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Molecular Species of Fish Muscle Lecithin*[1],*[2]

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A satisfactory separation of the molecular species of fish muscle lecithin was made by modifying the lecithin into diglyceride acetate for high performance reversed phase liquid chromatography (HPLC). The molecular species of each chromatographic peak was determined by fatty acid composition and by total acyl carbon number analysis subsequent to separation, using a thin layer chromatographic technique with silver nitrate impregnated silica gel plates (Ag^+-TLC). After plotting the relative retention time (RRT) of each molecular species from soybean, egg yolk, chum salmon, big-eyed tuna, Alaska pollack, and carp muscle lecithins semilogarithmically against the total acyl carbon number or the number of total double bonds of each molecular species, a set of parallel straight oblique lines was obtained. Thus we can express the molecular species in matrix relation by giving a variable integer x for the acyl carbon number and a variable integer y for the number of double bonds of each fatty acid in the molecular species. By using the correlations between the RRTs and the corresponding molecular species on semilogarithmic plots, it was concluded that an identification of molecular species from fish lipids could be done by RRTs from HPLC.

Lecithin has been studied by many workers concerning lipid changes during storage or processing of fish products.[1] However, these works were done mainly by analyzing the lipid composition or fatty acid composition, which might not be enough to elucidate the mechanisms of deterioration of foods caused by the changes in lipid. Therefore, a new method for determining molecular species of lecithin is necessary not only in the field of biochemistry, but also in the field of food chemistry.

Recently, HPLC method has been introduced by ŌSHIMA *et al.**[5] in order to analyze fish phospholipids which might have the most complicated molecular species composition among biological sources.

The purpose of this study was to develop the method of ŌSHIMA *et al.**[5] to determine the individual molecular species of fish phospholipid.

Experimental

Preparation of Lecithin

Total lipids were obtained from the fish muscle tabulated in Table 1, according to the method of BLIGH & DYER. Soybean lecithin was purchased from Wako Pure Chemical Industries, Ltd., Osaka, and egg yolk lecithin was kindly supplied by Asahi Chemical Industry Ltd., Tokyo. These total lipid and crude lecithin were subjected to a silica gel column chromatography which has been done successfully by LANDS *et al.*[2] for the purification of lecithin. Eluates were monitored by TLC. And lecithin of more than 95% purity were collected.

Preparation of Diglyceride Acetate from Lecithin

Pure lecithin was dephosphorylized with phospholipase C (*Clostridium perfringens*), according to the method of RENKONEN.[3] Diglyceride was prepared by preparative TLC from the dephosphorylized lipid. The developing solvent was *n*-hexane/diethyl ether (1 : 1, v/v).

Acetylation was performed by adding an appropriate amount of acetic anhydride to the solution of diglyceride in pyridine, and by standing it for 12 h

*1 Molecular Species of Marine Animal Lipid-I.
*2 Presented at the Japanese Society of Scientific Fisheries Meeting, Tokyo, April, 1982.
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*5 T. ŌSHIMA, S. WADA, and C. KOIZUMI: Presented at the annual meeting of the Japanese Society of Scientific Fisheries, Tsu, Japan, October, 1981.

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Table 1. Fish examined.

Species	Mean body length and weight	Locality of catch	Date of catch
Chum salmon (Summer)*[1] *Oncorhynchus keta*	65 cm, 3.5 kg, (1)*[2]	The offing of Akkeshi, Hokkaido	June 1980
Chum salmon (Fall)*[1] *Oncorhynchus keta*	72 cm, 4.5 kg, (1)	The Moheji River, Hokkaido	Nov. 1981
Big-eyed tuna *Parathunnus obesus*	110 cm, 20 kg, (1)	Purchased from the Market.	—
Alaska pollack *Theragra chalcogramma*	44 cm, 610 g, (10)	The Uchiura Bay, Hokkaido	Dec. 1981
Carp *Cyprinus carpio*	23 cm, 175 g, (5)	Cultured	Sep. 1980

*[1] Male. *[2] Nos. of individual used.

at room temperature.[4] The resulting diglyceride acetates were purified by the method of preparative TLC by using the solvent *n*-hexane/diethyl ether (75: 25, v/v). Finally, they were filtered through a 0.45 μ type FP-45 Fluoropore filter (Sumitomo Electric Industry, Ltd., Osaka) and subjected to HPLC.

HPLC Fractionation of the Molecular Species of Diglyceride Acetate Derived from Lecithin

The diglyceride acetates were fractionated into major molecular species on twin 8×250 mm LiChrosorb RP-18 columns wich were connected in series. A Hitachi Liquid Chromatograph Model 638-50 (Hitachi Ltd., Tokyo) equipped with a Shodex RI detector Model SE-11 (Showa Denko Ltd., Tokyo) was used. The eluting solvent used was isopropanol/acetone/methanol/acetonitrile (1: 1: 3: 4, v/v). Diglyceride acetates were dissolved into 5 volumes of tetrahydrofuran, and 25 μl of these solutions were applied to the column under room temperature (lower the better) and at a flow rate of 1.5 m*l*/min.

Identification of Molecular Species of Each Peak on HPLC

Peaks on HPLC chromatograms were numbered in sequence of elution. The fatty acid composition of each collected predominant peak was analized by gas chromatography. The analytical conditions for fatty acid were as follows.

Gas chromatograph: Hitachi 063, Column: Unisole 3000 (Gasukuro Kogyo Ltd., Tokyo), glass column 0.2×200 cm, Column temp.: 220°C, Detector: FID, Detector temp.: 250°C, Injection temp.: 280°C, Carrier gas: N_2, flow rate: 20 m*l*/min.

Methyl esters of fatty acids were prepared according to the method of CHRISTOPHER and GLASS described by PREVOT and MORDRET.[5] An aliquot amount (less than 20 mg) of lipid was dissolved in 1 m*l* *n*-hexane and 0.2 m*l* of methanolic 2N-NaOH solution was added. After shaking this mixture, it was stand for 20 s under 50°C and then 0.2 m*l* of methanolic 2N-HCl solution was added. The *n*-hexane layer was collected and then concentrated. Methyl esters prepared were subjected to a gas liquid chromatographic analysis.

The small peaks which have critical pairs were first subjected to Ag^+-TLC. The developing solvent used was benzene/diethyl ether (8: 2, v/v). The band obtained by Ag^+-TLC were then eluted with diethyl ether containing dotriacontane which was used as an internal standard, and then applied to fatty acid analysis and total acyl carbon number analysis. The analytical conditions for total acyl carbon number analysis were as follows.

Gas chromatograph: Hitachi 063, Column: OV-101 (Gasukuro Kogyo Ltd., Tokyo), steel column 0.3×50 cm, Column temp.: 300–330°C, programed as 1°C/min, Detector: FID, Detector temp.: 340°C, Injection temp.: 345°C, Carrier gas: N_2, flow rate: 60 m*l*/min.

Hydrolysis of Diglyceride Acetate Derived from Lecithin by Pancreatic Lipase

Fraction of the molecular species of 16: 0 in position 1 and 22: 6 in position 2 was collected by HPLC. This lipid (less than 5 mg) was then suspended by shaking vigorously in a mixture of 1 M tris-HCl buffer, pH 8 (1 m*l*), 2.2% $CaCl_2$ (0.1 m*l*), and 0.05% taurocholic acid sodium salt (0.25 m*l*) at 40°C for 1 min. Then, 40 mg of pancreatic lipase (Calbiochem, San Diego, Calif. 92112) was added to the mixture and the reaction was proceeded for 4 min at 40°C by shaking it

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virgorously. The reaction was stopped by adding 1 m*l* of ethanol and 1 m*l* of 6 N-HCl. The hydrolysate was extracted with diethyl ether and purified by using a preparative TLC with *n*-hexane/diethyl ether/formic acid (80: 20: 2, v/v) as developing solvent.

Results

Figs. 1–3 show chromatograms of diglyceride acetate derived from each lecithin sources on HPLC. Chromatography was regularly completed in about 2 h. The diglyceride composition of each collected predominant peak was easily determined by fatty acid analysis, as shown in Table 2. This table shows the results on big-eyed tuna lecithin as an example. Peak number 7 in Fig. 3-E is obviously ascribed to the diglyceride acetate composed of 16: 0 and 22: 6. We have considered that 22: 6 is bound in position 2 of the molecule since this peak was the most predominant, and it is said that highly unsaturated fatty acids such as 20: 5 or 22: 6 are usually dominantly bound in position 2.[8–10] In addition, after pancreatic lipase hydrolysis, only a trace amount

Table 2. Determination of lecithin molecular species of major component*

Fatty acid	Peak number		
	5	6	7
15: 0			trace
16: 0	25.6	48.4	53.8
17: 0			trace
18: 1	25.9	4.1	trace
20: 4			trace
20: 5	23.3	3.7	trace
22: 4			trace
22: 6	25.1	43.8	46.2
Molecular species	18: 1 / 20: 5	22: 6 / 16: 0	16: 0 / 22: 6

* Example of big-eyed tuna.

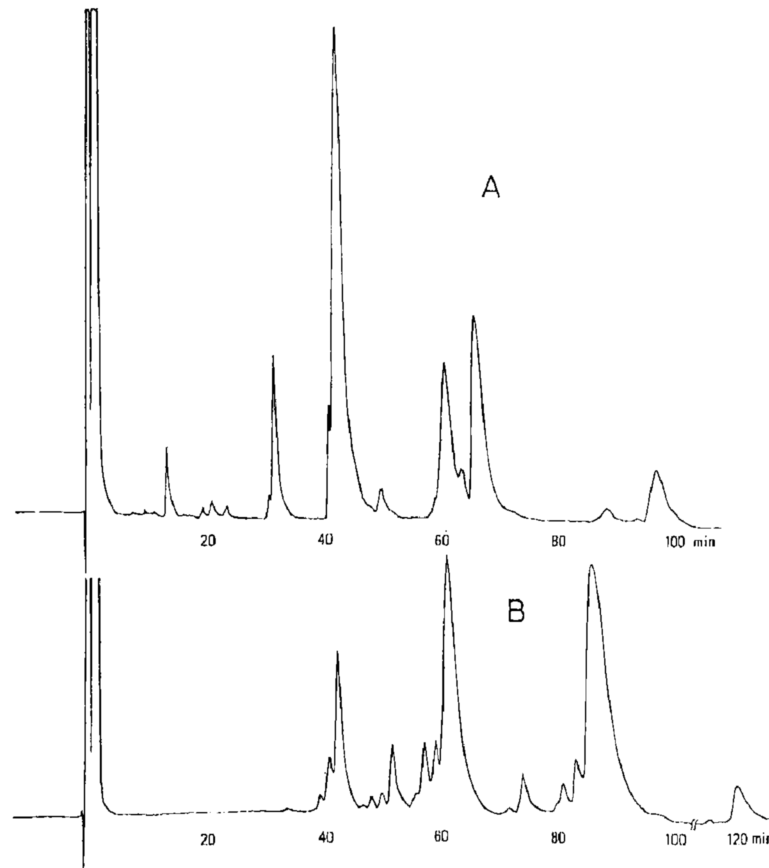

Fig. 1. HPLC chromatograms of soybean and egg yolk lecithin.
A: Soybean. B: Egg yolk.

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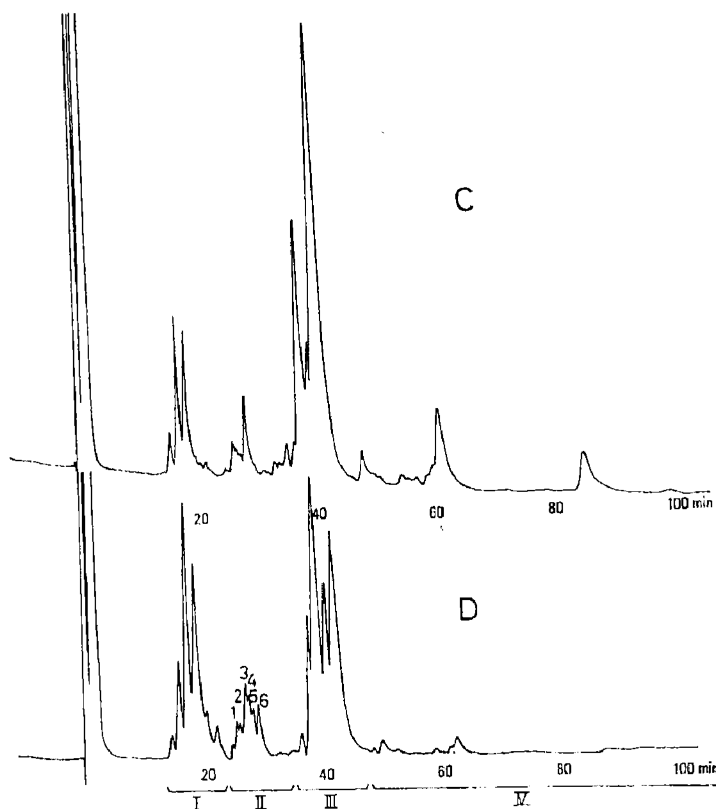

Fig. 2. HPLC chromatograms of chum salmon muscle lecithin.
C: Captured in summer. D: Captured in fall.

of 22: 6 was detected in the free fatty acid fraction (this fraction represents the fatty acid in position 1), although more than 70% of the lipid was hydrolized (determined by a densitometric method). Peak number 6 had the same combination as that in peak number 7, whereas 18: 1–20: 5 were considered as contaminants of peak number 5. Nevertheless in this case, 22: 6 was considered to be bound in position 1 in the molecule. Peak number 5 had an almost even amount of fatty acids of 16: 0, 18: 1, 20: 5 and 22: 6. Among these fatty acids, 16: 0 as well as 22: 6 were regarded as contaminants from peak number 6, considering that peak number 6 is larger than peak number 5. It was concluded that peak number 5 was the combination of 18: 1 and 20: 5. Molecular species from other biological sources were also determined in the same manner. The small peaks which have critical pairs were first subjected to Ag^+-TLC and separated according to their degree of unsaturation. In most of the samll peaks in the first half of the HPLC chromatograms of fish lecithin, only one band appeared on Ag^+-TLC plate. The complex combinations of molecular species were identified in the following manner. An example of determination of molecular species in the small peaks with critical pairs appeared in the first half of the HPLC chromatogram of chum salmon (captured in fall) is shown in Table 3 (also see Fig. 2-D). Small peak number 1 is obviously a combination of 20: 5 and 16: 1. Small peak number 2 is also a combination of 20: 5 and 16: 1 with 10% unidentified contaminants. The difference in retention time on HPLC between these two peaks was attributed to the differences in binding position of the fatty acid. It was considered that small peak number 1 had 20: 5 in position 1, whereas small peak number 2 had it in position 2 in the molecule, since molecular species which have a highly unsaturated fatty acid in position 2 are likely to elute later than the one which has the same fatty acid in position 1. Small peak number 3 is a combination of 20: 5 in position 1 and 14: 0 in position 2, and

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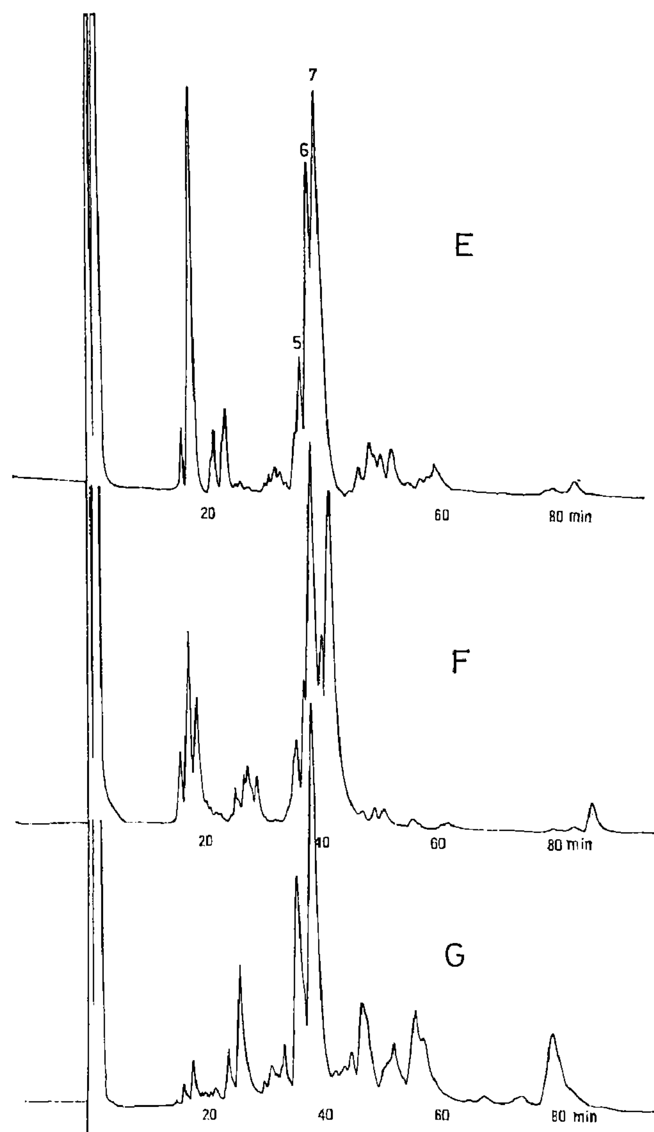

Fig. 3. HPLC chromatograms of big-eyed tuna, Alaska pollack and carp muscle lecithin.
E: Big-eyed tuna. F: Alaska pollack. G: Carp.

also 16: 1 in position 1 and 20: 5 in position 2 since this peak is composed of two combinations of total acyl carbon numbers of 34 and 36 respectively. However, the latter molecular species, i.e. 16: 1–20: 5, are considered to be contaminants from the previous peak. From the fatty acid composition and total acyl carbon number, three molecular species were presented in small peak number 4, i.e. combination of 14: 0 in position 1 and 20: 5 in position 2 for 63.3%; combination of 16: 1 in position 1 and 20: 5 in position 2 for 5.7%; and combination of 22: 6 in position 1 and 16: 1 in position 2 for 31.0%. Among these molecular species, 16: 1–20: 5 can be considered as contaminants from the two previous peaks. In small peak number 5, three molecular species, i.e. 14: 0 in position 1 and 20: 5 in position 2; 22: 6 in position 1 and 14: 0 in position 2; and 16: 1 in position 1 and 22: 6 in position 2 were identified in the same manner as that in small peak number 4. In this case, molecular species of 14: 0–20: 5 was considered to be a contaminant from the previous peak. In the case of small peak number 6, molecular species of 14: 0 in

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