA up to 36:8n-3 [23,24].

As a tentatively identified component, 26:7n-3 were found in Dinophyceae (*Heterocapsa niei*, 0.2% of total fatty acids; and *Amphidinium carterae*, in trace amounts), Prymnesiophyceae (*Pavlova pinguis*, up to 0.3%), and Criptophyceae (*Proteomonas sulcata* and *Phodomonas salina*, each in trace amounts) [25]. In the fatty acids of the dinoflagellate *Amphidinium carteae*, 26:7n-3 was found at a concentration of 3.2% in the heptaenoic + octaenoic acid concentrate [23], which corresponds to 0.064% of the total fatty acids. The same acid was also found at 1.40% in a concentrate of VLCPUFA with more than 3 olefinic bonds [24]. To the best of our knowledge, there has been no other report describing the occurrence of 26:7n-3 in nature. While the analogous 26:6n-3 was observed in vertebrate retina [5,6], murine testis [10], and ram spermatozoa [11], the fatty acids of these tissues were not reported to include 26:7n-3. Baltic herring [15], seal [17], and crustacea (Bathynellacea) [45,46] contain both analogous 26:6n-3 and homologous 28:7n-3, but not 26:7n-3.

Therefore, the roughscale sole flesh lipids are characterized by the occurrence of the rare VLCPUFA, 26:7n-3. This fatty acid seems to be formed from coexistent 24:6n-3 *via* two-carbon chain elongation followed by Δ5-desaturation. In GC analysis, unidentified minor peaks were observed between the peaks of 24:6n-3 and 26:7n-3 (Fig. 1). Roughscale sole preferentially feed on brittle star [54,55], which is usually rich in 24:6n-3 [20–22]. At this time, the occurrence of 26:7n-3 in brittle star is unknown. Although the concentrations of 26:7n-3 in the flesh lipids (0.3–1.3%) were not very high, there was no sample where 26:7n-3 was not found.

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Figure captions

Fig.1 Gas chromatogram of fatty acid methyl esters formed from roughscale sole flesh lipids (Restek FAMEWAX, 170 to 240°C at 4°C/min).

Fig. 2 Mass spectra of dimethyl disulfide (DMDS) adducts of 26:1 isomers formed by hydrazine hydrogenation of the roughscale sole 26:7 acid.

Fig. 3 Mass spectra of 4,4-dimethyloxazoline (DMOX) derivative (**a**) and methyl ester (**b**) of the roughscale sole 26:7 acid.

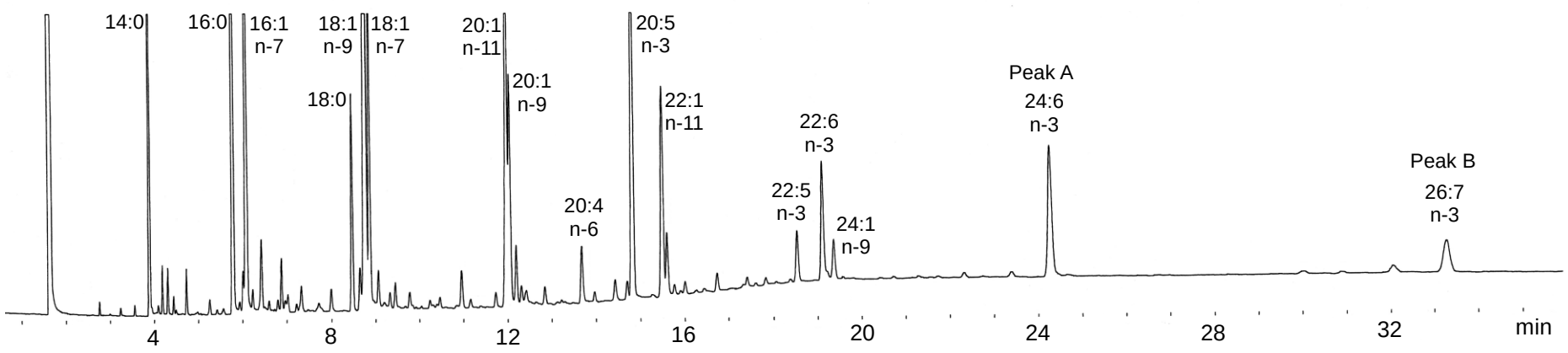

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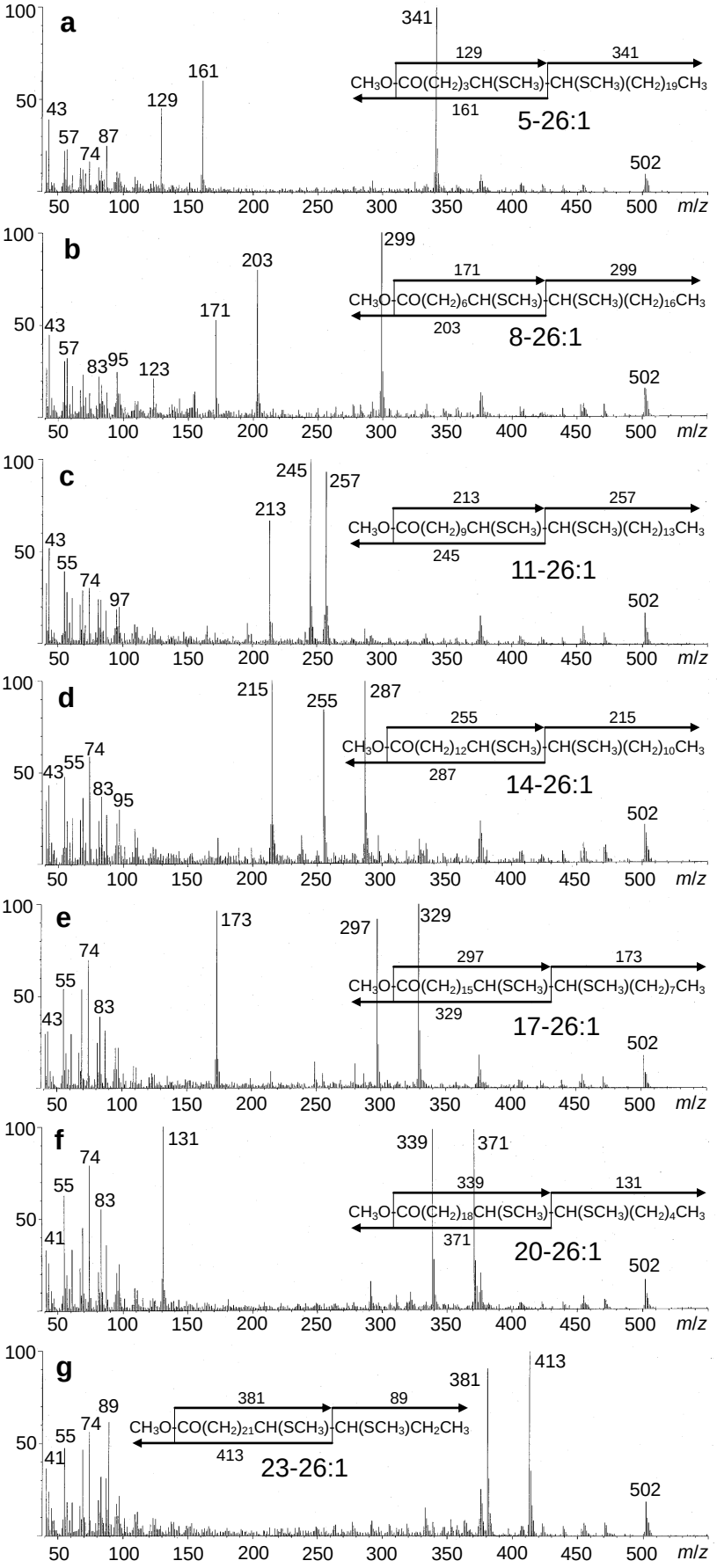
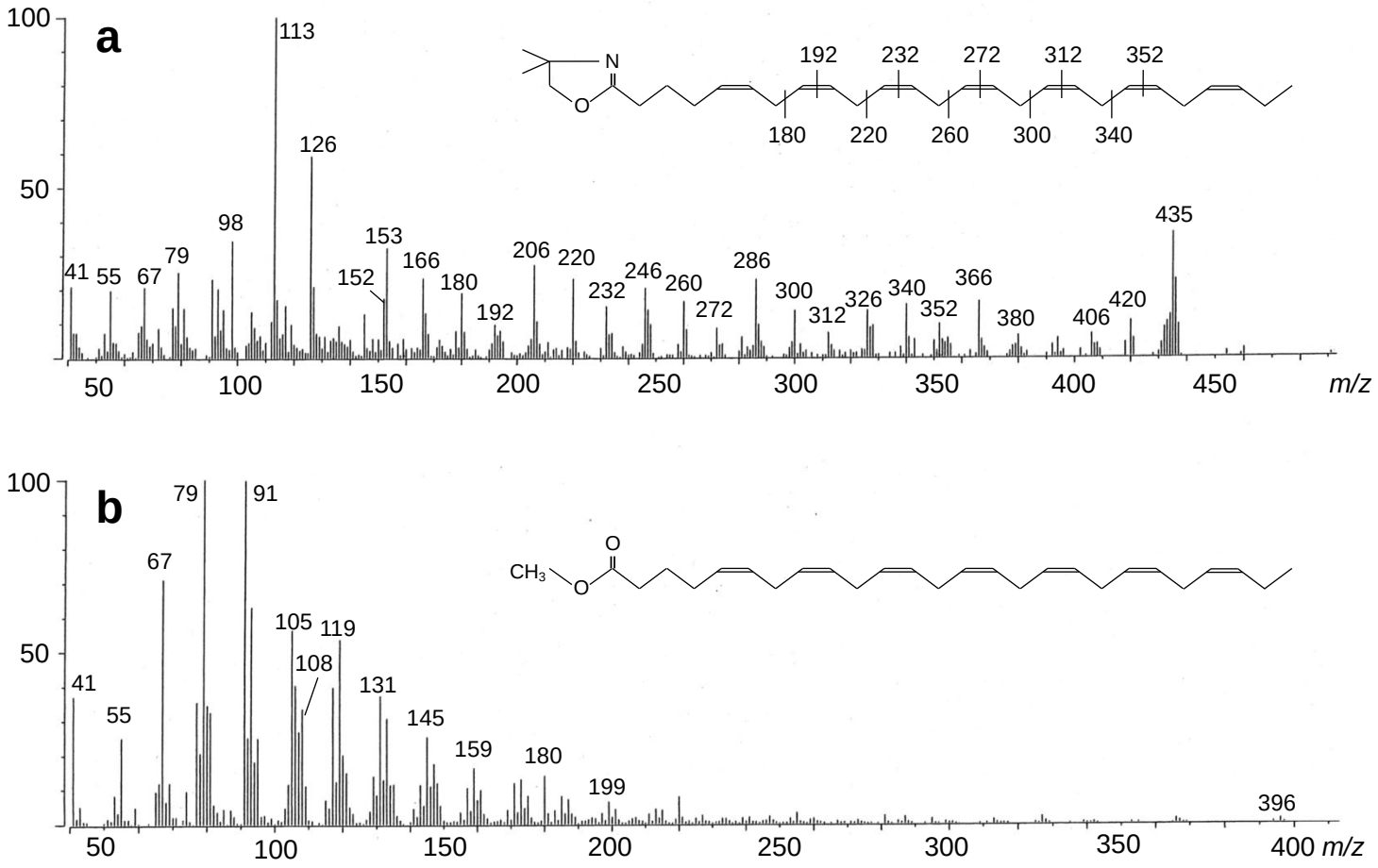
Figure 1



Fukuda & Ando
Figure 2



Fukuda & Ando
Figure 3

Table 1 Enrichment of 26:7 from the fatty acids of roughscale sole flesh lipids

Fatty acid	Intact[a]	Solvent partition	RP-TLC	Ag-TLC
Concentration (wt%)				
20:5n-3	7.7	48.4	0.4	ND
21:5n-3	0.2	1.1	0.5	ND
22:5n-3	0.7	2.0	6.8	ND
22:6n-3	2.0	15.7	ND	ND
24:6n-3	3.1	16.4	62.1	17.0
26:7n-3	1.3	10.4	27.7	83.0
Others	85.0	6.0	2.5	0.0

[a] Fatty acid methyl esters prepared from a Pacific sample (41.2 cm, 2.8 kg).

Table 2 Fatty acid composition of the roughscale sole flesh lipids (wt%)

Fatty acid	Composition[a]	Fatty acid	Composition
12:0	0.06 ± 0.02	18:4n-3	0.37 ± 0.06
14:0	5.97 ± 0.64	18:4n-1	0.10 ± 0.02
14:1n-5	0.28 ± 0.03	20:0	0.14 ± 0.03
iso-15:0	0.24 ± 0.08	20:1n-11+n-13	6.50 ± 1.38
anteiso-15:0	0.10 ± 0.04	20:1n-9	2.63 ± 0.34
15:0	0.31 ± 0.01	20:1n-7	0.76 ± 0.16
iso-16:0	0.12 ± 0.03	20:2n-6	0.18 ± 0.02
16:0	12.05 ± 1.44	20:3n-6	0.02 ± 0.02
16:1n-9	0.33 ± 0.04	20:4n-6	0.83 ± 0.13
16:1n-7	9.30 ± 0.65	20:3n-3	0.10 ± 0.02
16:1n-5	0.20 ± 0.03	20:4n-3	0.23 ± 0.03
iso-17:0	0.52 ± 0.19	20:5n-3	8.31 ± 0.46
anteiso-17:0	0.11 ± 0.03	22:0	0.07 ± 0.01
16:2n-4	0.15 ± 0.04	22:1n-11+n-13	3.07 ± 0.59
17:0	0.18 ± 0.02	22:1n-9	0.93 ± 0.14
16:3n-4	0.08 ± 0.03	22:1n-7	0.22 ± 0.05
17:1n-7	0.32 ± 0.02	22:2n-6	0.05 ± 0.03
16:4n-1	0.19 ± 0.09	21:5n-3	0.24 ± 0.05
18:0	2.12 ± 0.13	22:5n-6	0.13 ± 0.05
18:1n-13	0.69 ± 0.15	22:5n-3	0.80 ± 0.11
18:1n-9+n-11	23.54 ± 1.48	22:6n-3	2.88 ± 0.74
18:1n-7	4.34 ± 0.25	24:1n-9	0.75 ± 0.11
18:1n-5	0.60 ± 0.07	24:6n-3	3.75 ± 1.02
18:2n-6	0.33 ± 0.05	26:7n-3	0.69 ± 0.34
18:2n-4	0.13 ± 0.04	Others	3.18 ± 0.34
18:3n-6	0.02 ± 0.02		
18:3n-3	0.15 ± 0.03	Lipid content (%)[b]	30.5 ± 4.5

[a] Mean ± SD of the 5 individuals caught in the Pacific water and the Sea of Okhotsk around Hokkaido, Japan.

[b] Determined by gravimetry (% on the wet-weight base).

サメガレイ *Clidoderma asperrimum* の筋肉脂質における全-*cis*-5,8,11,14,17,20,23-ヘキサコサヘプタエン酸（26:7n-3）の存在

福田悠紀・安藤靖浩（北大院水）

北海道産サメガレイの筋肉脂質の脂肪酸を GC で分析したところ，炭素数 24 以上の領域に未知のピークが出現した。この成分を濃縮後，数種の誘導体に変換して GC-MS に供した結果，同成分は全-*cis*-5,8,11,14,17,20,23-ヘキサコサヘプタエン酸（26:7n-3）と同定された。総脂肪酸中の含有量は供試 5 個体の平均で 0.69±0.34%であった。サメガレイは，n-3 系および n-6 系超長鎖ポリエン酸の給源として一般的な高等動物の網膜，精子，ニシン油などには見られない 26:7n-3 を含む点で特徴的である。