Fatty Acids of Long Chain Length in Baltic Herring Lipids

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Abstract

The component fatty acids with carbon numbers exceeding 22 in flesh lipids of Baltic herring caught in May and September 1967 in the Turku archipelago have been studied. The total lipid content of the flesh of the herring was 3.5% on average in May and 7.2% on average in September. The fatty acids in the lipids were converted to methyl esters which were resolved and analyzed by urea adduct fractionation, thin layer chromatography and programmed temperature gas liquid chromatography (GLC). The lipids of the herring caught in May were found to contain 15 fatty acids with 24–32 carbon atoms, whereas the lipids of the herring caught in September were found to contain only nine fatty acids with 24–28 carbon atoms. The differences are probably due to nutritional factors. The long chain fatty acids in the lipids of the herring caught in September were isolated by preparative GLC and their structures were studied by UV spectroscopy before and after alkali isomerization, by IR spectroscopy and by GLC of their ozonization products. The identified acids were tetracosanoic, 15-tetracosenoic, 12,15,18,21-tetracosatetraenoic, 9,12,15,18,21-tetracosapentaenoic, 6,9,12,15,18,21-tetracosahexaenoic, 17-hexacosenoic, 11,14,17,20,23-hexacosapentaenoic, 8,11,14,17,20,23-hexacosahexaenoic and 4,7,10,13,16,19,22-octacosaheptaenoic acids. The proportion of the fatty acids containing over 22 carbon atoms in the lipids of fall herring is much higher than has been found earlier in the lipids of marine teleost fish; the reason may be due at least partly to differences in analytical methods.

Introduction

Most of the component fatty acids of fish lipids contain from 14 to 22 carbon atoms (1,2). The best known of the higher fatty acids in fish lipids is 15-tetracosenoic acid or nervonic acid (3). Japanese workers have found small amounts of C_{24}, C_{26} and C_{28} polyenoic acids in various fish lipids (4). Their analytical data do not, however, give a reliable picture of the structures of the acids because artifacts were probably formed in the isolation procedures (5). The occurrence of C_{24} polyenoic acids (6) and tetracosapentaenoic and tetracosahexaenoic acids (7,8) in fish lipids has been reported also by other investigators.

It is, however, possible that such long-chain fatty acids are more common in fish lipids than has been suspected for they easily remain unnoticed when isothermal gas liquid chromatography (GLC) is employed.

A long-chain polyenoic acid that has been isolated from flesh lipids of the Baltic herring (*Clupea harengus*) is *cis*-12, *cis*-15, *cis*-18, *cis*-21-tetracosatetraenoic acid (9,10a). Also several other long chain fatty acids such as tetracosatrienoic, tetracosapentaenoic, tetracosahexaenoic, hexacosatetraenoic and hexacosapentaenoic acid have been found in small amounts among the 70 component fatty acids of Baltic herring lipids. The presence of acids with even longer chains in herring lipids is indicated by the occurrence in the isolated lipids of both odd- and even-numbered higher normal alkanes up to C_{30} (10b).

The work described below deals with the isolation of fatty acids with carbon atoms exceeding 22 from Baltic herring lipids and their identification.

Experimental Procedures

The material examined consisted of flesh lipids from Baltic herring caught in the Turku Archipelago in the spring and autumn of 1967. The methods of isolation, the reagents employed and reference compounds were those described in an earlier investigation (10c).

The lipids were extracted from herring flesh by a modification of the method of Bligh and Dyer which, like the other analytical methods employed, was described in an earlier paper (10d). The lipids were treated with methanolic sulfuric acid (AOCS method Ce 2–66) to convert the fatty acids into their methyl esters. The methyl esters were resolved by precipitation with urea employing a modification of the method of Hashimoto et al. (11). The precipitation was performed partly at room temperature and partly at lower temperatures (see Table I). The procedures and operating conditions, when isothermal analyses of the ester mixtures were run at 210 C on a Barber-Colman M-10 gas chromatograph using a SE-30 column, were described earlier (10d). Most of the GLC analyses were, however, run on an Aerograph 204 Gas Chromatograph equipped with a hydrogen flame ionization detector and columns of Pyrex glass coils (⅛ × 72 in.) containing different packings. The flow rate of the carrier gas (nitrogen) was 30 ml/min. Runs on a column filled with siliconized Gas-Chrom P, 100–200 mesh, containing 1% SE-30 took place at 150–260 C employing a temperature rise of 2 C/min (long chain fatty acid methyl esters); those on a column filled with siliconized Gas-Chrom P, 100–200 mesh, containing 3% EGSS-X took place either isothermally at 185 C (long chain fatty acid methyl esters) or in the range 100–175 C with a temperature rise of 2 C/min (dimethyl alkanedicarboxylates produced by ozonolysis); and those on a column filled with Gas-Chrom P, 100–200 mesh, containing 10% Apiezon L grease took place in the range 100–175 C with a temperature rise of 2 C/min (short chain monocarboxylic acid methyl esters produced by ozonolysis).

Preparative GLC analyses were run on an Autoprep Model A 700 gas chromatograph using a catharometer as detector. The Pyrex glass coils containing the packing had an inside diameter of 0.9 cm and were 95 cm long. The packing was siliconized Chromosorb W, 80–100 mesh, containing 3% SE-30. The rate of flow of the carrier gas, helium, was 300 ml/min and the column temperature 220 C. The receivers were Pyrex glass flasks of the type described by Hardy and Keay (12), which were held in a bath at −40 C.

The fractions isolated by urea adduct formation and those isolated by preparative GLC were purified by passing them through a Florisil column and the purities of the resulting fatty acid methyl esters were checked by thin layer chromatography (TLC) on Silica Gel G plates using hexane-diethyl ether-glacial acetic acid (90:10:1 v/v/v) as solvent (10d).

Results

The fat content of a composite sample of the flesh of the Baltic herring caught in the spring 1967 was 3.5% and that of a composite sample of the flesh of the herring caught in the autumn of 1967 7.2%. The low fat content of the spring herring is in close agreement with the contents represented earlier, but the mean fat content of the autumn herring was clearly higher than the highest fat content, 6.7%, found earlier (10e). The latter fat content was found in herring caught early in November 1964.

The fatty acids in the lipids extracted from the two flesh samples were converted into their methyl esters which were fractionated by precipitation with urea. The fractions were then analyzed by TLC and GLC. The long chain fatty acids in the lipids of the spring herring (sample A) were fractionated and identified tentatively because the amounts of extracted lipids and fatty acids were very small. On the other hand, all the fatty acids containing more than the 22 carbon atoms that were present in the lipid samples from the autumn herring (sample B) were isolated by preparative GLC.

The experimental conditions and the amounts of various esters in the urea adduct fractions of the esters derived from the lipids of sample B are presented in Table I. The results of TLC analyses of samples of the various ester fractions on silica gel impregnated with silver nitrate are shown in Figure 1.

Illustrative chromatograms recorded on programmed temperature GLC of fatty acid methyl esters (sample B) on 1% SE-30 are shown in Figure 2. Chromatogram 2a refers to the total fatty acid methyl ester fraction before fractionation with urea, chromatogram 2b to the same sample after hydrogenation and chromatograms 2c and 2d to the first ester Fraction I and the unprecipitated ester Fraction IX, respectively.

TABLE I

Weights of Fatty Acid Ester Fractions Resolved by Precipitation With Urea After Conversion of the Fatty Acids of Lipids of Baltic Herring Caught in September (Sample B), Into Their Methyl Esters[a]

Fraction	Temperature, C	Added urea, g	Adjusted volume of solution, ml	Weight of ester fraction, g	Weight of ester fraction, %
I	22	20	280	1.860	8.3
II	22	10	280	2.531	11.2
III	22	7	280	2.872	12.8
IV	0	....	280	3.604	16.1
V	0	5	280	3.094	13.8
VI	0	....	175[b]	1.689	7.6
VII	−20	....	175	1.000	4.5
VIII	−20	....	75[b]	1.291	5.8
IX[c]				4.427	19.9
Total				22.368	100.0
Recovery					94.0

[a] The original sample of methyl esters weighed 23.704 g and was dissolved in methanol.
[b] Volume after concentration by evaporation under vacuum.
[c] Not precipitated by urea.

Relative retention times with respect to methyl oleate of fatty acid esters derived from samples A and B after they had been resolved by urea adduct fractionation and then subjected to isothermal GLC on 1% SE-30 and 3% EGSS-X columns are given together with their carbon numbers in Table II. The chain lengths of the esters were determined after hydrogenation and the number of double bonds were then determined from extrapolated plots of log retention time against carbon number for lower acids with different numbers of double bonds.

Long chain fatty acid esters in the fractions isolated by urea fractionation of the methyl esters derived from sample B were separated and isolated by preparative GLC. The purities of the isolated esters determined by analytical GLC are presented in Table III. Illustrative ultraviolet spectra of the separated $C_{24:6}$, $C_{26:6}$ and $C_{28:7}$ esters before and after alkali isomerization as described in AOCS method Cd 7–58 (see also Ref. 13) are shown in Figure 3.

Discussion

Ackman et al. (14), Sano and Murase (15) and Ueda and Shimonoseki (16) have employed urea adduct fractionation combined with GLC to separate methyl esters of fish oil fatty acids. Many-staged urea adduct fractionation at several temperatures

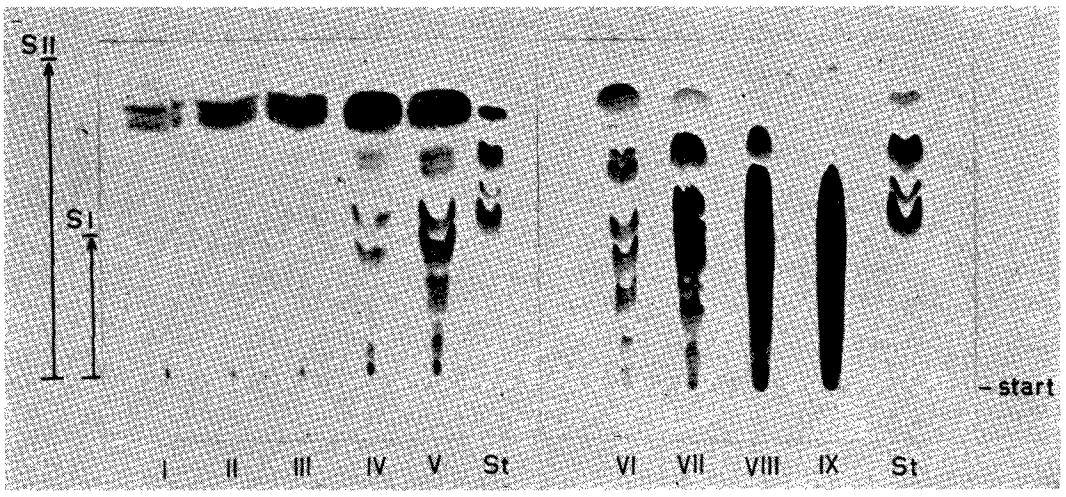

FIG. 1. TLC on Silica Gel G impregnated with silver nitrate of fractions of methyl esters of fatty acids isolated from flesh lipids (Sample B) of Baltic herring caught in September. The ester fractions were obtained by precipitating the esters from methanol with urea. Fractions I–VIII were successively precipitated ester fractions and Fraction IX consisted of the esters that were not precipitated (Table I). The standard ester mixture (St) contained methyl stearate, methyl oleate, methyl linoleate and methyl linolenate. Solvents: SI, a 95:5 (v/v) ethyl acetate-benzene mixture; SII, benzene.

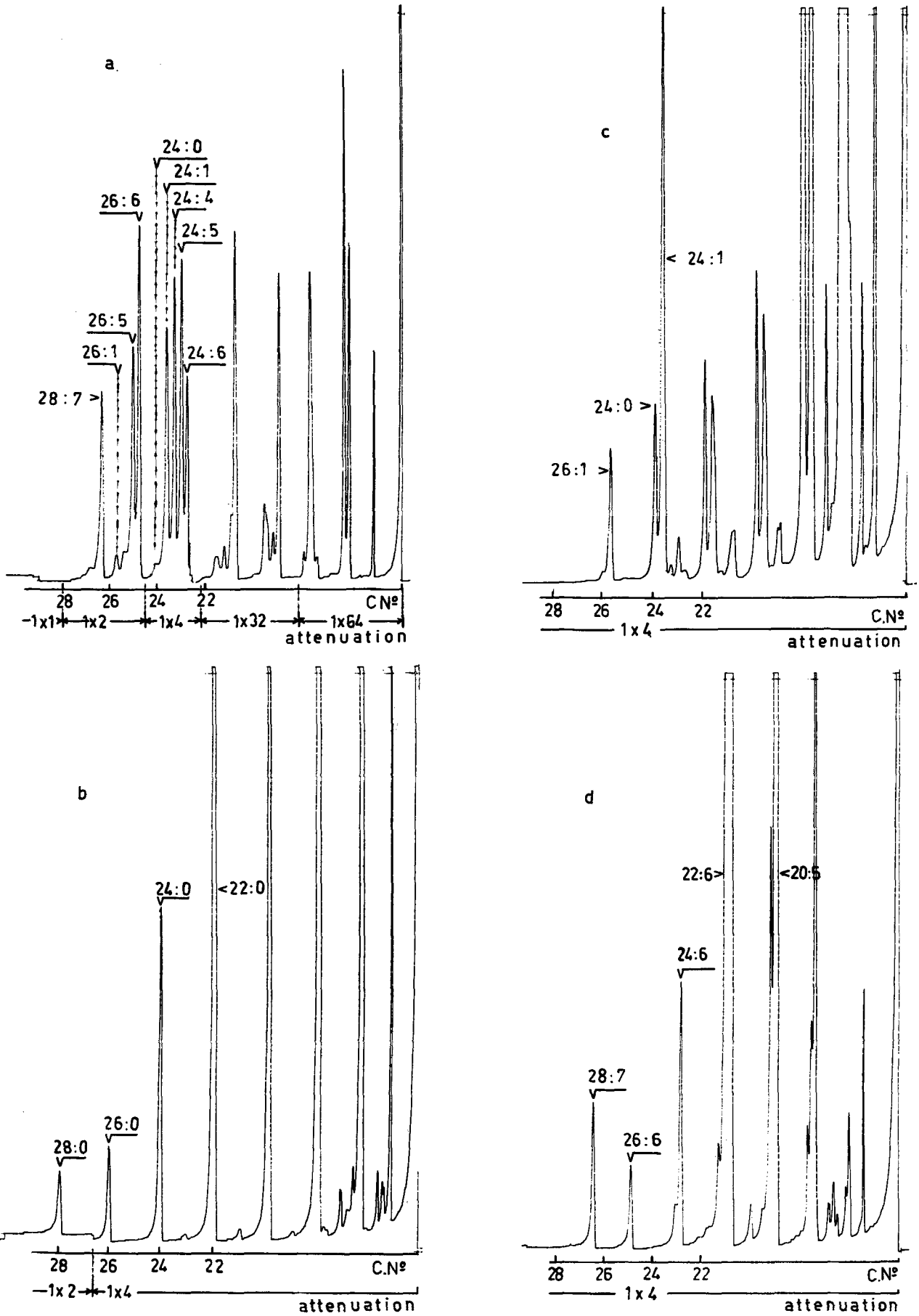

FIG. 2. Gas chromatograms of methyl esters of fatty acids isolated from flesh lipids (sample B) of Baltic herring caught in September. The esters were separated into fractions by precipitation with urea and the fractions were analyzed by GLC on 1% SE-30 employing a temperature rise of 2 C/min from 150 to 260 C. (a) Original mixture of fatty acid esters; (b) the same mixture after hydrogenation; (c) esters in first urea adduct precipitate (Fraction I); (d) ester Fraction IX.

TABLE II

Retention Times Relative to Methyl Oleate of Methyl Esters of Long-Chain Fatty Acids Isolated from Baltic Herring Lipids and their Carbon Numbers Determined by Gas-Liquid Chromatography on a 1% SE-30 Column at 210 C and on a 3% EGSS-X Column at 185 C

No. of carbon atoms[a]	1% SE-30 column at 210 C				3% EGSS-X column at 185 C		Acid
	Sample A		Sample B		Sample B		
	r[b]	Carbon No.	r[b]	Carbon No.	r[b]	Carbon No.	
24	.....	.....	7.40	24.00	4.80	24.00	24:0
24	6.60	23.63	6.70	23.69	5.45	24.78	24:1
24	6.03	23.38	6.03	23.36	10.0	26.85	24:4
24	5.60	23.12	5.50	23.08	11.8	27.46	24:5
24	.....	.....	5.05	22.80	13.6	28.00	24:6
25	7.82	24.20	.....	.....	.....	.....	25:5
26	.....	.....	12.6	25.69	10.4	27.06	26:1
26	11.5	25.43	.....	.....	.....	.....	26:4
26	10.6	25.20	10.3	25.07	22.6	29.78	26:5
26	9.90	24.97	9.40	24.79	24.0	30.90	26:6
27	14.8	26.20	.....	.....	.....	.....	27:5
28	22.2	27.48	.....	.....	.....	.....	28:4
28	20.0	27.15	.....	.....	.....	.....	28:5
28	18.0	26.83	.....	.....	.....	.....	28:6
28	15.8	26.43	15.6	26.39	51.0	32.73	28:7
30	37.7	29.19	.....	.....	.....	.....	30:5[c]
30	34.0	28.87	.....	.....	.....	.....	30:6[c]
32	64.0	30.84	.....	.....	.....	.....	32:6[c]

[a] Deduced from gas chromatogram of hydrogenated esters.
[b] r, Retention times.
[c] Number of double bonds tentatively deduced from extrapolated plots of log retention time versus number of carbon atoms.

and programmed temperature GLC proved to be effective methods for the fractionation and rapid analysis of the methyl esters of the long chain fatty acids in Baltic herring flesh lipids. By suitable variation of the concentration of urea and reduction of the temperature from room temperature to 0 C and from the latter temperature to −20 C, eight ester fractions were precipitated and a ninth fraction was recovered from the final filtrate (Table I). The weights of esters in the first eight fractions were fairly equal, but the unprecipitated esters (Fraction IX) amounted to about one fifth of the total esters. The overall yield of esters, 94%, may be considered good.

To follow the course of the fractionation of the esters by urea adduct formation, samples were analyzed by TLC on Silica Gel G impregnated with silver nitrate (Fig. 1) which resolves esters according to degree of unsaturation and by programmed temperature GLC on a SE-30 column (Fig. 2a–2d) which resolves esters according to chain length. It was established that there were esters of fatty acids containing more than 22 carbon atoms in all nine fractions, although certain trends were noted in their distribution.

The esters of saturated and monoenoic acids were concentrated in the first three urea adduct precipitates so that most of the esters of the saturated fatty acids were present in Fraction I, whereas the esters of the monoenoic acids precipitated in the order of decreasing chain length. Thus, for example, the ester of the C_{26} monoenoic acid was present only in Fraction I (Fig. 2c).

It was observed that the longest of the esters of long chain polyenoic acids that contained an equal number of double bonds was precipitated as an urea adduct before the others; this has been found earlier to be the rule for esters of polyenoic fatty acids (10f). As the acids in question are mostly of the linolenic acid type (terminal chain ω3; Table III) (10g), the adduct formation depends on the length of the chain from the carboxyl group to the first double bond. In Fraction VI this length was 11–12 carbon atoms, in Fractions VII–VIII 7–9 carbon atoms, and in Fraction IX, 4–5 carbon atoms. The unprecipitated long chain fatty acid esters (Fraction IX) were esters of $C_{28:7}$, $C_{26:6}$ and $C_{24:6}$ acids. In addition, this fraction contained relatively large amounts of esters of $C_{22:6}$ and $C_{20:5}$ acids (Fig. 2d).

The esters of the long chain polyenoic acids change readily when they are resolved by preparative GLC, although the changes can be largely avoided by a proper choice of experimental conditions. The extents of the changes are revealed by the purities of the isolated esters shown in Table III.

As seen in Table II, the lipids of the Baltic herring caught in spring and autumn differed in the nature of their component long chain fatty acids. Particularly noteworthy is that the fatty acids of the spring herring were much more varied than those of the autumn herring. Whereas the latter fatty acids included nine long chain acids from C_{24} to C_{28}, the

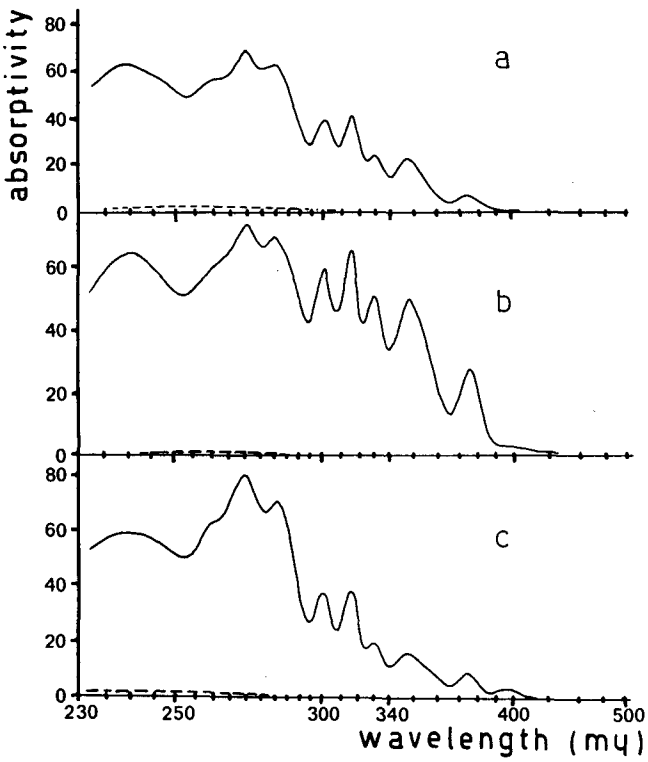

FIG. 3. Ultraviolet absorption spectra of methyl esters: a, tetracosahexaenoic acid; b, hexacosahexaenoic acid; and c, octacosaheptaenoic acid before (- - - -) and after (——) alkali-isomerization.

TABLE III

Structural Data for Long Chain Fatty Acids Isolated From Flesh Lipids of Baltic Herring Caught in September (Sample B)

No. of carbon atoms	No. of double bonds	Chain lengths of acids produced on ozonolysis		Locations of double bonds (from the carboxyl carbon)	Purity of methyl ester of parent acid, %	Per cent of total fatty acids[a]
		Monocarboxylic acid	Dicarboxylic acid			
24	0	..	....	....	95	0.1
24	1	9	15	15	94	1.1
24	4	3	12	12,15,18,21	91	1.3
24	5	3	9	9,12,15,18,21	95	1.4
24	6	3	6	6,9,12,15,18,21	94	0.9
26	1	9	17	17	100	0.1
26	5	3	11	11,14,17,20,23	96	0.5
26	6	3	8	8,11,14,17,20,23	100	0.7
28	7	6	4	4,7,10,13,16,19,22	91	0.4

[a] In the original mixture of unfractionated methyl esters.

former fatty acids included 15 long chain fatty acids from C_{24} to C_{32}. This difference is suspected to be due to differences in available food in different seasons and to the fact that the herring suffers from a shortage of food in the spring. The difference in the fatty acid composition in the spring and autumn herring may, on the other hand, be due to a different selective mobilization of the fatty acids which is reflected in the composition of the deposited fatty acids. The fatty acids $C_{25:5}$, $C_{26:1}$, $C_{27:5}$, $C_{28:4}$, $C_{28:5}$, $C_{28:6}$, $C_{28:7}$, $C_{30:5}$, $C_{30:6}$ and $C_{32:6}$ have not been previously found in fish oils.

The proportion of long chain component fatty acids was relatively high in the lipids of the autumn herring (Table III). As calculated from the gas chromatogram of the methyl esters of the hydrogenated fatty acids, the C_{24} acids amounted to 5.6%, the C_{26} acids to 2.5% and the C_{28} acids to 1.1% of the total acids. As percentages of the fresh fish flesh weight, the proportion of C_{24} acids was 0.40%, that of the C_{26} acids 0.18% and that of the C_{28} acids 0.08%. In an earlier study the C_{24} acids were found to represent 5.2% of the fatty acids in the lipids of Baltic herring caught in the winter (10h). Thus the content of C_{24} acids is much higher in lipids of herring caught in autumn and winter than has been found in the lipids of marine teleost fishes, where it varies from 1–2% of the total fatty acids (3,8).

Analytical data for nine long chain fatty acids isolated from the autumn herring lipids are shown in Table III. The chain lengths were determined by GLC after hydrogenation and the numbers of double bonds were deduced from retention times of the unhydrogenated fatty acid methyl esters and the ultraviolet spectra of the esters after alkali-isomerization. The locations of the double bonds counted from the carboxyl group based on these data and GLC data for samples oxidized with ozone are given in the fifth column. These reveal that the polyenoic acids have the methylene-interrupted double bond structure typical of fish oil fatty acids. The IR spectra of the isolated acids showed that, like other natural fatty acids, these acids have the *cis* configuration.

Table III further shows that the previously assumed location of the double bond at position 15 in tetracosenoic acid is correct (10i). The double bond in hexacosenoic acid is at position 17, and hence the terminal chain contains nine carbon atoms ($\omega 9$) like that in tetracosenoic acid and the acid is of the oleic acid type (17). The isolated polyenoic acids are of the linolenic acid type ($\omega 3$) with the single exception of octacosaheptaenoic acid, which is of the linoleic acid type ($\omega 6$). The octacosaheptaenoic acid is also exceptional in that, as revealed by the ultraviolet absorption spectrum of the alkali-isomerized sample, it contains seven double bonds, whereas at most six double bonds have previously been found in the component fatty acids of fish oils.

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[Received March 27, 1969]