Bull. Fac. Fish. Hokkaido Univ.
29(4), 378-385. 1978.

Molecular Species of Fish Muscle Lipids

I. Molecular species of triglyceride and phosphatidylcholine of sardine and rainbow trout

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Abstract

In order to determine the molecular species of triglyceride and phosphatidylcholine of fish muscle lipids directly, fractionation using silica gel impregnated with silver nitrate thin layer chromatography and fractionation using polar siloxane column (Silar-5CP) gas liquid chromatography was examined after conversion to their corresponding diglyceride acetate and TMS ether of diglyceride. Poor chromatograms—overlapping and low resolution—were obtained from the fish muscle triglyceride and phosphatidylcholine. On the other hand, the analysis of triglyceride and phosphatidylcholine from soybean lipids seemed to be very successful using both methods. It was presumed that the complexity of combination of the molecular species of fish muscle triglyceride and phosphatidylcholine causes difficulty, and that there is no other way except calculation depending upon its probability to determine their molecular species.

For this, a method for calculating the moleculer species combination of triglyceride and phosphatidylcholine by probability (based on a digital computer program using operational language FORTRAN 7000) is presented. Qualitative and quantitative results of gas liquid chromatographic analysis of fatty acids after their degradation by Grignard's reagent from C-1,3 position of triglyceride molecules, and after their stereospecific phospholipase A hydrolysis from C-2 position of phosphatidylcholine were used as main input data.

The results showed that for the triglyceride, di and tri component combinational types occupied nearly 100%, and among those $C_{16:0}/C_{20:5}$, $C_{16:0}/C_{22:6}$ were the relatively prominent combinations in sardine and $C_{16:0}/C_{18:1}$, $C_{18:1}/C_{16:0}$ combinations in rainbow trout. For the phosphatidylcholine, both sardine and rainbow trout contained $C_{16:0}/C_{22:6}$ combination as their main components.

Introduction

In spite of the increasing necessity to study the molecular species of fish muscle lipids, no study has been made concerning marine oil except the report of Bottino[1] who had studied about the composition of triglyceride (TG) of whale oil and cod liver oil by the combination of a silver nitrate impregnated thin layer chromatography (TLC) and gas liquid chromatography (GLC) method. A direct method by means of TLC, GLC and a new liquid phase of GLC i.e., Silar-5CP was examined to test whether it can fractionate the fish muscle lipid molecular species. On the other hand, an indirect method by means of computer calculation was examined. This

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indirect method was recently introduced by Koman *et al.*[2] and Wathelet *et al.*[3]. In this study, it was observed that this indirect method i.e., numerical method using a computer was the only applicable method for determining the molecular species of fish muscle lipids at present.

Materials and Methods

The materials used were sardine (*Sardinops melanosticta*) caught from the coast of Kamiiso, Hokkaido, in Sept. 1977 and rainbow trout (*Salmo gairdnerii irideus*) obtained from Nanae Fish Culture Experimental Station, Hokkaido University in the same month. The average body weight was 80 g and 340 g respectively.

Preparation of the fish muscle triglyceride and phosphatidylcholine

Total lipids were obtained from the muscle according to the method of Bligh and Dyer. A portion of the total lipid was applied to preparative TLC (Wako gel B-5), and the TG was prepared by scraping off the corresponding band on the TLC plate. The phosphatidylcholine (PC) was prepared from the other portion of total lipids using chromatographic technique according to the method of Lands *et al.*[4]. Soybean TG and PC were always used throughout the experiment as an analytical technique reference.

Hydrolysis of the fish muscle triglyceride and phosphatidylcholine

The prepared TG was partially hydrolyzed by the method of Brockerhoff[5] and 1,3-diglyceride (1,3-DG) was prepared by the preparative TLC on silica gel impregnated with 2% boric acid, from the hydrolyzed lipid. On the other hand, PC was hydrolyzed with phospholipase C (*Clostridium perfringens*), according to the method of Renkonen.[6] And 1,2-DG was prepared by the preparative TLC on silica gel impregnated with 2% boric acid, from the hydrolyzed lipid. The developing solvent was n-hexane: ethyl ether: acetic acid=50: 50: 1 (v/v). PC

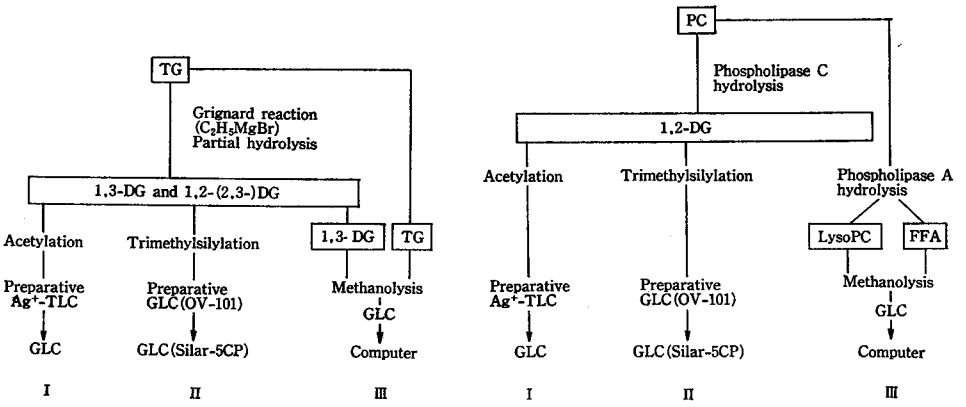

Fig. 1. Analysis of triglyceride and phosphatidylcholine molecular species.

was also hydrolyzed with phospholipase A (*Trimeresurus flavoviridis*) according to the method of Noda *et al.*[7]. Fatty acid from position 2 of PC and lyso PC which represents the fatty acid composition in position 1 of PC were separated in a small silicic acid column using ethyl ether for the fatty acid and chloroform: methanol=1:9 (v/v) for the lyso PC as the eluting solvent. The obtained hydrolysates were analyzed as follows (Fig. 1).

I. Separation of the molecular species of triglyceride and phosphatidylcholine by acetate derivative

Acetylation was performed by adding an appropriate amount of acetic anhydride to the solution of diglyceride in pyridine and by keeping them for 12 hours under room temperature. The diglyceride acetate was purifed by the method of preparative TLC. The following solvent was used; n-hexane: ethyl ether=75: 25 (v/v). The diglyceride acetates and TG were then applied to the silica gel plate impregnated with 8% silver nitrate (abbrev. Ag^+-TLC). The developing solvent used was benzene: ethyl ether=8:2 (v/v) for the TG. And benzene: chloroform: methanol 98:2:0.1 (v/v) used as a developing solvent for the diglyceride acetates. The individual bands found on the plate were scraped off to get the lipids for checking the carbon number distribution and the fatty acid composition.

Analytical condition of the fatty acid composition by gas liquid chromatography (Hitachi 063 Gas Chromatograph)

A portion of the scraped off lipids from the TLC plate was methanolyzed with 10% HCl-methanol in the sealed tube, and subjected to a GLC. The condition was as follows.
column: DEGS, steel column 100×0.3 cm, column temp.: 205°C, detector: FID, detector temp.: 240°C, injection temp.: 270°C, carrier gas: N_2, flow rate 40 ml/min.

Analytical condition of the carbon number distribution by gas liquid chromatography

Another portion of the scraped off lipids was subjected to a GLC. The condition was as follows.
column: OV–101, steel column 50×0.3 cm, column temp.: 300~330°C, 1°C/min, detector: FID, detector temp.: 335°C, injection temp.: 370°C, carrier gas: N_2, flow rate 60 ml/min.

II. Separation of the molecular species of triglyceride and phosphatidylcholine using the trimethylsilyl ether derivative

Trimethylsilylation was performed by adding one volume of trimethylchlorosilane and two volumes of 1,1,1,3,3,3-hexamethyldisilazan to the solution of diglyceride in pyridine. The reaction continued for 3 hours at room temperature. The trimethylsilylated derivatives of the diglyceride were then analyzed by GLC under the following condition.
column: Silar-5CP, glass column 100×0.3 cm, column temp.: 270°C, detector: FID, detector temp.: 300°C, injection temp.: 330°C, carrier gas: N_2, flow rate 60 ml/min.

III. Determination of the molecular species of triglyceride and phosphatidylcholine using a computer technique

A. Input data for the triglyceride.
   ① Number of fatty acid component.
   ② Fatty acid composition of 1.3-DG which represents the fatty acid composition of the original fatty acid composition of the TG in positions 1 and 3.
   ③ Fatty acid composition in position 2 of the original TG (3×Total fatty acid composition of the original TG−2×Fatty acid composition in positions 1 and 3).
   ④ Lowest limit of the printed combinations.

B. Input data for the phosphatidylcholine.
   ① Number of fatty acid component.
   ② Fatty acid composition of position 1.
   ③ Fatty acid composition of position 2.
   ④ Lowest limit of the printed combinations.

①, ② and ③ were read by a photo tape reader. And ④ by a teletype. ②, ③ and ④ are in mole %. The computer program outline is shown in Fig. 2.

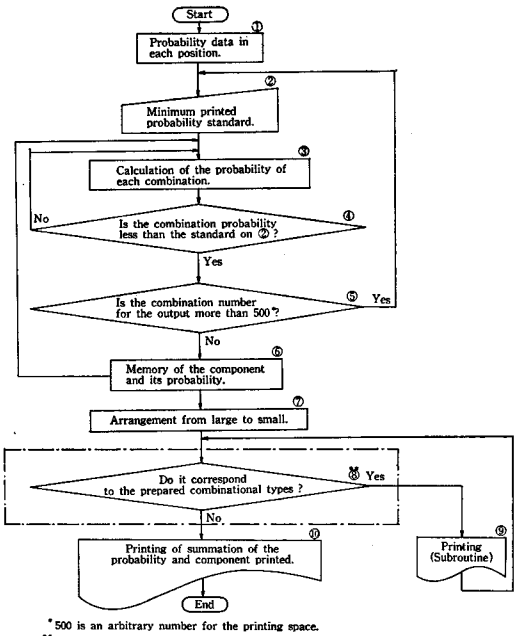

Fig. 2 Program outline.

Results and Discussion

In contrast to the good separation and simple composition of each band of soybean TG and diglyceride acetates prepared from PC, those of the fish muscle of sardine and rainbow trout did not show good separations on Ag^+-TLC, and the

composition of each band was complicated, almost impossible to determine the molecular species. Also, the separation of the molecular species by the trimethylsilyl ether derivative on GLC did not work well when it came to sardine and rainbow trout muscle lipids even though preparative GLC by carbon number was performed previously. As a conclusion it was observed that the chromatographic method is presently not well developed in separating the very complicated molecular species of the fish muscle TG and PC. However, this method is applicable for the determination of molecular species of vegetable oil such as the soybean TG and PC. For this reason, introducing the probability calculation by a computer was the only selective method among the examined methods. The authors tried to apply the method of Koman *et al.*,[2] in this study but could not get the computer program.

Table 1. *Combinational type printed (triglyceride).*

Combinational type	Sardine	Rainbow trout	Soybean
A, A, A	1.6	4.5	16.6
A, A, B + A, B, A	21.4	37.8	40.2
A, B, C	28.0	31.4	41.1
Sum of the probability printed	51.0	73.7	97.9
Printed component number	192	144	66

in mole % (≧0.2%)

Table 2. *Combinational type printed (phosphatidylcholine).*

Combinational type	Sardine	Rainbow trout	Soybean
A, A	22.1	41.2	39.5
A, B + B, A	70.7	53.4	60.2
Sum of the probability printed	92.8	94.6	99.7
Printed component number	41	42	22

in mole % (≧0.2%)

Table 3. *Molecular species of dicomponent combinational type.*

Position 1, 3	Position 2	Sardine	Rainbow trout
16:0* or 18:1	16:0	4.7(1.0)	9.0(3.4)
16:0 or 18:1	18:1	2.8(0.6)	12.6(4.8)
16:0 or 20:5	16:0	4.7(1.0)	—
14:0 or 16:0	14:0	3.7(0.8)	1.1(0.4)
16:0 or 16:1	16:0	3.7(0.8)	4.2(1.6)
16:1 or 18:1	18:1	1.9(0.4)	7.4(2.8)
16:0 or 20:5	16:0	3.7(0.8)	—
16:0 or 22:6	16:0	2.8(0.6)	—
18:1 or 18:2	18:1	—	5.8(2.2)
16:0 or 18:2	16:0	—	3.2(1.2)
16:0 or 16:1	16:1	2.8(0.6)	2.1(0.8)
16:1 or 18:1	16:1	1.9(0.4)	2.1(0.8)
.	.	.	.
.	.	.	.
.	.	.	.
16:0	20:5	2.8(0.6)	—
18:1	16:0	2.8(0.6)	5.5(2.1)
16:0	14:0	2.8(0.6)	0.5(0.2)
16:0	16:1	1.9(0.4)	1.6(0.6)
16:0	18:1	1.9(0.4)	5.0(1.9)
16:0	22:6	1.9(0.4)	—
18:1	16:1	1.0(0.2)	2.6(1.0)
.	.	.	.
.	.	.	.
.	.	.	.

( ) in mole % among the whole combinational type.
* Number of carbon atoms and double bonds of fatty acid.

Therefore in this study, a simplified program for the mini computer which has a function more than JIS–7000 standard was designed. The simplified form of the calculated result is shown in Tables 1 and 2. As was evident from Table 1, the molecular species was very complicated in sardine muscle TG (192 components) and rainbow trout muscle TG (144 components) compared with that of soybean TG (66 components). In addition to that, the molecular species which had an amount less than 0.2% occurred in a considerable amount in sardine muscle TG (100.0–51.0 =49.0%) and rainbow trout muscle TG (100.0–73.7=26.3%) in contrast to that of soybean (100.0–97.9=2.1%). As shown in Table 2, the same tendency was observed in the molecular species of PC. Comparison between the molecular species of sardine muscle TG and that of rainbow trout muscle TG is shown in Tables 3 and 4. Remarkable differences were observed between these two TG. For example, $C_{16:0}$ or $C_{18:1}/C_{18:1}$ combinational types were very rich in rainbow trout muscle TG (Table 3), whereas on the other hand $C_{16:0}/C_{20:5}$ and $C_{16:0}/C_{22:6}$ types were very rich in sardine muscle TG (Table 4). In PC molecular species comparison (Table 5), differences in the amount of $C_{16:0}/C_{16:0}$, $C_{16:0}/C_{20:5}$ and $C_{16:0}/C_{22:6}$ combinational types were considerable. But in the case of $C_{16:0}/C_{22:6}$ combinational types, more than 10% amount exist in rainbow trout muscle PC, while there was almost none in rainbow trout muscle TG.

Table 4. *Molecular species of tricomponent combinational type.*

Position 1 or 3	Position 2	Sardine	Rainbow trout
14:0*	16:0	1.4	—
14:0	20:5	1.4	—
16:0	14:0	7.9	—
16:0	16:1	7.9	—
16:0	18:0	—	2.5
16:0	18:1	7.8	19.7
16:0	18:2	—	2.6
16:0	18:3	—	3.2
16:0	20:5	12.1	—
16:0	22:5	—	2.6
16:0	22:6	8.6	—
16:1	16:0	3.6	2.6
16:1	18:1	1.4	7.0
16:1	20:5	2.9	—
16:1	22:6	4.3	—
18:1	14:0	2.9	3.2
18:1	16:0	5.7	17.8
18:1	16:1	2.9	7.6
18:1	18:0	—	6.4
18:1	18:2	—	8.9
18:1	18:3	—	7.6
18:1	20:5	5.7	—
18:1	22:5	—	6.4
18:1	22:6	2.9	—
18:2	16:1	—	1.9
20:5	14:0	2.8	—
20:5	16:0	9.3	—
20:5	16:1	2.8	—
20:5	18:1	4.3	—
20:5	22:6	1.4	—

in mole %
Sum of the tricomponent combinational type (each$\geq$0.2%) corresponds to 100%.
* Number of carbon atoms and double bonds of fatty acid.

Differences in the composition of the molecular species of TG and PC between the two fish muscles seem to be due to the differences between salt-water fish and fresh-water fish. Yet, to make a definite conclusion, research on a number of fish has to be done.

But, if the presented computer program is applied in different ways such as for observing the changes in the molecular species of fish muscle TG and PC during storage, or during processing, it will surely give some information to solve the problems caused by lipid decomposition.

Acknowledgement

The authors wish to thank Professor R. Kawashima and Assistant Professor, Y. Sakamoto, Faculty of Fisheries, Hokkaido University, for their helpful advice on this study.

Table 5. *Molecular species of sardine and rainbow trout phosphatidylcholine.*

Position 1 \ Position 2	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:2	20:3	20:5	22:1	22:3	22:4	22:5	22:6
14:0*		0.6 1.3			0.2					0.4					0.7 0.3
15:0		0.4													0.2
16:0	1.0 1.9	17.4 39.7	2.8	0.5 0.9	7.7 5.6	0.6 3.0	0.3 0.3	0.5 0.4	0.4	12.2 1.7	2.3 0.6	0.5 0.2	0.4	1.5 0.3	22.3 10.6
18:0		0.8 1.8			0.4 0.3					0.6					1.0 0.5
18:1		1.4 3.2	0.2		0.6 0.5	0.2				1.0					1.8 0.9
18:2		0.3													
20:4		0.3													
20:5		1.4 3.3	0.2		0.6 0.5	0.2				1.0					1.9 0.9
22:1		0.4 0.8								0.2					0.5 0.2
22:3		0.3 0.6													0.4
22:6	0.3	2.4 5.5	0.4		1.1 0.8	0.4				1.7 0.2	0.3			0.2	3.1 1.5

in mole %
Upper: Sardine, Lower: Rainbow trout, Probability≧0.2%
* Number of carbon atoms and double bonds of fatty acid.

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