Analysis of Very Long Chain Polyenoic Fatty Acids by High Performance Liquid Chromatography and Gas Chromatography–Mass Spectrometry with Chemical Ionization

Preparative high performance liquid chromatography of fatty acid methyl esters from herring on a cation-exchange column containing silver ions is described. The polyenoic fatty acid fraction obtained was further separated on a polar capillary gas chromatographic column. The structures of the individual methyl esters were identified by mass spectrometry with chemical ionization, comparing the m/z 109 and m/z 151 ions characteristic of ω-3 and ω-6 double bonds. The even polyenoic acids as large as 32:6ω-3, the odd acids (21:5ω-3, 21:6ω-3, 25:5, and 27:5), and a minor component (28:7ω-3, previously undetected in marine fish) were identified.

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Studies of saturated very long chain fatty acids (chain length > C_{22}) have been published (1–3). The occurrence in biological materials of very long chain polyunsaturated fatty acids (VLCPUFA) that have more than two unsaturated bonds has also been reported. In 1970, the presence of VLCPUFA in herring (*Clupea harengus*) was first mentioned (4), and within 15 years, these compounds were detected in other vertebrate tissues, such as retina and the human brain with Zellweger Syndrome and other similar diseases (1,5,6). VLCPUFA having chain lengths as long as C_{30} were also found in marine sponges (7).

A common problem in the analysis of these fatty acids is the separation and identification of the components, especially the minor ones. For example, VLCPUFA represent 0.01–0.1% of the total fatty acids in the retina. Various procedures for enriching fatty acids with VLCPUFA, such as silver nitrate–impregnated thin-layer chromatography (TLC), have been tested. Ion-exchange high performance liquid chromatography (HPLC) has allowed the separation of fatty acids according to the number of unsaturated bonds (8). Ion-exchange HPLC has been applied only to fatty acids having chain lengths < C_{22}.

Pure VLCPUFA or their mixture are not commercially available. Therefore, the focus of this study was on the acquisition of a mixture of polyenoic fatty acids enriched with VLCPUFA.

EXPERIMENTAL

Fatty acid methyl esters (FAME) were prepared by the basic transesterification of the total lipids that had been extracted from fish using a mixture of chloroform and methanol (9). Oxygen was removed from the HPLC mobile phases — methanol (solvent A) and acetonitrile (solvent B) — by sparging with helium. Butylated hydroxytoluene was added to other solvents (70 mg/L). All the vessels and the rotary evaporator were flushed with nitrogen before use.

Silver-ion-exchange HPLC (8): Preparative HPLC was performed in a gradient LC system G-I (Shimadzu, Tokyo, Japan) with two LC-6A pumps (4 mL/min), an SCL-6A system controller, an SPD UV detector (208 nm), an SIL-1A sample injector, and a C-R3A data processor. A 30 cm × 7.9 mm semipreparative column packed with a strongly acidic cation-exchange resin (–SO_3H groups, SCR-101H, 10-μm spherical particles, Shimadzu, Tokyo, Japan) was employed. After 8.1 mg FAME was injected, a linear gradient was performed from 0% B to 50% B over 30 min. The individual classes of FAME (from zero to four [and more] unsaturated bonds) were collected, evaporated, and weighed.

Gas Chromatography–Mass Spectrometry–Chemical Ionization (GC–MS–CI): Fraction IV from the ion-exchange HPLC of FAME (Figure 1) was separated and identified using a Finnigan MAT 1020 B (Finnigan MAT, San Jose, California). A 15 m × 0.25 mm Supelcowax 10 column with a film thickness of 0.25 μm (Supelco, Gland, Switzerland) was used. Injection temperature was 100 °C. The temperature program was as follows: 100 °C for 1 min, then 100 °C to 230 °C over 6.5 min, 230 °C to 280 °C over 25 min, and 280 °C for 10 min. The carrier gas was helium at a linear flow rate of 70 cm/s. Methane was used as the chemical-ionization reagent. The spectra were scanned within *m/z* 60–600.

RESULTS AND DISCUSSION

The greatest problem is choosing a suitable natural material. Several practical sources exist (for example, sponges) that contain VLCPUFA with unsaturated bonds separated by more than one methylene group (7). Other sources are mammalian sperm and testes, the retina of vertebrates, and Zellweger Syndrome–afflicted human brain. The first two of these sources are not usually available, and the excision of the retina and brain re-

quires surgical knowledge and skill. For these reasons, the herring was used, which could be bought frozen (−18 °C) in the supermarket.

A distinct separation of methyl esters of the saturated, monoenoic, and dienoic acids can be seen in Figure 1. Trienoic acids could also be separated. A different situation, however, occurred with tetra-, penta-, hexa-, and heptaenoic acids. The chromatographic method described in the literature (8) easily separated all the above-mentioned acids except for the heptaenoic acids, which were not present in the sample. On the basis of the published data (5,6) for the separation of FAME using TLC on silica gel to obtain long and short chains, we assume that, in our case, an opposite phenomenon occurs. Because of a great variability in the chain length and as a result of a great number of CH_2 groups in long chains, a more-rapid elution occurs. Thus, the hexaenoic VLCPUFA elute with the pentaenoic PUFA. In spite of that, the method is suitable for quickly obtaining a fraction enriched with the tetra-, penta-, hexa-, and heptaenoic unsaturated acids.

The correlation between the detector response and the actual mass of the polyenoic fraction was good. As measured by detector response, the polyenoic fraction was 19.3% of the starting material. The measured mass of the isolated polyenoic fraction was 1.9 g, which was 23.5% of the mass of the starting material.

Figure 2 shows the GC–MS pattern of fraction IV. On the basis of our previous experience (10) with the chromatography of diglycerides that have mostly 39 carbon atoms and six unsaturated bonds (for example, dilinolenoyl diglyceride), a polar capillary column was used. A GC–MS–CI record of the ions *m/z* 109 and 151 was obtained that could be used to determine the position of unsaturated bonds (11). Baseline separation was obtained for those compounds using the polar capillary column, whereas the C_{30} acids were eluted effectively as one peak from a nonpolar capillary column (12). On the basis of the scans of the ions *m/z* 109 and 151, the positions of the unsaturated bonds, such as ω-6, were determined in peaks D and X; the other FAME had these bonds at position ω-3. This structure was also confirmed by the spectra of the individual peaks; the spectrum of peak Y is shown in Figure 3. The proportion of the intensities of the ions *m/z* 109–151 was >1, which confirmed the structure ω-3. In the original work with the herring (4), this position isomer was not found; on the contrary, only the ω-6 isomer was detected, and it was reported as a minor component. The equivalent chain length (ECL) values and the relative percentages of the identified methyl esters are given in Table I. The ECL values increased as the fractional chain length (FCL) values gradually decreased. For pentaenes with unsaturated bonds in position ω-3, the FCL values decreased from 1.76 (20:5ω-3) to

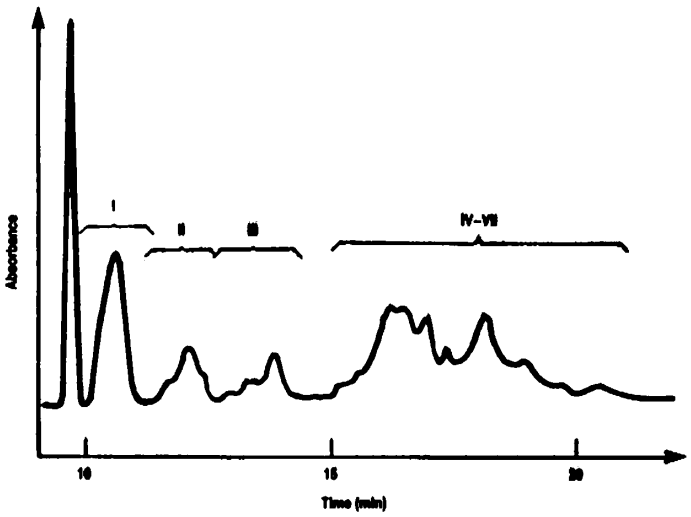

FIGURE 1: Ion-exchange HPLC of FAME from herring. Chromatographic conditions are given in the text.

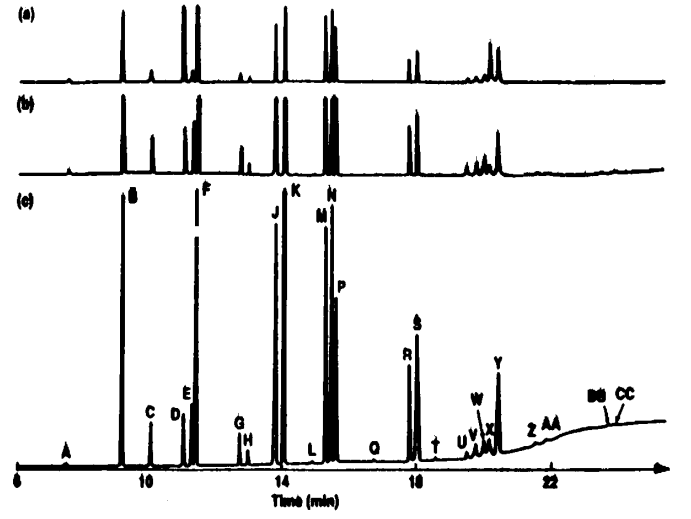

FIGURE 2: GC–MS–CI of FAME from fraction IV (ion-exchange HPLC chromatogram shown in Figure 1). Shown are (a) *m/z* 151, (b) *m/z* 109, and (c) the total ion chromatogram. Chromatographic conditions are given in the text.

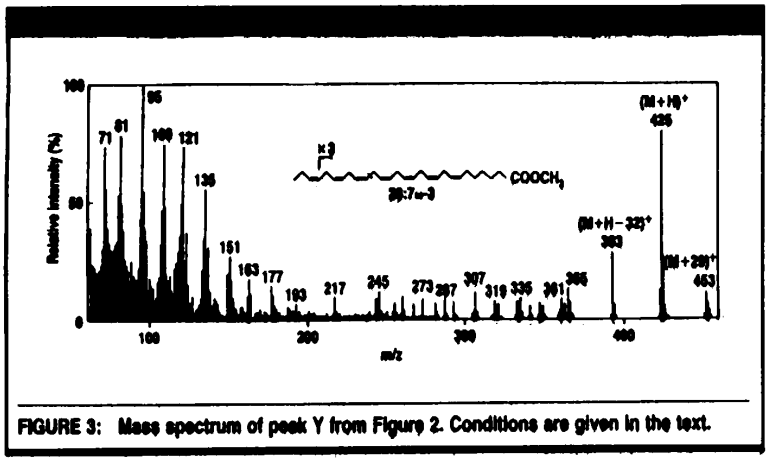

FIGURE 3: Mass spectrum of peak Y from Figure 2. Conditions are given in the text.

1.70 (30:5ω-3). This behavior could be explained by a decreasing contribution of the unsaturated-bond polarity with the increasing chain length (the 20:5 pentaene has a much lower number of nearly nonpolar CH_2 groups compared with the 30:5 pentaene). We were not able to identify peaks L and BB, but we assumed that they were FAME.

CONCLUSION

The enriched PUFA fraction, including VLCPUFA, will be investigated further using reversed-phase HPLC. The use of the comparatively simple method of ion-exchange HPLC linked with GC–MS–CI yielded the following new results. The odd-numbered PUFA were identified (peaks G, H, Q, and T in Figure 2c) as well as the even-numbered PUFA. Also, for the first two peaks, the corresponding positions of the unsaturated bonds were determined. The unusual PUFA (18:5ω-3, 21:6ω-3, and 28:7ω-3) were shown to be identifiable after preconcentration. The last of the above-mentioned acids was detected in fish oil for the first time. However, 28:7ω-6 was found to be only a minor component. The use of GC–MS–CI enables a rapid separation and identification of PUFA.

TABLE I: Fatty Acid Composition*			
Fatty Acid	Peak**	ECL	%+
16:4w-3	A	17.55	0.09
18:4w-3	B	19.55	6.39
18:5w-3	C	20.15	1.00
20:4w-6	D	21.12	1.17
20:4w-3	E	21.58	1.44
20:5w-3	F	21.76	32.43
21:5w-3	G	22.73	0.74
21:6w-3	H	22.99	0.26
22:5w-3	J	23.72	5.79
22:6w-3	K	24.03	25.97
24:4w-3	M	25.56	5.68
24:5w-3	N	25.74	6.09
24:6w-3	P	25.98	3.91
25:5++	Q	26.72	0.04
26:5w-3	R	27.72	2.16
26:6w-3	S	27.96	3.04
27:5++	T	28.70	0.04
28:4w-3	U	29.55	0.17
28:5w-3	V	29.70	0.43
28:6w-3	W	29.96	0.57
28:7w-6	X	30.17	0.43
28:7w-3	Y	30.31	1.97
30:5w-3	Z	31.70	0.08
30:6w-3	AA	31.94	0.09
32:6w-3	CC	33.94	0.04

* determined as FAME by means of capillary GC-MS with chemical ionization
**peaks shown in Figure 2c
+percentage of total fatty acids analyzed
++identified by retention time

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