Title	A Novel of Approach for the Identification of Lipid Molecular Species : Application of High Performance Liquid Chromatography on Fish Muscle Lecithin Molecular Species Analysis
Author(s)	Koretaro, Takahashi; 高橋, 是太郎
Degree Grantor	北海道大学
Degree Name	博士(水産学)
Dissertation Number	乙第2740号
Issue Date	1984-12-25
Doc URL	https://hdl.handle.net/2115/42778
Type	doctoral thesis
File Information	takahashi_thesis.pdf

A NOVEL APPROACH FOR THE IDENTIFICATION OF LIPID MOLECULAR SPECIES

Application of High Performance Liquid Chromatography on Fish Muscle Lecithin Molecular Species Analysis

KORETARO TAKAHASHI

1984

ACKNOWLEDGMENTS

The author is deeply indebted to Prof. Kōichi Zama under whose supervision this investigation was conducted. His ideas and advices throughout the study were invaluable.

The useful suggestions of Prof. Tōru Takagi in preparation of this manuscript are greatly appreciated.

Associate Prof. Mutsuo Hatano kindly accepted the reviewer. Sincere gratitude to Associate Prof. Kōzō Takama for guiding and for drawing the authors attention to this problem.

This work would not have been possible without the help of Mr. Tsugihiko Hirano.

The author was favored to have the assistance of Mr. Hideaki Ebina.

Mr. Makoto Egi did the computer programming.

Thanks are due to Dr. Masahiko Kunimoto and to Mr. Seiichi Ando for the encouragement.

CONTENTS

NOMENCLATURE AND ABBREVIATIONS

22:6	represents a fatty acid with a chain length of 22 carbon atoms with six double bonds
GLC	Gas liquid chromatography
HPLC	High performance liquid chromatography
TLC	Thin layer chromatography
PL	Phospholipid
PC	Lecithin, Phosphatidylcholine
PS	Phosphatidylserine
PE	Phosphatidylethanolamine
NP	Non phospholipid
DG	Diglyceride
ST	Sterol
FFA	Free fatty acid
TG	Triglyceride
MS	Molecular species
RRT	Relative retention time
CN	Total acyl carbon number
DB	Number of total double bonds
PN	Partition number
ECN	Equivalent carbon number
EC	Effective carbon number
Acyl	Acyl group
E	Glycerol residue
$a \in A$	a belongs to A
μ	Chemical potential
DM	Dark muscle
WM	White muscle
PCA	Principal component analysis
ACC.%	Accumulative value of the contribution.

GENERAL INTRODUCTION

Analytical techniques or instruments have been developed for fatty acid analysis. Resultingly, many knowledge has been achieved on the study of fatty acid analysis.

In contrast to this, the study of molecular species, namely, the study of the intact or unmodified lipid is still away behind of development. This might be due to the limited and time consuming conventional techniques represented by silver nitrate impregnated thin layer chromatography (Ag^+ -TLC) followed by gas liquid chromatographic analysis (GLC) (such examples are too numerous to mention). Generally speaking, Ag^+ -TLC can separate only up to heptaene. And GLC can analyze quantitatively only up to 700~800 molecular weight. If the sample contains highly unsaturated fatty acids such as 20:4, 20:5 or 22:6, the degree of unsaturation will drastically increase and exceeds the analytical limit of the Ag^+ -TLC.

Fats and oils from marine sources are rich in highly unsaturated fatty acids. Accordingly, it is almost impossible to analyze the molecular species from these sources by the conventional method.1)

High performance liquid chromatograph has become the most expected instrument in separating the nonvolatile or high molecular weight compound. Since there are significant amount of triglycerides in marine lipid that exceed the molecular weight of 800 which is the limit of GLC, the utilization of high performance liquid chromatography (HPLC) is expected to separate the molecular species of lipids from these sources. The reversed-phase type HPLC 2~4) is becoming the main analytical type in separating the most analogous compounds. 5) The earlier workers in this field tried to characterize the chromatographic rules such as partition number (PN) 6,7) or effective carbon number (EC) 8) which are defined as $PN = CN - 2 \cdot DB$ and $EC = CN - DB$, respectively, in the elution of triglyceride or lecithin molecular species. CN is the total acyl carbon number and DB is the total double bonds in the molecule. This empirical equations were useful in predicting the approximate retention value (in practical cases, retention time or retention volume) though these equations lacked the theoritical background. In the same year, Plattner et al. 9,10) has proposed equivalent carbon number (ECN) which is the same concept with PN. And recently, in accordance with the improvement of separation on HPLC, theoritical carbon number (TCN) has been proposed by Perkins et al. 11) In 1982, the generalized form of ECN (or PN) and EC has been presented by Compton et al. 12) that can be written as $I_u = I_s - CD_b$, where I_s is the carbon number of the standard alkane, D_b is the total double bonds in the molecule and I_u is the index observed for unsaturated triglyceride and phospholipid molecular species by the coefficient of C.

In this study, the formulae that control the sequence of elution of lipid molecular species on reversed-phase HPLC are proposed. The theoritical aspect of the presented formulae has been discussed in relation to ECN (or PN). And it is demonstrated that the formulae proposed in this study might be invariant rules.

Lecithin (phosphatidylcholine) were analyzed by HPLC from various sources including those from fish muscle. Though there are some reports concerning the phospholipid molecular species analysis by HPLC from land sources,[13~20] the study done by the author might be the only example that have worked on the phospholipid molecular species analysis on HPLC from fish lipid except the conventional way of analysis based on the probability simulation done by Oshima et al.[21~25]

In chapter I, it is demonstrated that the modification of lecithin into diglyceride acetate via diglyceride is necessary for the reversed-phase type HPLC analysis in order to have a sufficient separation between the critical pairs that have the same ECN, PN or EC. And a new matrix model is proposed instead of the ECN, PN or EC concept.

In chapter II, the proposed matrix model that exhibits the chromatographic rules of diacyl type molecular species is developed into the rules for triacyl type molecular species such as triglycerides. And the physicochemical background, as well as the theoritical relationship between the ECN (or PN) and the new matrix model, is demonstrated.

In chapter III, the lipid molecular species identification software is designated for the personal computer. The accumulated data were fully utilized for the actual identification since the equation that regulates the sequence of molecular species inevitably contains errors due to the deviation of relative retention time (RRT) on HPLC.

In chapter IV, characteristics of several kinds of fish including cartilaginous fish, as well as fresh water fish, are discussed from the view point of muscle lecithin.

In the final chapter, summary and conclusion of this work is described with a few supplemental discussion.

CHAPTER I

IDENTIFICATION OF LECITHIN MOLECULAR SPECIES ON REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

A New Concept that Helps the Molecular Species Determination

A satisfactory separation of lecithin molecular species from natural sources including those from marine sources was made by modifying the lecithin into diglyceride acetate for HPLC analysis.

In this chapter, the necessity of modification of lecithin into diglyceride acetate through the hydrolysis by phospholipase C and the subsequent acetylation by acetic anhydride are discussed. And also the discovery of a new empirical equation instead of the former ECN or PN for the identification of molecular species of lecithin is discussed.

Section 1. Experimental

Preparation of Lecithin

Total lipids were obtained from the fish muscle tabulated in Table I-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 column chromatography which has been successfully done by Lands et al.,[26)] namely, aliquot amount of total lipid was dissolved

Table I-1. Fish examined

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

* Male. ** Nos. of individual used.

into double volume of diethyl ether/ethanol (9:1, v/v) and then applied to the silica gel column. And elutions were done through diethyl ether/ethanol (9:1, v/v), diethyl ether/ ethanol (1:1, v/v), ethanol/methanol (9:1, v/v) and finally by 100% methanol. The 100% methanol fraction was collected with a fraction collecter, monitored by TLC, and lecithin of more than 95% purity was collected.

Purification of Diglyceride Acetate from Lecithin

Pure lecithin was hydrolyzed with phospholipase C (*Clostridium perfringence*, P-L Biochemical Inc., Milwaukii), according to the method of Renkonen.[27)] It was done in the following manner. First, 50 to 100 mg of lecithin was dissolved in 10 to 15 ml of diethyl ether. Then, 10 to 15 ml of 1 molar Tris buffer (pH 7.3) including calcium chloride with 5 mg of phospholipase C was added to the ether solution. The head space gas of the container was filled with nitrogen gas and the hydrolysis of lecithin was continued for four hours under room temperature. The hydrolysate was washed several times with water and diglyceride was purified by a preparative TLC from this hydrolysate. 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 hours at room temperature.[28)] 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 0.45μ 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 8x250 mm LiChrosorb RP-18 (Merck, West Germany) columns which 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 five 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 ml/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 analyzed by gas chromatography. The analytical conditions for fatty acids were as follows:

Gas chromatograph: Hitachi 063, Column: Unisole 3000 (Gasukuro Kōgyō Ltd., Tokyo), Glass column 0.2x200 cm, Column temp.: 220°C, Detector: FID, Detector temp.: 250°C, Injection temp.: 280°C, Carrier gas: N_2 , Flow rate: 20 ml /min.

Methyl esters of fatty acids were prepared according to the method of Christopher and Glass described by Prevot and Mordret.[29)] An aliquot amount (less than 20 mg) of lipid was dissolved in 1 ml n-hexane and 0.2 ml of methanolic 2N-NaOH solution was added. After shaking this mixture, it was stand for 20 seconds under 50°C and then 0.2 ml of methanolic 2N-HCl solution was added. The n-hexane layer was collected and then concentrated. Methyl esters prepared as described above were subjected to a GLC.

The small peaks which have critical pairs were first subjected to Ag^+-TLC. The developing solvent used was benzene / diethyl ether (4:1, v/v).30) 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 Kōgyō Ltd., Tokyo), Steel column 0.3x50 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 ml/min.

Hydrolysis of Diglyceride Acetate Derived from Lecithin by Pancreatic Lipase

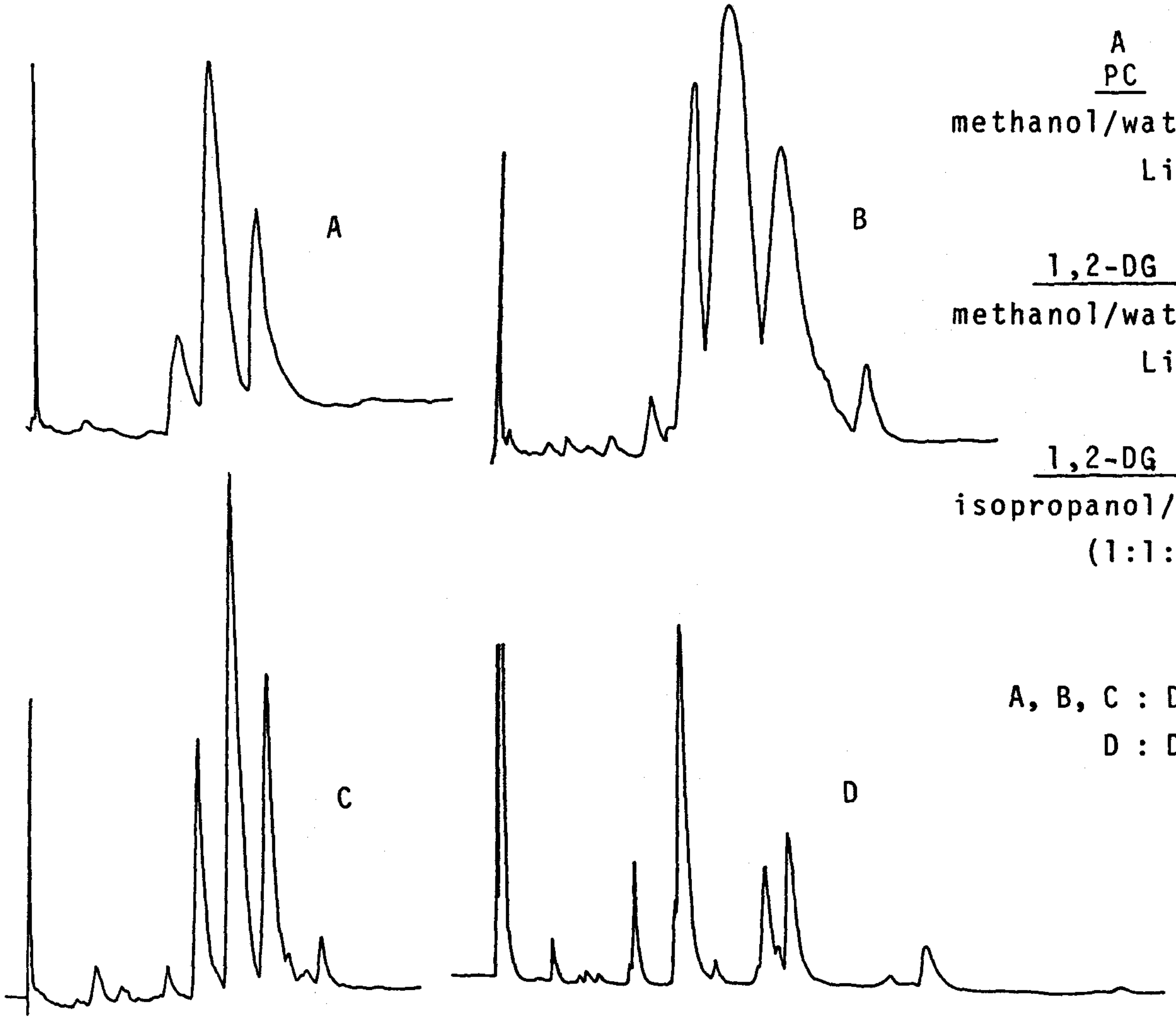
Fraction of the molecular species of 16:0 in position 1 and 22:6 in position 2, that is, (16:0)(22:6) was collected by HPLC. This lipid (less than 5 mg) was then suspended by shaking vigorously in a mixture of 1 molar Tris-HCl buffer, pH 8 (1ml), 2.2% calcium chloride (0.1 ml), and 0.05% sodium taurochorate (0.25 ml) 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 processed for 4 min at 40°C by shaking it vigorously. The reaction was stopped by adding 1 ml of ethanol and 1 ml of 6N-HCl. The hydrolysate was extracted with diethyl ether and purified by using a preparative TLC with n-hexane/diethyl ether/formic acid (40:10:1, v/v) as developing solvent. These procedures were the modified form of the experiment done by Kosugi et al.31)

Section 2. Results

Figure I-1 shows the comparison of separation on HPLC by the differences in the molecular form which has the same acyl combination. Examples here are of soybean lecithin:

A : Intact lecithin,
Developing solvent: methanol/water (95:5, v/v)
methanol 100, gradient, UV detector.
B : 1,2-Diglyceride i.e. the hydrolysate of lecithin,
Developing solvent: methanol/water (95:5, v/v)
methanol 100, gradient, UV detector.
C : 1,2-Diglyceride acetate derived from lecithin,
Developing solvent: methanol/water (97:3, v/v)
methanol 100, gradient, UV detector.
D : 1,2-Diglyceride acetate derived from lecithin,
Developing solvent: isopropanol/acetone/methanol/
acetonitrile (1:1:3:4, v/v), RI detector.

As it is clear from this figure, the acetate form is the best in separation on HPLC followed by diglyceride. The intact lecithin is the worst among these three. It is considered that the very high polarity of phosphorylchorine group interferes with the interactions between the acyl groups and the stationary phase of HPLC. Although the polarity of hydroxy group is not so high as those of phosphorylcholine, this group also interferes with the interactions between the acyl groups and the stationary phase, resulting on the poor separation on HPLC as shown in chromatograms A and B. The combination of four solvents are better than that of two solvents for the elution of



A
PC

B
1,2-DG derived from PC

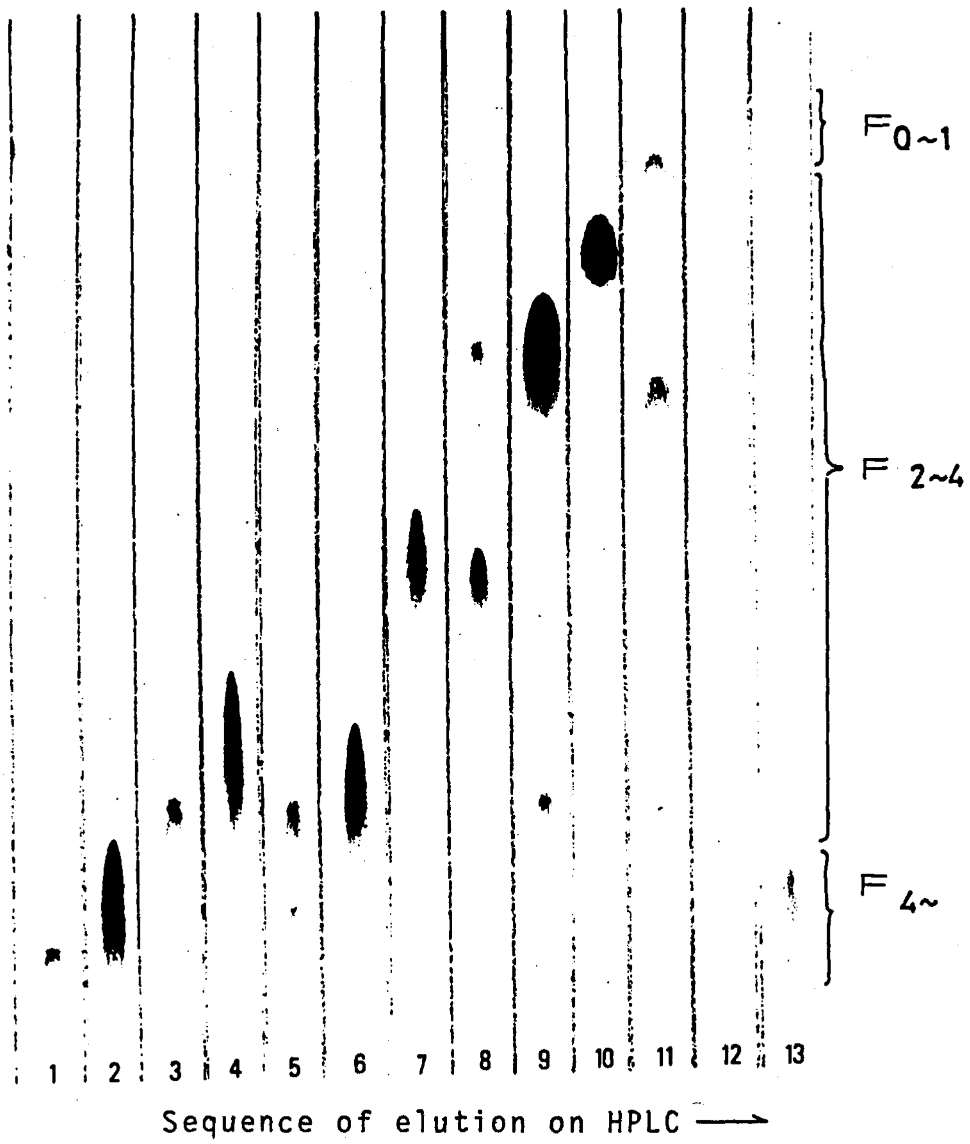
methanol/water (95:5, v/v)→methanol 100
Linear Gradient 90 min

C
1,2-DG acetate derived from PC
methanol/water (97:3, v/v)→methanol 100
Linear Gradient 60 min

D
1,2-DG acetate derived from PC
isopropanol/acetone/methanol/acetonitrile
(1:1:3:4, v/v), Isocratic

A, B, C : Detected by UV detector, 215 nm
D : Detected by RI detector, 20°C

Fig. I-1. Comparison of separation on HPLC by the differences in the molecular form.



F : Number of double bonds.
Developing solvent: benzen/diethyl ether (4:1, v/v)

Fig. I-2. Ag^+-TLC of diglyceride acetate derived from soybean lecithin separated by HPLC.

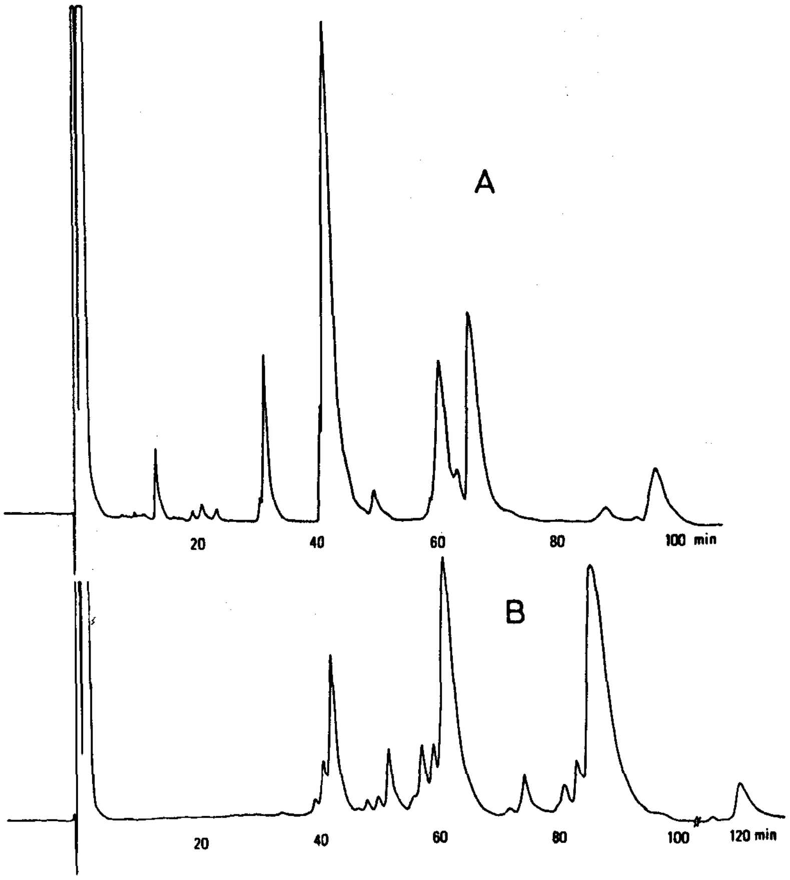
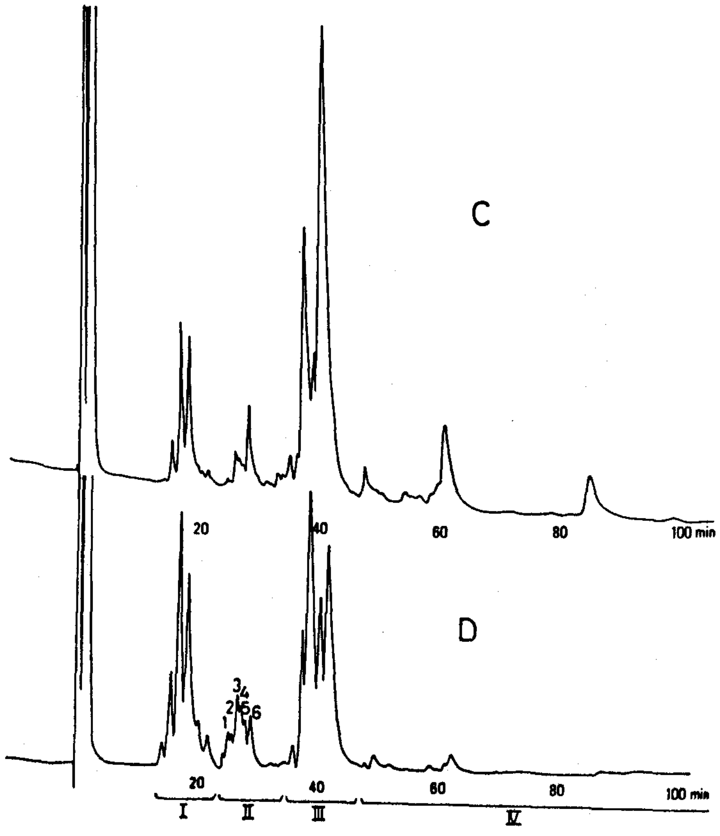


Fig. I-3. HPLC chromatograms of soybean and egg yolk lecithin.
A: Soybean. B: Egg yolk.



I: Molecular species composed of highly unsaturated fatty acids such as (20:5)(20:5), (20:5)(22:6) and (22:6)(22:6).

III: Molecular species composed of generally found fatty acids such as 16:0 or 18:1 with combinations of 20:5 or 22:6, that is, (16:0)(20:5), (16:0)(22:6), (18:1)(20:5) and (18:1)(22:6).

II & IV: Others.

Fig. I-4. HPLC chromatograms of chum salmon muscle lecithin.
C: Captured in summer. D: Captured in fall.

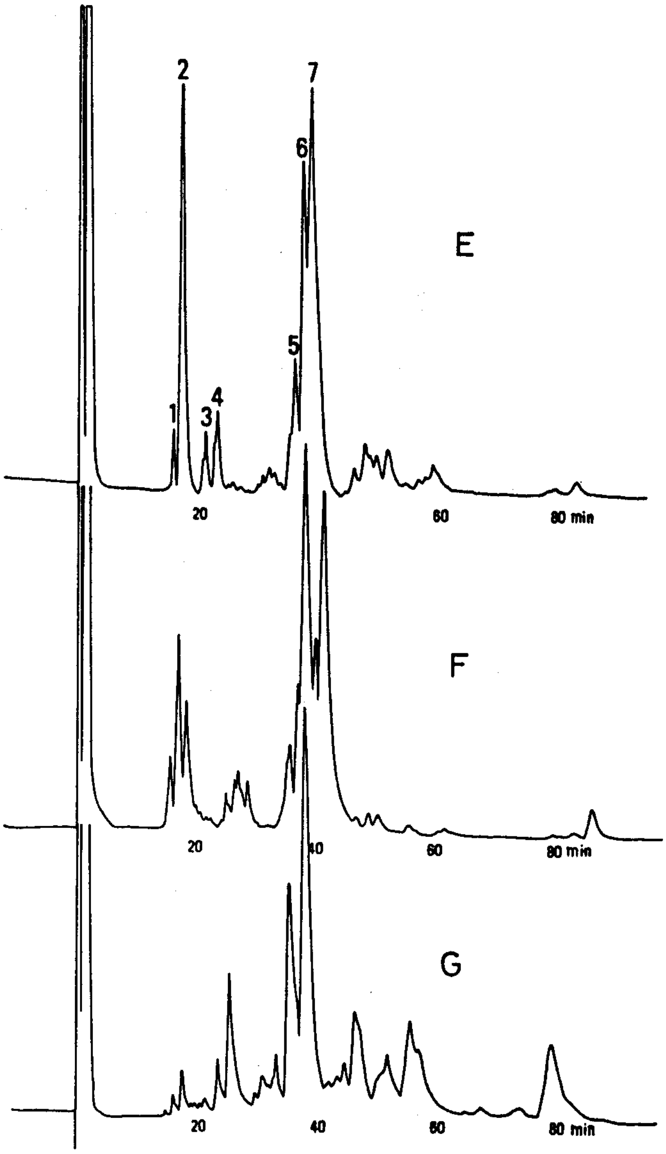


Fig. I-5. HPLC chromatograms of big-eyed tuna, Alaska pollack and carp muscle lecithin.
E: Big-eyed tuna. F: Alaska pollack. G: Carp.

Table I-2. Determination of molecular species of major component*

Peak number Fatty acid	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)	22:6 16:0	16:0 22:6

* Example of big-eyed tuna in Fig. I-5E.

diglyceride acetate as shown in chromatograms C and D. For the diglyceride acetate analysis, this isocratic condition was used throughout the experiment.

Figure I-2 shows the Ag^+-TLC chromatograms of the collected fractions on HPLC. It is numbered in sequence of elution. This is an example of diglyceride acetate from soybean lecithin. As seen in this chromatogram, this type of column packing i.e. the reversed phase column elutes the polyunsaturated types first followed by tri, di, mono and finally saturated types. This is the basic characteristics of this type of column.

Figures I-3~5 show chromatograms of diglyceride acetates derived from several kinds of lecithin sources on HPLC. Chromatography was regularly completed in about two hours. The diglyceride composition of each predominant peak collected was easily determined by fatty acid analysis, as shown in Table I-2. This table shows the results on big-eyed tuna lecithin as an example. Peak number 7 in Fig. I-5E is obviously ascribed to the diglyceride acetate composed of 16:0 and 22:6. It is considered that 22:6 is bound in position 2 of the molecule since this peak is 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.[1,32~36) In addition, after pancreatic lipase hydrolysis, only a trace amount 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 has the same combination as that in peak number 7,

Table I-3. Determination of lecithin molecular species of minor component*

Peak number Fatty acid	1	2	3	4	5	6
14:0			21.3	30.6	34.6	44.6
16:1	53.0	47.7	28.3	19.0	19.6	9.2
20:5	47.0	52.3	50.4	36.2	15.6	8.3
22:6				14.2	30.2	37.9
Carbon number **						
34			42.6	63.3	32.2	10.8
36	95.<	90.0	57.4	5.7	41.7	89.2
38		10.0		31.0	26.1	
Carbon number **						
34			20:5 14:0 42.6	14:0 20:5 63.3	14:0 20:5 32.2	14:0 20:5 10.8
36	20:5 16:1 95.<	16:1 20:5 90.0	16:1 20:5 57.4	16:1 20:5 5.7	22:6 14:0 41.7	14:0 22:6 89.2
38				22:6 16:1 31.0	16:1 22:6 26.1	

in relative %

* Example of chum salmon (Fall)　　** Total acyl carbon number

whereas (18:1)(20:5) is considered as contaminants of peak number 5. Nevertheless in this case, 22:6 is considered to be bound in position 1 in the molecule. Peak number 5 has 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 are regarded as contaminants from peak number 6, considering that peak number 6 is larger than peak number 5. It is concluded that peak number 5 is 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 small 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 I-3 (also see Fig. I-4D). 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 differences in retention time on HPLC between these two peaks is attributed to the differences in binding position of the fatty acid. It is considered that small peak number 1 has 20:5 in position 1, whereas small peak number 2 has 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 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 are 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 moleclar species, (16:1)(20:5) can be considered as contaminant 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 is identified in the same manner as that in small peak number 4. In this case, molecular species of (14:0)(20:5) is considered to be a contaminant from the previous peak. In the case of small peak number 6, molecular species of 14:0 in position 1 and 20:5 in position 2 are considered to be the contaminants from the previous peak. By analyzing the data in this way, molecular species of all other small peaks were identified.

The relative retention times (RRTs) of all peaks were determined by dividing the retention time of each peak by the retention time of (16:0)(22:6). In the case of soybean lecithin, (16:0)(18:2) was used as a reference peak and the RRT were recalculated against (16:0)(22:6) by using the RRT

Table I-4. Relation between relative retention time on HPLC and molecular species of soybean lecithin

PN	MS	Rt	RRT*
26	18:2 18:3	27.6	70.4
28	18:2 18:2	37.6	95.8
"	18:1 18:3	39.2	99.9
"	16:0 18:3	42.0	107.1
30	18:1 18:2	51.4	131.0
"	16:0 18:2	55.2	140.7
"	18:0 18:3	60.0	152.9
31	17:0 18:2	62.8	160.1
32	18:1 18:1	73.8	188.1
"	20:1 18:2	"	"
"	16:0 18:1	79.6	202.9
"	18:0 18:2	"	"
"	16:0 16:0	83.6	213.0
34	18:0 18:1	113.2	288.6
"	18:2 20:0	"	"

Abbreviations:

PN:Partition number. MS:Molecular species. RRT:Relative retention time. Rt:Retention time.

* $\left|\begin{matrix}16:0\\22:6\end{matrix}\right|$ is used as the reference peak.

Table I-5. Relation between relative retention time on HPLC and molecular species of egg yolk lecithin

PN	MS	Rt	RRT
28	16:1 18:2	41.2	100.0
"	18:2 18:2	"	"
"	14:0 18:2	"	"
26	16:0 22:6	"	"
28	16:0 18:3	47.0	114.1
"	18:1 20:4	"	"
"	16:0 20:4	50.4	122.2
30	16:0 22:4	54.4	132.0
"	16:1 18:1	55.6	134.9
"	18:1 18:2	"	"
"	16:0 18:2	59.2	143.7
28	18:0 22:6	"	"
31	15:0 18:1	72.4	175.7
"	16:0 17:1	"	"
"	17:0 18:2	"	"
30	18:0 20:4	"	"
32	18:1 18:1	79.0	191.7
"	18:0 22:4	"	"
"	16:0 18:1	83.6	202.9
"	18:0 18:2	"	"
34	18:0 18:1	121.8	295.1

Abbreviations:
PN:Partition number, MS:Molecular species, RRT:Relative retention time.
Rt:Retention time.

Table I-6. Relation between relative retention time on HPLC and main molecular species of fish muscle lecithin

Chum salmon (Summer)		Chum salmon (Fall)		Big-eyed tuna		Alaska pollack		Carp	
MS	PN/RRT	MS	PN/RRT	MS	PN/RRT	MS	PN/RRT	MS	PN/RRT
20:5 20:5	20 37.3	20:5 20:5	20 37.5	20:5 22:6	20 41.2	20:5 20:5	20 37.2	20:5 22:6	20 42.5
20:5 22:6	20 40.7	20:5 22:6	20 40.4	22:6 22:6	20 44.6	20:5 22:6	20 40.4	22:6 22:6	20 46.2
22:6 22:6	20 44.2	22:6 22:6	20 44.1	20:4 22:6	22 55.2	22:6 22:6	20 44.3	16:1 20:5	24 62.5
14:0 22:6	24 69.7	18:1 20:5	26 87.0	22:5 20:5	22 55.2	18:1 20:5	26 86.7	18:2 20:5	24 62.5
16:0 20:5	26 92.2	20:5 16:0	26 89.5	22:5 22:6	22 60.5	20:5 16:0	26 89.8	16:1 22:6	24 67.7
18:1 22:6	26 92.2	16:0 20:5	26 91.9	18:1 22:6	26 93.5	22:6 18:1	26 89.8	18:2 22:6	24 67.7
22:6 16:0	26 97.1	18:1 22:6	26 91.9	16:0 20:5	26 93.5	16:0 20:5	26 92.0	14:0 22:6	24 67.7
16:0 22:6	26 100.0	22:6 16:0	26 96.7	22:6 16:0	26 96.8	18:1 22:6	26 92.0	18:1 20:5	26 88.3
		16:0 22:6	26 100.0	16:0 22:6	26 100.0	22:6 16:0	26 97.1	16:0 20:5	26 93.7
				16:0 22:5	28 134.9	16:0 22:6	26 100.0	18:1 22:6	26 93.7
						16:0 18:1	32 216.6	16:0 22:6	26 100.0
						14:0 20:1	32 216.6	16:0 20:4	28 124.0

Abbreviations:

PN: Partition number. MS: Molecular species. RRT: Relative retention time.

Table I-7. Relation between relative retention time on HPLC and molecular species of fish muscle lecithin

Chum salmon (Summer)		Chum salmon (Fall)		Big-eyed tuna		Alaska pollack		Carp	
MS	PN/RRT	MS	PN/RRT	MS	PN/RRT	MS	PN/RRT	MS	PN/RRT
20:4 22:6	22 50.6	18:3 22:6	22 48.7	22:6 16:1	24 65.0	16:1 20:5	24 61.0	18:3 22:6	22 50.7
22:5 20:5	22 50.6	20:4 22:6	22 48.7	18:2 22:6	24 66.9	18:2 20:5	24 61.0	20:4 20:5	22 52.3
22:5 22:6	22 52.8	22:5 20:5	22 48.7	14:0 22:6	24 71.1	14:0 20:5	24 64.2	22:5 22:6	22 54.5
16:1 20:5	24 60.8	20:5 16:1	24 59.1	22:4 22:6	24 71.1	16:1 22:6	24 65.9	20:4 22:6	22 56.4
14:0 20:5	24 64.8	20:5 18:2	24 59.1	16:1 20:4	26 71.1	18:2 22:6	24 65.9	16:1 22:5	26 79.4
16:1 22:6	24 65.8	16:1 20:5	24 60.8	20:4 22:5	24 74.0	14:0 22:6	24 69.9	18:2 22:5	26 79.4
22:6 14:0	24 67.4	18:2 20:5	24 60.8	22:6 17:1	25 78.4	22:5 16:0	28 115.7	15:0 22:6	25 79:4
15:0 20:5	25 77.8	20:5 14:0	24 62.2	22:5 22:5	24 78.4	16:0 20:4	28 115.7	17:1 22:6	25 79.4
17:1 22:6	25 82.3	14:0 20:5	24 64.2	17:2 20:4	25 78.4	16:0 22:5	28 120.3	16:1 20:4	26 82.7
14:0 22:5	26 82.3	22:6 16:1	24 64.2	17:2 22:5	25 78.5	20:1 20:5	28 125.0	18:2 20:4	26 82.7
15:0 22:6	25 84.4	16:1 22:6	24 67.1	17:1 22:6	25 82.7	18:0 20:5	28 137.5	18:1 22:5	28 111.7
20:5 18:1	26 87.2	22:6 14:0	24 67.1	15:0 22:6	25 84.7	20:1 22:6	28 137.5	16:0 16:2	28 111.7
18:1 20:5	26 90.2	14:0 22:6	24 69.5	20:5 18:1	26 84.7	18:1 16:1	30 137.5	18:1 20:4	28 115.8
20:5 16:0	26 90.2	16:1 22:5	26 77.8	18:1 20:5	26 88.2	18:1 18:2	30 137.5	16:0 22:5	28 119.3
22:6 18:1	26 90.2	15:0 20:5	25 77.8	22:6 18:1	26 91.3	14:0 18:1	30 150.0	16:0 22:4	30 133.8
16:0 22:5	28 121.9	17:1 22:6	25 77.8	20:5 16:0	26 91.3	16:0 16:1	30 150.0	20:1 22:6	28 133.8
16:0 20:4	28 126.6	20:5 18:1	26 82.2	20:4 16:0	28 116.7	16:0 18:2	30 150.0	18:1 16:1	30 138.4
17:0 22:6	27 126.6	22:5 16:0	28 116.6	17:0 22:6	27 116.7	18:0 22:6	28 150.0	18:1 18:2	30 138.4
20:1 20:5	28 126.6	16:0 22:5	28 119.9	18:1 22:5	28 116.7	18:1 16:0	32 207.4	16:0 18:2	30 148.4
20:1 22:6	28 140.6	16:0 20:4	28 124.6	16:0 20:4	28 124.6	20:1 14:0	32 207.4	16:0 16:1	30 148.4
18:0 20:5	28 140.6	20:1 20:5	28 124.6	22:5 16:0	28 130.0	16:0 20:1	34 301.9	18:0 22:6	28 148.4
18:1 16:1	30 143.6	18:0 20:5	28 133.3	22:6 18:0	28 143.2	18:0 18:1	34 301.9	18:0 20:4	30 179.4
18:0 22:6	28 152.5	18:1 16:1	30 137.2	18:0 22:6	28 148.1			18:1 18:1	32 195.3
14:0 18:1	30 152.5	14:0 18:1	30 142.9	18:1 18:1	32 189.6			16:0 18:1	32 210.1
16:0 16:1	30 152.5	16:0 16:1	30 142.9	18:1 16:0	32 206.1				
24:1 20:5	32 265.7			16:0 18:1	32 215.5				

Abbreviations:
PN: Partition number. MS: Molecular species. RRT: Relative retention time.

data in egg yolk lecithin. The RRT of each molecular species is summerized in Tables I-4~7.

Section 3. Discussion

We have plotted the RRT of each molecular species semilogarithmically against the partition number (PN) defined as PN=CN-2·DB where CN is the total acyl carbon number and DB is the total double bonds since PN is proportional to the logarithm of RRT.37) A general expression for this can be written as:

$$PN \propto \log(RRT) \qquad (1)$$

After plotting the RRT of each molecular species semilogarithmically, a similar correlation was obtained, that is:

$$PN \propto \log(RRT)+\Delta Q \qquad (2)$$

(2) is similar to (1), and if we formulate (2) into an equation:

$$PN= P\cdot\log(RRT)+\Delta Q \qquad (3)$$

where P becomes the slope of the oblique line, and ΔQ becomes the intersept on the ordinate. This equation (3) suggests that PN is not only a function of RRT alone, but also a function including ΔQ.

Back to the definition of the PN, that is, PN=CN-2·DB, the left member in (3) can be changed as:

$$CN-2\cdot DB= P\cdot\log(RRT)+\Delta Q \qquad (4)$$

So, RRT can be expressed as follows:

$$RRT= 10^{\frac{CN-2\cdot DB-\Delta Q}{P}} \qquad (5)$$

If we put ΔQ as $Q_1+\alpha$, equation (5) may be written as:

$$RRT= 10^{\frac{CN-2\cdot DB-(Q_1+\alpha)}{P}} \quad (Q_1 \text{ is a minimum of } \Delta Q) \qquad (6)$$

where P and Q are invariables and α is a variable range of Q_1. By letting CN and DB be invariables, RRT becomes a function of α which is:

$$RRT = f(\alpha) \qquad (7)$$

Back to equation (4), by putting ΔQ as $Q_1 + \alpha$, the following equation can be obtained:

$$CN-2\cdot DB = P\cdot\log(RRT)+Q_1+\alpha \qquad (4')$$

If we let DB be invariable and rearrange the expression for CN, (4') will become:

$$CN = P\cdot\log(RRT)+\alpha+Q_2 \qquad (Q_2 = Q_1+2\cdot DB) \qquad (8)$$

On the other hand, if we let CN be invariable and rearrange the expression for DB, (4') will become:

$$DB = -\frac{P}{2}\log(RRT)-\frac{\alpha}{2}+Q_3 \qquad (Q_3 = -\frac{1}{2}(Q_1-CN)) \qquad (9)$$

These two functions, i.e. (8) and (9) have a deviation which are α for the former and $-\alpha/2$ for the latter. Alfa is considered to be a factor that has a small but significant effect on RRT, and it may be due to the positional isomers such as between 1 and 2 positions in the molecule, or by the large differences in number of double bonds between the two acyl group in the molecule. This leads us to the model of a matrix since it is convenient to distinguish the positional isomers or the bias in the number of double bonds between the two acyl groups in the molecule. So in order to simplify the equation, we can induce the following equations from (8) and (9) respectively under the conditions of:

$$CN' = \begin{vmatrix} x & d_1 \\ c_2 & d_2 \end{vmatrix} \qquad (c_2,\ d_1 \text{ and } d_2 \text{ are invariables})$$

$$DB' = \begin{vmatrix} c_1 & y \\ c_2 & d_2 \end{vmatrix} \qquad (c_1,\ c_2 \text{ and } d_2 \text{ are invariables})$$

$$CB' = P_1 \cdot \log(RRT) + q_1 \qquad (8')$$

$$DB' = P_2 \cdot \log(RRT) + q_2 \qquad (9')$$

by analyzing the RRT data of each molecular species.

After plotting the RRT of each molecular species from the source examined against the total acyl carbon number or the number of total double bonds of each molecular species, the RRT plots of molecular species laid almost on a straight line by giving a variable integer x for the carbon number and a variable integer y for the number of double bonds of each acyl group in the molecular species, when we express the molecular species in matrix relation. The oblique line is almost parallel to each other as shown in Fig I-6. By applying these correlations between the RRT and the corresponding molecular species under the condition of matrix for the determination of molecular species, it is suggested that an unidentified molecular species can be predicted from RRT on HPLC even if it has the most complicated composition of molecular species such as fish muscle lecithin.

Another method for HPLC analysis of phospholipid molecular species was published by Patton et al.[19)] To our surprise, the sequence of each molecular species in elution on HPLC, when the RRTs are plotted against the total acyl carbon number from our biological material, are the same as common molecular species of rat liver which was analyzed by Patton et al.[19)] despite the fact that the analytical conditions are significantly different. This suggests that, although there are differences in retention time or in RRT among the different conditions on HPLC, the sequence in

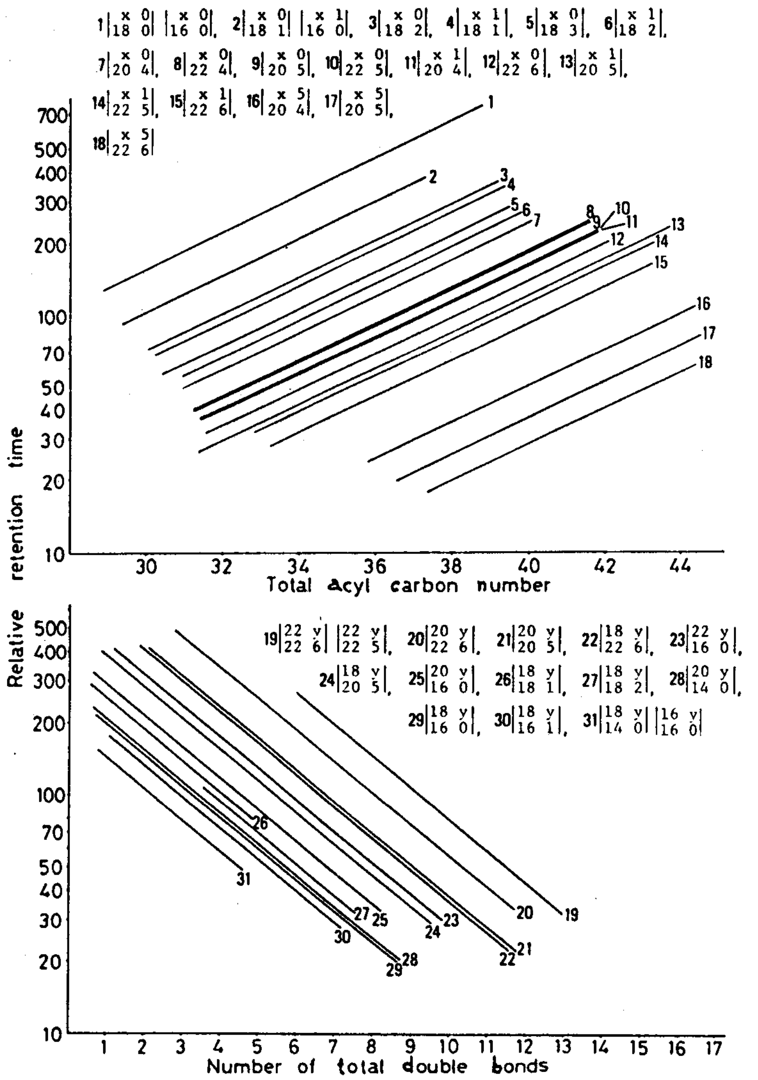


Fig. I-6. Relation between relative retention time and total acyl carbon number and relation between relative retention time and total double bonds on HPLC of lecithin.

elution of each molecular species might be unchangeable. This leads us to a conclusion that in HPLC, the sequence in elution might be controlled by a fixed correlation, that is matrix relation. By accepting this idea, we can expand this matrix model to triglycerides. Details will be discussed in the next chapter.

CHAPTER II

A NOVEL APPROACH FOR THE IDENTIFICATION OF TRIGLYCERIDE MOLECULAR SPECIES ON REVERSED-PHASE HIGH PERFOMANCE LIQUID CHROMATOGRAPHY

In chapter I, two rules in the elution of diglyceride acetate derived from lecithin on HPLC were discovered, that is:

$$CN= P_1 \cdot \log(RRT)+Q_1 \qquad CN= \begin{vmatrix} x & d_1 \\ c_2 & d_2 \end{vmatrix} \qquad (1)$$

$$DB= P_2 \cdot \log(RRT)+Q_2 \qquad DB= \begin{vmatrix} c_1 & y \\ c_2 & d_2 \end{vmatrix} \qquad (2)$$

where P is the slope and Q is the intercept on the ordinate of the semilogarithmic plots of the RRTs of molecular species against CN or DB. c and d are acyl carbon number and number of double bonds in each acyl group, respectively. x and y are variables of acyl carbon number and number of double bonds, respectively.

The structure of diglyceride acetate is:

$$\left[\begin{array}{l} -\text{Acyl}_1 \\ -\text{Acyl}_2 \\ -\text{CO}\cdot\text{CH}_3 \end{array}\right. \qquad (\text{ Acyl : esterified fatty acid }) \qquad (3)$$

And the structure of triglyceride is:

$$\left[\begin{array}{l} -\text{Acyl}_1 \\ -\text{Acyl}_2 \\ -\text{Acyl}_3 \end{array}\right. \qquad (4)$$

The difference between (3) and (4) is at position 3 in the molecule. But $CO \cdot CH_3$ in (3) can also be considered as the shortest form of acyl group.

So: $\begin{bmatrix} \text{Acyl}_1 \\ \text{Acyl}_2 \\ \text{Acyl}_3 \end{bmatrix} \ni \begin{bmatrix} \text{Acyl}_1 \\ \text{Acyl}_2 \\ \text{CO}\cdot\text{CH}_3 \end{bmatrix}$ (where $\ni$ means "include")

therefore, (1) and (2) can be rewritten as:

$$CN = P_1 \cdot \log(RRT) + Q_1 \qquad CN = \begin{vmatrix} x & d_1 \\ c_2 & d_2 \\ 2 & 0 \end{vmatrix} \qquad (1)'$$

$$DB = P_2 \cdot \log(RRT) + Q_2 \qquad DB = \begin{vmatrix} c_1 & y \\ c_2 & d_2 \\ 2 & 0 \end{vmatrix} \qquad (2)'$$

since (2, 0) in position 3 exhibits the acyl (acetyl) group which has two carbons and no double bond. So we can conclude that (1) and (2) are members of the general rule for triglycerides under the condition of $\begin{vmatrix} x & d_1 \\ c_2 & d_2 \\ 2 & 0 \end{vmatrix}$ or $\begin{vmatrix} c_1 & y \\ c_2 & d_2 \\ 2 & 0 \end{vmatrix}$. By adapting the general expressions to triglyceride, the following equations can finally be obtained.

$$CN = P_1 \cdot \log(RRT) + Q_1 \qquad CN = \begin{vmatrix} x & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix} \qquad (5)$$

$$DB = P_2 \cdot \log(RRT) + Q_2 \qquad DB = \begin{vmatrix} c_1 & y \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix} \qquad (6)$$

In this chapter, the theory that has proved mathematically, is verified by the actual experiment.

Section 1. Experimental

Preparation of Triglyceride

Linseed oil and olive oil were purchased from Wako Pure Chemical Industries, Ltd., Osaka. Rapeseed oil was obtained from a commercial source. Cacao butter was supplied by Yunokawa Seiyaku Co. Ltd., Hakodate, Hokkaido, Japan. "Ogonori" (*Gracilaria verrucosa*) was collected at the shore of Taisei-chō, Hokkaido, Japan. Total lipid was extracted from "Ogonori" using chloroform/methanol (1:2, v/v) with the use of an ultra-turrax for comminution.

Triglycerides from these oils were purified by a preparative TLC using n-hexane/diethyl ether (4:1, v/v) as the developing solvent.

HPLC Fractionation of the Moleclar Species of Triglyceride

The purified triglycerides were filtered through a 0.45μ type FP-45 Fluoropore filter (Sumitomo Electric Industry, Ltd., Osaka) and subjected to HPLC. Separation of triglycerides by HPLC has been achieved on LiChrosorb RP-18 (Merck, West Germany) twin 8x250 mm columns. These columns were connected in series. The instruments used consisted of a Hitachi 638-50 Liquid Chromatograph (Hitachi Ltd., Tokyo) equipped with a Shodex SE-11 RI detector (Showa Denko Ltd., Tokyo). The eluting solvent used was acetone/acetonitrile (3:1, v/v). Triglycerides were dissolved in chloroform at 5μg/25μl and applied to the column under room temperature (20~22°C). The flow rate was 1.5 ml/min.

Identification of Molecular Species of Each Peak on HPLC.

Peaks on HPLC chromatograms were numbered in sequence of elution. The fatty acid composition and the total acyl carbon number of each collected predominant peak was analyzed by GLC as previously shown in chapter I.

Section 2. Results

Figure II-1 shows chromatograms of triglycerides on HPLC. The acyl combination of each predominant peak collected was determined by fatty acid analysis and total acyl carbon number anaylsis as shown in Table II-1. This table shows the results on linseed oil in Fig. II-1 as an example. Peak number 1 in Fig. II-1 is triglyceride composed of 18:3 alone, i.e., (18:3,18:3,18:3) because over 98% of fatty acid of this fraction is 18:3, in addition, the total acyl carbon number of this peak is mostly 54. Peak number 2 is considered to be the combination of one mol of 18:2 and two mols of 18:3, that is, (18:2,18:3,18:3). This is supported by the data of total acyl carbon number of this fraction. Peak number 3 is mainly (18:3,18:2,18:2) for the same reasons in peak number 2. Peak number 4 is (18:1,18:3,18:3) with 4~10% contaminants because 9.7% of 18:2 is detected as overlaps from the previous peak. Peak number 5 has at least two contaminants, i.e. 18:1 and 18:2. This peak is concluded to be (16:0,18:3,18:3) from the data of total acyl carbon number. All other peaks were indentified in the same manner.

The relative retention times (RRTs) of all peaks were determined by dividing the retention time of each peak by that of triolein. When the amount of triolein was very small and or the peak overlapped considerably, purified triolein was added to the sample as an internal standard. Predominant or reliable peaks were selected (Table II-2) to plot the RRT of individual triglycerides on a semilogarithmic graph paper. Results are shown in Fig. II-2A. The RRT were also

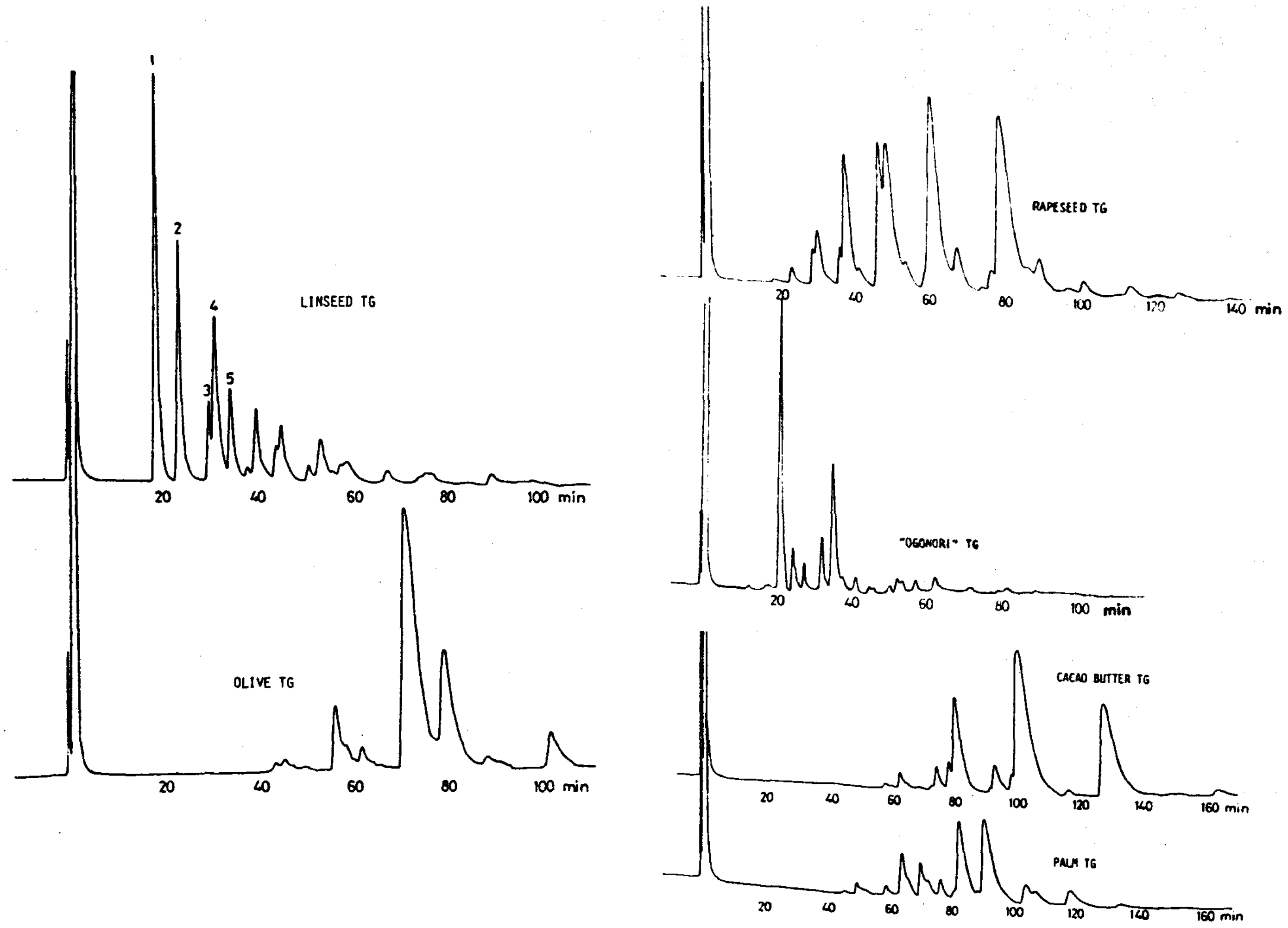


Fig. II-1. HPLC chromatograms of triglycerides from natural sources.

Table II-1. Determination of triglyceride molecular species*

	Peak number				
	1	2	3	4	5
Fatty acid					
16:0					29.6
18:1				28.9	3.7
18:2		33.4	63.2	9.7	1.1
18:3	98.0<	66.6	36.8	61.4	65.5
Carbon number**					
52					98.0<***
54	98.4***	98.3***	96.6***	96.8***	
Molecular species	18:3 18:3 18:3	18:3 18:3 18:2	18:3 18:2 18:2	18:3 18:3 18:1	16:0 18:3 18:3

in relative %

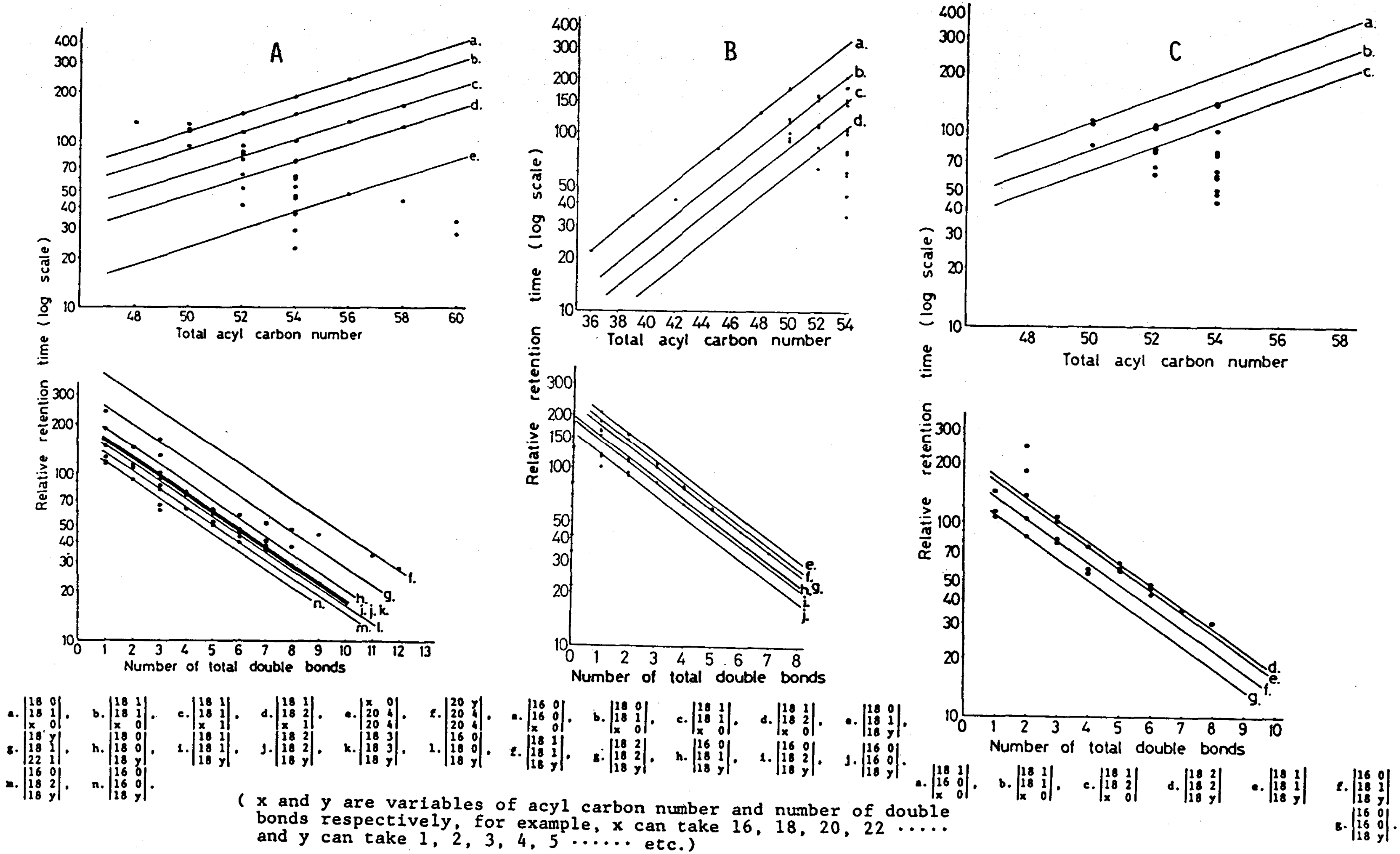
* Example of linseed oil. ** Total acyl carbon number.

*** Percentage by weight.

Table II-2. Relationship between relative retention time and molecular species of triglyceride from natural sources

Rapeseed oil		Linseed oil		Cacao butter		Palm oil		Ogonori		Olive oil	
RRT	Molecular species	RRT	Molecular species	RRT	Molecular species	RRT	Molecular species	RRT	Molecular species	RRT	Molecular species
28.6	(18:3) X 2(18:2)	22.5	(18:3) X 3	91.2	(16:0) X 2(18:2)	62.3	(18:2) X 2(16:0)	27.8	(20:4) X 3	125.4	(16:0) X 2(18:1)
35.9	(18:2) X 2(18:3)	28.6	(18:3) X 2(18:2)	117.5	(16:0) X 2(18:1)	74.7	(18:1) X 2(18:2)	32.8	(20:4) X 2(20:3)	144.8	(18:1) X 2(18:0)
37.5	(18:3) X 2(18:1)	35.5	(18:2) X 2(18:3)	136.2	(18:1) X 2(18:0)	83.6	(16:0) (18:1) (18:2)	37.0	(20:4) X 2(14:0)		
45.0	(18:2) X 3	36.8	(18:3) X 2(18:1)	143.7	(18:0) X 2(18:2)	92.1	(16:0) X 2(18:2)	43.8	(20:4) X 2(18:1)		
46.8	(18:1) (18:2) (18:3)	40.1	(18:3) X 2(16:0)	146.8	(16:0) (18:0) (18:1)	128.6	(16:0) X 3	47.8	(20:4) X 2(16:0)		
58.4	(18:2) X 2(18:1)	44.4	(18:2) X 3	184.8	(18:0) X 2(18:1)						
61.0	(18:1) X 2(18:3)	46.5	(18:1) (18:2) (18:3)	238.5	(18:0) (18:1) (20:0)						
76.3	(18:1) X 2(18:2)	51.0	(16:0) (18:2) (18:3)								
85.5	(16:0) (18:1) (18:2)	58.4	(18:2) X 2(18:1)								
100.0	(18:1) X 3	61.0	(18:1) X 2(18:3)								
112.4	(18:1) X 2(16:0)	76.6	(16:0) (18:1) (18:3)								
122.4	(18:1) (18:2) (22:1)	92.6	(16:0) (18:0) (18:3)								
128.3	(18:1) X 2(20:1)	100.0	(18:1) X 3								
143.9	(18:1) X 2(18:0)										
160.0	(18:1) X 2(22:1)										
162.8	(isomer)										

RRT : relative retention time when (18:1) X 3 is used as the reference peak.



(x and y are variables of acyl carbon number and number of double bonds respectively, for example, x can take 16, 18, 20, 22 ..... and y can take 1, 2, 3, 4, 5 ...... etc.)

Fig. II-2. Relation between relative retention time and total acyl carbon number and relation between relative retention time and total double bonds on HPLC of triglycerides. A: Plant sources in this study. B: Data of E.G. Perkins et al. in J. Am. Oil Chem. Soc., 58, 867~872 (1981) and Lipids, 17, 460~463 (1982). C: Data of C. Merritt et al. in J. Am. Oil Chem. Soc., 59, 422~432 (1982).

calculated from the chromatograms in the reports of Perkins et al.[11,38] (see Table II-3 in the next section) and those of Merritt et al.,[39] and plotted on a semilogarithmic graph paper as shown in Fig. II-2B and Fig. II-2C, respectively. As it is clear from these figures, the sequence in elution might be controlled by a fixed correlation, that is, a matrix relation, though the analytical conditions are different among the three figures in Fig. II-2. If we express these by empirical equations, the two equations described previously (equations (5) and (6) in section 1) can be obtained.

In Fig. II-2, it is observed that even triglycerides from plant sources show complicated sets of oblique lines. This is due to the extreme increase in probability in combination of fatty acids. For example, if we have four kinds of fatty acids, there is a probability that diglyceride or lecithin might have a maximum of $4^2=16$ kinds of acyl combinations. But in case of triglycerides, this will drastically increase up to $4^3=64$ kinds of acyl combinations. More specifically, in case of fish lipid, for instance if we have ten kinds of fatty acids, there is a probability that diglyceride or lecithin might have a maximum of $10^2=100$ kinds of acyl combinations, while in case of triglyceride, $10^3=1000$ combinations might be possible. The author has tried to analyze the molecular species of triglycerides from fish muscle. But the theoritical plate of the HPLC column is still not enough to separate the very complicated combination of molecular species of triglyceride from marine sources. Recently, column packing with 3-μm particles has been developed by Dong et al.[40] for triglyceride analysis from vegitable sources. Though the life of this type of column packing is shorter than the coventional 5-μm particle type, it is expected to give a high resolusion chromatograms.

Section 3. Discussion

The chromatograms of Perkins et al.11,38) were introduced in this study in order to calculate the RRT of each peak on HPLC since it is based on 28 kinds of known triglyceride standards purchased from Nu-Chek-Prep (Elysian, MN) and Supelco (Supelco, Bellefonte, PA). RRT of each peak on Perkins et al.'s11,38) chromatograms were calculated by dividing the retention time of each peak by that of triolein. And multiple regression was performed against RRT using Personal computer Model PC-8001 (NEC, Tokyo) to link the two concepts, that is , ECN or PN and the new matrix model. The commonly used computer program for the multiple regression41) was written in N-BASIC available for most of the NEC personal computers.

Theoritical Relations between the ECN (or PN) and the New Matrix Model

We will express the molecular species of triglyceride in a matrix form as it is written previously such as $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$. When a so called functional group X (in this case $-CH_2-$) is added to $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$, then $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$ will become $\begin{vmatrix} c_1{+}X & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$. The theory of Martin42) was:

$$\frac{\Delta\mu_B}{RT} = \frac{\Delta\mu_A}{RT} + \frac{\Delta\mu_X}{RT} \qquad (1)$$

$$\ln\left(\frac{\alpha_B}{\alpha_A}\right) = \frac{\Delta\mu_X}{RT} \qquad (2)$$

(μ: Chemical potential, R: Gas constant, T: Absolute temperature, B: Substance which contains group X in addition to substance A, α: Partition coefficient)

If we substitute A as $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$ and B as $\begin{vmatrix} c_1{+}X & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$ in equations (1) and (2), the following equations can be obtained:

$$\frac{\Delta\mu\begin{vmatrix} c_1+X & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}}{R\cdot T} = \frac{\Delta\mu\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}}{R\cdot T} + \frac{\Delta\mu_X}{R\cdot T} \tag{3}$$

$$\ln\left(\frac{\alpha\begin{vmatrix} c_1+X & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}}{\alpha\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}}\right) = \frac{\Delta\mu_X}{R\cdot T} \tag{4}$$

On the other hand, the general rule for the liquid chromatography was:

$$V_r = \alpha V_s$$

(V_r: Total adjusted retention volume, V_s: Volume of the liquid stationary phase, α: Partition coefficient)

So,

$$V_r\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix} = \alpha\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}\cdot V_s \tag{5}$$

$$V_r\begin{vmatrix} c_1+X & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix} = \alpha\begin{vmatrix} c_1+X & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}\cdot V_s \tag{6}$$

From equations (5) and (6),

$$\frac{V_r\begin{vmatrix} c_1+X & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}}{V_r\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}} = \frac{\alpha\begin{vmatrix} c_1+X & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}}{\alpha\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}} \tag{7}$$

The right member in equation (7) is a partition ratio which corresponds to retention value, namely:

$$\frac{\alpha\begin{vmatrix} c_1+X & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}}{\alpha\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}} = RRT \tag{8}$$

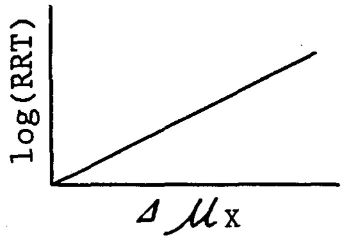
By substituting equation (8) into (4),

$$\ln(RRT) = \frac{\Delta\mu_X}{R\cdot T}$$

$$\log(RRT) = \frac{\Delta\mu_X}{2.303\ R\cdot T}$$

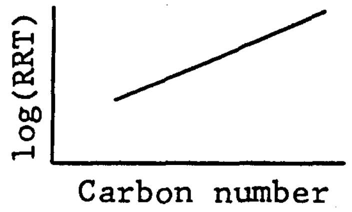
$$\therefore \log(\mathrm{RRT}) \propto \Delta\mu_X \qquad (9)$$

If equation (9) is held, the following correlation should hold.



$$\log(\mathrm{RRT}) = k\cdot\Delta\mu_X \qquad (10)$$

And if we let the x-axis be the carbon number:



$$\log(\mathrm{RRT}) = P_1 C + q_1 \qquad (11)$$

where P becomes the slope and q becomes the intercept on the ordinate. This equation (11) is exactly a modified form of the emperical equations:

$$\mathrm{CN} = P_1\cdot\log(\mathrm{RRT}) + Q_1 \qquad \mathrm{CN} = \begin{vmatrix} x & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$$

$$\mathrm{DB} = P_2\cdot\log(\mathrm{RRT}) + Q_2 \qquad \mathrm{DB} = \begin{vmatrix} c_1 & y \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$$

namely, the new concept of the chromatographic rule discovered in this study.

So far, the physicochemical background of this new concept has been demonstrated by introducing the theory of Martin.42)

In the same way, for all of the functional groups added to the triglyceride $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$, equation (11) should hold, namely:

$$\log(RRT)=P_1 \cdot C_1+q_1 \quad (12)$$

$$\log(RRT)=P_2 \cdot C_2+q_2 \quad (13)$$

$$\log(RRT)=P_3 \cdot C_3+q_3 \quad (14)$$

$$\log(RRT)=P_1' \cdot D_1+q_4 \quad (15)$$

$$\log(RRT)=P_2' \cdot D_2+q_5 \quad (16)$$

$$\log(RRT)=P_3' \cdot D_3+q_6 \quad (17)$$

Careful considerations should be done here.

I. If we take notice of one hydrocarbon chain of the triglyceride molecule, $-CH_2-$ and $-CH=CH-$ can be considered as physicochemical functional groups. And adding to those, the differences in arrangement of these units might affect the total chemical potential of the triglyceride molecule and this should also be considered (We will call this as Ω factor).

II. From the view point of stereo specific structure, unless three carbon chains are the same, we should consider about the degree in bias between the positional isomers.

III. Specific fatty acids such as iso, trans or hydroxy fatty acids might also affect the total chemical potential of the triglyceride molecule.

So, strictly speaking, we can conclude that the chemical potential of the triglyceride molecule is the resultant of all physicochemical functional groups in the molecule. If we do not consider about the affection of III since these fatty acids seldom appear, the chemical potential (μ) of triglyceride $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$ can be written as follows:

$$\mu = g\{f(c_1, d_1, \Omega_1), f(c_2, d_2, \Omega_2), f(c_3, d_3, \Omega_3)\} \quad (18)$$

where Ω is an Ω factor, μ is the chemical potential given by the hydrocarbon chain. And g is the function of chemical potential given by the differences in positional isomers. Finally, we have reached to the function (18) that sums up the chemical potential of the triglyceride molecule, and this (18) is at the same time, an equation that controls the sequence in elution on HPLC. This will be discussed in chapter V again.

We will derive ECN (or PN) from function (18). When we neglect the factor numbered II, and suppose there is no iso or trans or hydroxy fatty acids, we can rewrite equation (18) as:

$$\mu = f(c_1, d_1, \Omega_1) + f(c_2, d_2, \Omega_2) + f(c_3, d_3, \Omega_3) \qquad (19)$$

Adding to it, if we neglect the Ω factor, equation (19) will become:

$$\begin{aligned} \mu &= f(c_1, d_1) + f(c_2, d_2) + f(c_3, d_3) \\ &= f(c_1 + c_2 + c_3,\ d_1 + d_2 + d_3) \\ &= f(CN, DB) \qquad (20) \end{aligned}$$

This equation (20) exactly corresponds to the equation of ECN (or PN). Another way of demonstrating the theoritical relation between the new concept and ECN (or PN) is as follows. If we do not consider about the Ω factor and also the iso, trans and hydroxy fatty acids, we can add both members from equation (12) to (17), namely:

$$6 \cdot \log(RRT) = P_1 \cdot C_1 + P_2 \cdot C_2 + P_3 \cdot C_3 + P_1' \cdot D_1 + P_2' \cdot D_2 + P_3' \cdot D_3 + q_1 + q_2 + q_3 + q_4 + q_5 + q_6 \qquad (21)$$

And if we put Pn=1/6 Pn, P'n=1/6 P'n, $\sum q/6 = Q$, the following equation can be obtained:

$$\log(RRT) = P_1 \cdot C_1 + P_2 \cdot C_2 + P_3 \cdot C_3 + P_1' \cdot D_1 + P_2' \cdot D_2 + P_3' \cdot D_3 + Q \qquad (22)$$

This equation (22) can be considered as the first order

Table II-3. Relative retention time calculated from the HPLC chromatograms of E.G. Perkins et al. in J. Am. Oil Chem. Soc., 58, 867~872 (1981) and on Lipids, 17, 460~463 (1982)

Molecular species	RRT*	Molecular species	RRT*
LaLaLa	21.9	M 0 P	101.1
tri-13:0	33.9	S 0 L	104.1
L L Le	34.3	S P L	108.7
M M M	42.1	P 0 0	113.1
L L L	44.8	S 0 M	117.3
L 0 L	58.7	P 0 P	120.1
L L P	63.3	P P P	132.9
L 0 0	76.7	S 0 0	144.2
tri-15:0	81.8	S S L	145.0
P L 0	83.7	S 0 P	152.7
P P L	89.5	S 0 0	155.3
P L P	90.7	S P 0	162.3
L P P	94.2	S P P	179.8
0 0 0	100.0	S 0 S	208.1

Abbrev.
La : Lauryl, L : Linoleyl, Le : Linolenyl,
M : Myristyl, 0 : 0leyl, P : Palmityl.

*RRT : Relative retention time when 000 is used as the reference peak.

Table II-4. Multiple regression analysis of the data in Table II-3

```
DATE  74/03/19
TIME  08:04:25
Perkins T.G. DATA POSITIONAL 6-dim
------------------------------------------------------------------------------
** MULTIPLE REGRESSION ANALYSIS ** PC=8001 N-BASIC  **  By MAKOTO EGI

INPUT PARAMETERS
No. OF SAMPLE     : 28
NO. OF VARIABLE   : 6
RAW(0)/STD(1)       0

INPUT DATA
SAMPLE     X(  1)   X(  2)   X(  3)   X(  4)   X(  5)   X(  6)       Y
  1       12.000   12.000   12.000    0.000    0.000    0.000  134.020
  2       13.000   13.000   13.000    0.000    0.000    0.000  152.992
  3       18.000   18.000   18.000    2.000    2.000    3.000  153.502
  4       12.000   14.000   14.000    0.000    0.000    0.000  162.399
  5       18.000   18.000   18.000    2.000    2.000    2.000  165.098
  6       18.000   18.000   18.000    2.000    1.000    2.000  176.832
  7       18.000   18.000   16.000    2.000    2.000    0.000  190.108
  8       18.000   18.000   18.000    2.000    2.000    1.000  188.446
  9       15.000   15.000   15.000    0.000    0.000    0.000  191.241
 10       16.000   18.000   18.000    0.000    2.000    1.000  192.238
 11       16.000   16.000   18.000    0.000    0.000    2.000  195.147
 12       16.000   18.000   16.000    0.000    2.000    0.000  195.725
 13       18.000   16.000   16.000    2.000    0.000    0.000  197.370
 14       18.000   18.000   18.000    1.000    1.000    1.000  199.964
 15       14.000   18.000   16.000    0.000    1.000    0.000  200.439
 16       18.000   18.000   18.000    0.000    1.000    2.000  201.709
 17       18.000   16.000   18.000    0.000    0.000    2.000  203.586
 18       16.000   18.000   18.000    0.000    1.000    1.000  205.309
 19       18.000   18.000   14.000    0.000    1.000    0.000  206.893
 20       16.000   18.000   16.000    0.000    1.000    0.000  207.917
 21       16.000   16.000   16.000    0.000    0.000    0.000  212.314
 22       18.000   18.000   18.000    0.000    1.000    1.000  215.858
 23       18.000   18.000   18.000    0.000    0.000    2.000  216.098
 24       18.000   18.000   16.000    0.000    1.000    0.000  218.345
 25       18.000   18.000   18.000    0.000    1.000    1.000  219.078
 26       18.000   16.000   18.000    0.000    0.000    1.000  220.992
 27       18.000   16.000   16.000    0.000    0.000    0.000  225.438
 28       18.000   18.000   18.000    0.000    1.000    0.000  231.785

AVERAGE   16.714   16.929   16.643    0.464    0.821    0.786  195.387
STD.DEV    1.887    1.669    1.716    0.823    0.758    0.901   23.625

CORRELATION MATRIX
           X(  1)   X(  2)   X(  3)   X(  4)   X(  5)   X(  6)       Y
 X(  1)    1.000    0.720    0.719    0.384    0.339    0.426    0.559
 X(  2)    0.720    1.000    0.715    0.258    0.696    0.323    0.507
 X(  3)    0.719    0.715    1.000    0.244    0.363    0.690    0.466
 X(  4)    0.384    0.258    0.244    1.000    0.476    0.327   -0.399
 X(  5)    0.339    0.696    0.363    0.476    1.000    0.205   -0.119
 X(  6)    0.426    0.323    0.690    0.327    0.205    1.000   -0.133
      Y    0.559    0.507    0.466   -0.399   -0.119   -0.133    1.000

================================================================================
USE VAR No.   1
USE VAR No.   2
USE VAR No.   3
USE VAR No.   4
USE VAR No.   5
USE VAR No.   6

REGRESSION EXPRESSION
Y =     5.57391 * X( 1 )
        5.90109 * X( 2 )
        7.00699 * X( 3 )
      -11.63260 * X( 4 )
      -13.40241 * X( 5 )
      -15.40738 * X( 6 )
      -85.77448

Y & Y' FOR EACH SUBJECT
SAMPLE No. :  1   Y :  134.02026   Y' :  136.00953   ERROR :  -1.98927
SAMPLE No. :  2   Y :  152.99240   Y' :  154.49153   ERROR :  -1.49913
SAMPLE No. :  3   Y :  153.50175   Y' :  150.60940   ERROR :   2.89236
SAMPLE No. :  4   Y :  162.39894   Y' :  161.82571   ERROR :   0.57323
SAMPLE No. :  5   Y :  165.09805   Y' :  166.01677   ERROR :  -0.91872
SAMPLE No. :  6   Y :  176.83197   Y' :  179.41918   ERROR :  -2.58721
SAMPLE No. :  7   Y :  190.10791   Y' :  192.81754   ERROR :  -2.70963
SAMPLE No. :  8   Y :  188.44559   Y' :  181.42415   ERROR :   7.02144
SAMPLE No. :  9   Y :  191.24089   Y' :  191.45554   ERROR :  -0.21466
SAMPLE No. : 10   Y :  192.23793   Y' :  193.54152   ERROR :  -1.30359
SAMPLE No. : 11   Y :  195.14716   Y' :  193.13677   ERROR :   2.01038
SAMPLE No. : 12   Y :  195.72546   Y' :  194.93491   ERROR :   0.79055
SAMPLE No. : 13   Y :  197.36952   Y' :  197.82017   ERROR :  -0.45064
SAMPLE No. : 14   Y :  199.96397   Y' :  206.45916   ERROR :  -6.49518
SAMPLE No. : 15   Y :  200.43900   Y' :  197.18949   ERROR :   3.24951
SAMPLE No. : 16   Y :  201.70874   Y' :  202.68438   ERROR :  -0.97564
SAMPLE No. : 17   Y :  203.58627   Y' :  204.28460   ERROR :  -0.69833
SAMPLE No. : 18   Y :  205.30927   Y' :  206.94393   ERROR :  -1.63466
SAMPLE No. : 19   Y :  206.89253   Y' :  205.47116   ERROR :   1.42137
SAMPLE No. : 20   Y :  207.91684   Y' :  208.33732   ERROR :  -0.42048
SAMPLE No. : 21   Y :  212.31425   Y' :  209.93754   ERROR :   2.37672
SAMPLE No. : 22   Y :  215.85762   Y' :  218.09176   ERROR :  -2.23414
SAMPLE No. : 23   Y :  216.09785   Y' :  216.08679   ERROR :   0.01107
SAMPLE No. : 24   Y :  218.34457   Y' :  219.48515   ERROR :  -1.14057
SAMPLE No. : 25   Y :  219.07765   Y' :  218.09176   ERROR :   0.98589
SAMPLE No. : 26   Y :  220.99203   Y' :  219.69198   ERROR :   1.30006
SAMPLE No. : 27   Y :  225.43834   Y' :  221.08537   ERROR :   4.35297
SAMPLE No. : 28   Y :  231.78545   Y' :  233.49913   ERROR :  -1.71369

TABLE OF THE ANALYSIS OF VARIANCE
         -------------------------------------------------------
           SOURCE           S.S        D.F.     M.S.     F-RATIO
         -------------------------------------------------------
          REGRESSION15446.93051         6 2574.48842  298.30483
           RESIDUAL    181.23829       21    8.63039
             TOTAL  15628.16881        27
         -------------------------------------------------------
MULTIPLE CORRELATION COEFFICIENT       0.99418
COEFFICIENT OF DETERMINATION          0.98840
```

combination of C_1, C_2, C_3, D_1, D_2, D_3 against log(RRT). The author have performed the multiple regression analysis by using the chromatograms of Perkins et al.[11,38] The RRT calculated from the chromatograms exhibited by Perkins et al.[11,38] are shown in Table II-3. And the results calculated by the multiple regression analysis are shown in Table II-4. This multiple regression analysis program is the commonly used software from "Personal Computer Program Library Vol. 3" published by Kōgakutosho, Ltd., Tokyo.[41] "INPUT DATA" in Table II-4 are the data of RRT (written as column Y), and acyl carbon number in position 1 (written as X(1)), position 2 (written as X(2)), position 3 (written as X(3)), and also number of double bonds in position 1 (written as X(4)), position 2 (written as X(5)), position 3 (written as X(6)) from Table II-3. "REGRESSION EXPRESSION" corresponds to equation (22) and the coefficients of each term are determined. "Y & Y" is the actual RRT and the predicted RRT by multiple regression analysis, respectively. In this case, positional isomers such as $\begin{vmatrix} 16 & 0 \\ 16 & 0 \\ 18 & 2 \end{vmatrix}$ and $\begin{vmatrix} 16 & 0 \\ 18 & 2 \\ 16 & 0 \end{vmatrix}$ can be distinguished (see sample PPL and PLP in Table II-3). But if the positional isomers cannot be distinguished, each coefficient i.e. Cn and Dn will become equivalent. So if we rewrite Pn as P and P'n as P', the following equation can be derived from equation (22):

$$\begin{aligned} \log(RRT) &= P(C_1+C_2+C_3) + P'(D_1+D_2+D_3)+Q \\ &= P(CN) + P'(DB) + q \qquad (23) \end{aligned}$$

This equation (23) is actually the same with the definition of ECN (or PN) since P'/P corresponds to the coefficient -2 in ECN (or PN).

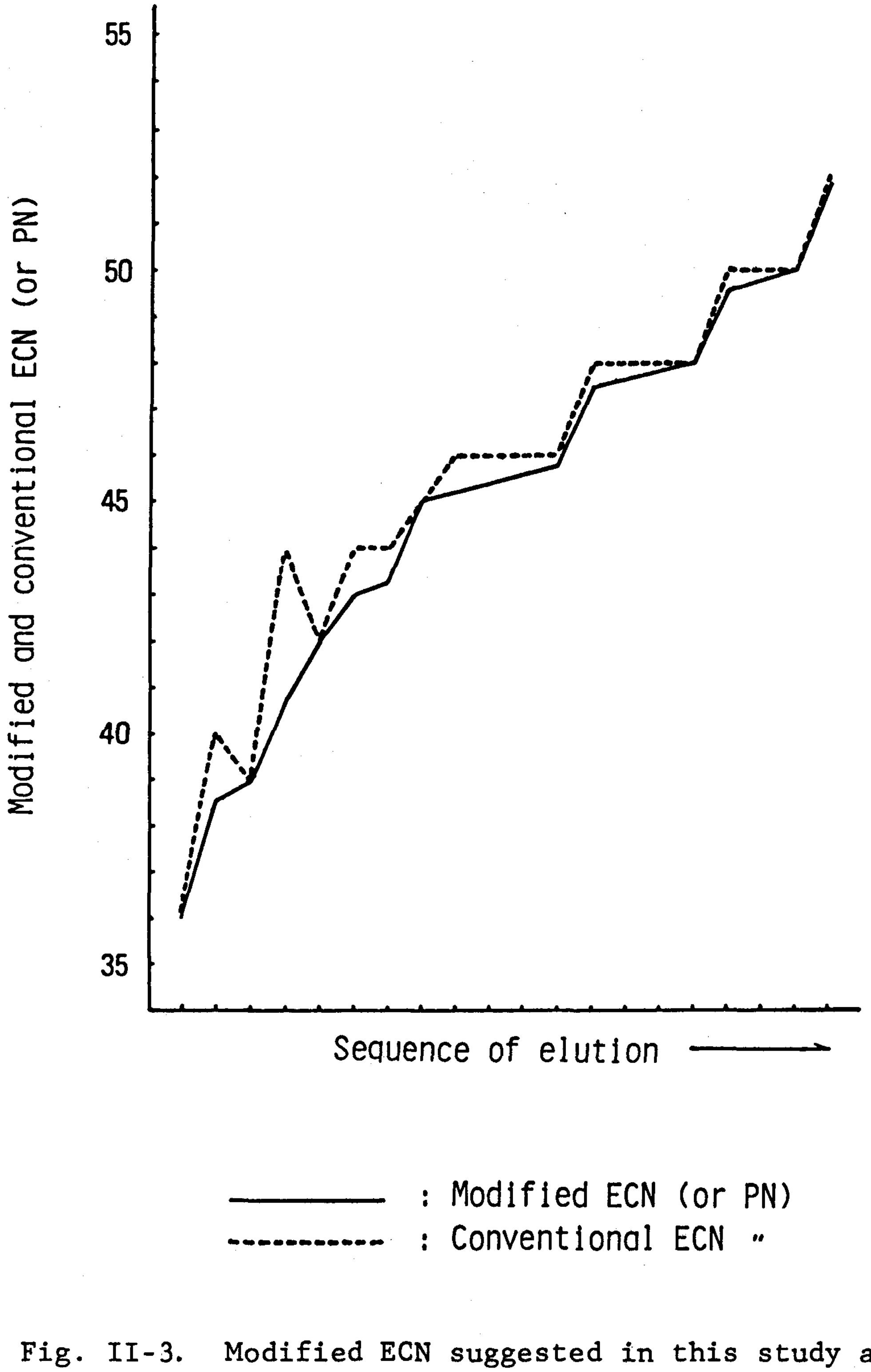


Fig. II-3. Modified ECN suggested in this study and the conventional ECN in relation to the sequence of elution of triglyceride species on HPLC.

Finally, we have induced the conclusion of ECN (or PN) from Martin's theory.

Back to the results in Table II-4 "REGRESSION EXPRESSION" was 5.57391 for C_1, 5.90109 for C_2, 7.00699 for C_3, -11.63260 for D_1, -13.40241 for D_2 and -15.40738 for D_3. Accordingly, if we do not consider about the positional isomers, from equation (23):

$$P'/P = \frac{-(11.63260+13.40241+15.40738)}{5.57391+5.90109+7.00699} = -2.1882 \fallingdotseq -2.2$$

This ratio i.e. -2.2 is near but not equal to the ECN (or PN) definition ratio which is -2.

<u>Limitation of the ECN (or PN) Concept</u>

By changing the equation from (22) to (23), the limitation in positional isomers will be released. For example, $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$ and $\begin{vmatrix} c_2 & d_2 \\ c_1 & d_1 \\ c_3 & d_3 \end{vmatrix}$ cannot be distinguished in equation (23). And what is more important, $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$ and $\begin{vmatrix} c_1 & d_2 \\ c_2 & d_1 \\ c_3 & d_3 \end{vmatrix}$ cannot be distinguished also after changing the equation (23). Adding to this, the ECN definition ratio was an integral number which is -2. This implies that a lot of molecular species exist that have the same ECN. If we use the regression coefficient on equation (23) which is -2.2, at least it is possible to distinguish the following case that has the same ECN (=38):

$$\begin{vmatrix} 18 & 1 \\ 20 & 4 \\ 22 & 6 \end{vmatrix} \quad (A) \qquad\qquad \begin{vmatrix} 16 & 0 \\ 20 & 4 \\ 22 & 6 \end{vmatrix} \quad (B)$$

Namely, by using coefficient -2.2, ECN' of (A) becomes 60-2.2x11=35.8 and ECN' of (B) becomes 58-2.2x10=36.0. The author have performed a sequence simulation on HPLC of Table II-3 by using equation (23). Results are shown in Fig. II-3. As it is clear from this Fig. II-3, all the molecular species in Table II-3 become irreversible (shown as a solid line).

In conclusion, we might say the coefficient of the ECN should not be an integral number, and this coefficient is affected by the analytical conditions employed. This was also supported by the latest work done by Toya et al.43)

The matrix model might be the invariant rules that controls the sequence in elution of molecular species on HPLC.

CHAPTER III

LECITHIN MOLECULAR SPECIES IDENTIFICATION PROGRAM FOR THE PERSONAL COMPUTER

So far, the matrix model, namely, the formulae that control the sequence in elution of individual molecular spesies on HPLC have described.

The next step of this study was the efficient progress in the molecular species indentification on HPLC chromatograms by utilizing these formulae. A personal computer was introducted for this purpose. NEC personal computer Model PC-8001 (NEC Ltd. Tokyo) was used.

Section 1. Lecithin Molecular Species Identification Program Version I.

At first, the slopes of the formulae (formulae (8') and (9') in chapter I) were set by analyzing the data of lecithin molecular species on HPLC that had been accumulated. And the intercepts of the same formulae were all determined by the regression analysis. The identification program was designed by using these slopes and intercepts. Though this identification program was a concrete form of the chromatographic rules presented in chapter I (equations (8') and (9')), there were some identification errors. It was observed that in some cases, specifically in the case of tetraene group, there were some deviations as shown in Fig. III-1 resulting in the errors for the molecular species identification. The main reasons were considered to be the following two:

1. When the peaks overlap, it is hard to determine the RRTs of positional isomers.
2. Both $\omega 3$ and $\omega 6$ type fatty acids exist in tetraene groups that make the large deviation of RRT.

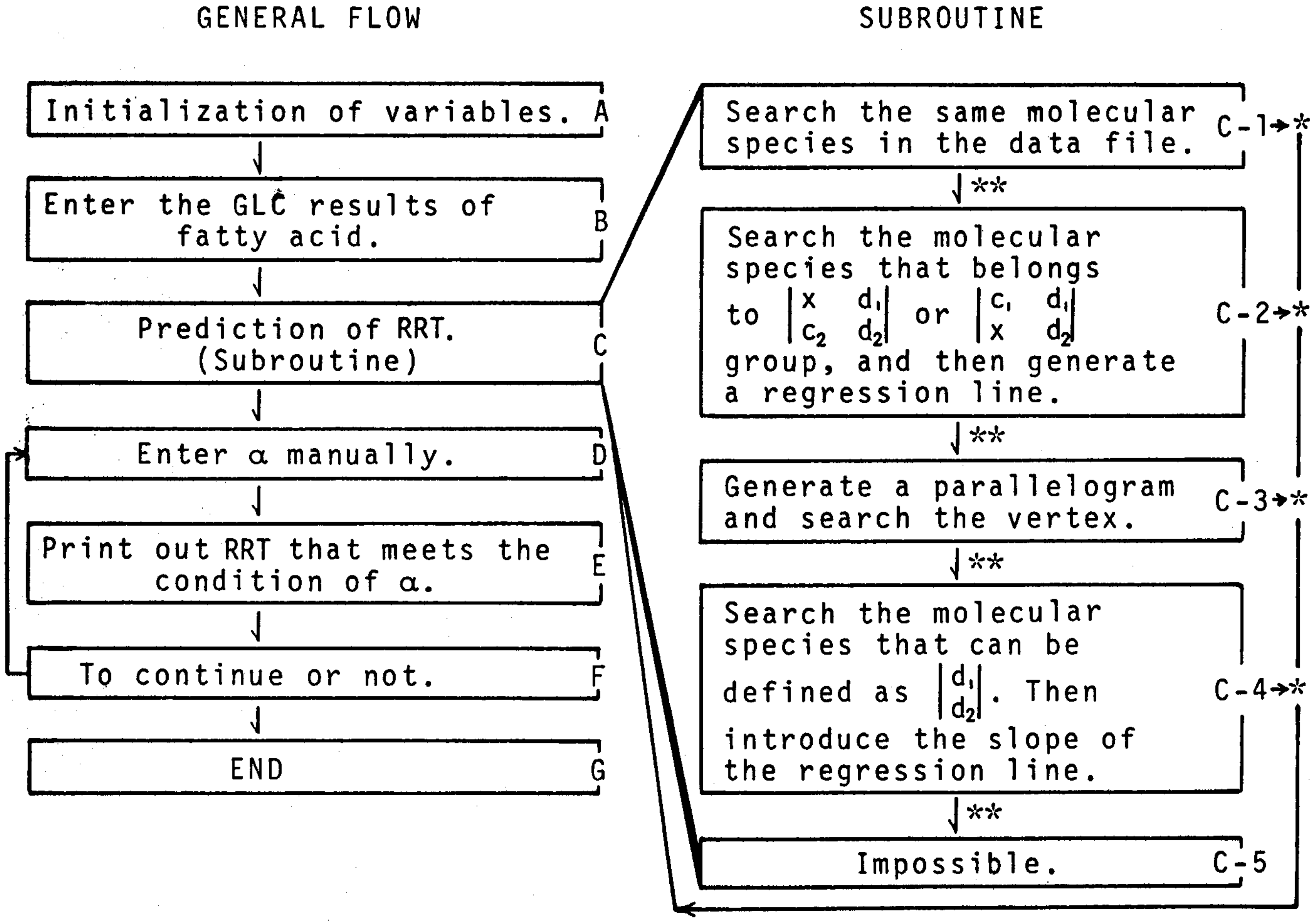
So, it can be concluded that the thorough development of instruments i.e. the column, the pump etc. of HPLC are required in order to clearify the RRT differences in positional isomers as well as the differences in ω isomers, and to complete the molecular species indentification program that exactly reflexes the equations ((8') and (9')).

Section 2. Lecithin Molecular Species Identification Program Version II.

Though it was found to be hard to design a computer program that strictly follows the chromatographic rules presented in this study due to the inadequate imformation of RRT between the isomers or the inadequate reproducibility of RRT on the present HPLC, a more practical identification program based on the collation with the deposit data were designed. It runs as follows as designated in Scheme III-1:

At first, the fatty acid composition data of the collected peak on HPLC was entered (shown as B in Scheme III-1) after initialization (shown as A). Then the identification subroutine starts running according to the priority order of identification that goes as follows:

1. Search the same molecular species in the data file and when there is, then print out the RRT of it that meets the condition of α which restricts the range of RRT that has entered from the keybord manually (shown as C-1 and D, E). This is called "PREDICTED BY S. T. D. RRT" in Program III-1.
2. If not in the case of 1, search the molecular species that belongs to $\begin{vmatrix} x & d_1 \\ c_2 & d_2 \end{vmatrix}$ (or $\begin{vmatrix} c_1 & d_1 \\ x & d_2 \end{vmatrix}$) group, and then generate a regression line as shown in Fig. III-2. After that, print out the RRT of it that meets the condition of α (shown as C-2 and D, E). This is called "Liner prediction routine" as well as "RREDICTED BY 3-VAR. LINER METHOD" in Program III-1.
3. If not the cases of 1 and 2, search the molecular



* Searched. ** Not searched.

Scheme III-1. Flow chart of the lecithin molecular species identification program for the personal computer.

```
1 PRINT "IF YOU WANT TO ONLY PREDICT ONE M.S. RRT THEN ELASE ' OF LINE 75":DELET
E 1:END
40 '            D.G.      RRT PREDICT PROGRAM LAST VERSION ( EVERY WAY )
60 WIDTH80,25:CONSOLE,,0,1:COLOR7,0,1:PRINT CHR$(12):LOCATE 10,10 :PRINT "MOLECU
LER SPECIES OF DI-GLYCERIDE DETERMINATION HELPER":LOCATE 10,12:PRINT "PROGRAMED
BY M.E.                     1983.10.18.":LINE(16,36)-(130,54),PSET,B
70 LOCATE 15,18:PRINT "INITIALIZE PHASE":         GOSUB 7000
72 PRINT CHR$(12)
75 'INPUT C1,D1,C2,D2:GOSUB 1000:PRINT PR,ME$(M):GOTO 75
80 FOR I=1 TO 10 :FC(I)=0:FD(I)=0:FP(I)=0:FOR J=1 TO 10:MM(I,J)=0:ML(I,J)=0:NEXT
 J:NEXT I:PRINT CHR$(12)
90 PRINT :INPUT "SAMPLE NAME IS ";SN$:INPUT "HPLC PEAK NO. IS ";NS:PRINT :INPUT"
INCLUDING  F.A. NO.";N
100 PRINT :FOR I=1 TO N
110 PRINT "F.A. NO.";I:INPUT"CARBON NO:";FC(I):INPUT"DOUBLE BOND NO: ";FD(I):PRI
NT :NEXT I:PRINT CHR$(12)
120 PRINT "SAMPLE NAME  : ";SN$:PRINT :PRINT :PRINT "HPLC PEAK NO.  :";NS:PRINT
:PRINT "CALCULATING NO. ";S:S=S+1:PRINT :PRINT "INCLUDING F.A. OF THIS PEAK ":PR
INT
130 FOR I=1 TO N:PRINT "F.A. NO. ";I;:PRINT "      ";FC(I);":";FD(I);"          ":
NEXT I:PRINT :PRINT "OK":PRINT
140 INPUT "REAL RRT OF THIS PEAK";RT:PRINT :PRINT "NOW CALCULATING PHASE":PRINT
150 FOR I1=1 TO N:FOR J1=1 TO I1:C1=FC(I1):C2=FC(J1):D1=FD(I1):D2=FD(J1):GOSUB 1
000:MM(I1,J1)=PR:ML(I1,J1)=M:NEXT J1:NEXT I1
160 BEEP:PRINT "CALCULATION ENDED":PRINT
170 INPUT "ENTER MAX. OF ALPHA";K:PRINT
180 FOR I=1 TO N:FOR J=1 TO I:IF ABS(MM(I,J)-RT)<K THEN PRINT "|";FC(I);":";FD(I
);"|":PRINT "|";FC(J);":";FD(J);"|";"     P.RRT=";MM(I,J);"  ALPHA=";MM(I,J)-RT:P
RINT    :PRINT"                      METHOD :";ME$(ML(I,J)):PRINT
190 NEXT J:NEXT I
200 AN$="yes":INPUT "ANY MORE PREDICTION ";AN$:IF AN$="yes" THEN 170
210 AN$="yes":INPUT "ANY MORE CALCULATION ";AN$:IF AN$="yes" THEN 80
900 GOTO 80
1000 '---------------------- PR Calculation -----------------------------------
1010 K=0:K1=0:K2=0:PR=0:M=1
1015 IF D1>D2 THEN C=C1:D=D1:C1=C2:D1=D2:C2=C:D2=D
1020 IF (D1=D2 AND C1>C2)THEN C=C1:D=D1:C1=C2:D1=D2:C2=C:D2=D
1030 FOR I=1 TO SN:IF D1=SD(I,2)AND D2=SD(I,4) THEN K=K+1:SS(K)=I:NEXT I ELSE NE
XT I
1070 FOR I=1 TO K:IF C1=SD(SS(I),1) AND C2=SD(SS(I),3) THEN PR=SD(SS(I),5):M=2:G
OTO 1200 ELSE NEXT I
1080 FOR I=1 TO K:IF C1=SD(SS(I),1) THEN K1=K1+1:S1(K1)=SS(I)
1085 IF C2=SD(SS(I),3) THEN K2=K2+1:S2(K2)=SS(I)
1090 NEXT I
1110 IF K1>=2 OR K2>=2 THEN GOSUB 4000:GOTO 1200
1130 IF FO(D1,D2,1)=1 THEN T1=LOG(SD(FO(D1,D2,4),5))*43.429:T2=(C1-SD(FO(D1,D2,4
),1))*FO(D1,D2,2):T3=(C2-SD(FO(D1,D2,4),3))*FO(D1,D2,3):PR=10^((T1+T2+T3)/100):M
=5:GOTO  1200
1145 IF  K<>0 THEN PR=10^((A1*(C1+C2)+QQ(D1,D2))/100):M=3:GOTO 1200
1150 M=1:PR=0
1200 RETURN
4000 '------------------------ Liner prediction routine ----------------------
4005 SX=0:SY=0:XX=0:YY=0:XY=0:M=4
4010 IF K1>=2 THEN FOR I=1 TO K1 :X=SD(S1(I),1)+SD(S1(I),3):Y=LOG(SD(S1(I),5))*4
3.43:SX=SX+X:SY=SY+Y:XX=XX+X*X:YY=YY+Y*Y:XY=XY+X*Y:NEXT I:KS=XX-SX*SX/K1:LS=XY-S
X*SY/K1:MS=YY-SY*SY/K1:A4=LS/KS:B4=SY/K1-A4*SX/K1:R4=LS/SQR(LS*MS)
4015 SX=0:SY=0:XX=0:YY=0:XY=0:P1=(C1+C2)*A4+B4:P1=10^(P1/100)
4020 IF K2>=2 THEN FOR I=1 TO K2 :X=SD(S2(I),1)+SD(S2(I),3):Y=LOG(SD(S2(I),5))*4
3.43:SX=SX+X:SY=SY+Y:XX=XX+X*X:YY=YY+Y*Y:XY=XY+X*Y:NEXT I:KS=XX-SX*SX/K2:LS=XY-S
X*SY/K2:MS=YY-SY*SY/K2:A5=LS/KS:B5=SY/K2-A5*SX/K2:R5=LS/SQR(LS*MS)
4025 P2=(C1+C2)*A5+B5:P2=10^(P2/100)
4030 IF K1>K2 THEN PR=P1 ELSE IF K1<K2 THEN PR=P2
4990 RETURN
7000 '----------------------- PR Calculation Initialize ----------------------
7005 SN=69:K=0:K1=0:K2=0:PR=0:T=24:A1=7.66589:PR=0
7010 DIM SD(SN,5),SS(15),FO(6,6,4),DS(T,2),Q3(T),QQ(7,7),FC(10),FD(10),FP(10),MM
(10,10),ML(10,10)
7020 FOR I=1 TO SN:FOR J=1 TO 5:READ SD(I,J):NEXTJ:NEXT I
7030 FOR I=1 TO T:FOR J=1 TO 2:READ DS(I,J):NEXT J:READ Q3(I):QQ(DS(I,1),DS(I,2)
)=Q3(I):NEXT I
7040 FOR I=1 TO 5:READ ME$(I):NEXT I
7050 FOR I=1 TO 5:READ DO,DT:FO(DO,DT,1)=1:FOR II=2 TO 4:READ FO(DO,DT,II):NEXT
II:NEXT I
7060 FOR I=1 TO 2:FOR J=1 TO 2:READ P$(I,J):NEXT J:NEXT I
9040 RETURN
10000 '------ DATA SET OF STD. D.G. RRT ( MEAN OF DATA )  FROM D.G. BOOK ----
10010 DATA 16,0,16,0,213
10020 DATA 14,0,18,1,141.8,18,0,18,1,295.2,16,0,18,1,208.786,15,0,18,1,175.7,16,
0,17,1,175.7,16,0,16,1,148.45,14,0,20,1,212,16,0,20,1,301.9
10030 DATA 14,0,18,2,100,16,0,16,2,111.7,16,0,18,2,145.7,17,0,18,2,167.9,18,0,18
,2,202.9,20,0,18,2,288.6
10040 DATA 16,1,18,1,138.26,18,1,18,1,191.175
10050 DATA 16,1,18,2,100,18,1,18,2,135.45,20,1,18,2,188.1
10060 DATA 16,0,18,3,110.6,18,0,18,3,152.9
10070 DATA 16,0,20,4,122.057,16,0,22,4,132.9,18,0,20,4,177.55,18,0,22,4,191.7
10080 DATA 18,1,18,3,99.9
10090 DATA 18,2,18,2,97.9
10100 DATA 14,0,20,5,63.85,15,0,20,5,77.8,16,0,20,5,91.5667,14,0,22,5,82.3,16,0,
22,5,122.288,18,0,20,5,137.133
10110 DATA 16,1,20,4,76.9,18,1,20,4,114.95
10120 DATA 18,2,18,3,70.4
10130 DATA 14,0,22,6,68.9143,15,0,22,6,82.8333,16,0,22,6,98.77,17,0,22,6,121.65,
18,0,22,6,147.65
10140 DATA 16,1,20,5,60.84,18,1,20,5,86.8125,18,1,22,5,114.2,20,1,20,5,125.4,24,
1,20,5,265.7,16,1,22,5,78.6
10150 DATA 17,2,20,4,78.4,18,2,20,4,82.7
10160 DATA 16,1,22,6,65.95,17,1,22,6,80.12,18,1,22,6,91.825,20,1,22,6,137.3
10170 DATA 18,2,20,5,60.85,17,2,22,5,78.5,18,2,22,5,79.4
10180 DATA 18,2,22,6,66.8333
10190 DATA 20,4,20,5,52.3,20,4,22,5,74
10200 DATA 18,3,22,6,49.7
10210 DATA 20,5,20,5,37.33,20,5,22,5,51.5,22,5,22,5,78.4
10220 DATA 20,4,22,6,52.725,22,4,22,6,71.1
10230 DATA 20,5,22,6,41.04,22,5,22,6,55.7667
10240 DATA 22,6,22,6,44.68
10500 '-------------------- Q3 DATA -------------------------------------------
10510 DATA 0,0,-11.369,0,1,-28.418,0,2,-45.810,1,1,-47.709,1,2,-63.869,0,3,-56.9
3,0,4,-72.255,0,5,-78.303,1,4,-85.423,2,3,-91.215,0,6,-91.9381,1,5,-97.0845,2,4,
-99.553,1,6,-110.012,2,5,-113.455,2,6,-124.747,4,5,-134.785,3,6,-136.999,5,5,-14
9.348
10520 DATA 4,6,-151.552,5,6,-160.477,6,6,-172.366,1,3,-76.01549,2,2,-77.8345
10600 '-------------------- METHODS -------------------------------------------
10610 DATA "IMPOSSIBLE TO PREDICT.","PREDICTED BY S.T.D. RRT","PREDICTED BY (D1,
D2) METHOD.","PREDICTED BY 3-VAR. LINER METHOD.","NETWORK PREDICTION"
10700 '-------------------- NETWORK DATA --------------------------------------
10710 DATA 0,2,7.62355,5.77032,12,0,4,8.13790,1.84811,23,0,5,8.24843,5.51194,29,
1,5,8.01821,5.56167,43,2,5,8.00000,5.77800,58
10720 '---------------------- NAME OF VARIABLWE ------------------------------
10730 DATA "C1","D1","C2","D2"
10800 STOP
10900 END
```

Program III-1. Lecithin molecular species identification program for the personal computer, written in N-BASIC.

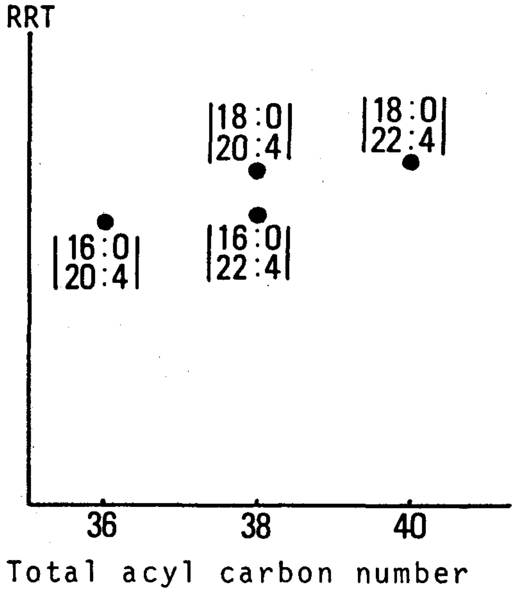


Fig. III-1. Deviations in tetraene and saturate combination group.

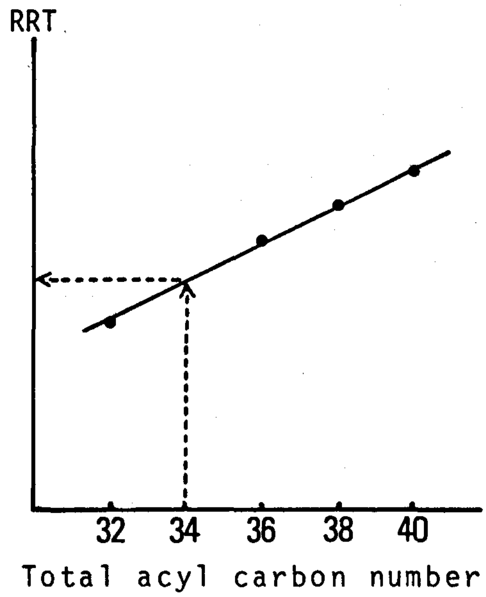


Fig. III-2. RRT prediction by the regression line.

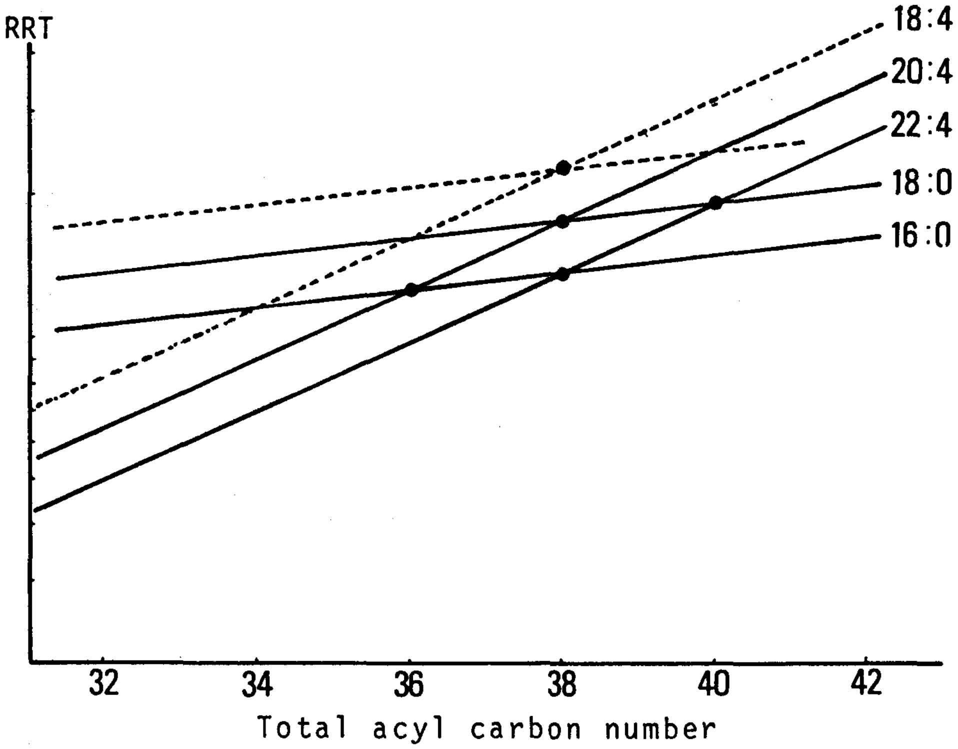


Fig. III-3. RRT prediction by the vertex of a parallelogram.

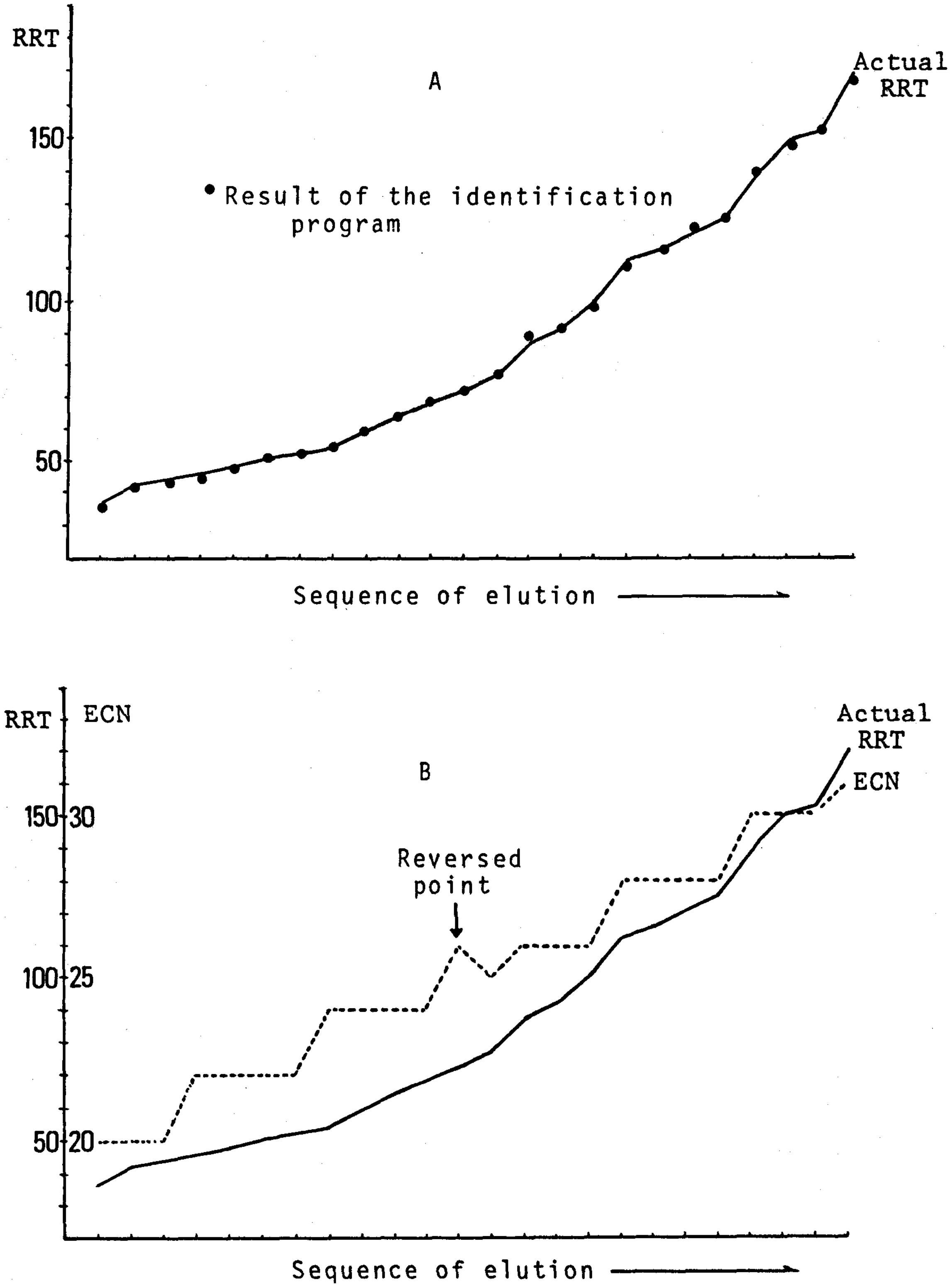


Fig. III-4. Results (RRT prediction) of the lecithin molecular species identification program in relation to the sequence of elution on HPLC. Sample is of sardine muscle.

species that can be defined as $\begin{vmatrix} d_1 \\ d_2 \end{vmatrix}$ and then search the molecular species that can generate a parallelogram (network) as shown in Fig. III-3, and print out the RRT of the vertex molecular species on the parallelogram that meets the condition of α (shown as C-3 and D, E). This is called "NETWORK PREDICTION" in Program III-1.

4. If not the cases of 1, 2 and 3, search the molecular species that can be defined as $\begin{vmatrix} d_1 \\ d_2 \end{vmatrix}$ as it has been done in 3. Then, introduce the slope and the intercept (shown as Q3 in Program III-1) of the generalized regression line and print out the RRT that meets the condition of α (shown as C-4, D, E). This is called "PREDICTED BY (D1, D2) METHOD" in Program III-1.
5. Impossible (shown as C-5).

Finally, it goes back to the main routine that continues or ends up the identification program. The whole view of the program is shown in Program III-1. The results obtained by this program were compared with the results obtained from the actual analysis of sardine muscle done by enormous labor. Fig. III-4A illustrates the good agreement between the actual analytical results shown by solid line and the identification via personal computer shown by dots. And for the comparsion, ECN (or PN) is also shown (shown by the broken line in Fig. III-4B). None of the reversed point in RRT was observed in this example by the presented identification program making good contrast with those of ECN (or PN).

Although, in some cases, there might be some rare missidentification, this personal computer program is sure to be of great help in predicting the molecular species that are generally found.

CHAPTER IV

MOLECULAR SPECIES OF FISH MUSCLE LECITHIN

So far, the formulae that control the sequence of elution of lipid molecular species on HPLC have been demonstrated. It has been proven that the matrix model presented is invariant. And this matrix model forms the bases of molecular species identification of muscle lecithin from fish sources that will be discussed in this chapter.

The characteristics of each fish from the view point molecular species of muscle lecithin is discussed with some supplemental analysis such as principal component analysis.

Section 1. Experimental

Total lipids were obtained from the fish muscle tabulated in Table IV-1 according to the method of Bligh & Dyer. Neutral lipid composition and phospholipid composition were measured by the Iatroscan-Chromarod Method.[44~47)] The developing solvent used were n-hexane/diethyl ether/formic acid (85:15:0.5, v/v) for the former and chloroform/methanol/ammonia/water (70:30:2:3, v/v) for the latter. Phospholipid content was determined by multiplying 25 to the phosphorus content of the lipid which had been determined by Fiske-Sabbarow method. Preparation of pure lecithin, hydrolysis of lecithin into diglyceride, and derivation to diglyceride acetate from diglyceride were done in the same manner as shown in chapter I. HPLC fractionation and identification of molecular species of each peak on HPLC were

Table IV-1. Fish examined

Samples	Mean body length and weight. Locality of catch.	Date of catch.	
Sardine *Sardinops melanosticta*	20.0cm, 80g Kamiiso, Hokkaido.	July 1982	A
	17.1cm, 43g Kamiiso, Hokkaido.	Oct. 1983	
Mackerel *Scomber japonicus*	41.8cm, 647g Kamiiso, Hokkaido	July 1982	
	32.2cm, 409g Todohokke, Hokkaido	Oct. 1983	
Big-eyed tuna, Frozen *Parathunnus obeus*	110.0cm, 20kg from the market	-	
	100.0cm, 24kg Indian sea	-	
Brown sole *Limanda ferruginea*	23.7cm, 169g Kikonai, Hokkaido	Oct. 1982	B
	21.2cm, 207g Kamiiso, Hokkaido	May 1983	
Sand flounder *Linanda punctatissima*	18.3cm, 65g Kamiiso, Hokkaido	Dec. 1982	
	18.6cm, 139g Abuta, Hokkaido	May 1983	
Rock fish *Sebastes schlegeli*	19.6cm, 187g Toi, Hokkaido	Oct. 1982	C
	27.0cm, 471g Kamiiso, Hokkaido	July 1983	
Alaska pollack *Theraga chalcogramma*	44.0cm, 610g Uchiura bay, Hokkaido	Dec. 1981	
	41.7g, 509g Shikabe, Hokkaido	Jan. 1984	
Chum salmon, Male *Oncorhyncus keta*	65.0cm, 3500g Akkeshi, Hokkaido	June 1980	
	42.7cm, 1367g 45°59'-49°29'N, 167°07'-175°30'E	Aug. 1983	
Chum salmon, Female *Oncorhyncus keta*	46.8cm, 1593g 45°59'-49°29'N, 167°07'-175°30'E	"	
Blue shark *Prionace grauca*	114.5cm, 8.5kg 38°30'-39°30'N, 155°00'E	June 1982	D
Mackerel shark *Lamna cornubica*	88.5cm, 10.2kg 41°30'-43°00'N, 175°30'E	July 1982	
Carp *Cyprinus carpio*	23.0cm, 175g from the market	Sep. 1980	E
Rainbow trout *Salmo gairdnerii irideus*	33.8cm, 455g Nanae, Hokkaido	Sep. 1982	
	38.0cm, 780g Nanae, Hokkaido	May 1983	

A: Migratory fish. B: Bottom fish. C: Hard to classify.

D: Cartilaginous fish. E: Fresh water fish.

also done in the same manner though a new matrix model is available in identifying the molecular species in order to verify the results.

Results and Discussion

Section 2. Characteristics of Muscle Lecithin of Fish

The yield of total lipid and the percentage of each lipid class against total lipid are shown in Table IV-2 and Table IV-3. Fatty acid composition of diglyceride acetates which represent the fatty acid composition of lecithin are shown in Table IV-4 and Table IV-5. Samples shown in these tables were subjected to the lecithin molecular species analysis.

HPLC chromatograms of each fish are shown in Figs. IV-1~6 and the detected molecular species of lecithin in sequence of elution on HPLC with the percentage data and those of mg/100 g muscle are shown in Figs. IV-7~32.

As illustrated in Fig. IV-1, the HPLC chromatogram of diglyceride acetate of fish muscle lecithin can be devided into four molecular species groups, that is, I: molecular species composed of highly unsaturated fatty acids such as 20:5 or 22:6, for instance (20:5)(20:5), (20:5)(22:6) and (22:6)(22:6), III: molecular species composed of generally found fatty acids such as 16:0 or 18:1 with combinations of 20:5 or 22:6, that is (20:5)(18:1), (18:1)(20:5), (22:6)(18:1), (18:1)(22:6), (20:5)(16:0), (16:0)(20:5), (22:6)(16:0) and (16:0)(22:6), and II and IV: others. As it is evident from the chromatograms (see Figs. IV-1~6), groups I and III accounts for at least 60% of the molecular species examined. Specifically, as shown in Fig. IV-1 and Fig. IV-7, groups I and III of the sardine white muscle

Table IV-2. Lipid composition of fish muscle examined, captured in 1980~1982

Lipid / Sample	Yield *	Non-phospholipid**				Phospholipid **		
		TG	FFA	ST	NP others	PC	PS+PE	PL others
Sardine (DM)	26.8	92.5	0.4	0.4	1.7	3.7	1.8	trace
Sardine (WM)	4.3	83.1	0.5	1.8	2.0	8.6	2.6	0.2
Mackerel (DM)	5.8	72.5	0.9	0.3	0.3	4.6	8.5	2.6
Mackerel (WM)	0.9	32.0	2.7	2.5	1.2	33.2	9.4	5.6
Big-eyed tuna	0.7	13.4	0.2	1.7	trace	59.4	9.0	0.5
Brown sole	1.7	62.2	5.4	2.3	0.7	16.0	3.1	3.4
Sand flounder	1.3	64.1	0.9	2.0	4.2	18.8	1.1	0.6
Rock fish	1.4	58.8	2.5	0.5	0.3	18.5	3.8	5.0
Alaska pollack	1.0	42.7	11.4	45.6	trace	81.2	trace	1.7
Blue shark	0.6	1.1	0.2	6.0	1.3	56.5	20.1	6.2
Mackerel shark	2.0	48.9	2.2	1.9	1.5	23.1	8.5	13.9
Carp	1.6	55.5	trace	6.7	trace	29.1	12.4	2.7
Rainbow trout	3.3	72.7	1.1	0.5	0.1	11.7	5.0	0.4

See the abbreviations in the opening.

*g/100g muscle. **g/100g total lipid.

Table IV-3. Lipid composition of fish muscle examined, captured in 1983~1984

Lipid / Sample	Yield*	Non-phospholipid**				Phospholipid **		
		TG	FFA	ST	NP others	PC	PS+PE	PL others
Sardine (DM)	3.3	53.8	1.9	2.1	1.2	11.2	27.7	2.1
Sardine (WM)	0.8	29.2	0.3	4.8	6.3	12.1	44.3	5.0
Mackerel (DM)	20.0	90.9	0.2	0.4	1.3	2.4	4.5	0.3
Mackerel (WM)	8.5	92.0	trace	0.6	0.9	4.6	0.6	1.3
Big-eyed tuna	0.5	6.4	3.8	13.4	2.8	11.7	57.8	4.1
Brown sole	0.9	20.0	trace	6.0	2.1	17.5	54.0	0.4
Sand flounder	1.7	62.9	2.5	3.0	3.4	7.9	18.6	1.7
Rock fish	1.6	79.0	trace	1.0	0.2	3.5	13.4	2.9
Alaska pollack	0.7	3.6	3.0	9.8	2.6	26.7	47.0	7.3
Rainbow trout	2.5	69.8	1.0	1.1	0.2	8.9	17.6	1.4

For chum salmon, see Table IV-9.
See the abbreviations in the opening.
*g/100g muscle. **g/100g total lipid.

Table IV-4. Fatty acid composition of diglyceride acetate derived from lecithin of fish muscle, captured in 1980~1982

Sample / Fatty acid	Sardine DM	Sardine WM	Mackerel DM	Mackerel WM	Big-eyed tuna	Brown sole	Sand flounder	Rock fish	Alaska pollark	Chum* salmon	Blue shark	Maskerel shark	Carp	Rainbow trout
12:0														0.07
14:0	1.09	1.48	0.98	1.39	0.72	2.00	1.54	1.11	2.69	3.27	1.04	1.30	1.11	0.97
15:0		0.37	0.26	0.28	0.63	0.68	0.90	0.34	0.31	0.69				0.17
16:0	18.42	32.35	30.82	24.42	25.60	22.70	29.14	26.50	41.88	35.19	22.82	34.80	32.28	29.70
16:1	0.74	1.78	2.13	0.75	0.48	1.95	1.83	6.51	0.57	0.45	5.40	3.60	2.59	3.30
17:0	0.82	0.52	0.56	0.52	1.46	1.22	1.09	1.33	0.37	1.04	0.45	trace		0.39
17:1	0.48	0.50	0.45		0.79	1.11	0.90	0.91	0.49		0.43	trace		0.32
18:0	5.99	1.11	0.90	4.29	0.92	1.93	1.41	1.63	0.82	0.78	8.93	2.80	3.08	3.46
18:1	18.69	6.80	4.22	12.09	7.24	10.24	8.27	16.49	12.32	6.26	10.04	11.30	16.71	29.75
18:2	1.09	1.84	0.45	1.34	0.56	0.21		0.66	0.46	0.33	0.77	0.60	9.06	8.60
19:1	0.45	0.63	0.21	0.32		0.33		0.21		0.35	0.35	trace		0.18
19:2		1.44	0.34			1.31			0.29	0.56				
20:0			1.26				0.97 **			0.71	1.15**	trace	1.16 **	
20:1	1.93	0.94		1.15		1.29**		0.56**	1.65 **			2.00		1.31
20:2											1.45	trace		1.13
20:3		0.30											0.82	1.32
20:4	3.50	2.71	1.96	3.37	5.27	7.43	6.97	3.54	0.88	1.07	3.60	4.80	3.85	0.86
20:5	10.47	13.98	18.64	11.08	4.58	31.60	27.59	13.83	16.79	8.79	10.92	11.30	6.39	1.79
22:1												trace		0.10
22:2						0.24								0.05
22:3		0.62	0.37			0.66					0.13	trace		0.07
22:4	1.37	0.45	0.42	1.14	5.75	0.38	0.89	0.83			1.70	trace		0.31
22:5	3.39	1.76	1.11	2.16	0.44	4.51	2.73	1.34	0.55	0.89	6.01	2.50	1.17	0.83
22:6	31.57	30.42	34.90	35.70	45.56	10.69	15.77	24.21	19.93	39.62	24.56	24.90	20.67	14.69
24:2														0.56
24:3														0.06
24:6						0.30					0.24		1.11	
Unknown						0.21								

* Feeding migration, Male.

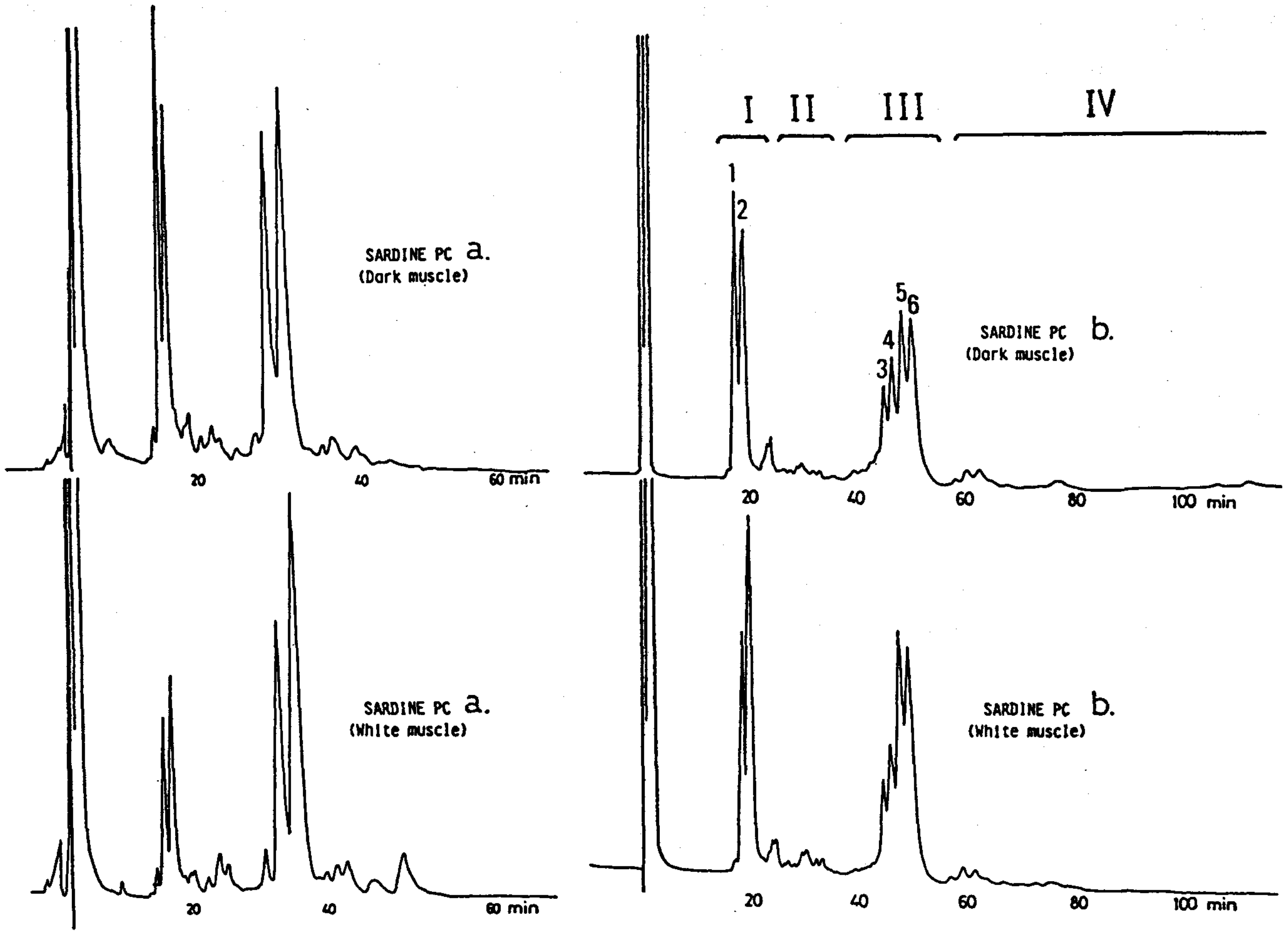
**Ether 20:0 or 20:1

DM: Dark muscle. WM: White muscle.

Table IV-5. Fatty acid composition of diglyceride acetate derived from lecithin of fish muscle, captured in 1983~1984

Fatty acid \ Sample	Sardine DM	Sardine WM	Mackerel DM	Mackerel WM	Big-eyed tuna	Brown sole	Sand flounder	Rock fish	Alaska pollack	Rainbow trout
14:0	1.08	1.20	0.92	0.83	0.75	2.77	1.77	1.03	2.13	1.27
15:0	0.41	0.43	0.33	0.31	0.49	1.07	0.85	0.32	0.34	0.22
16:0	24.09	36.38	21.15	28.17	33.29	34.11	31.22	33.41	29.01	36.73
16:1	1.43	1.25	1.42	1.34	1.00	7.55	2.39	3.76	1.96	3.01
17:0	0.51	0.50	0.78	0.43	1.75	1.78	1.11	0.74	0.46	0.23
17:1	0.41	0.33	0.57	0.69	0.84	0.84	0.91	0.64	0.36	0.15
18:0	0.88	0.71	5.44	3.05	2.28	2.38	1.70	1.99	0.74	1.81
18:1	5.02	2.11	15.93	10.24	23.02	11.31	10.77	18.93	13.34	12.81
18:2	0.49	0.60	1.89	0.99	0.67	0.46	0.70	1.40	1.23	5.97
19:1 or 18:4	0.17		0.51	0.47	0.31		0.33	0.37	0.45	0.63
19:2	0.17		0.70			0.28		0.23	0.49	0.25
20:0	0.11	0.33	1.30	0.23	0.12	1.05	1.86			0.34
20:1		0.12		0.72	0.98	0.07	2.24	0.75	3.02	0.15
20:2		0.11		0.14	0.33	0.08	0.06	0.21	0.14	0.30
20:3	2.99	0.12	4.26	0.13	0.13	4.64	0.17	0.18	0.06	2.99
20:4	0.15	2.35		3.74	5.96	0.19	4.70	3.87	2.21	0.29
20:5	18.85	14.20	16.41	16.78	3.74	18.53	25.12	10.02	24.46	5.90
22:1						0.11	0.27			
22:3	0.21	0.10	0.25		0.28	1.32	0.70	0.19	0.24	0.22
22:4	0.91	0.71	0.80	0.70	2.51	0.35	0.41	0.47	0.11	0.25
22:5	1.08	0.98	2.46	1.85	0.43	3.71	2.62	1.18	0.56	1.04
22:6	36.06	37.24	24.68	29.11	21.06	7.13	9.89	20.26	18.68	25.35
others	4.98	0.23	0.20	0.08	0.06	0.28	0.21	0.05	0.01	0.09

DM: Dark muscle. WM: White muscle.



I: Groups composed of highly unsaturated fatty acids, that is, 1:(20:5)(22:6), 2:(22:6)(22:6).

III: Groups composed of highly unsaturated fatty acids and generally found fatty acids, that is, 3:(20:5)(16:0), 4:(16:0)(20:5), 5:(22:6)(16:0) and 6:(16:0)(22:6).

II&IV: Others.

Fig. IV-1. HPLC chromatograms of sardine muscle lecithin.
a: Captured in 1982. b: Captured in 1983.

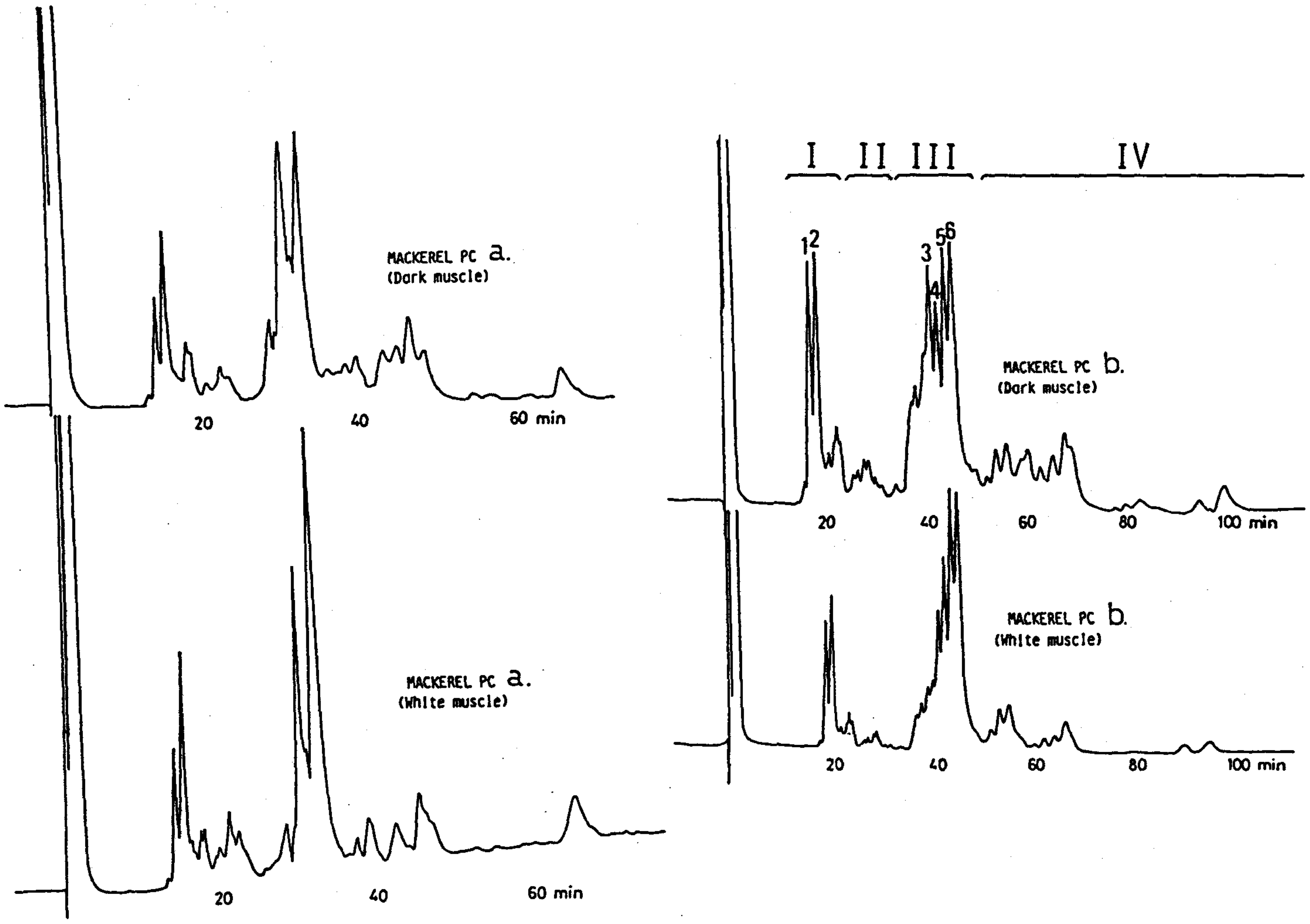


Fig. IV-2. HPLC chromatograms of mackerel muscle lecithin.
a: Captured in 1982. b: Captured in 1983.
Numbers as in Fig. IV-1.

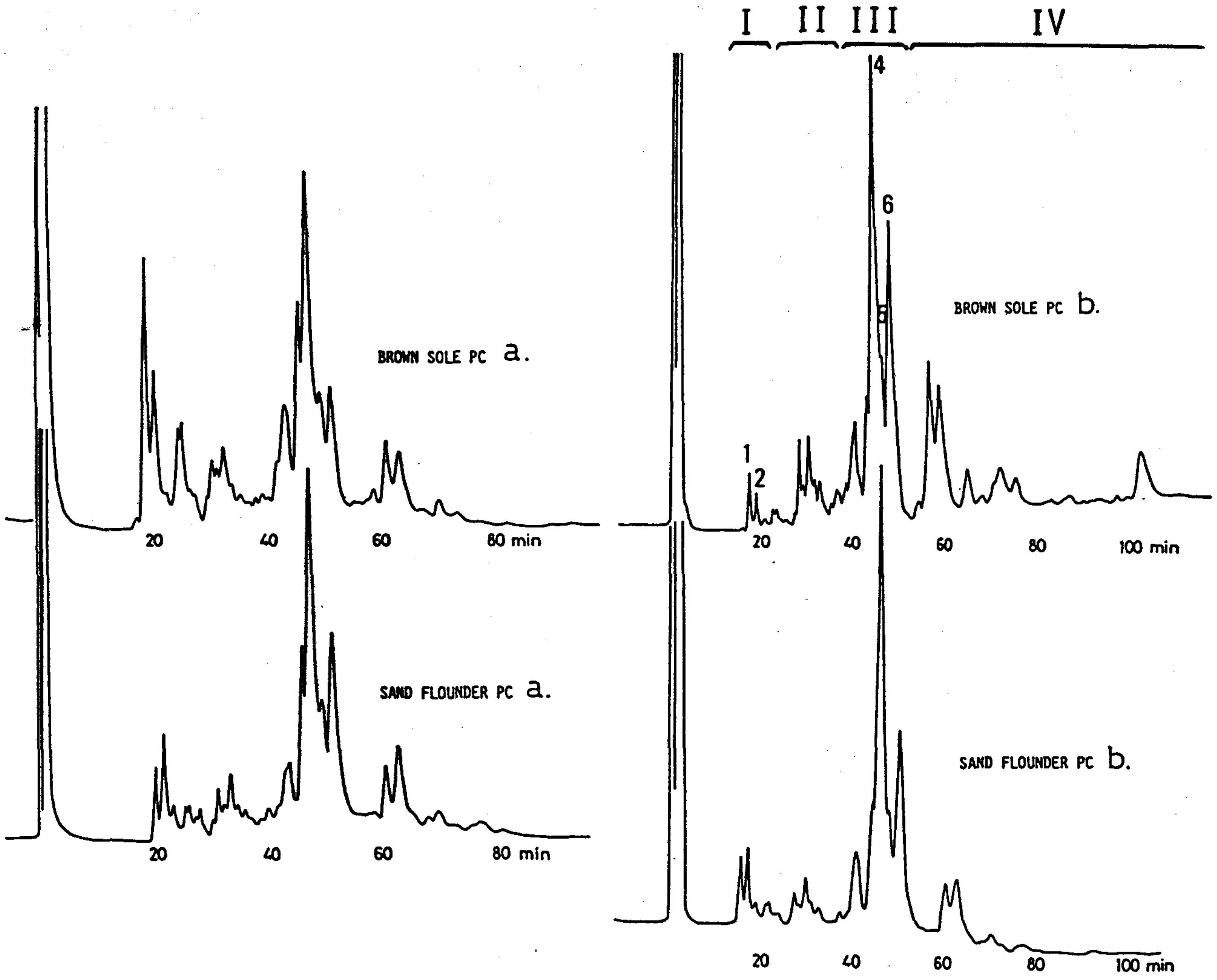


Fig. IV-3. HPLC chromatograms of brown sole and sand flounder muscle lecithin.

a: Captured in 1982. b: Captured in 1983.

Numbers as in Fig. IV-1.

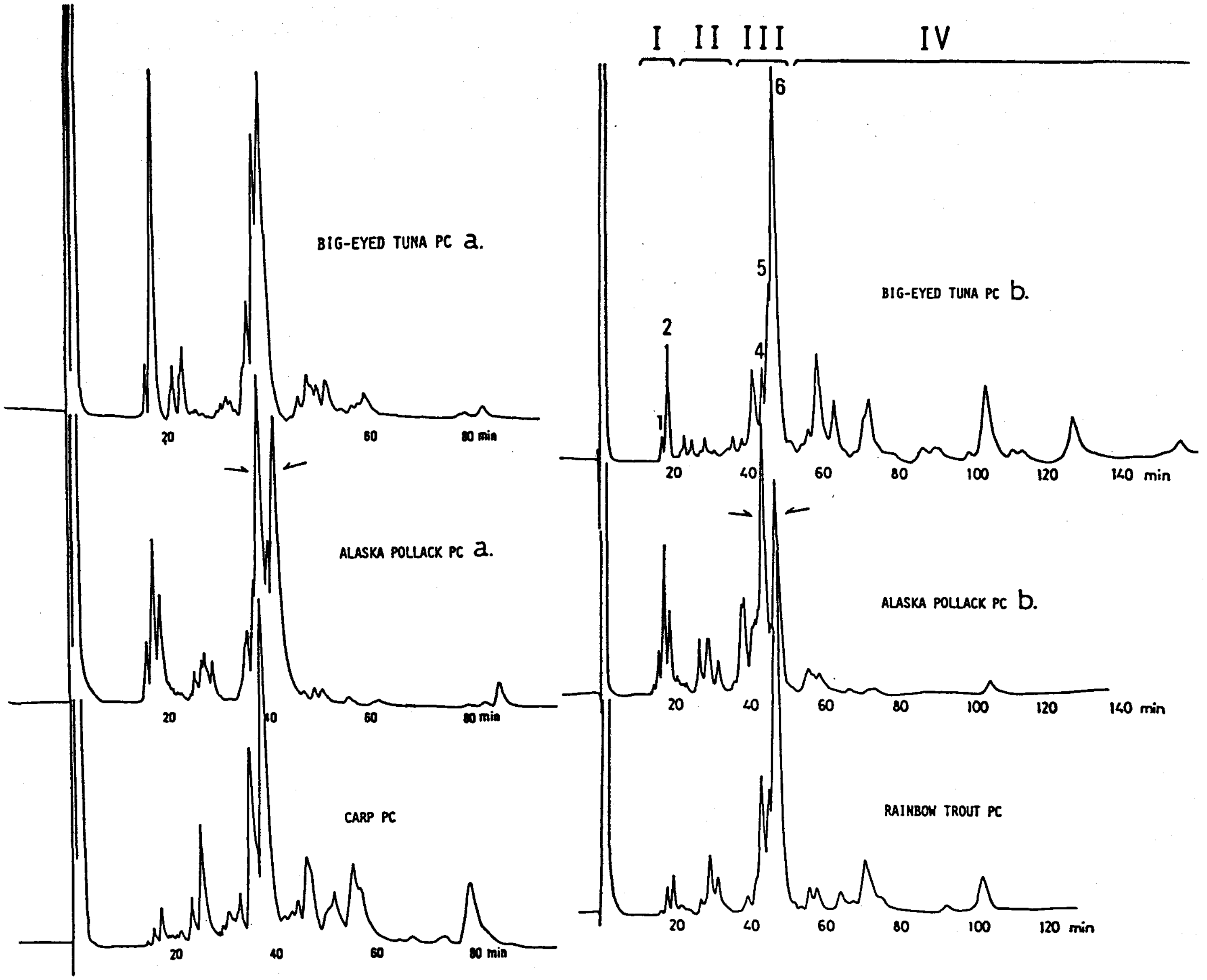


Fig. IV-4. HPLC chromatograms of big-eyed tuna, alaska pollack, carp and rainbow trout muscle lecithin. a: Captured in 1981. b: Captured in 1984.
Carp is the sample of 1980 and rainbow trout is the sample of 1983.
Numbers as in Fig. IV-1.

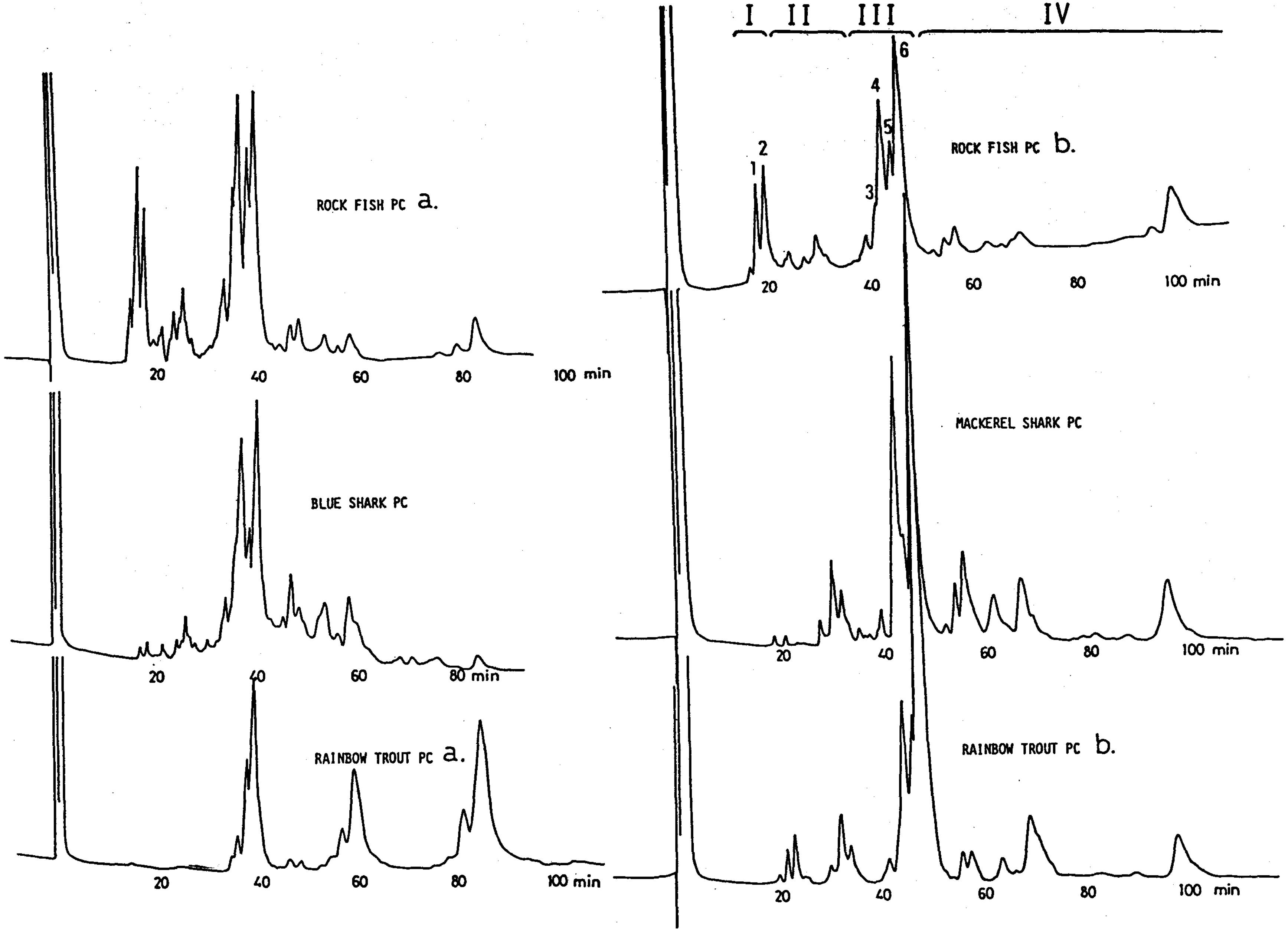


Fig. IV-5. HPLC chromatograms of rock fish, blue shark, mackerel shark and rainbow trout muscle lecithin.

a: Captured in 1982. b: 1983.

Blue shark and mackerel shark are the samples of 1982. Numbers as in Fig. IV-1.

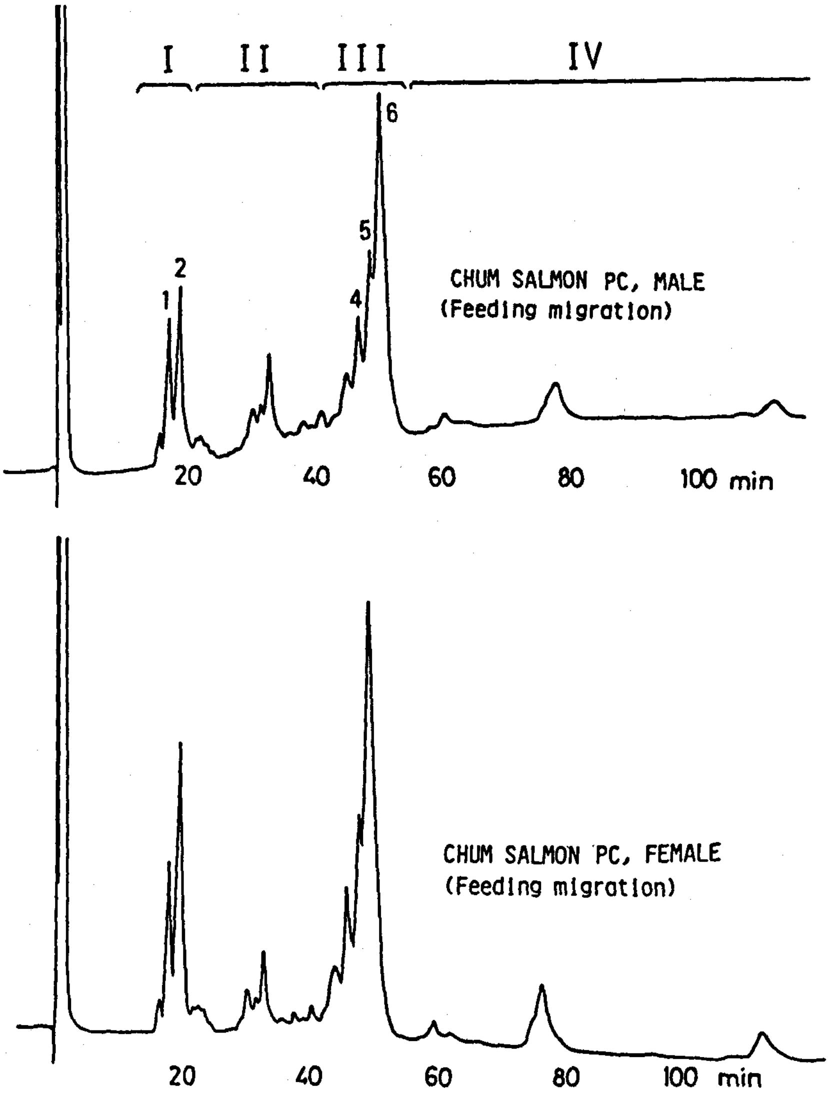


Fig. IV-6. HPLC chromatograms of chum salmon muscle lecithin
Both are the samples of 1983.
Numbers as in Fig. IV-1.

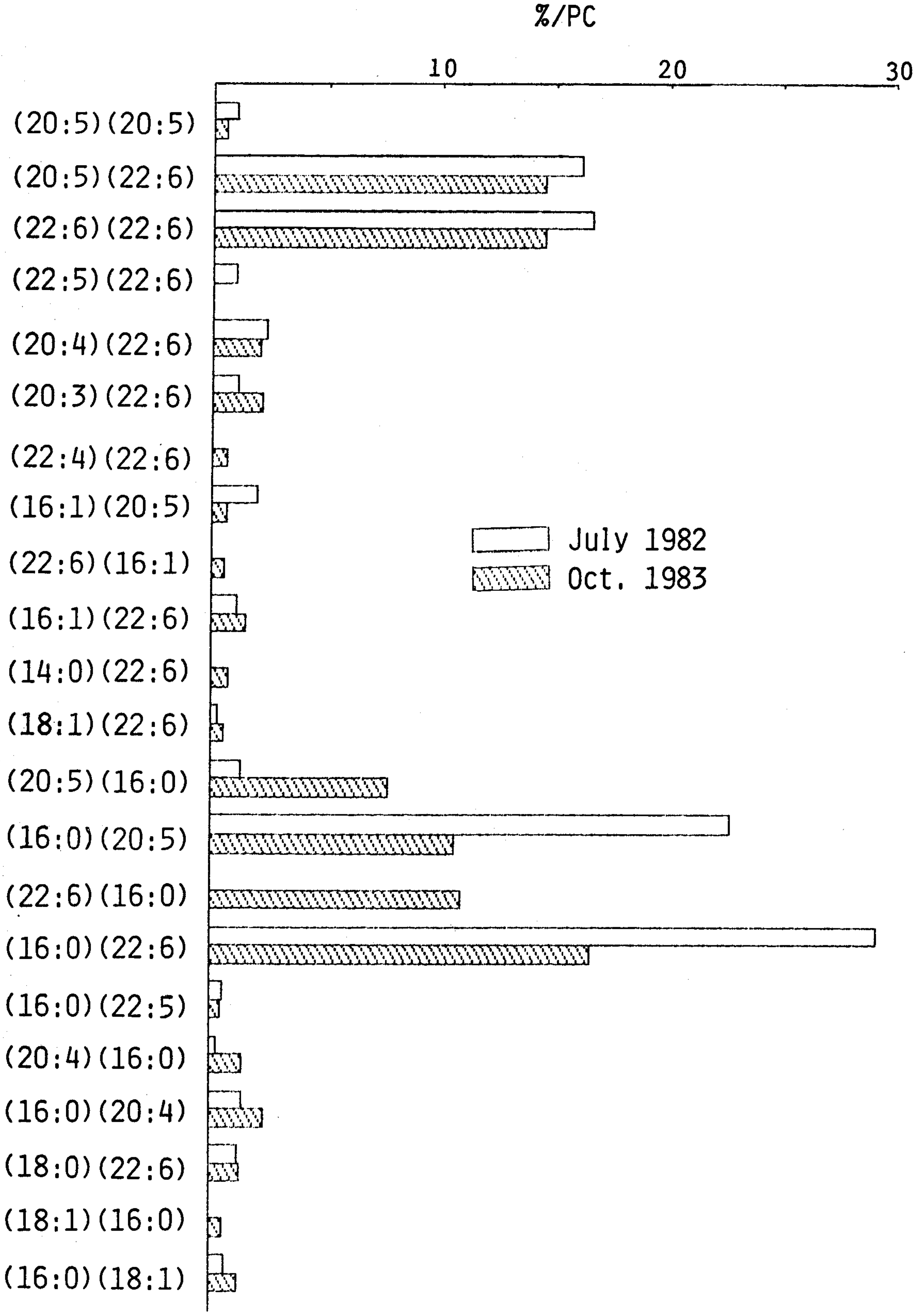


Fig. IV-7. Molecular species composition of sardine dark muscle lecithin, in relative %.

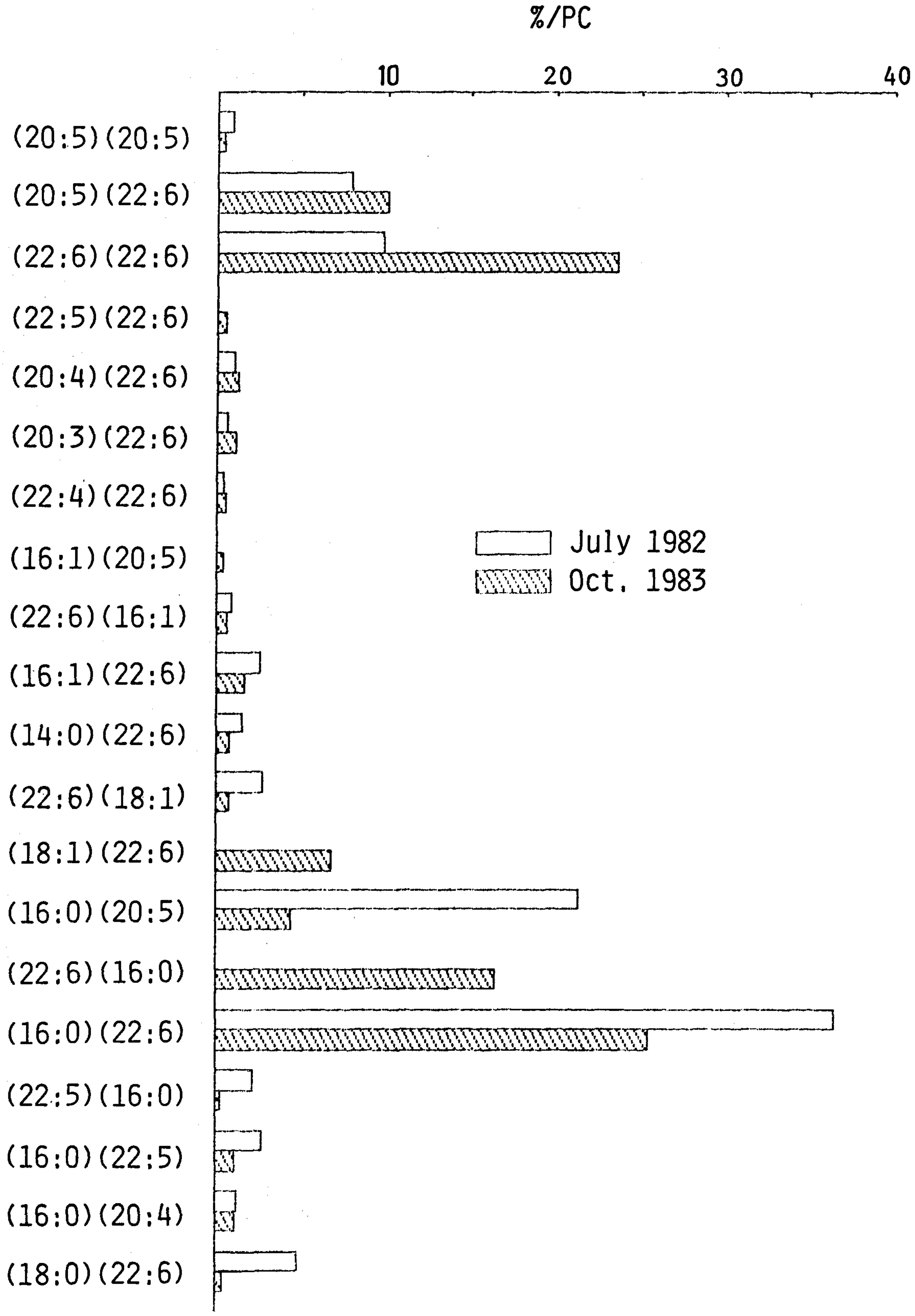


Fig. IV-8. Molecular species composition of sardine white muscle lecithin, in relative %.

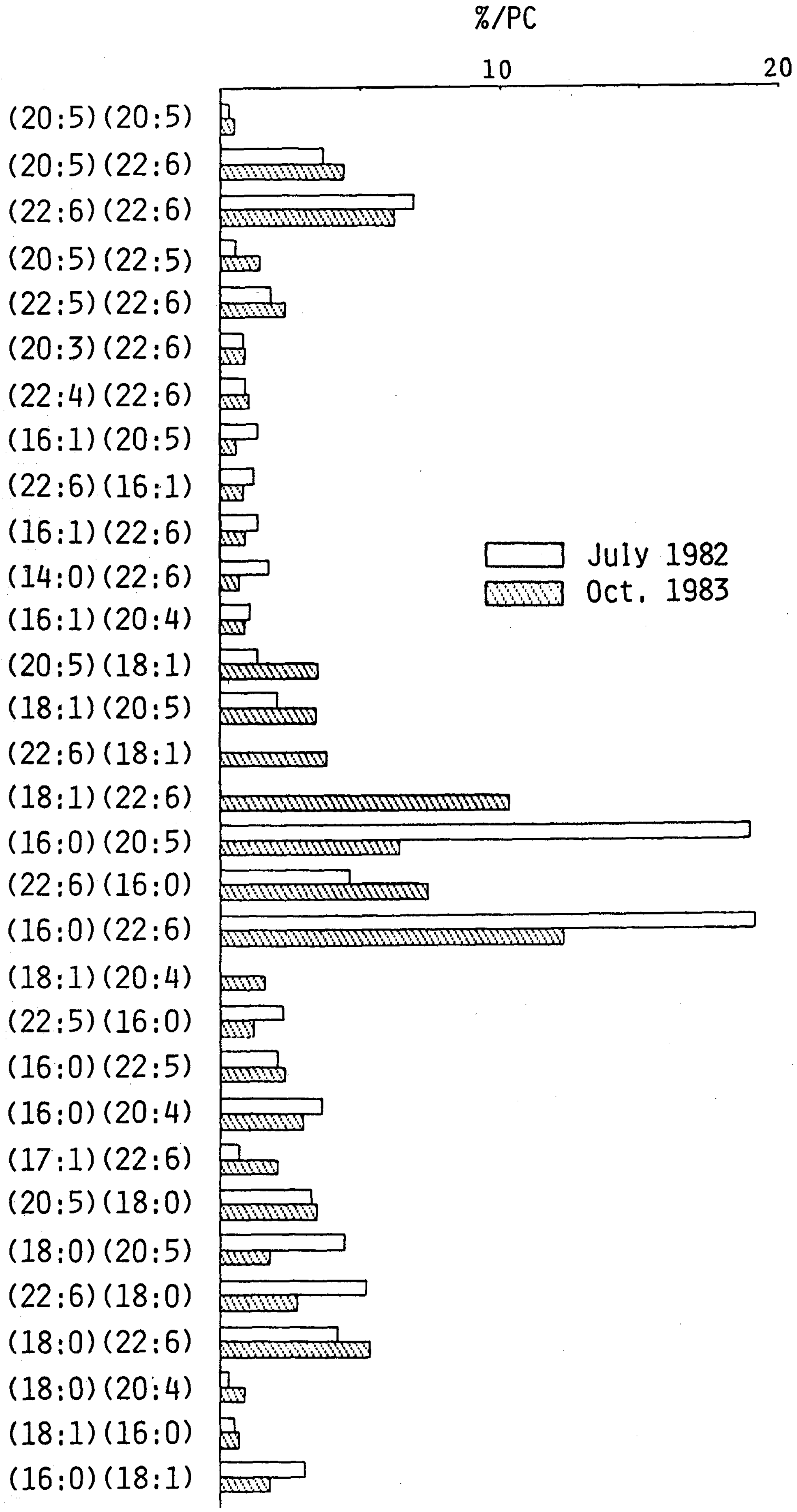


Fig. IV-9. Molecular species composition of mackerel dark muscle lecithin, in relative %.

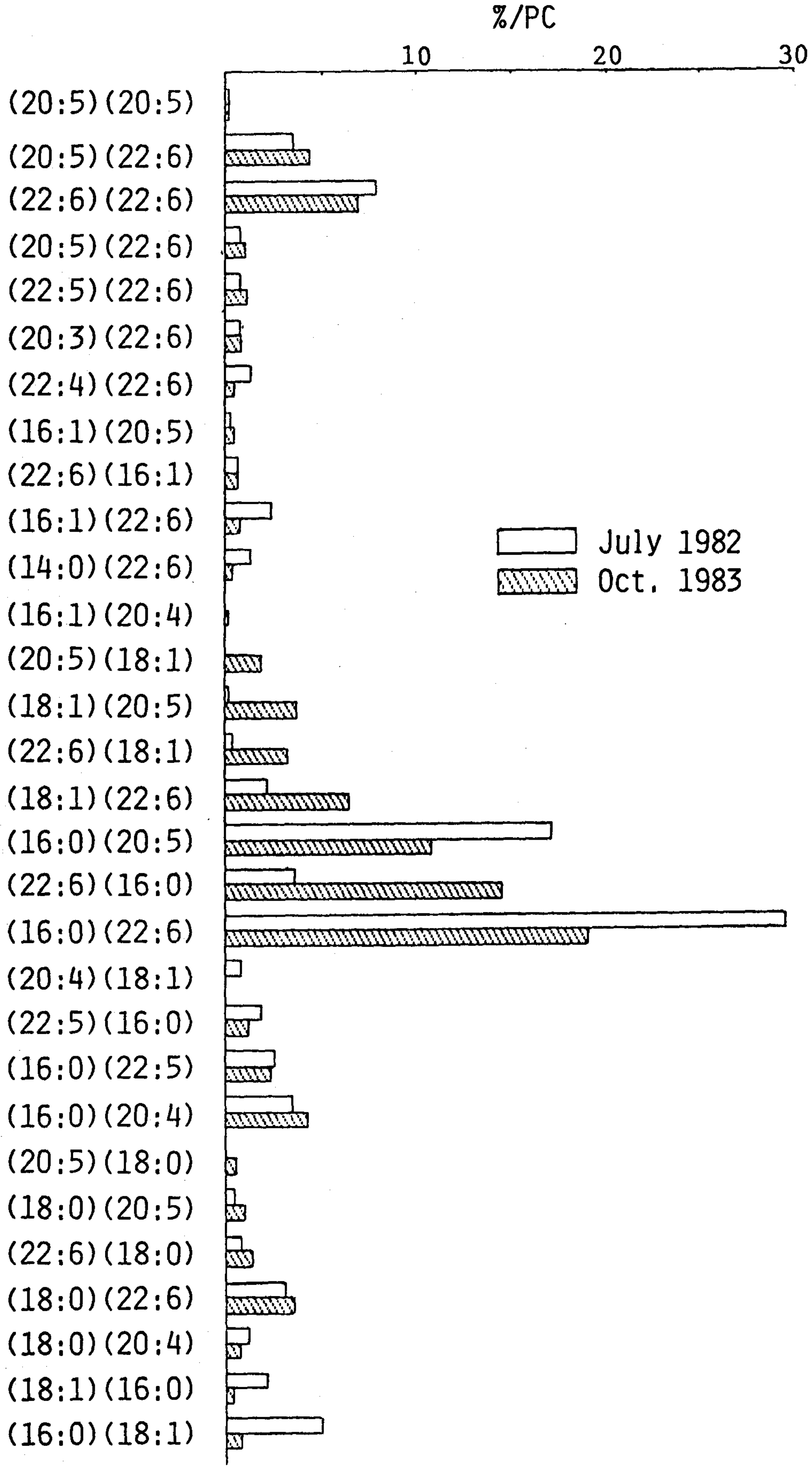


Fig. IV-10. Molecular species composition of mackerel white muscle lecithin, in relative %.

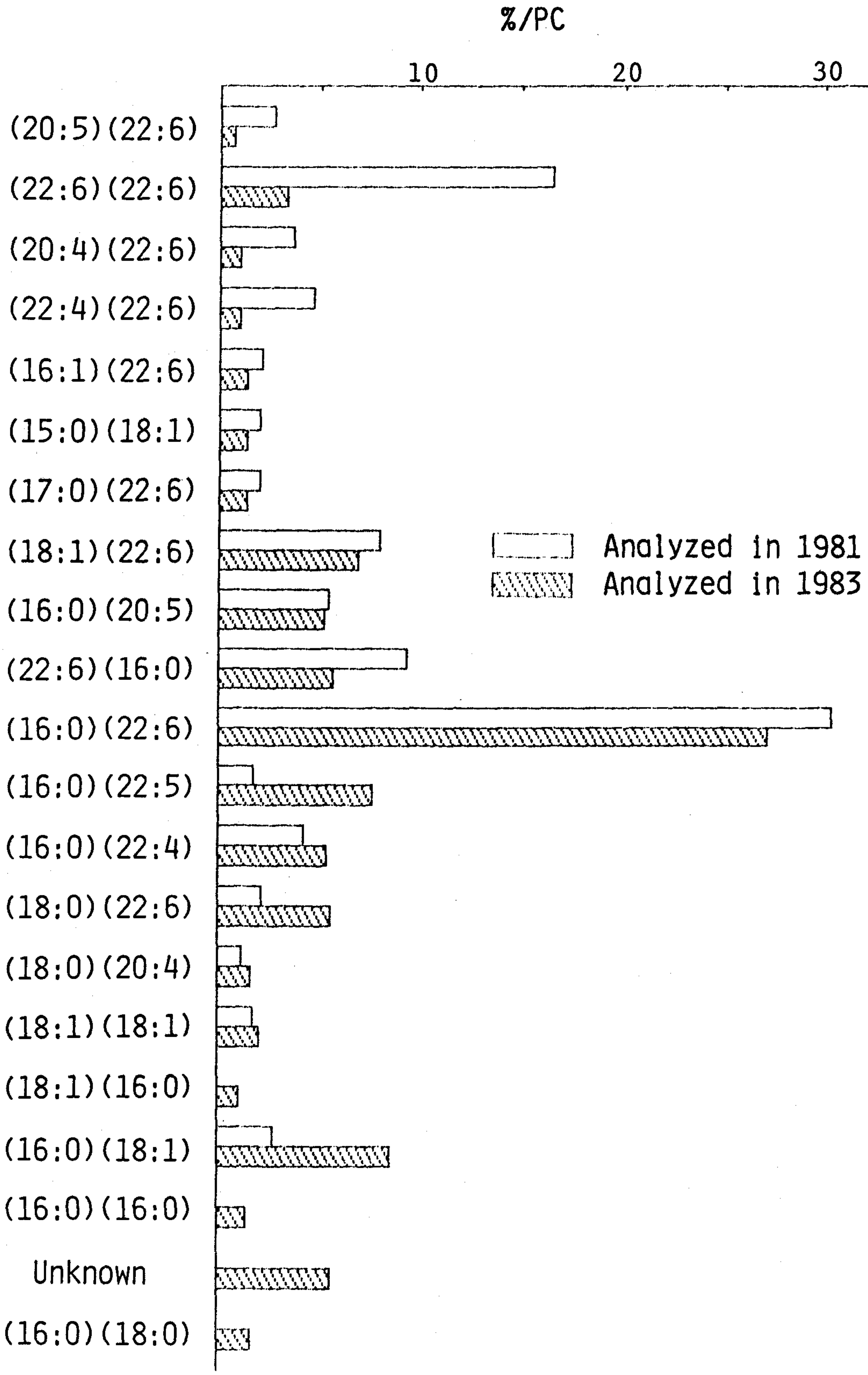


Fig. IV-11. Molecular species composition of big-eyed tuna muscle lecithin, in relative %.

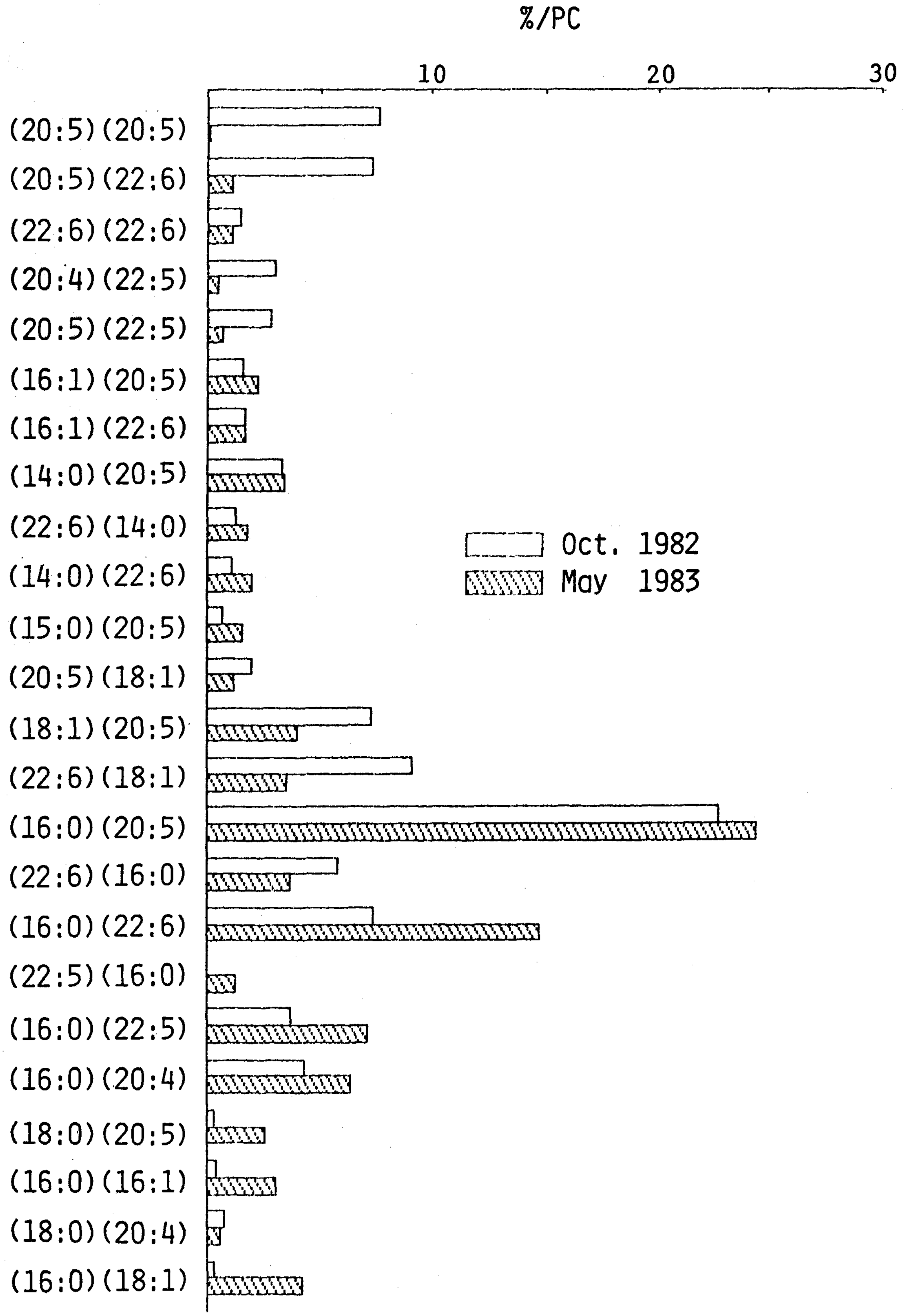


Fig. IV-12. Molecular species composition of brown sole muscle lecithin, in relative %.

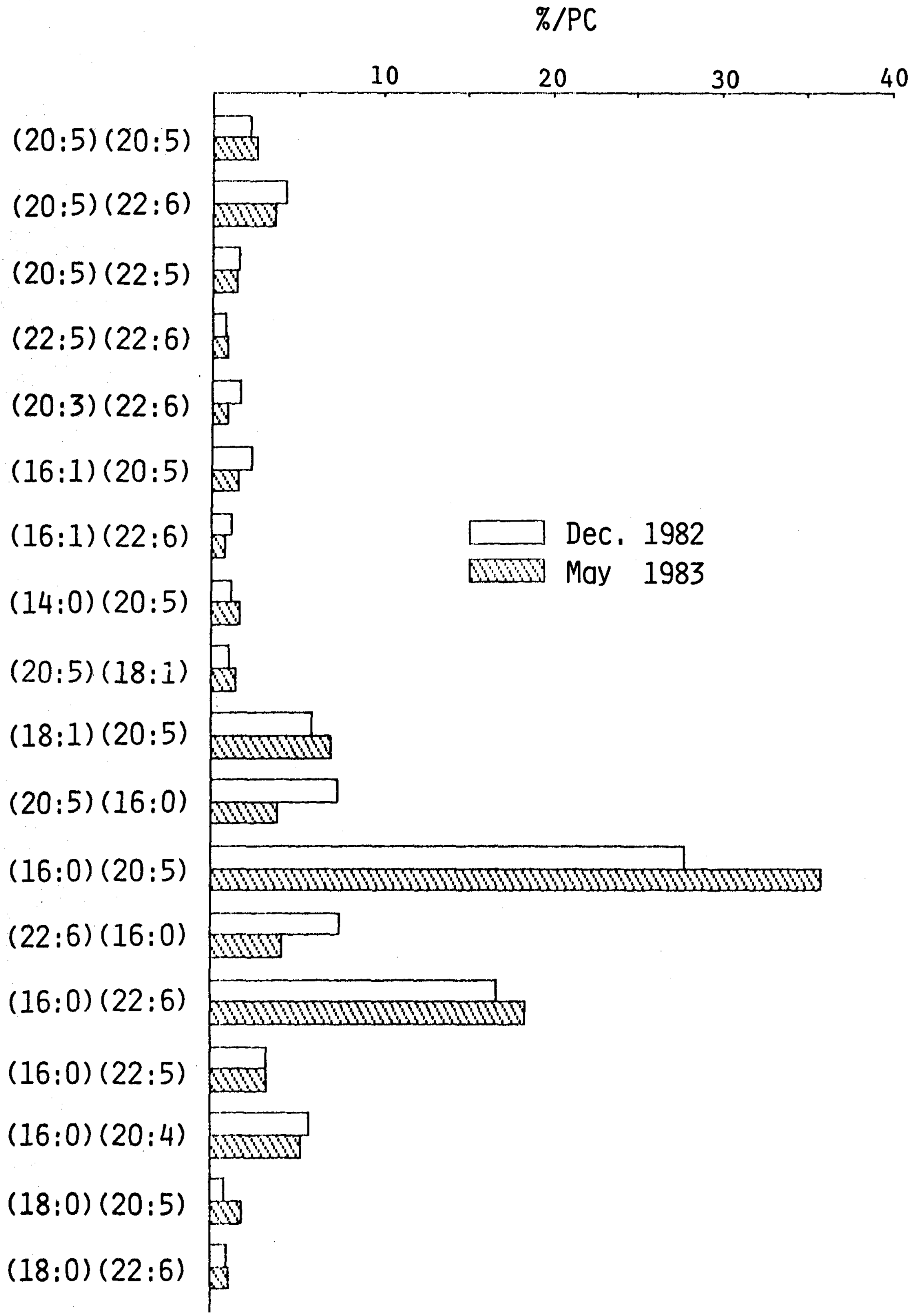


Fig. IV-13. Molecular species composition of sand flounder muscle lecithin, in relative %.

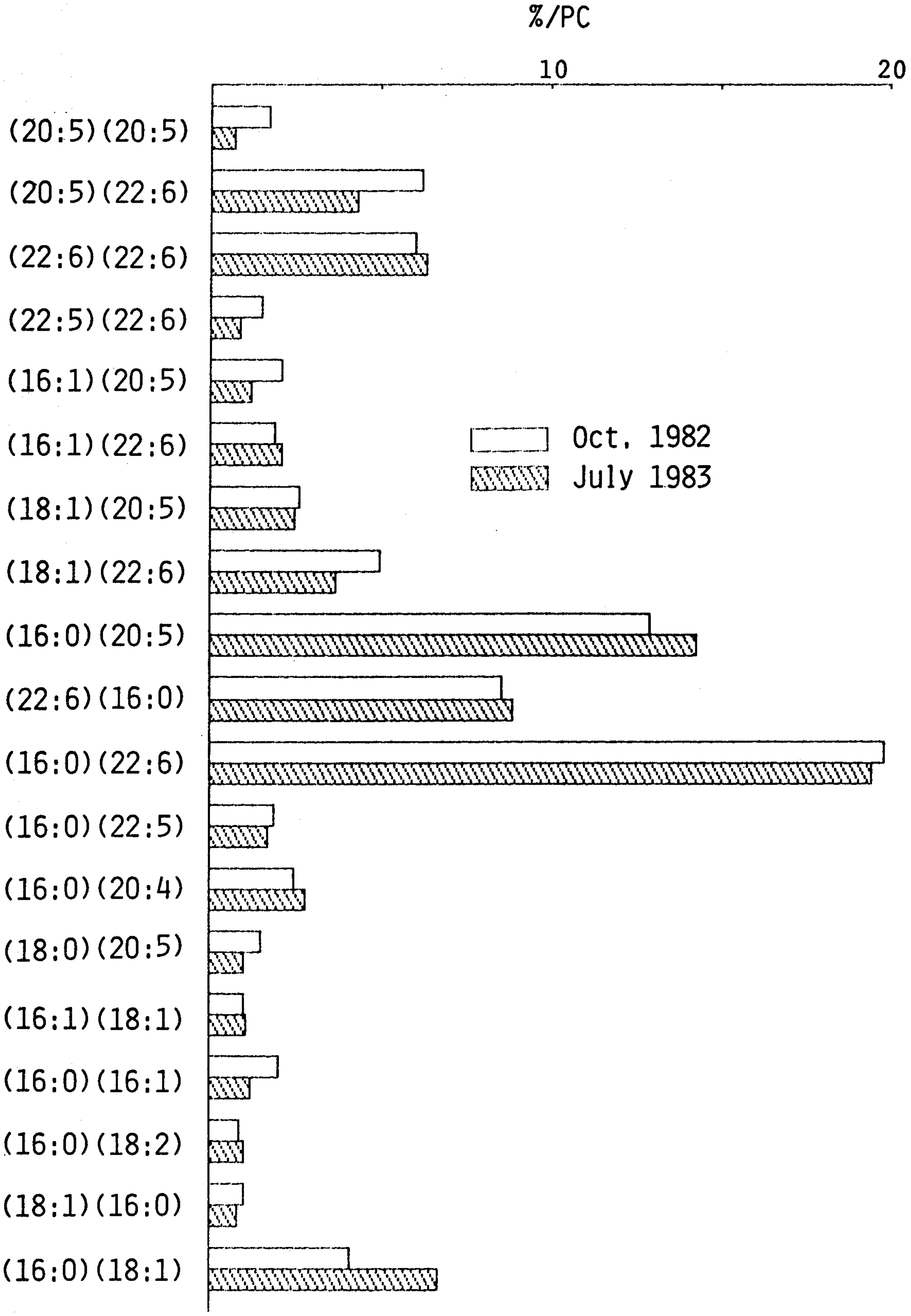


Fig. IV-14. Molecular species composition of rock fish muscle lecithin, in relative %.

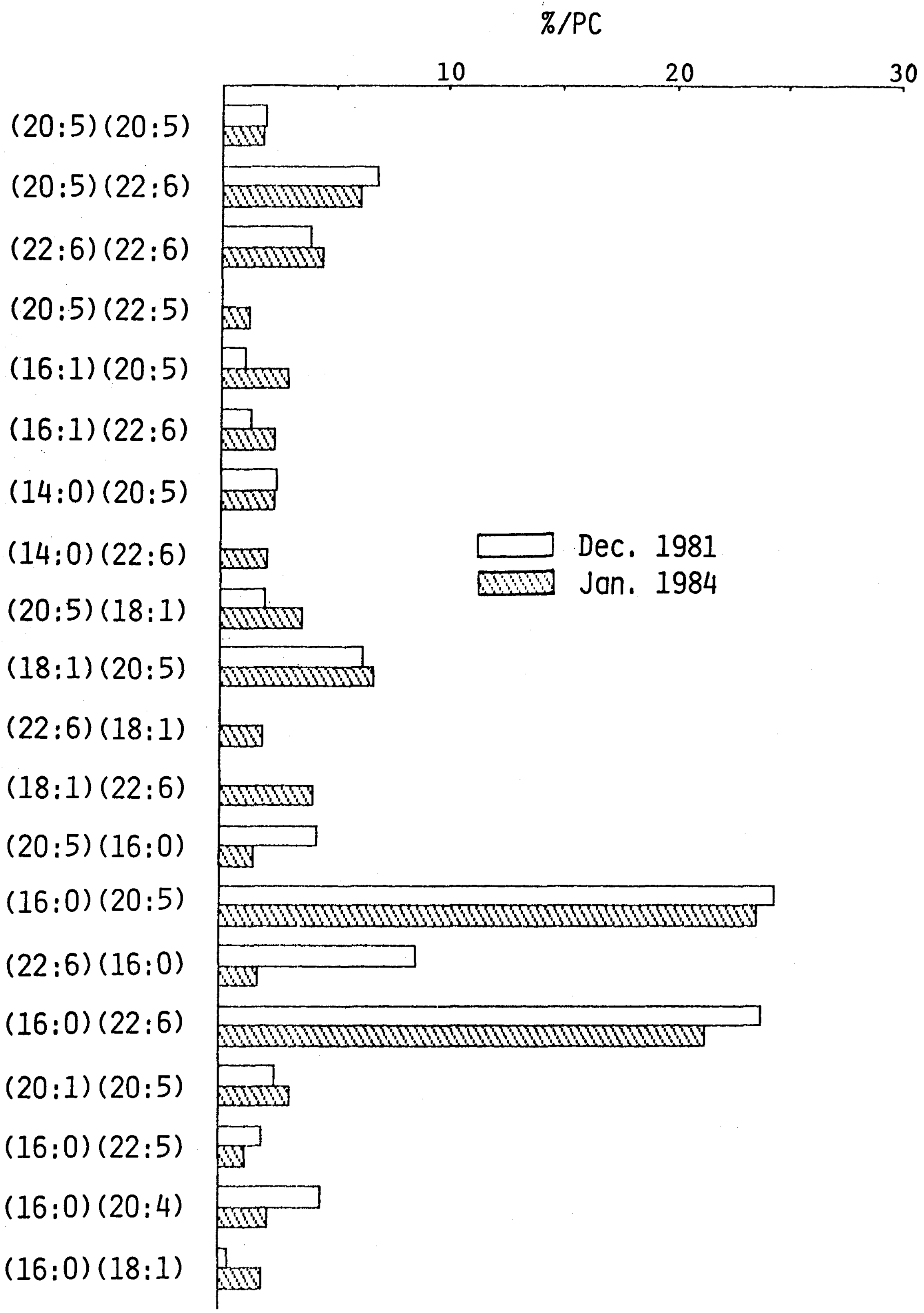


Fig. IV-15. Molecular species composition of Alaska pollack muscle lecithin, in relative %.

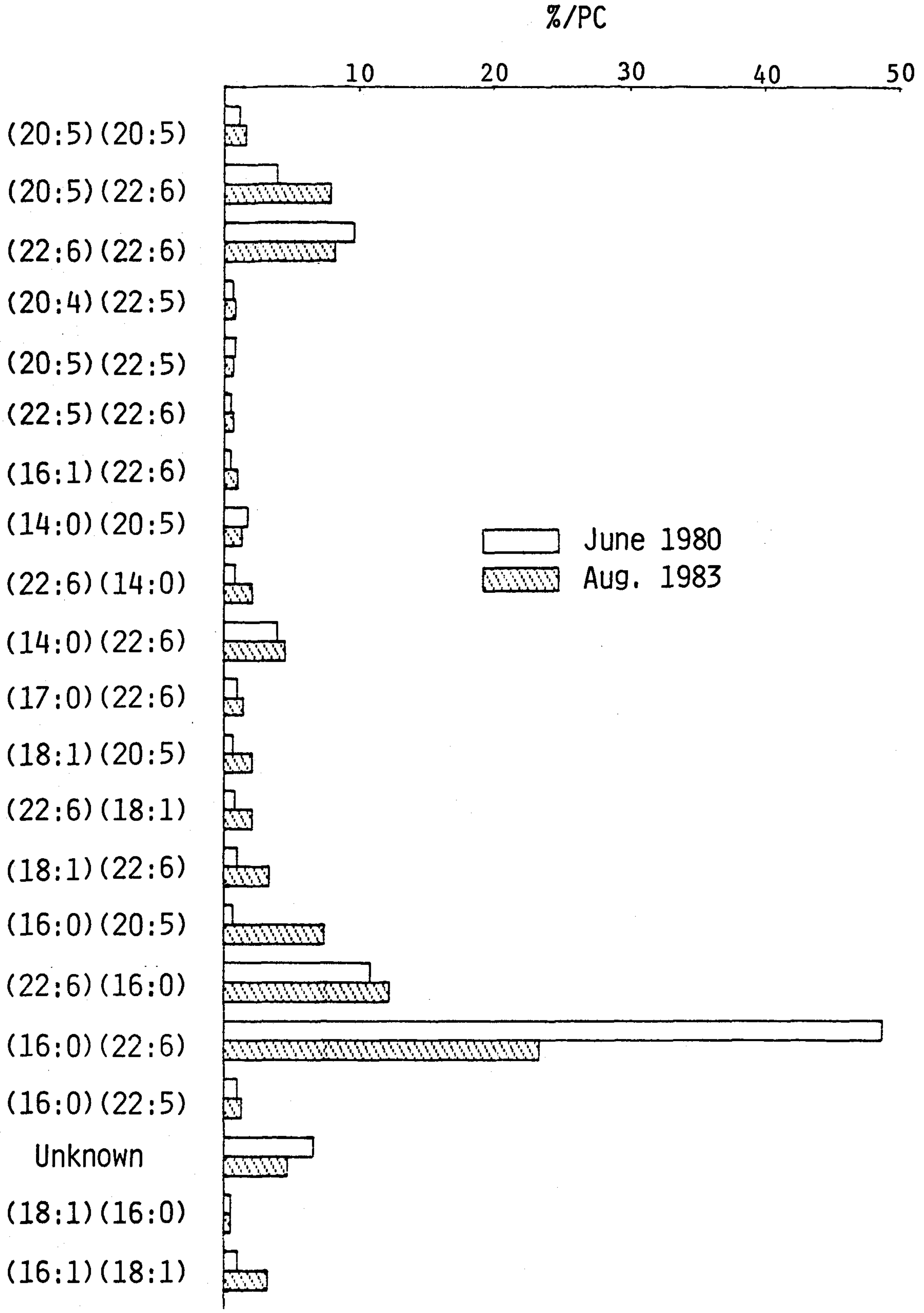


Fig. IV-16. Molecular species composition of male chum salmon muscle lecithin at the stage of feeding migration, in relative %.

Fig. IV-17. Molecular species composition of female chum salmon muscle lecithin at the stage of feeding migration, in relative %.

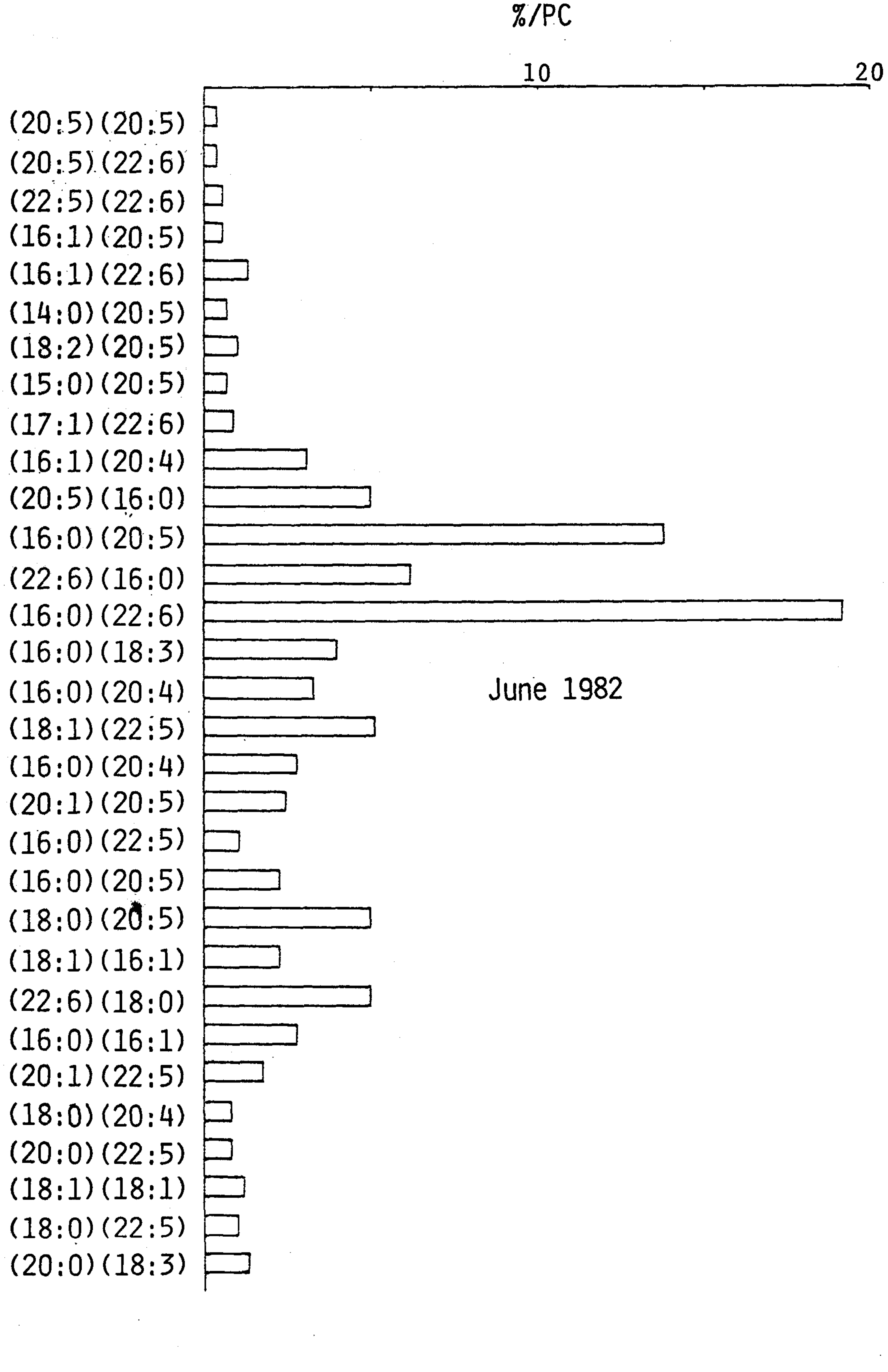


Fig. IV-18. Molecular species composition of blue shark muscle lecithin, in relative %.

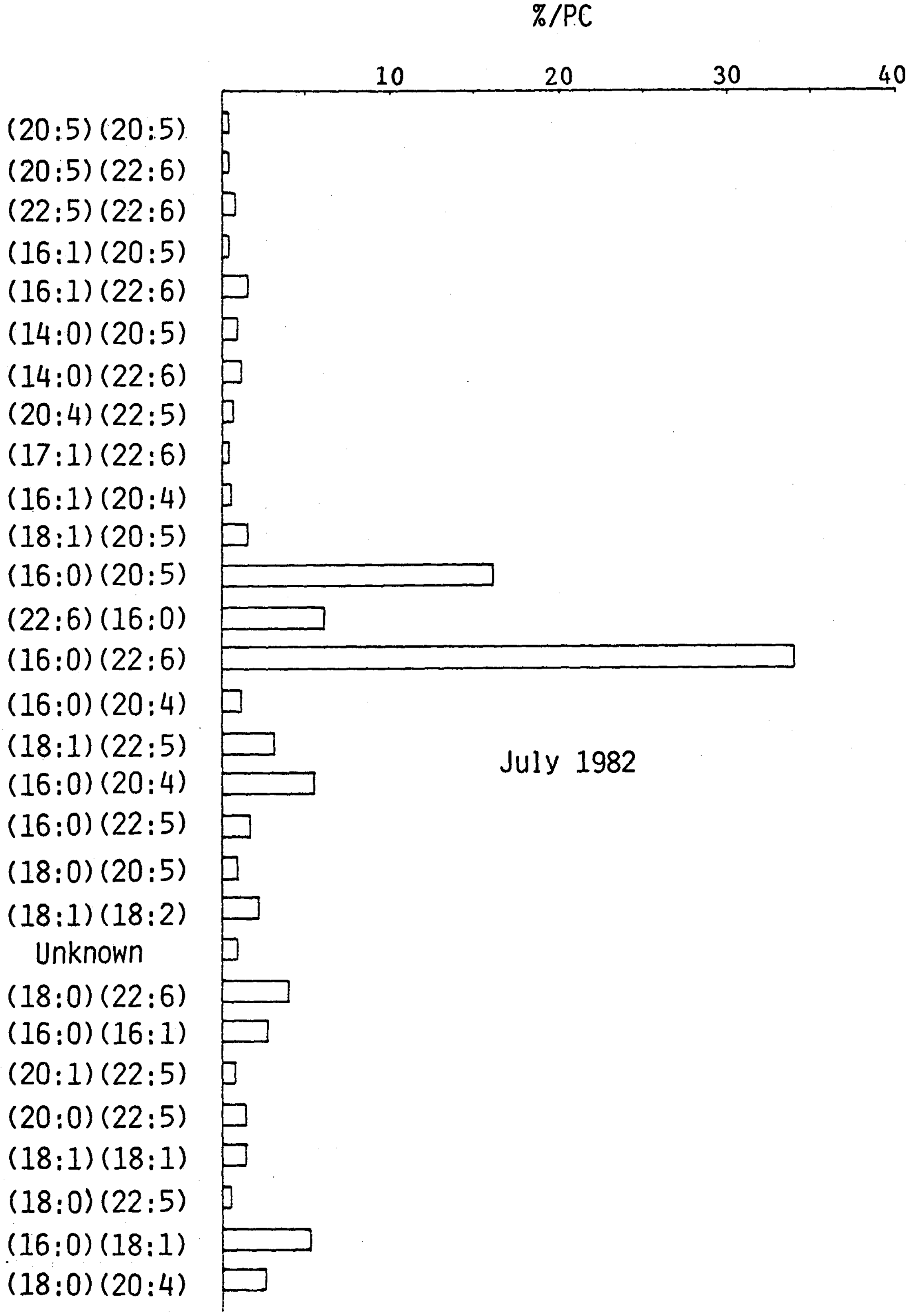


Fig. IV-19. Molecular species composition of mackerel shark muscle lecithin, in relative %.

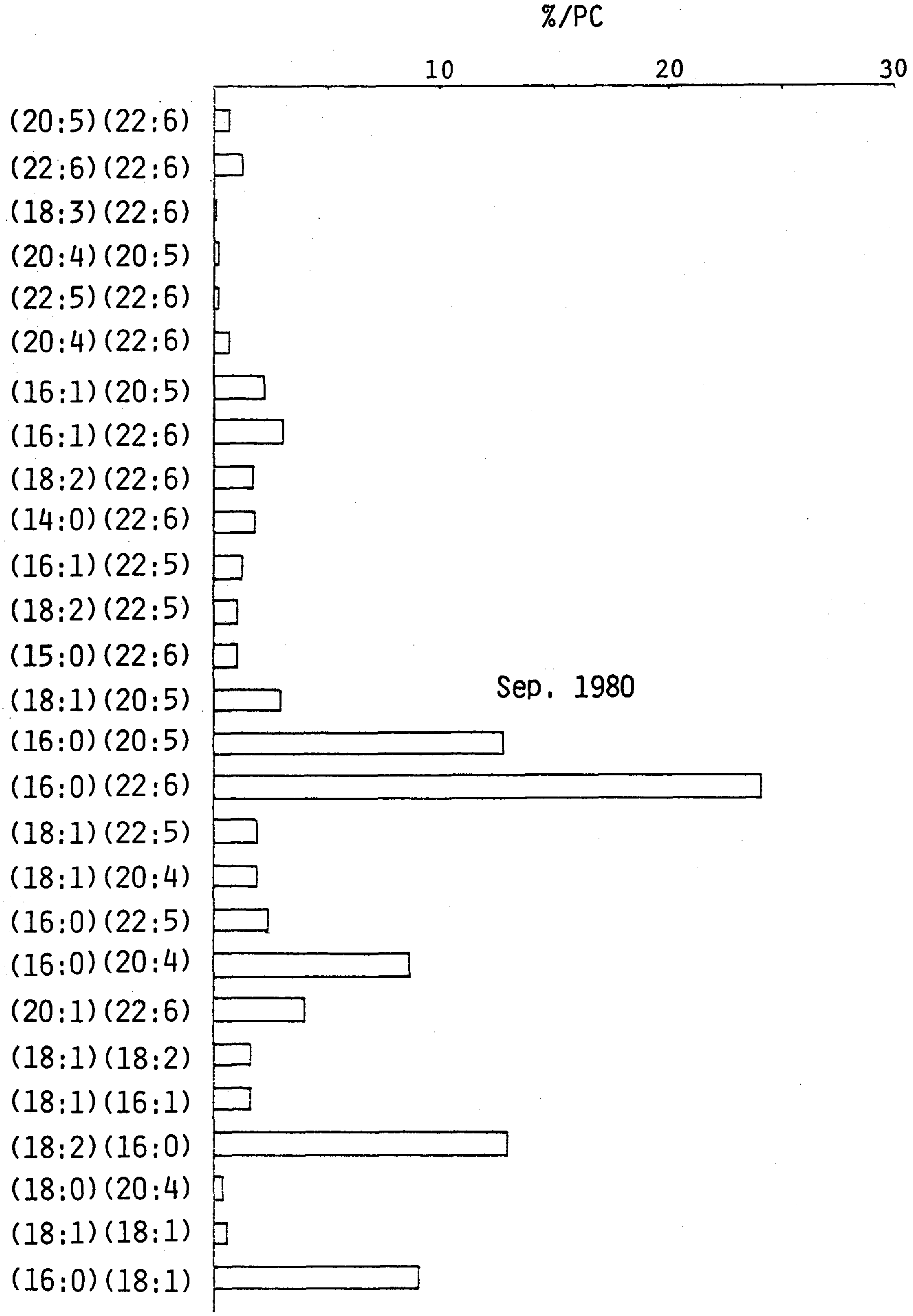


Fig. IV-20. Molecular species composition of carp muscle lecithin, in relative %.

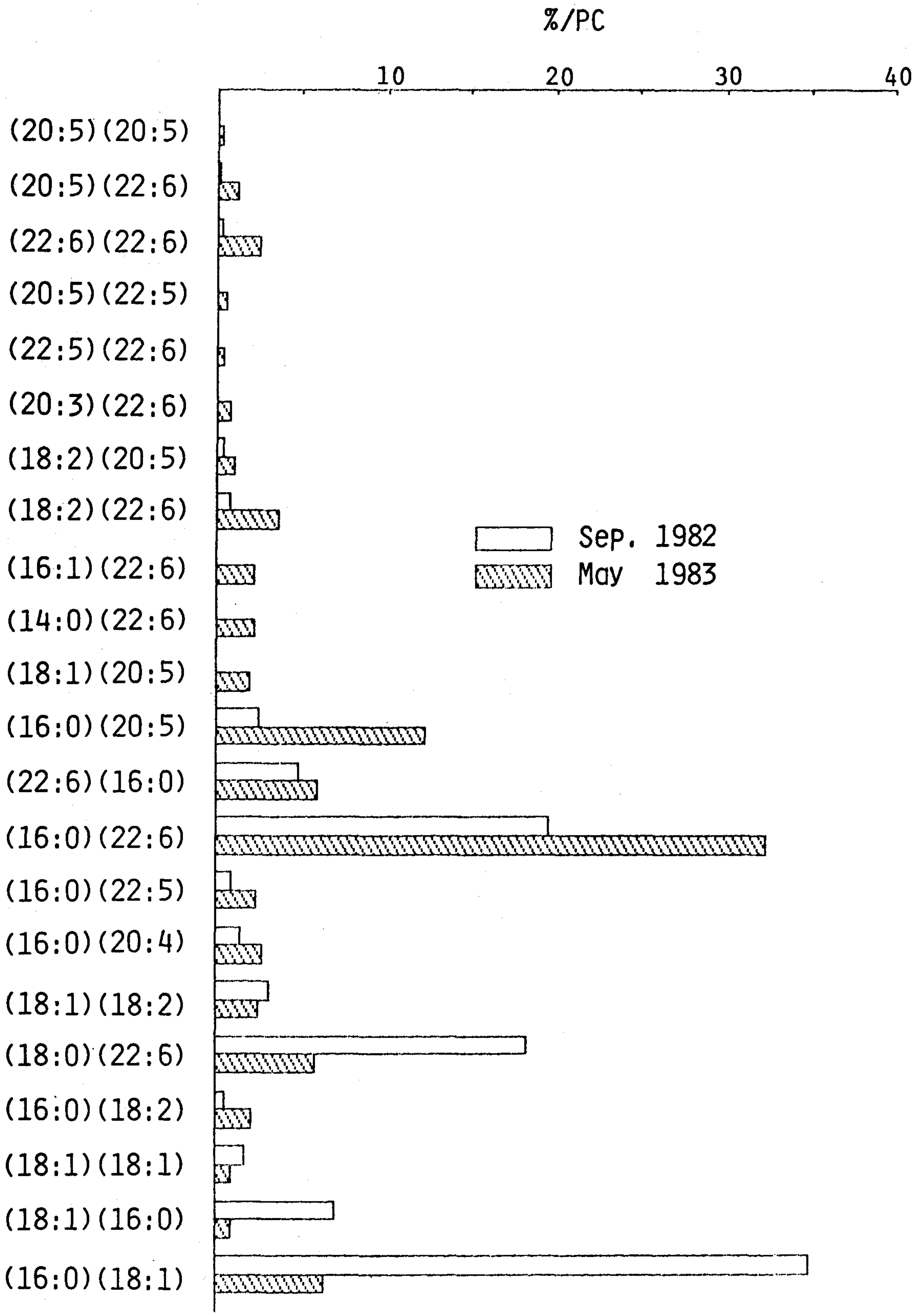


Fig. IV-21. Molecular species composition of rainbow trout muscle lecithin, in relative %.

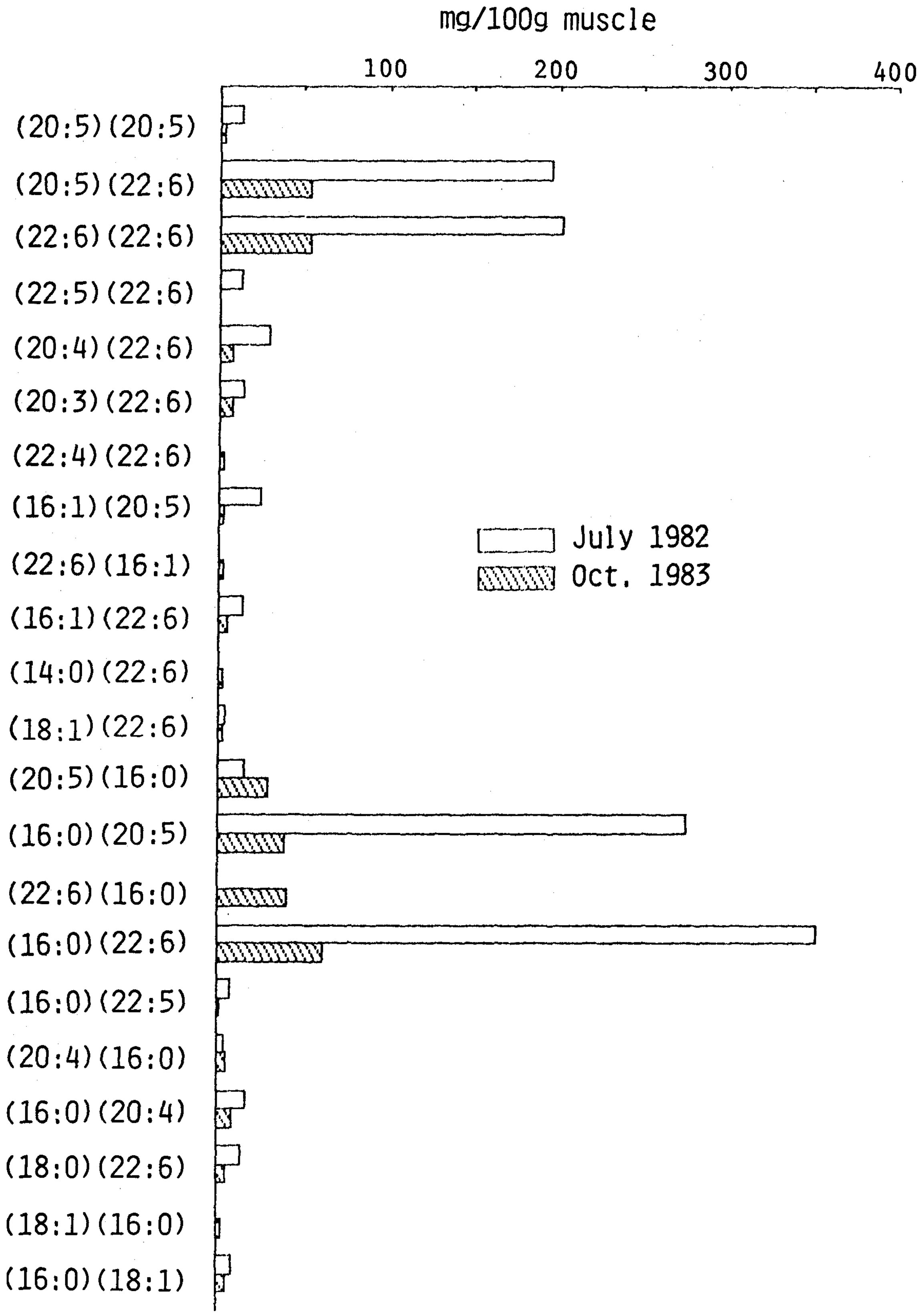


Fig. IV-22. Amount of each molecular species of sardine dark muscle lecithin, in mg/100g muscle.

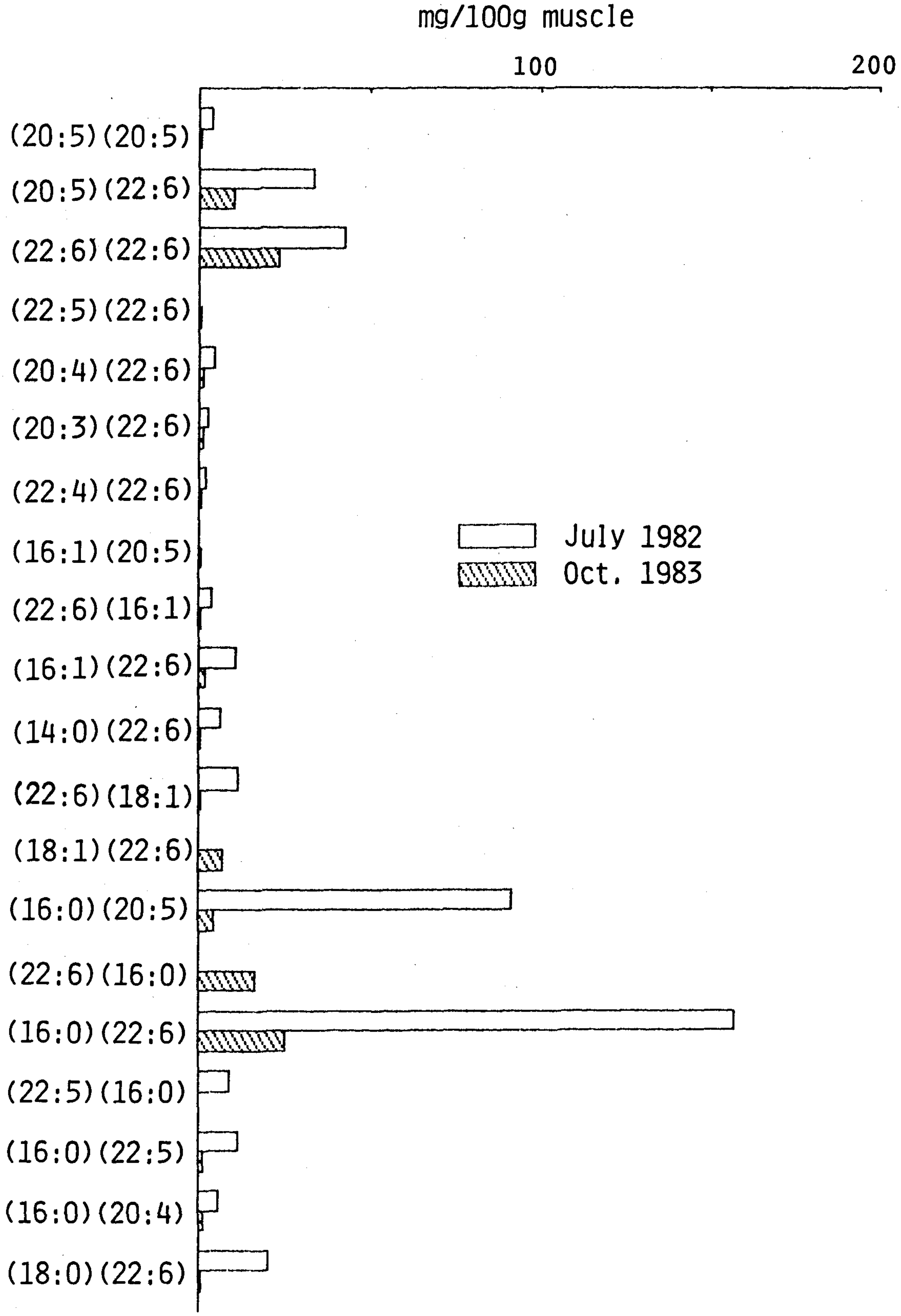


Fig. IV-23. Amount of each molecular species of sardine white muscle lecithin, in mg/100g muscle.

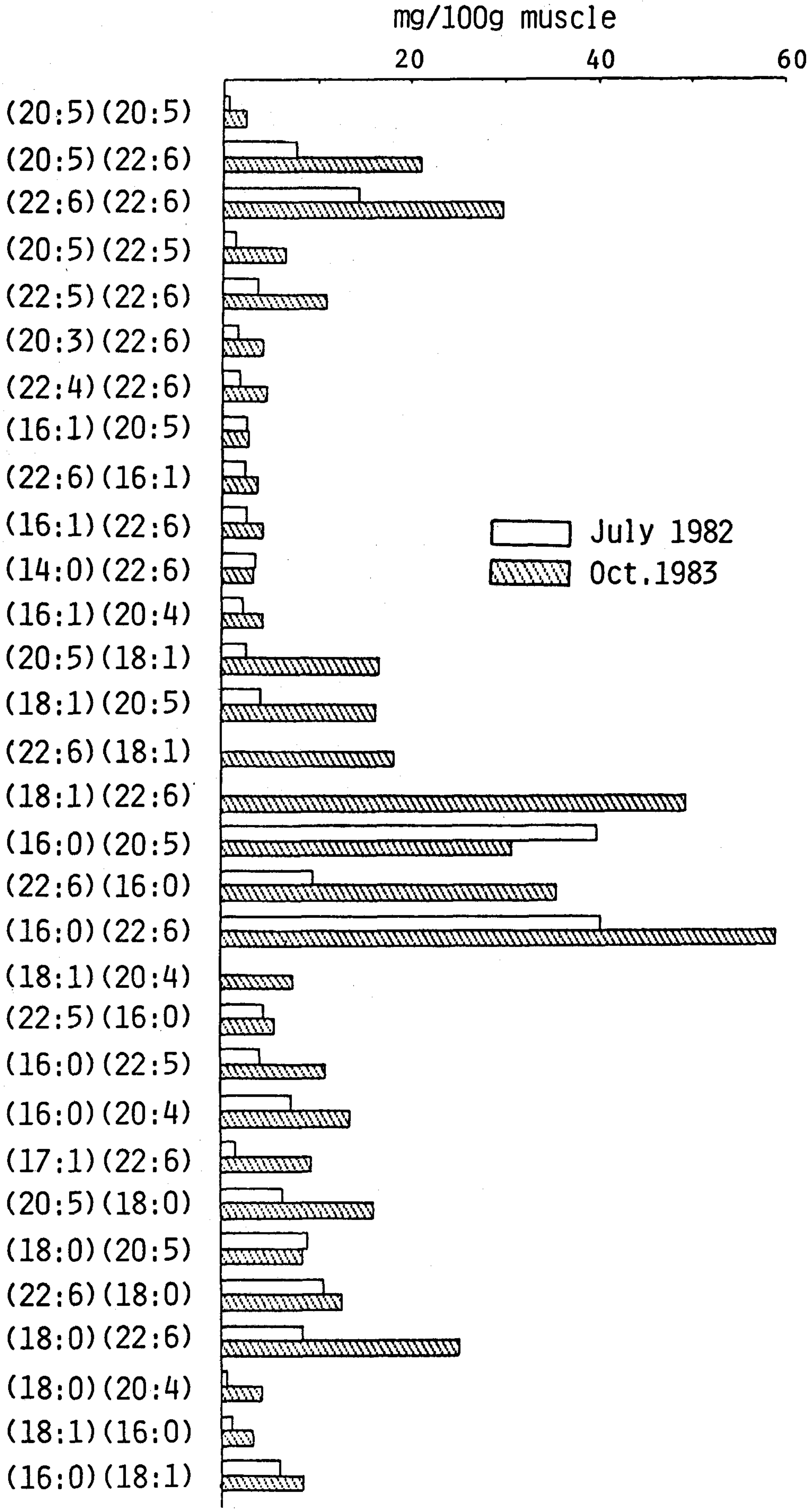


Fig. IV-24. Amount of each molecular species of mackerel dark muscle lecithin, in mg/100g muscle.

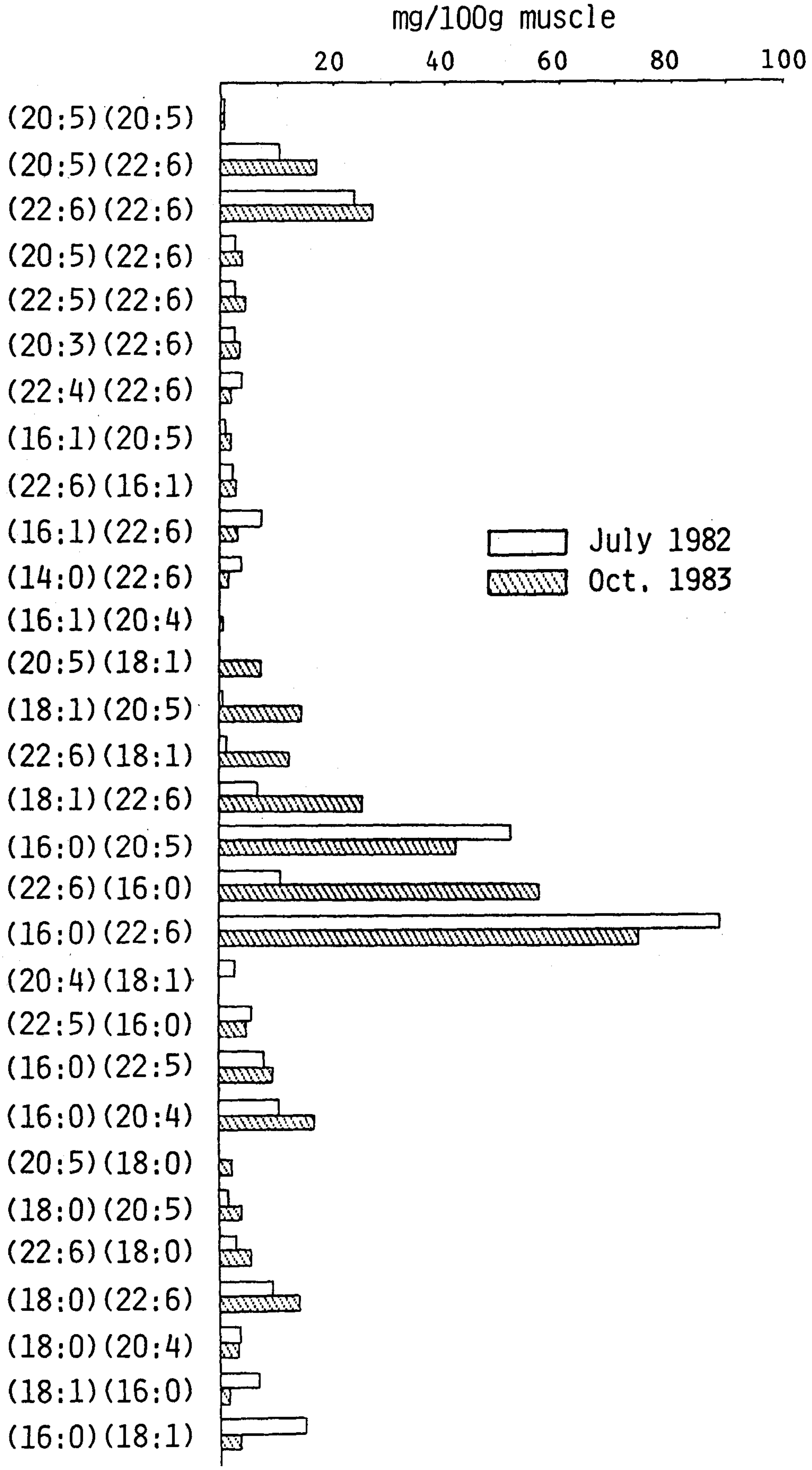


Fig. IV-25. Amount of each molecular species of mackerel white muscle lecithin, in mg/100g muscle.

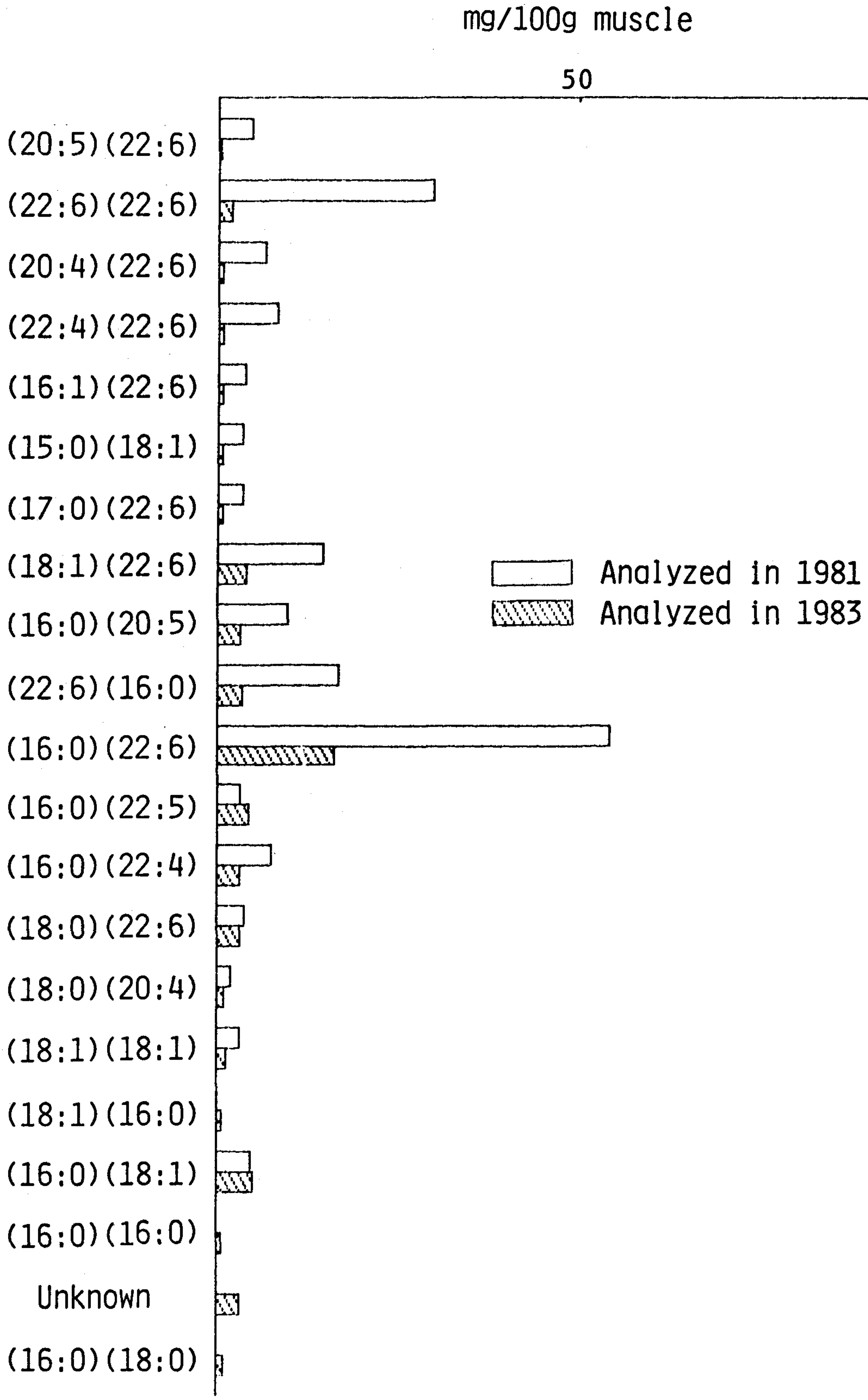


Fig. IV-26. Amount of each molecular species of big-eyed tuna muscle lecithin, in mg/100g muscle.

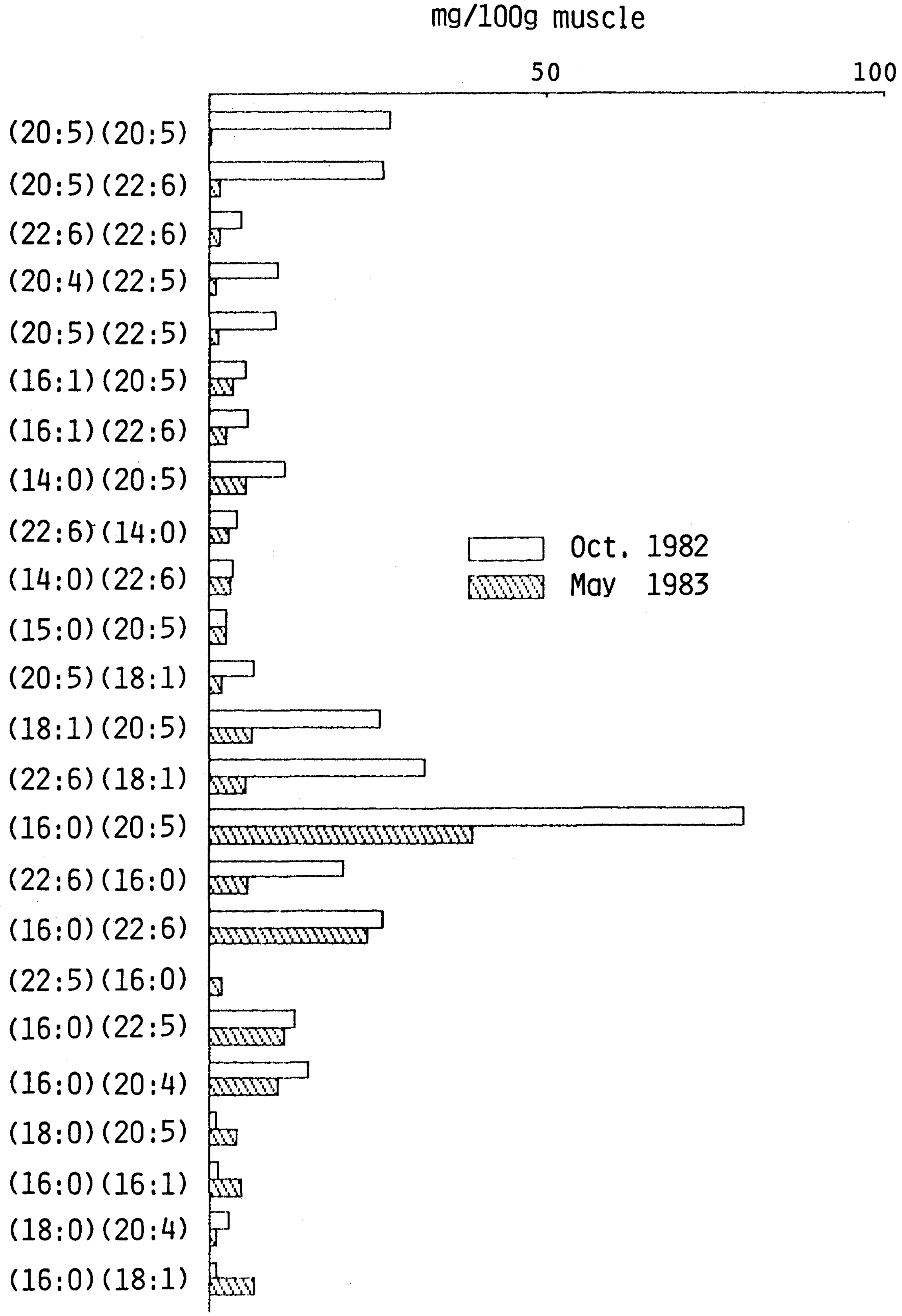


Fig. IV-27. Amount of each molecular species of brown sole muscle lecithin, in mg/100g muscle.

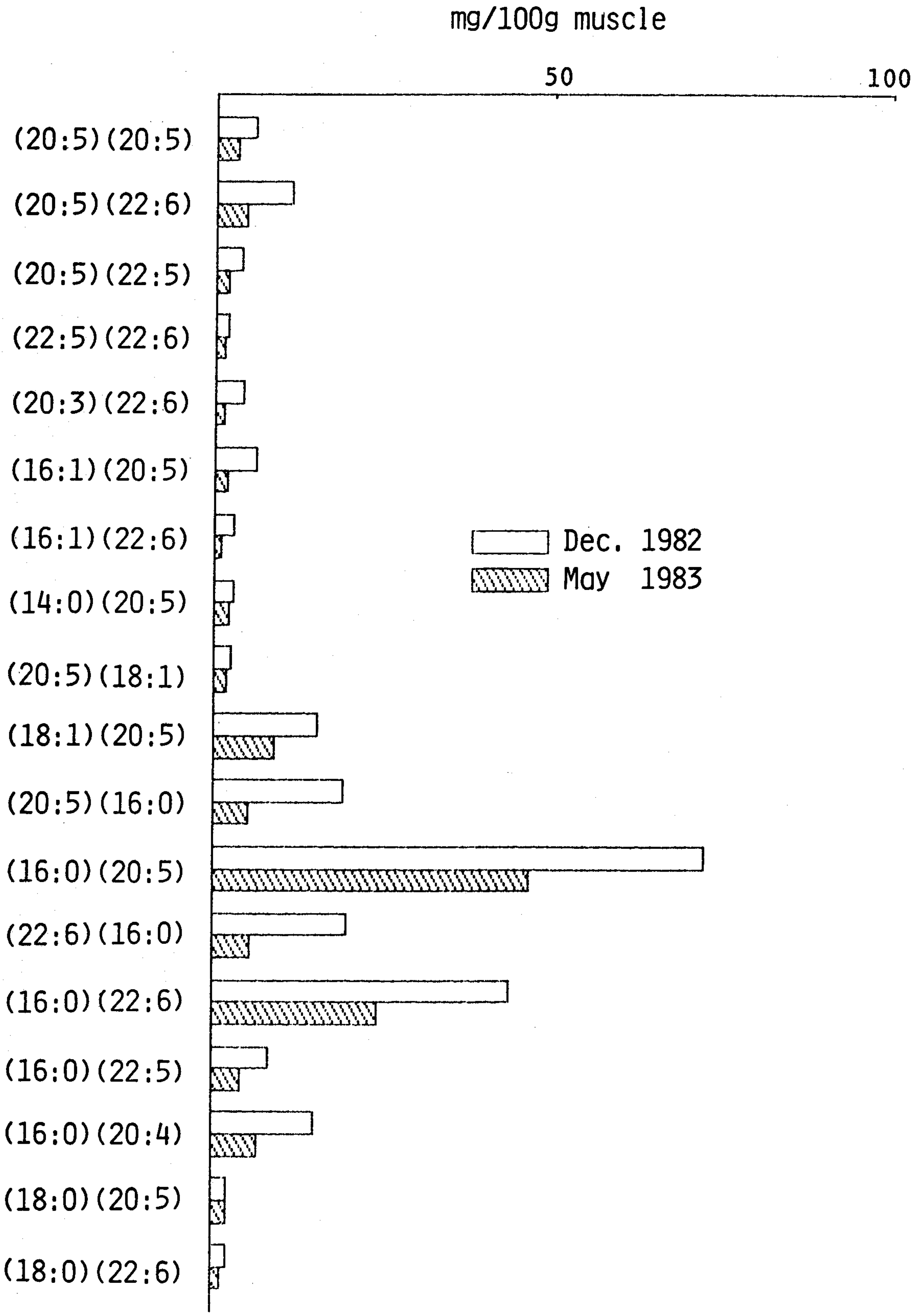


Fig. IV-28. Amount of each molecular species of sand flounder muscle lecithin, in mg/100g muscle.

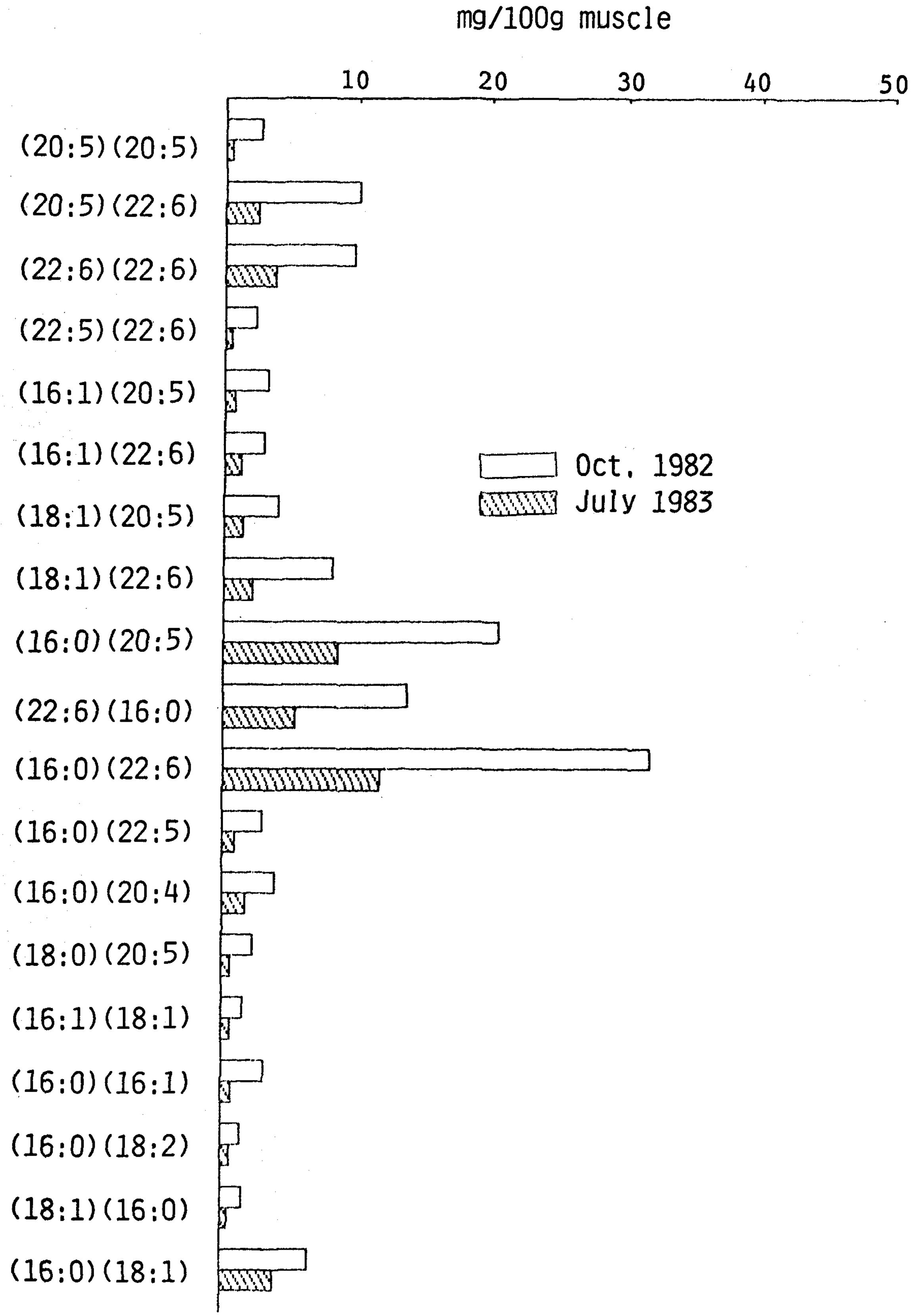


Fig. IV-29. Amount of each molecular species of rock fish muscle lecithin, in mg/100g muscle.

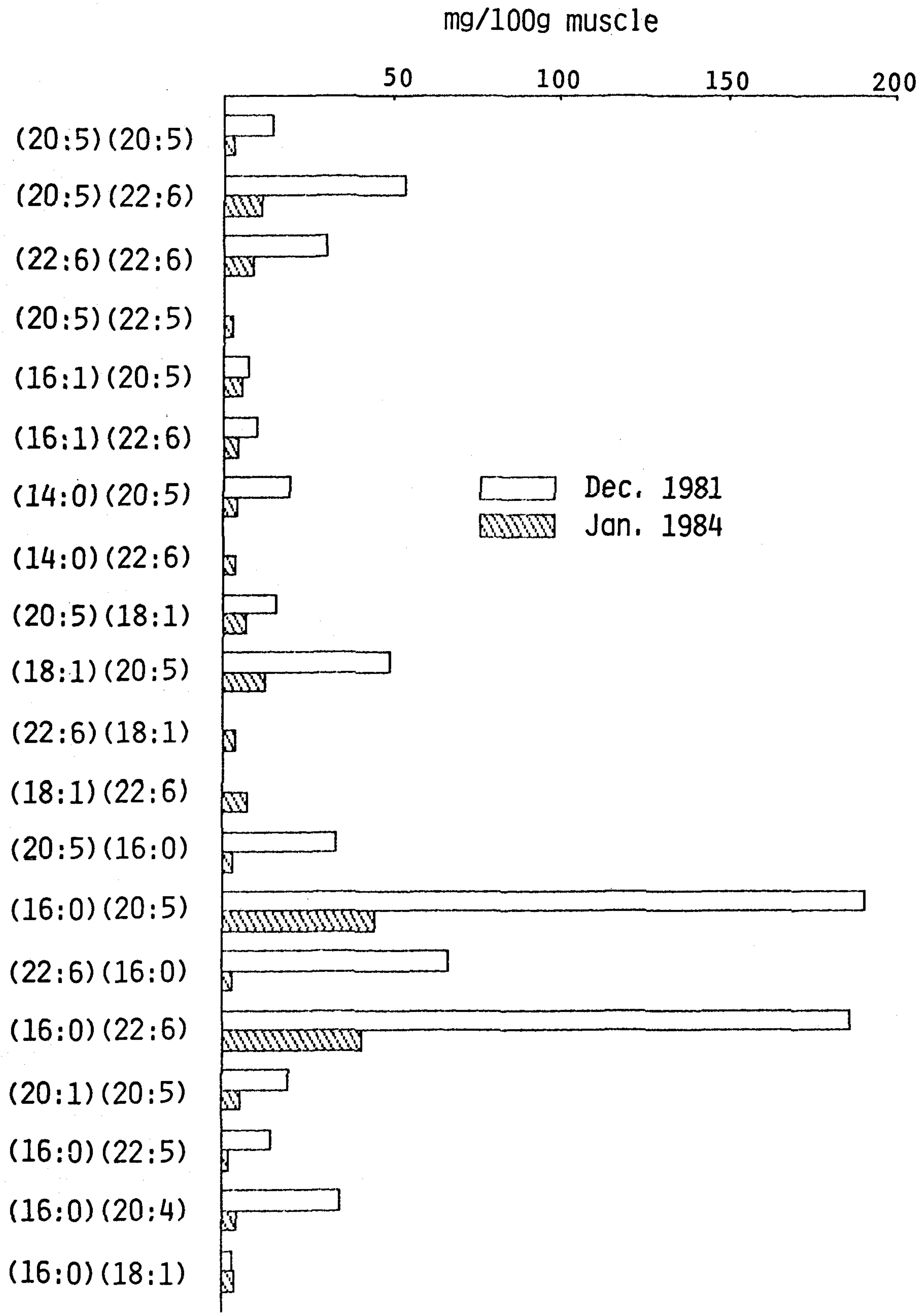


Fig. IV-30. Amount of each molecular species of Alaska pollack muscle lecithin, in mg/100g muscle.

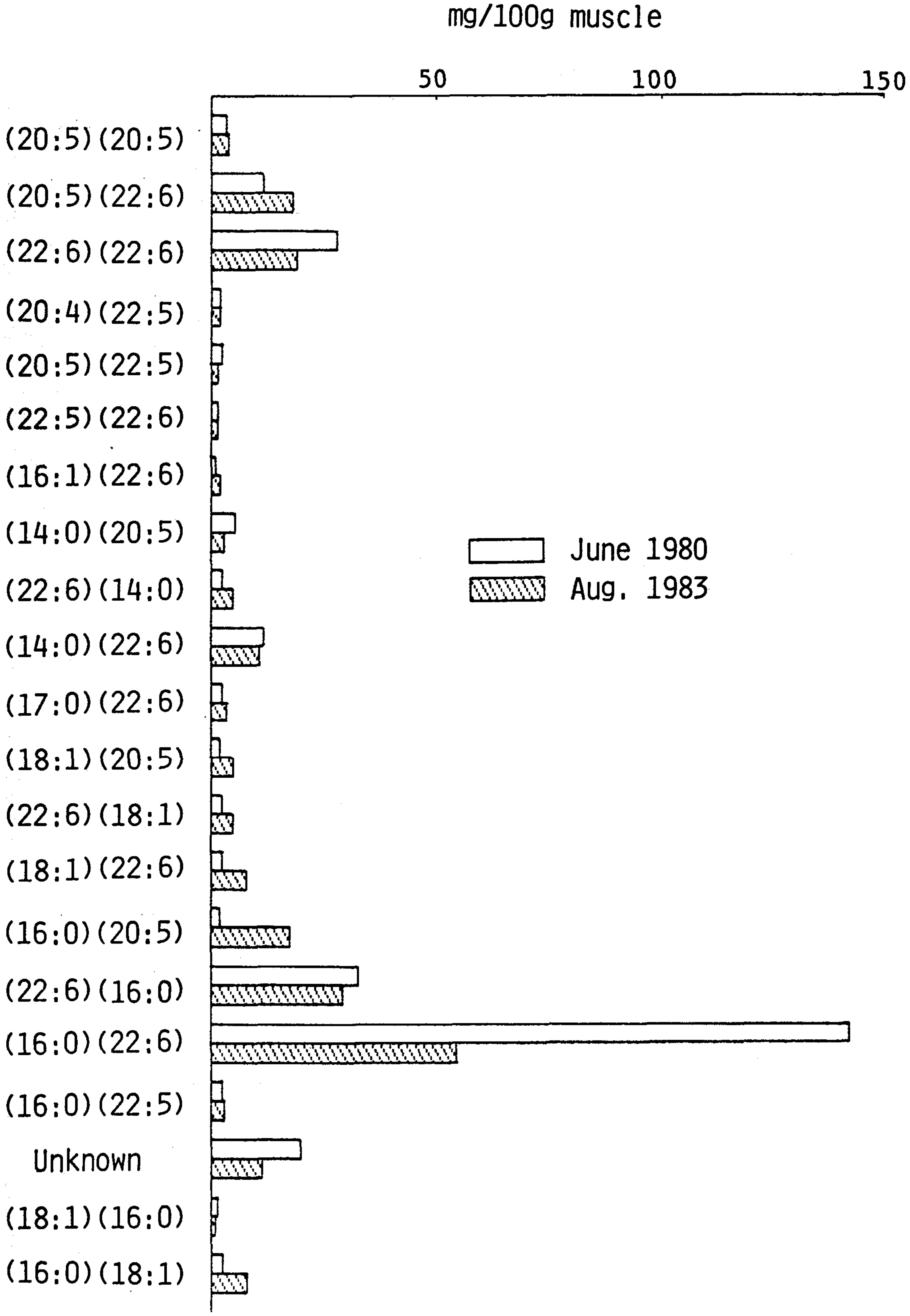


Fig. IV-31. Amount of each molecular species of male chum salmon muscle lecithin, in mg/100g muscle, at the stage of feeding migration.

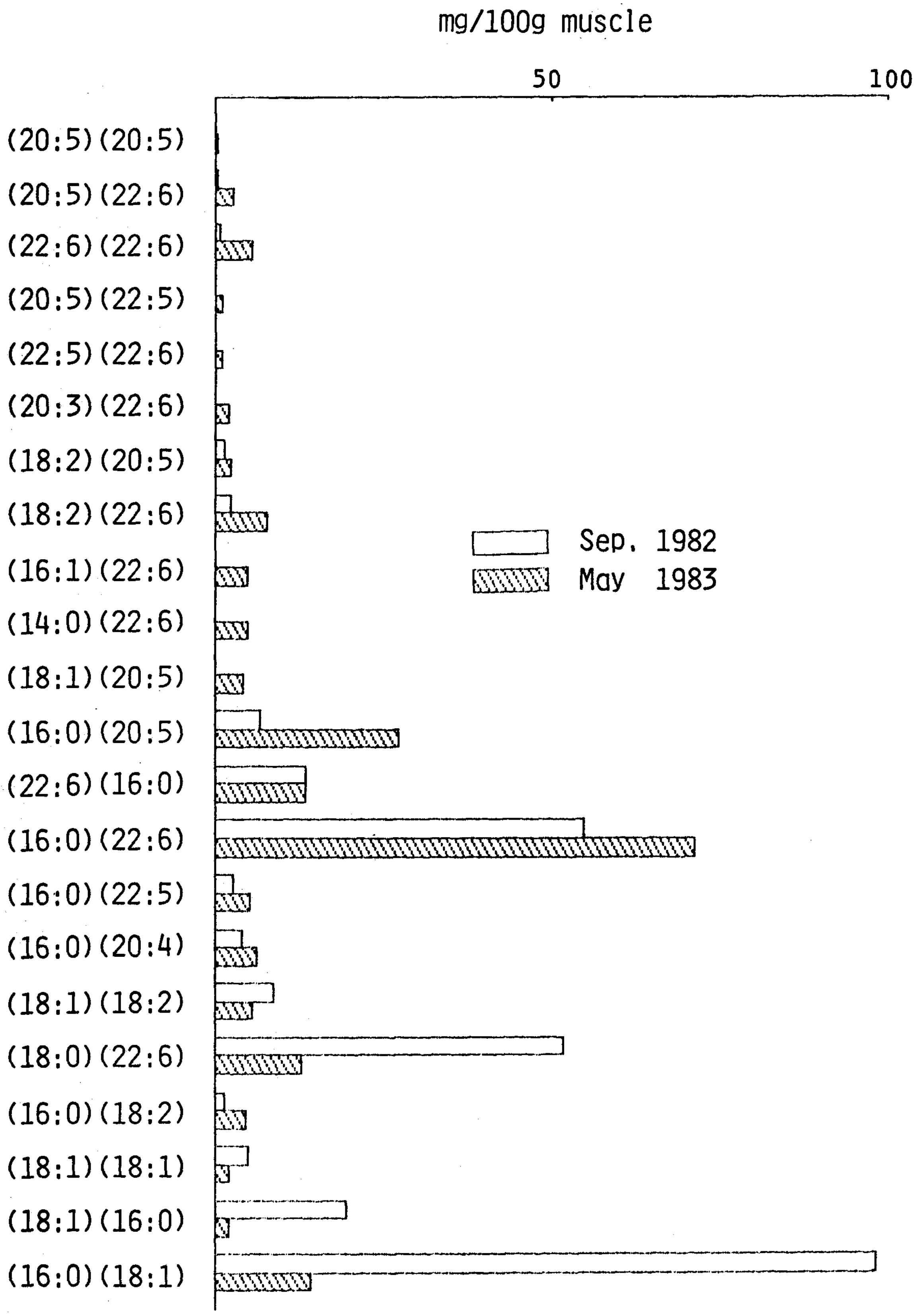


Fig. IV-32. Amount of each molecular species of rainbow trout muscle lecithin, in mg/100g muscle.

accounts for about 87~88%. These two groups might control or represents the characteristics of lecithin of fish muscle.

The left side chromatograms in Fig. IV-1 are the sardines captured in summer (July, 1982) and the right side chromatograms are of those captured in fall (Oct. 1983). Though a supplementary experiment should be done to give a conclusion, sardines captured in summer might have the tendency to lose the molecular species which combines the highly unsaturated fatty acid in position 1 such as (22:6)(16:0) and (20:5)(16:0) compared with those captured in fall.

Figure IV-2 shows the chromatograms of mackerels captured in the same day with sardine. The characteristics of this fish is the very complicated composition in group IV especially in dark muscle. Molecular species of (16:0)(22:5), (16:0)(20:4), (17:1)(22:6), (20:5)(18:0), (18:0)(20:5), (22:6)(18:0) and (18:0)(22:6) can be named in this group (see also Fig. IV-9).

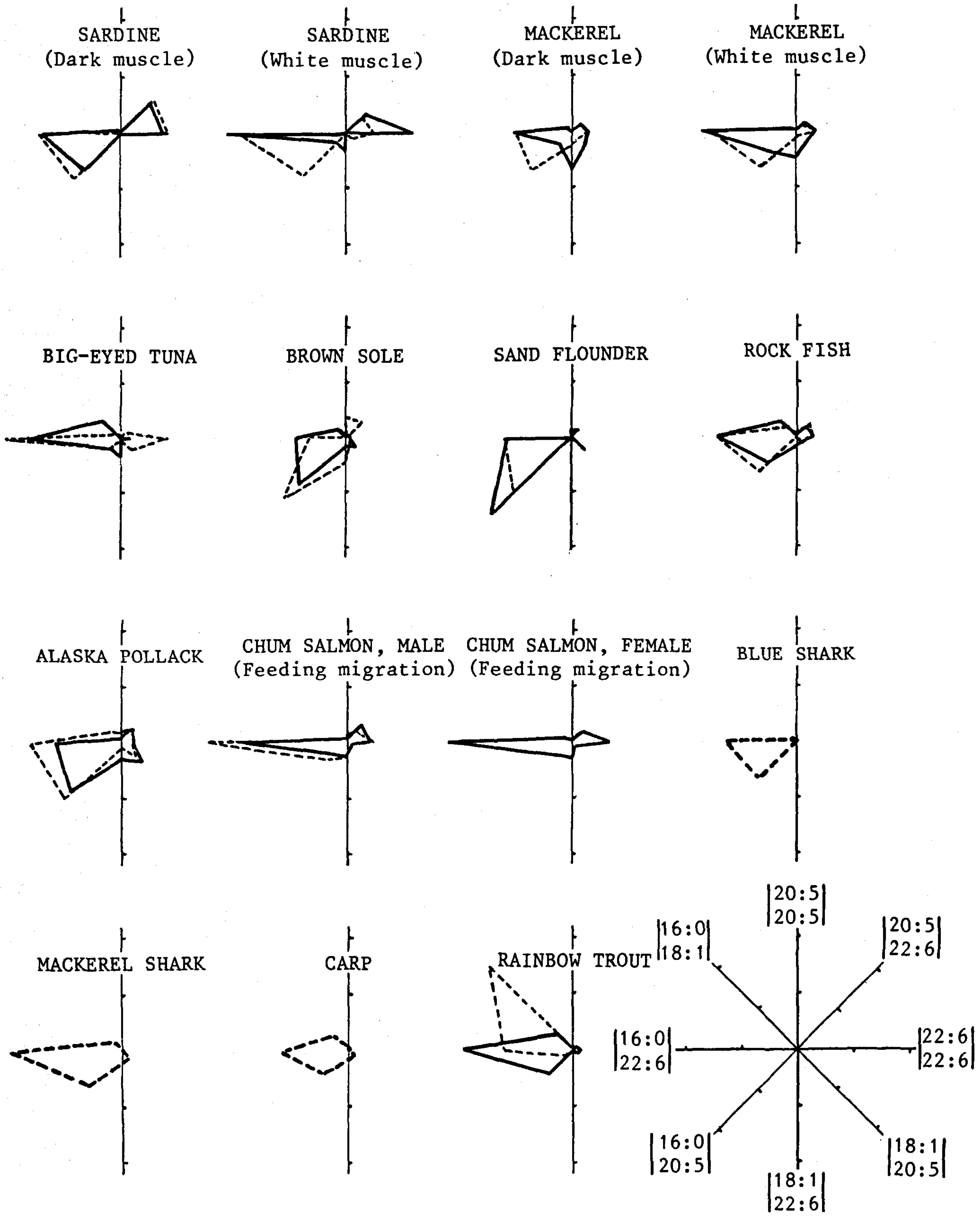
Figure IV-3 shows the chromatograms of bottom fish captured in October, December and May. These fish are extremely outstanding since the most predominant component of these fish is (16:0)(20:5) instead of (16:0)(22:6) unlike other fish examined (see also Fig. IV-12 and Fig. IV-13).

The chromatograms of big-eyed tuna, Alaska pollack, carp, rainbow trout are shown in Fig. IV-4, and the chromatograms of rock fish, blue shark, mackerel shark, rainbow trout are shown in Fig. IV-5. Throughout the chromatograms in Fig. IV-4 and Fig. IV-5, (16:0)(22:6) is the most predominant peak though in case of Alaska pollack, it contains (16:0)(22:6) and (16:0)(20:5) almost equally (shown by arrows in Fig. IV-4, see also Fig. IV-15 and Fig. IV-30.). The similarity among fresh water fish and cartilaginous fish is the very few content of group I i.e. the molecular species composed of highly unsaturated fatty acids such as (20:5)(20:5), (20:5)(22:6) and (22:6)(22:6) (see also Figs. IV-18~21), although the former contains less amount of molecular species that contains 20:5. The content of group I differs significantly in the case of big-eyed tuna. This is seemed to be the result of differences in days of frozen storage since other freshly prepared migratory fish examined contains group I abundantly.

Figure IV-6 shows the chromatograms of chum salmon. As it is evident from this Fig. IV-6, chum salmons under feeding migration are extremely rich in (16:0)(22:6) (see also Fig. IV-16 and Fig. IV-17).

Observations throughout Figs. IV-7~32 give information that the molecular species with the combinations of saturated fatty acids such as (16:0)(16:0) and (16:0)(18:0) are found only in big-eyed tuna. The reason why those are not found in other fish is considered that the melting point of (16:0)(16:0) or (16:0)(18:0) is higher than other molecular species observed so that the liquid crystal conformation of cell membrane composed of these saturated molecular species might be convenient for the warm environment and inconvenient for the cold environment.

It has been widely accepted that the amount of phospholipid of muscle is almost constant. But the expressions by mg/100g muscle of each lecithin molecular



One scale corresponds to 10%/lecithin.

Fig. IV-33. Radar charts of the main molecular species of fish muscle lecithins, in relative %.
Solid lines are the ones captured in 1983~1984, and the broken lines are the ones captured in 1980~1982.

species (Figs. IV-22~32) shows that even the amount of lecithin changes in a considerable degree.

As it is observed throughout the figures, predominant molecular species are usually composed of 16:0, 18:1, 20:5 and 22:6 such as (20:5)(20:5), (20:5)(22:6), (22:6)(22:6), (18:1)(20:5), (18:1)(22:6), (16:0)(20:5), (16:0)(22:6) and (16:0)(18:1). Figure IV-33 illustrates the amount of (20:5)(20:5), (20:5)(22:6), (22:6)(22:6), (20:5)(18:1)+(18:1)(20:5), (22:6)(18:1)+(18:1)(22:6), (20:5)(16:0)+(16:0)(20:5), (22:6)(16:0)+(16:0)(22:6) and (18:1)(16:0)+(16:0)(18:1) by radar charts. These charts are shown in sequence of elution (clockwise) and the original data have been employed from Figs. IV-7~21. As it is evident from this Fig. IV-33, the predominant peaks composed of 16:0, 18:1, 20:5 and 22:6 well characterize the characteristics of each fish. Bottom fish such as brown sole and sand flounder is of outstanding owing to the ratio of (16:0)(20:5) and (16:0)(22:6). All other fish except Alaska pollack contains (16:0)(22:6) as the most predominant component while Alaska pollack contains both component almost equally. From the view point of seasonal variation, rainbow trout shows an extraordinary change. It is considered that the closed environment of this fish forced the changes of lecithin for the adaptation. Fatty fish such as sardine and mackerel also show considerable changes compared with other marine fish examined.

Section 3. Characterization of Each Fish by Principal Component Analysis

Principal component analysis (PCA) is well known as the most representative way of multivariate analysis. PCA gives informations on allover relative relations of multivariate data of mulitiple samples at the same time.

Fig. IV-34 illustrates the dispersion of data on the second dimensional plane (X_1 ,X_2 co-ordinate) as an example of explanation of PCA. If we consider new axes on the same plane such as Z_1 and Z_2, the projection of each data (shown by arrow) on Z_1 axis well represents the characteristics of this set of data. Namely, we can decrease the dimension from second (plane) to first (line) with the smallest information loss. In this example, variates are only two. But if we expand this concept on multiple variate (multiple dimensions), and then if we decrease the dimension with the smallest information loss, such as, up to third or second dimension, this will become a so called PCA. In this study, the multiple dimension is decreased to second demension for the ease of comprehension of the whole view of data. Fig. IV-35 shows how to interpret the print out from the computer on PCA analysis. The axis named as large number 1 is the first principal component and the axis named as large number 2 is the second principal component. First principal component has the meaning of "the axis that represents the data most," and second principal component has the meaning of "the axis that represents the data next to the first principal component". Vectors shown as broken lines stand for

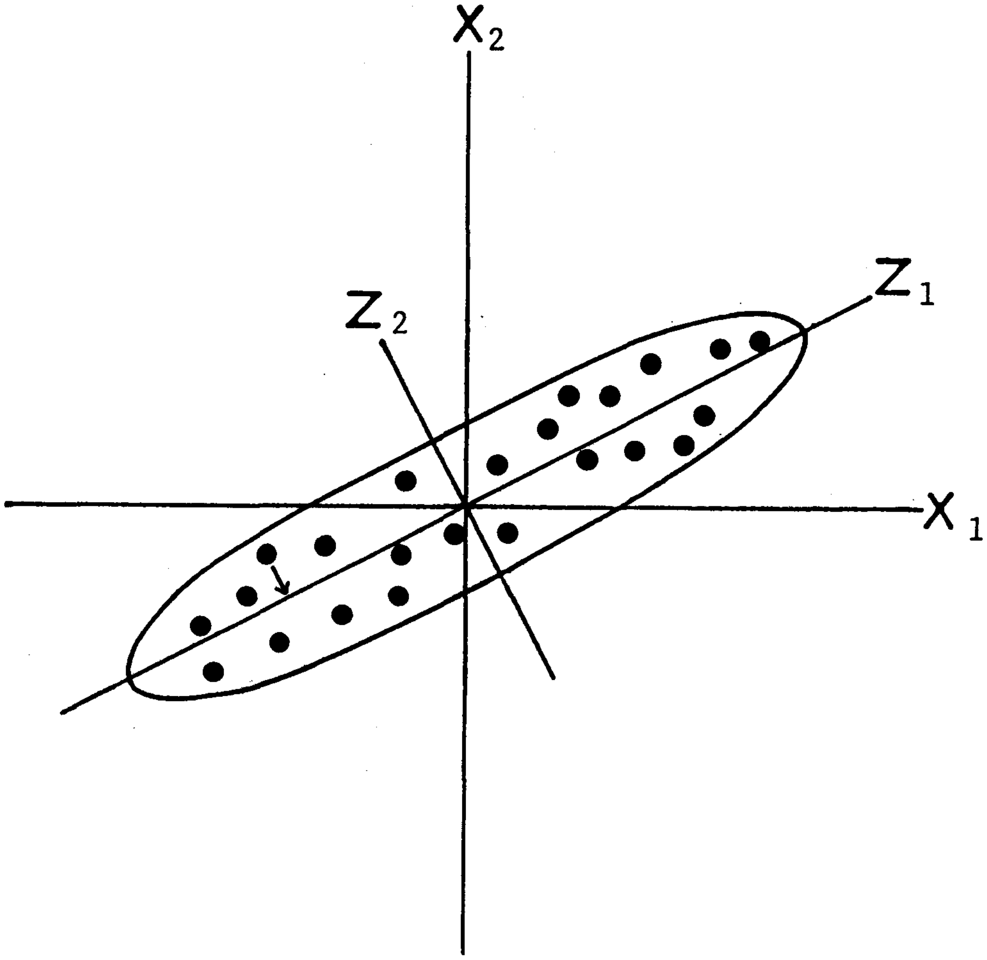
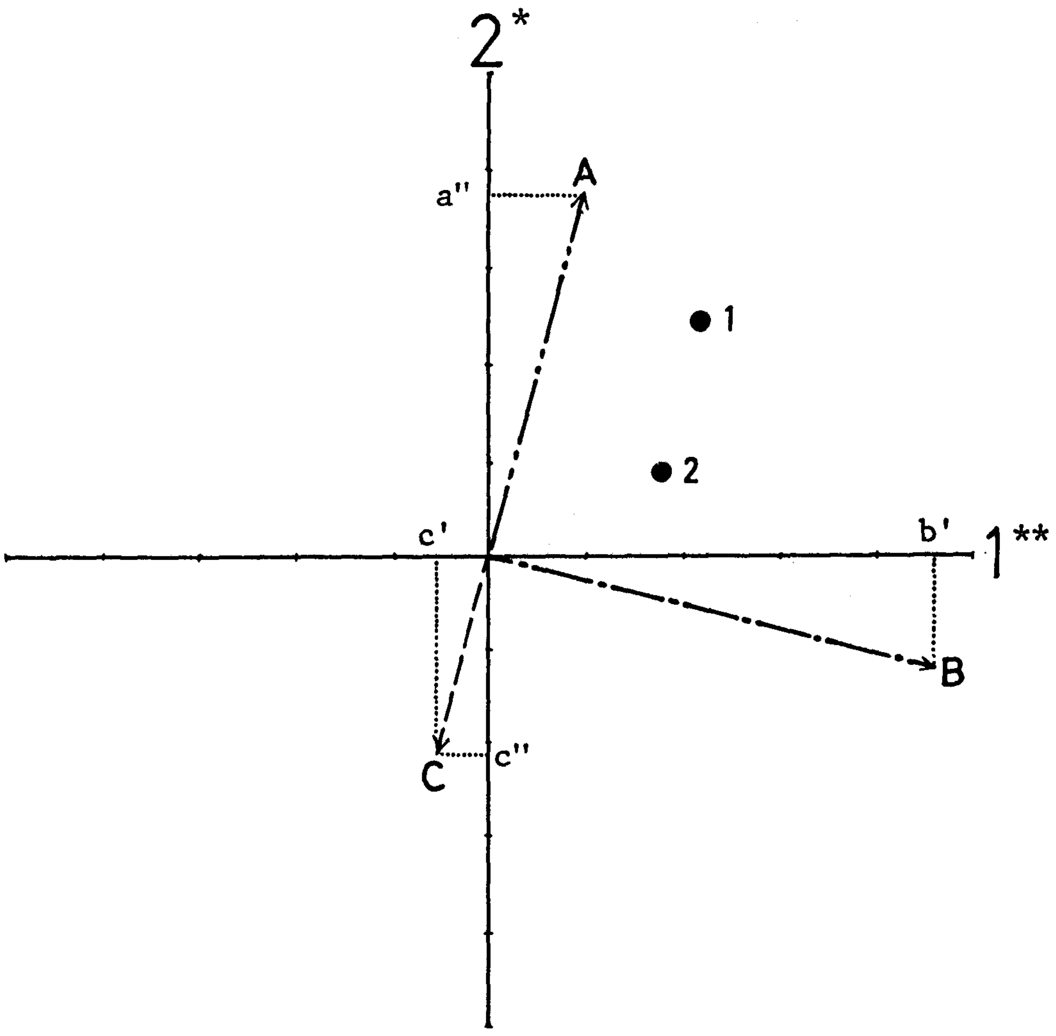


Fig. IV-34. Simplest form of PCA as an example. The arrow shows the projection of each data on the axis of first principal component.



Component A: Number 1 > Number 2

Component B: Number 1 = Number 2

Component C: Number 1 < Number 2

* Second principal component.

** First principal component.

a": Scalar of the eigenvector of component A on second principal component.
b': Scalar of the eigenvector of component B on first principal component.
c': Scalar of the eigenvector of component C on first principal component.
c": Scalar of the eigenvector of component C on second principal component.

Fig. IV-35. Interpretation of the results of PCA as an example

Table IV-6. Print out of the mean and the standard deviation of the amount of all fish muscle lecithin examined, and print outs of correlation matrix among the main molecular species, eigenvalues as well as contributions and finally component loadings

```
***** PRINCIPAL COMPONENT ANALYSIS *****
Number of Variables  8

Number of Sample  26

VARIABLE              MEAN          S.D.
  1                   3.539         5.895
  2                  19.969        38.480
  3                  22.352        39.263
  4                  23.121        57.776
  5                   8.587        15.364
  6                  39.974        46.670
  7                  93.237        76.259
  8                  11.161        22.591

========================================================================

 < Correlation Matrix >

          1        2        3        4        5        6        7        8
  1     1.000
  2     0.455    1.000
  3     0.279    0.965    1.000
  4     0.434    0.980    0.935    1.000
  5     0.170   -0.052   -0.019   -0.031    1.000
  6     0.503    0.030   -0.139   -0.011   -0.052    1.000
  7     0.361    0.802    0.784    0.755   -0.080    0.307    1.000
  8    -0.197   -0.112   -0.106   -0.075   -0.101   -0.181   -0.034    1.000


TRACE =   8

========================================================================


COMPONENT        *EIGENVALUE          %              ACC.%
  1                   3.860        48.252          48.252
  2                   1.532        19.155          67.407
  3                   1.088        13.602          81.009
  4                   0.874        10.929          91.938
  5                   0.472         5.906          97.843
  6                   0.133         1.658          99.501
  7                   0.036         0.446          99.947
  8                   0.004         0.053         100.000

**<COMPONENT LOADING >

      C O M P O N E N T
        1        2        3        4        5        6        7        8
  1   0.540    0.649    0.039    0.231    0.464    0.128    0.008    0.005
  2   0.983   -0.136    0.028   -0.022    0.059   -0.087    0.034   -0.052
  3   0.935   -0.299    0.114   -0.074   -0.041   -0.014    0.126    0.032
  4   0.958   -0.171    0.053    0.009    0.095   -0.159   -0.123    0.021
  5  -0.024    0.243    0.854    0.407   -0.212   -0.028   -0.002   -0.002
  6   0.160    0.838   -0.413    0.110   -0.248   -0.165    0.029    0.005
  7   0.873    0.030   -0.200    0.062   -0.367    0.233   -0.047   -0.003
  8  -0.155   -0.461   -0.360    0.794    0.042   -0.026    0.014    0.001
```

* Eigenvalues were calculated by the Jacobi method, and the iteration was 60 on this PCA.

** Component loading corresponds to the scalar of the eigenvector on PCA The row and the column are variable numbers same with those in the next figure (Fig. IV-36).

component loadings. For example, suppose vector A stands for water content, B for protein content and C for lipid content, water content is almost negatively correlative and at the same time, no correlation with protein content. If we have data number 1 and number 2, we can get the following informations:

Water content : number 1 > number 2
Lipid content : number 1 < number 2
Protein content : number 1 = number 2

By introducing this PCA, the whole view of all fish examined can be summerized in one or two planes with the minimum loss of information.

The computer program used for PCA was the modified program of "Personal Computer Library, Vol. 3".48) The original program was written for NEC PC-8001 personal computer. But it was modified for NEC PC-8801 mkII personal computer, for instance, the arrangement of the program was changed into N-88 BASIC, and the character mode was changed into graphic mode.

Table IV-6 shows the print out from the computer. In this Table, "Number of Variables 8" are the main molecular species which have been discussed in the radar chart on Fig. IV-33, namely, those corresponds to the footnote in Table IV-7 shown as "Row". "Number of Sample 26" are the fish examined and those are also shown in the footnote of Table IV-7. "MEAN" is the average amount of each molecular species mg/100g muscle. "S.D." is standard deviation. "Correlation Matrix" shows the correlation of each variable (molecular species). "EIGENVALUE" is calculated by the Jacobi method

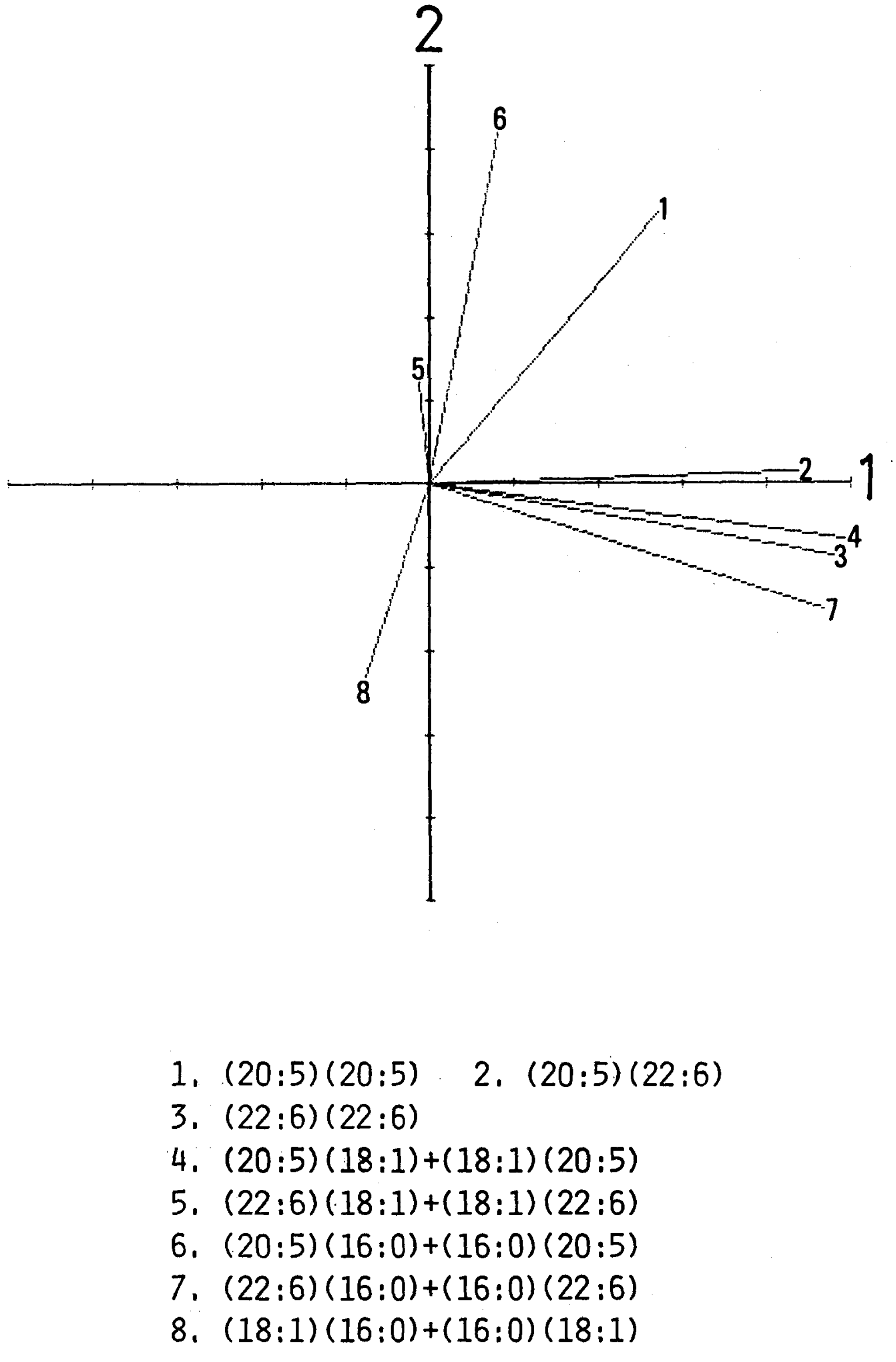
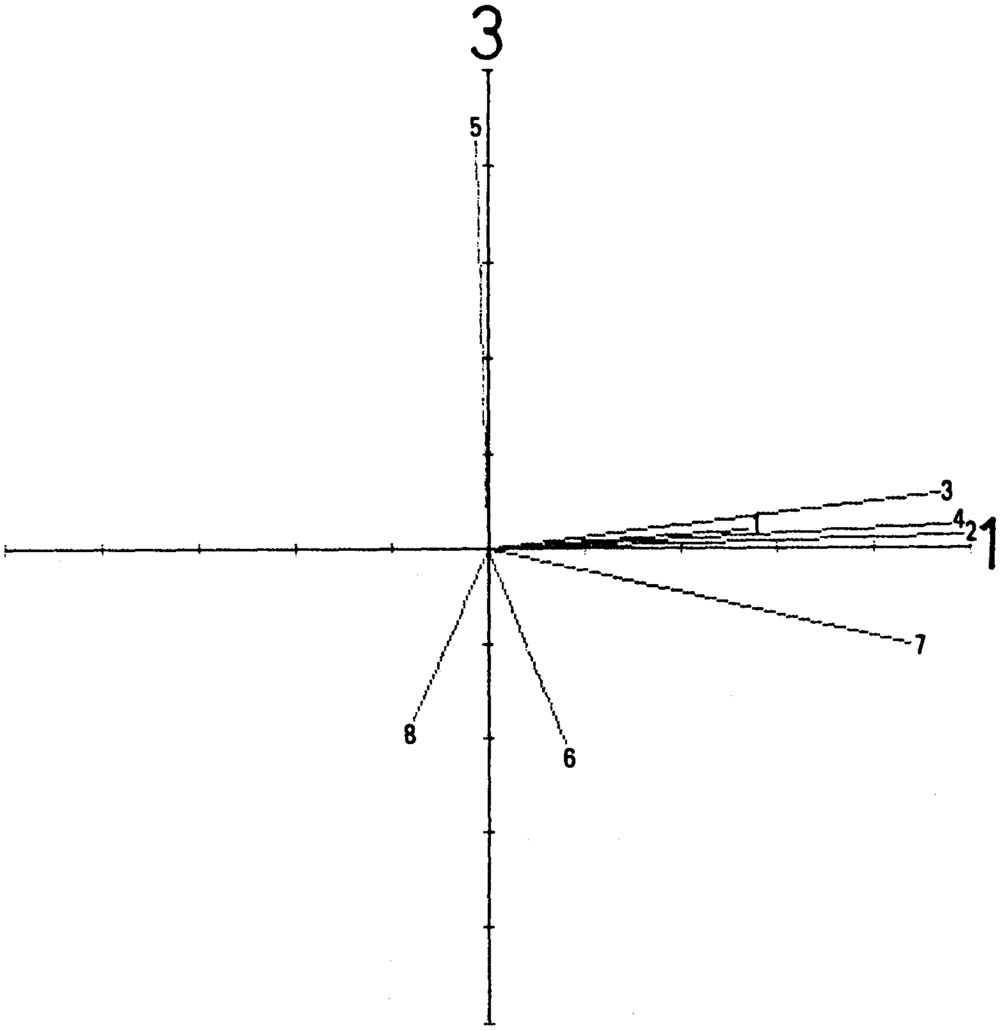


Fig. IV-36. Eigenvectors of major molecular species on the first and second principal component plane on PCA.



1. (20:5)(20:5)   2. (20:5)(22:6)
3. (22:6)(22:6)
4. (20:5)(18:1)+(18:1)(20:5)
5. (22:6)(18:1)+(18:1)(22:6)
6. (20:5)(16:0)+(16:0)(20:5)
7. (22:6)(16:0)+(16:0)(22:6)
8. (18:1)(16:0)+(16:0)(18:1)

Fig. IV-37. Eigenvectors of major molecular species on the first and third principal component plane on PCA.

Table IV-7. Print out of the principal loading (component score) on PCA.

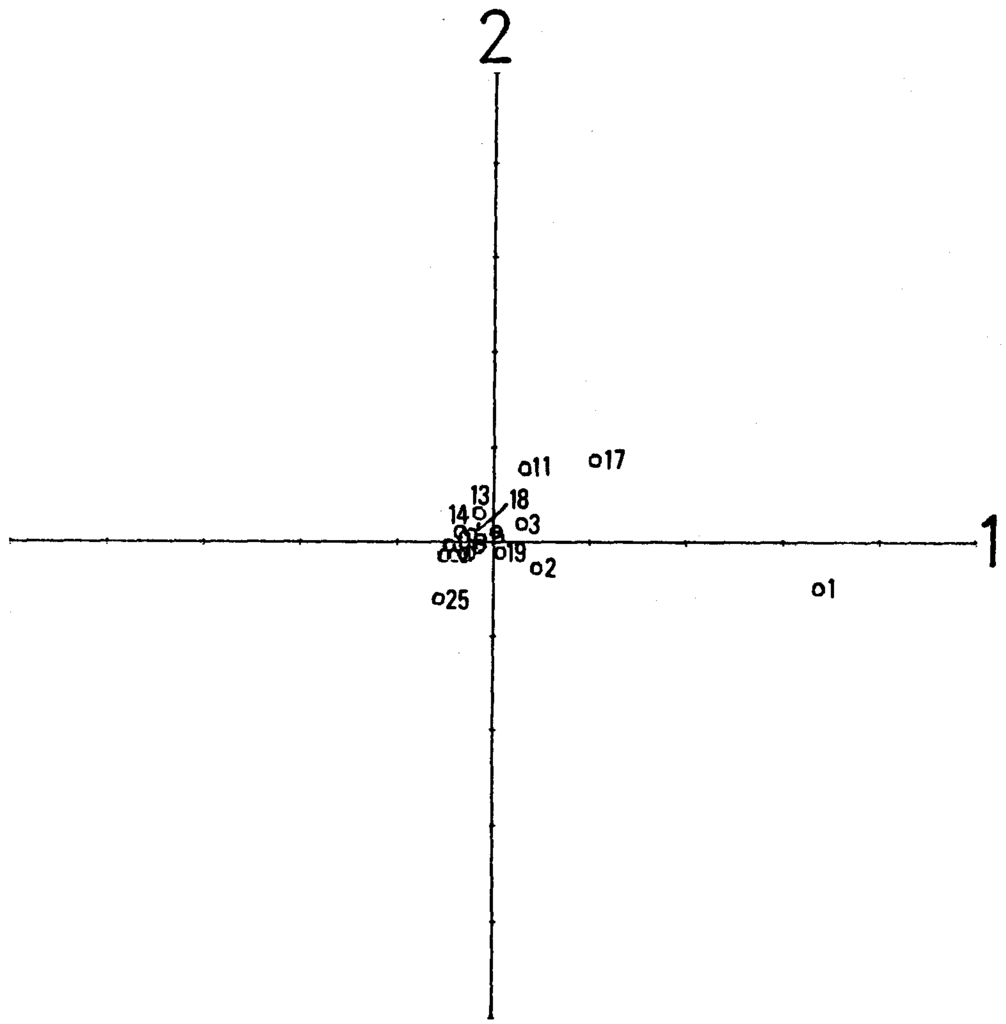
```
<PRINCIPAL LOADING>

          P R I N C I P A L
        1          2          3          4          5          6          7          8
  1   16.821     -2.524      0.185     -0.190      0.270     -0.056     -0.015      0.002
  2    2.228     -1.392      0.073     -0.592      0.269     -0.050      0.002     -0.008
  3    1.467      0.958     -0.860     -0.464     -0.499      0.074      0.113     -0.006
  4   -1.526     -0.714      0.478     -0.705      0.140      0.003      0.043      0.002
  5   -1.489     -0.246     -0.368     -0.503      0.053     -0.100      0.020      0.004
  6    0.151      0.536      3.374      1.515     -0.349     -0.129     -0.012     -0.002
  7   -0.810     -0.248     -0.373      0.264     -0.347     -0.013      0.050      0.005
  8    0.198      0.315      1.597      0.490     -0.836      0.010     -0.018     -0.000
  9   -1.240     -0.655      0.715     -0.310     -0.173      0.055      0.045      0.012
 10   -2.616     -0.746      0.241     -0.569      0.276     -0.042     -0.019     -0.002
 11    1.607      3.878      1.328      1.226      1.545      0.156      0.007      0.002
 12   -2.224     -0.105     -0.034     -0.402      0.052     -0.164     -0.019      0.002
 13   -0.773      1.472     -0.742     -0.400      0.136     -0.148     -0.015     -0.000
 14   -1.787      0.511     -0.326     -0.624      0.297     -0.133     -0.015      0.001
 15   -1.540     -0.215      0.244     -0.223      0.237     -0.011      0.005     -0.005
 16   -2.454     -0.635      0.123     -0.640      0.320     -0.065     -0.009     -0.002
 17    5.297      4.346     -2.244      0.512     -0.650     -0.086     -0.005      0.000
 18   -1.100      0.453      0.285     -0.200      0.128     -0.144     -0.014      0.000
 19    0.382     -0.350      0.022     -0.371     -0.232      0.465     -0.022      0.001
 20   -0.608     -0.193      0.462     -0.055      0.096      0.105      0.017     -0.010
 21   -1.260     -0.513      0.417     -0.401      0.082      0.070      0.019      0.002
 22   -1.550      0.282     -0.457     -0.646     -0.118      0.050     -0.039     -0.001
 23   -0.667      0.204     -1.129      0.214     -0.500      0.177     -0.073      0.000
 24   -2.052     -0.663     -0.545     -0.052      0.061     -0.057     -0.026      0.002
 25   -2.343     -2.996     -1.932      3.310      0.286     -0.026      0.017     -0.001
 26   -1.610     -0.561     -0.515     -0.185     -0.046      0.059     -0.033      0.000
```

Row

1. SARDINE PC (Dark muscle), July 1982
2. SARDINE PC (Dark muscle), Oct. 1983
3. SARDINE PC (White muscle), July 1982
4. SARDINE PC (White muscle), Oct. 1983
5. MACKEREL PC (Dark muscle), July 1982
6. MACKEREL PC (Dark muscle), Oct. 1983
7. MACKEREL PC (White muscle), July 1982
8. MACKEREL PC (White muscle), Oct. 1983
9. BIG-EYED TUNA PC, Frozen 1981
10. BIG-EYED TUNA PC, Frozen 1983
11. BROWN SOLE PC, Oct. 1982
12. BROWN SOLE PC, May 1983
13. SAND FLOUNDER PC, Dec. 1982
14. SAND FLOUNDER PC, May 1983
15. ROCK FISH PC, Oct. 1982
16. ROCK FISH PC, July 1983
17. ALASKA POLLACK PC, Dec. 1981
18. ALASKA POLLACK PC, Jan. 1984
19. CHUM SALMON PC, MALE, June 1980
20. CHUM SALMON PC, MALE, Aug. 1983
21. CHUM SALMON PC, FEMALE, Aug. 1983
22. BLUE SHARK PC, June 1982
23. MACKEREL SHARK PC, July 1982
24. CARP PC, Sep. 1980
25. RAINBOW TROUT PC, Sep. 1982
26. RAINBOW TROUT PC, May 1983

Column

1. (20:5)(20:5)
2. (20:5)(22:6)
3. (22:6)(22:6)
4. (20:5)(18:1)+(18:1)(20:5)
5. (22:6)(18:1)+(18:1)(22:6)
6. (20:5)(16:0)+(16:0)(20:5)
7. (22:6)(16:0)+(16:0)(22:6)
8. (18:1)(16:0)+(16:0)(18:1)



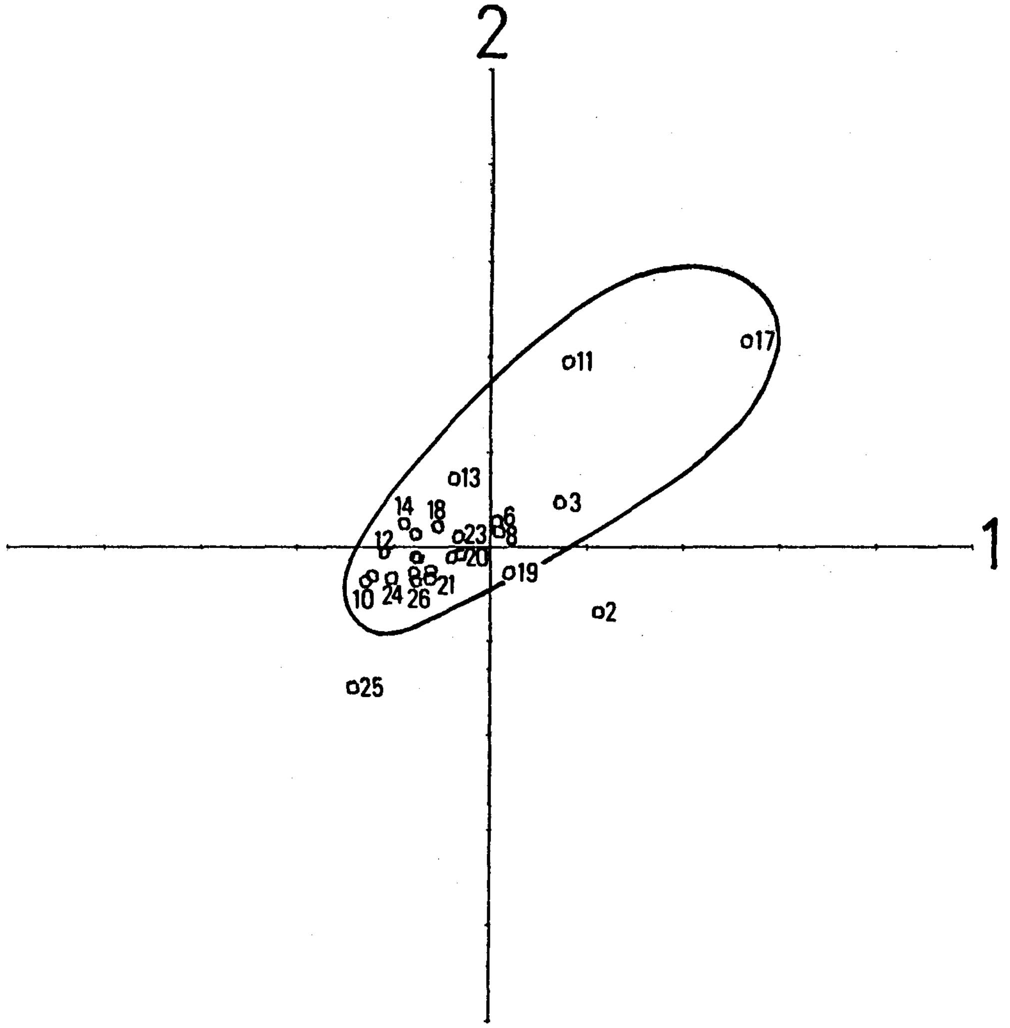
1. SARDINE PC (Dark muscle), July 1982
2. SARDINE PC (Dark muscle), Oct. 1983
3. SARDINE PC (White muscle), July 1982
4. SARDINE PC (White muscle), Oct. 1983
5. MACKEREL PC (Dark muscle), July 1982
6. MACKEREL PC (Dark muscle), Oct. 1983
7. MACKEREL PC (White muscle), July 1982
8. MACKEREL PC (White muscle), Oct. 1983
9. BIG-EYED TUNA PC, Frozen 1981
10. BIG-EYED TUNA PC, Frozen 1983
11. BROWN SOLE PC, Oct. 1982
12. BROWN SOLE PC, May 1983
13. SAND FLOUNDER PC, Dec. 1982
14. SAND FLOUNDER PC, May 1983
15. ROCK FISH PC, Oct. 1982
16. ROCK FISH PC, July 1983
17. ALASKA POLLACK PC, Dec. 1981
18. ALASKA POLLACK PC, Jan. 1984
19. CHUM SALMON PC, MALE, June 1980
20. CHUM SALMON PC, MALE, Aug. 1983
21. CHUM SALMON PC, FEMALE, Aug. 1983
22. BLUE SHARK PC, June 1982
23. MACKEREL SHARK PC, July 1982
24. CARP PC, Sep. 1980
25. RAINBOW TROUT PC, Sep. 1982
26. RAINBOW TROUT PC, May 1983

Fig. IV-38. Plots of principal loading on the first and second principal component plane on PCA.
Refer to the eigenvectors in Fig. IV-36 as background of this plane.

from correlation matrix. "%" shows the contribution for the information of the whole data of each principal component and "ACC.%" is the accumulative value of these "%". "< COMPONENT LOADING >" is the scalar of the eigenvector (see Fig. IV-35).

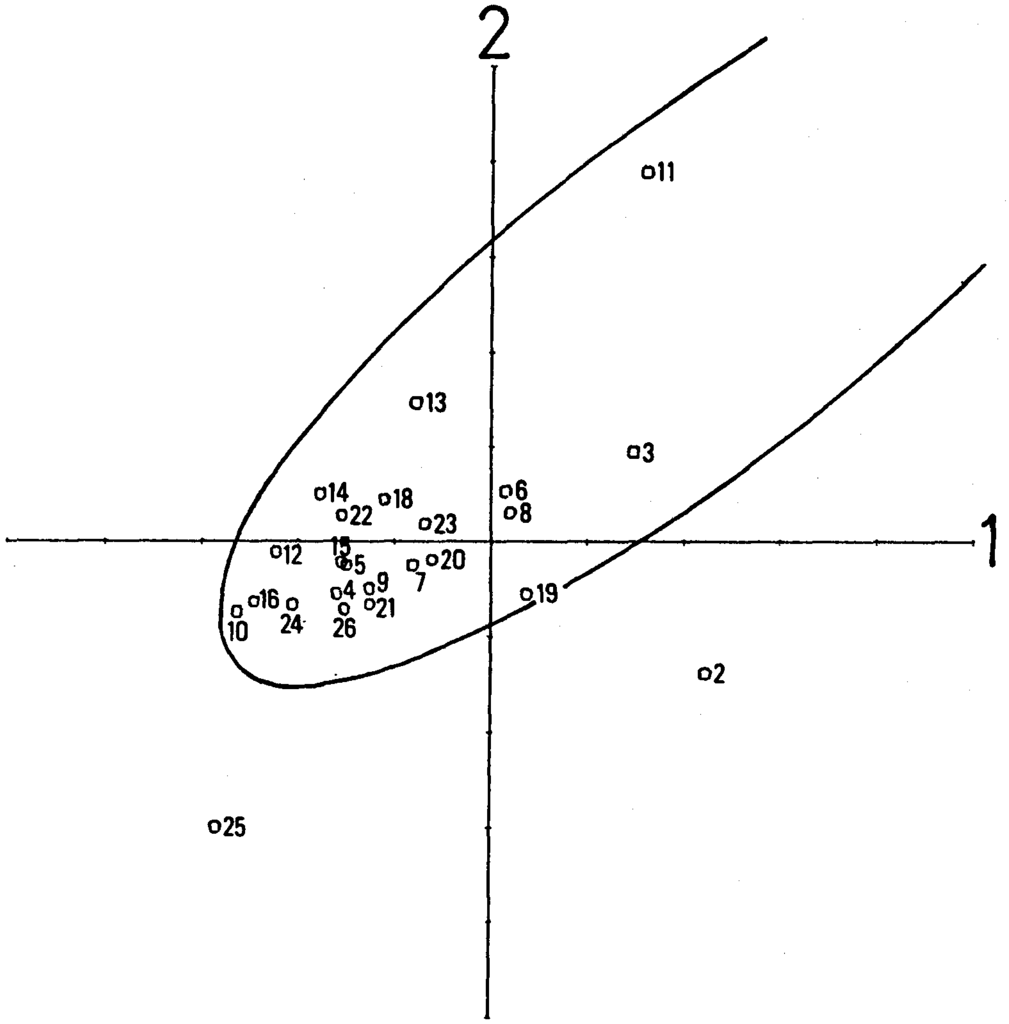
In Fig. IV-36, eigenvectors of major molecular species are shown as small numbers on the first and second principal component plane. These are drawn up from the print out of "< COMPONENT LOADING >" on columns 1 and 2 in Table IV-6. And in Fig. IV-37 eigenvector of the same molecular species are generated on the first and third principal component plane. And these are drawn up from the print out of "< COMPONENT LOADING >" in columns 1 and 3 in Table IV-6.

"< PRINCIPAL LOADING >" in Table IV-7 is the print out of principal loading of each fish which shows the position on the PCA plane. For example, the cell in the 1-th row, the 1-th column shows the distance on the first principal component, and the cell in the 1-th row, the 2-th column shows the distance on the second principal component. These principal loadings were plotted on the first and second principal component plane as shown in Figs. IV-38~40 and on the first and third principal component plane as shown in Figs. IV-44~46 in different scales. Eigenvector of Fig. IV-36 is for Fig. IV-38 and that of Fig. IV-37 is for Fig. IV-44. As shown in Fig. IV-38, sardine dark muscle has a drastic seasonal change (points number 1 and number 2). And among the white flesh fish, Alaska pollack has a large seasonal change (points number 17 and number 18). The movement of point number 1 to point number 2 is the direction of



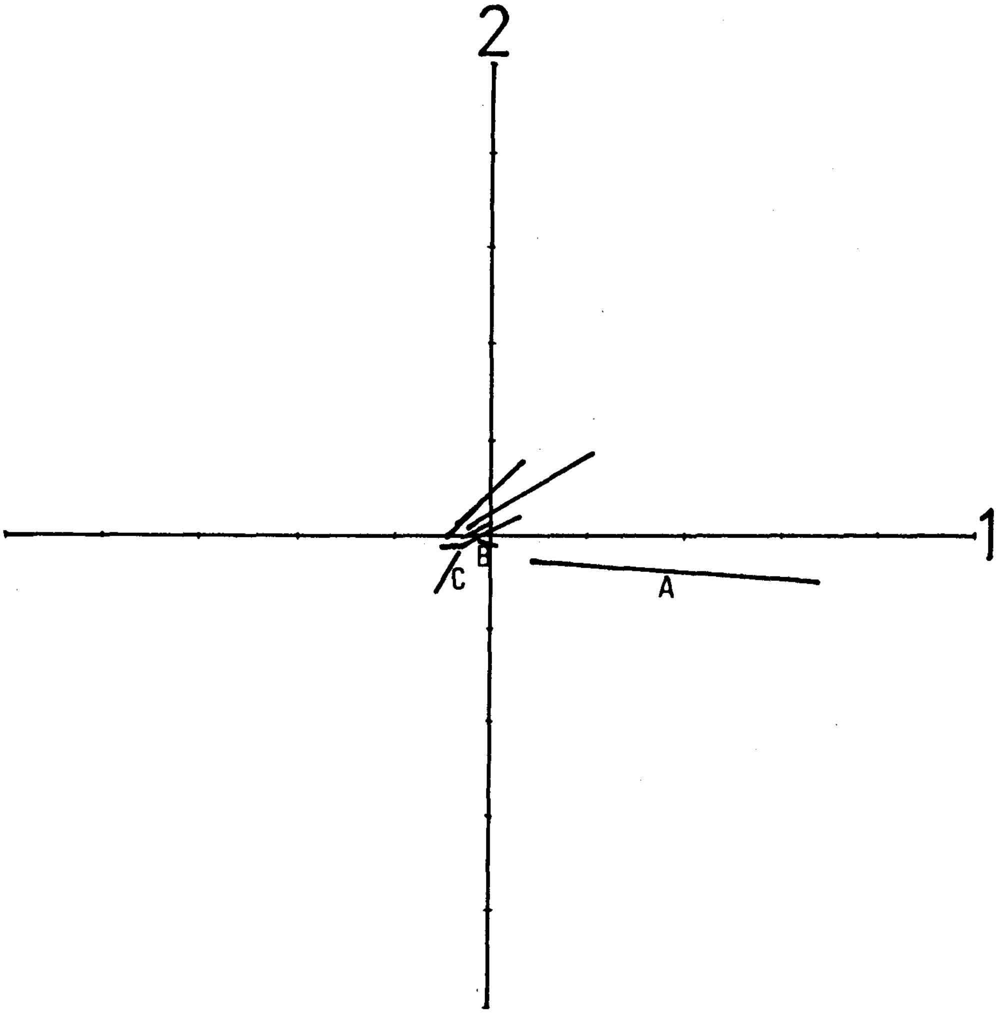
1. SARDINE PC (Dark muscle), July 1982
2. SARDINE PC (Dark muscle), Oct. 1983
3. SARDINE PC (White muscle), July 1982
4. SARDINE PC (White muscle), Oct. 1983
5. MACKEREL PC (Dark muscle), July 1982
6. MACKEREL PC (Dark muscle), Oct. 1983
7. MACKEREL PC (White muscle), July 1982
8. MACKEREL PC (White muscle), Oct. 1983
9. BIG-EYED TUNA PC, Frozen 1981
10. BIG-EYED TUNA PC, Frozen 1983
11. BROWN SOLE PC, Oct. 1982
12. BROWN SOLE PC, May 1983
13. SAND FLOUNDER PC, Dec. 1982
14. SAND FLOUNDER PC, May 1983
15. ROCK FISH PC, Oct. 1982
16. ROCK FISH PC, July 1983
17. ALASKA POLLACK PC, Dec. 1981
18. ALASKA POLLACK PC, Jan. 1984
19. CHUM SALMON PC, MALE, June 1980
20. CHUM SALMON PC, MALE, Aug. 1983
21. CHUM SALMON PC, FEMALE, Aug. 1983
22. BLUE SHARK PC, June 1982
23. MACKEREL SHARK PC, July 1982
24. CARP PC, Sep. 1980
25. RAINBOW TROUT PC, Sep. 1982
26. RAINBOW TROUT PC, May 1983

Fig. IV-39. Plots of principal loading on the magnified first and second principal component plane on PCA.
Refer to the eigenvectors in Fig. IV-36 as background of this plane.



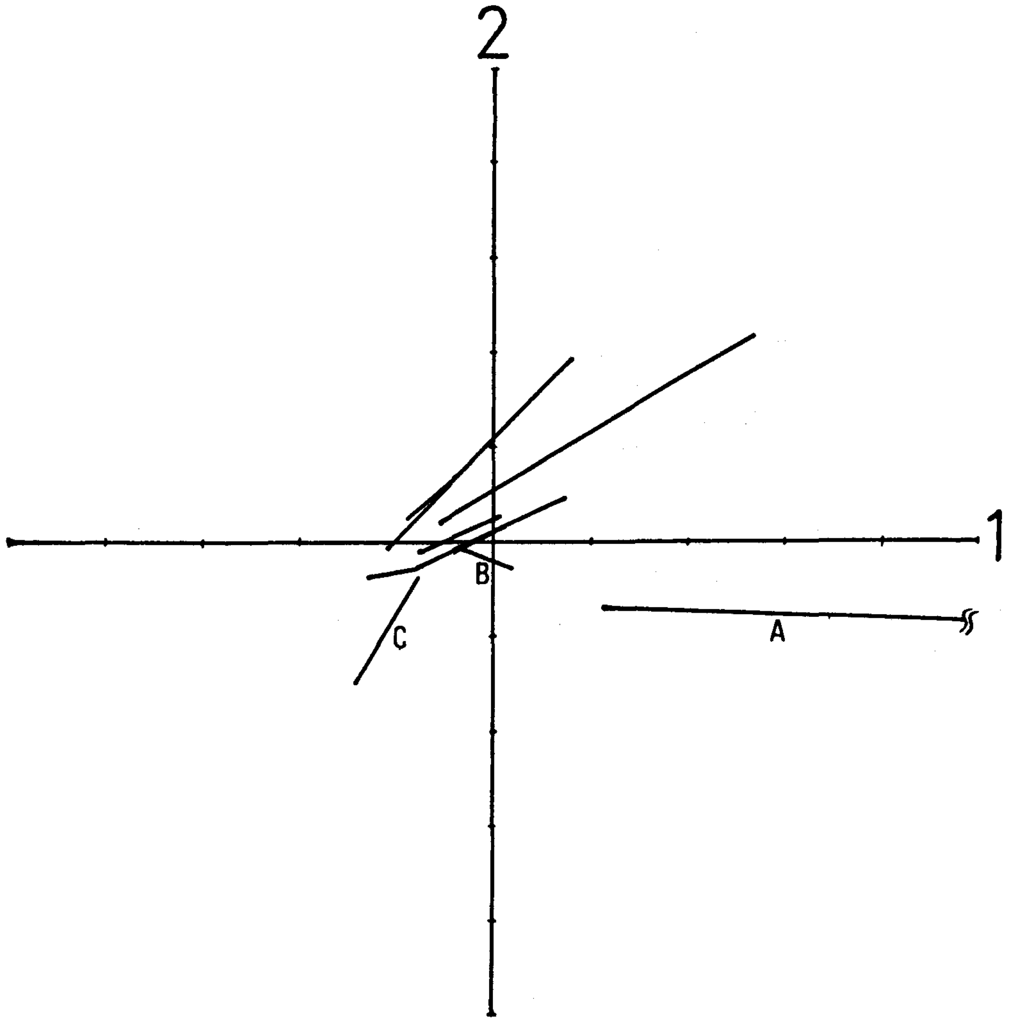
1. SARDINE PC (Dark muscle), July 1982
2. SARDINE PC (Dark muscle), Oct. 1983
3. SARDINE PC (White muscle), July 1982
4. SARDINE PC (White muscle), Oct. 1983
5. MACKEREL PC (Dark muscle), July 1982
6. MACKEREL PC (Dark muscle), Oct. 1983
7. MACKEREL PC (White muscle), July 1982
8. MACKEREL PC (White muscle), Oct. 1983
9. BIG-EYED TUNA PC, Frozen 1981
10. BIG-EYED TUNA PC, Frozen 1983
11. BROWN SOLE PC, Oct. 1982
12. BROWN SOLE PC, May 1983
13. SAND FLOUNDER PC, Dec. 1982
14. SAND FLOUNDER PC, May 1983
15. ROCK FISH PC, Oct. 1982
16. ROCK FISH PC, July 1983
17. ALASKA POLLACK PC, Dec. 1981
18. ALASKA POLLACK PC, Jan. 1984
19. CHUM SALMON PC, MALE, June 1980
20. CHUM SALMON PC, MALE, Aug. 1983
21. CHUM SALMON PC, FEMALE, Aug. 1983
22. BLUE SHARK PC, June 1982
23. MACKEREL SHARK PC, July 1982
24. CARP PC, Sep. 1980
25. RAINBOW TROUT PC, Sep. 1982
26. RAINBOW TROUT PC, May 1983

Fig. IV-40. Plots of principal loading on the highly magnified first and second principal component plane on PCA.
Refer to the eigenvectors in Fig. IV-36 as background of this plane.



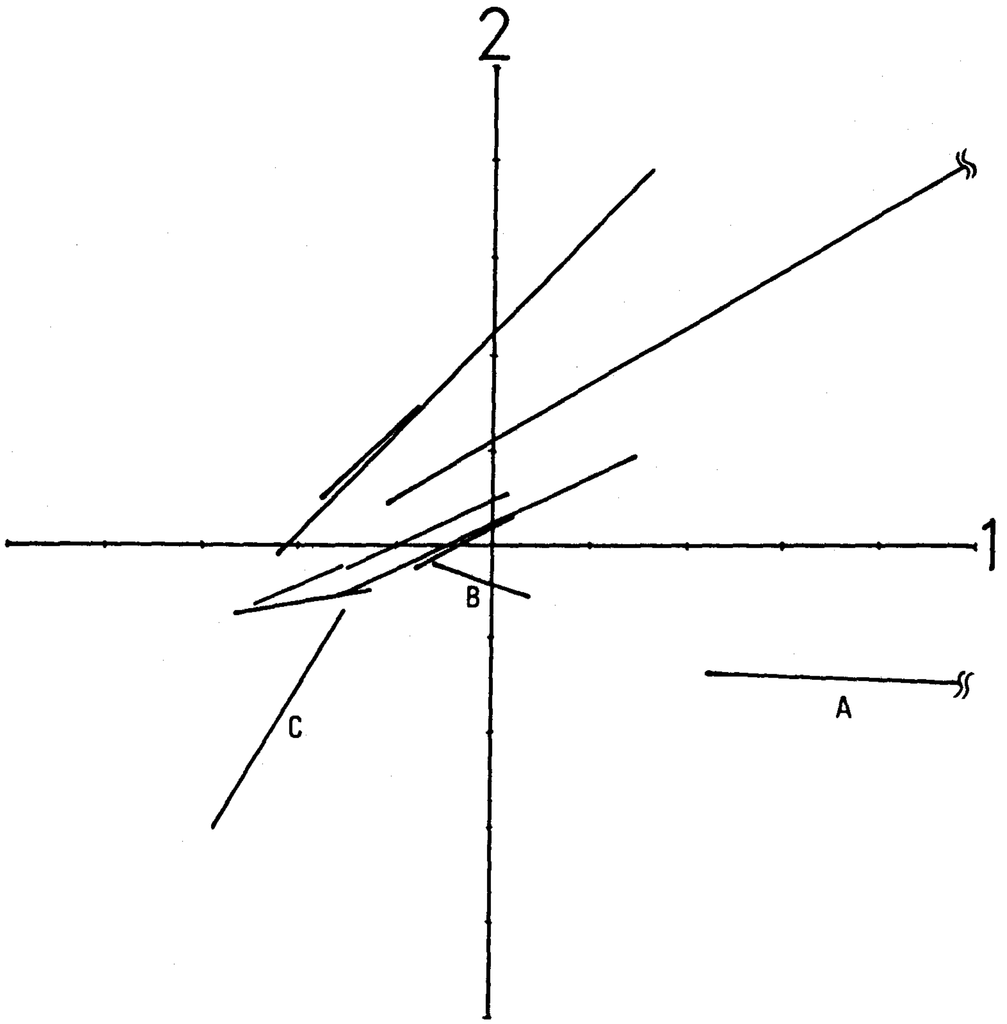
Line A: SARDINE PC (Dark muscle)
Line B: CHUM SALMON PC, MALE
Line C: RAINBOW TROUT PC

Fig. IV-41. Movement of the plots between the two different seasons on the same plane in Fig. IV-38.



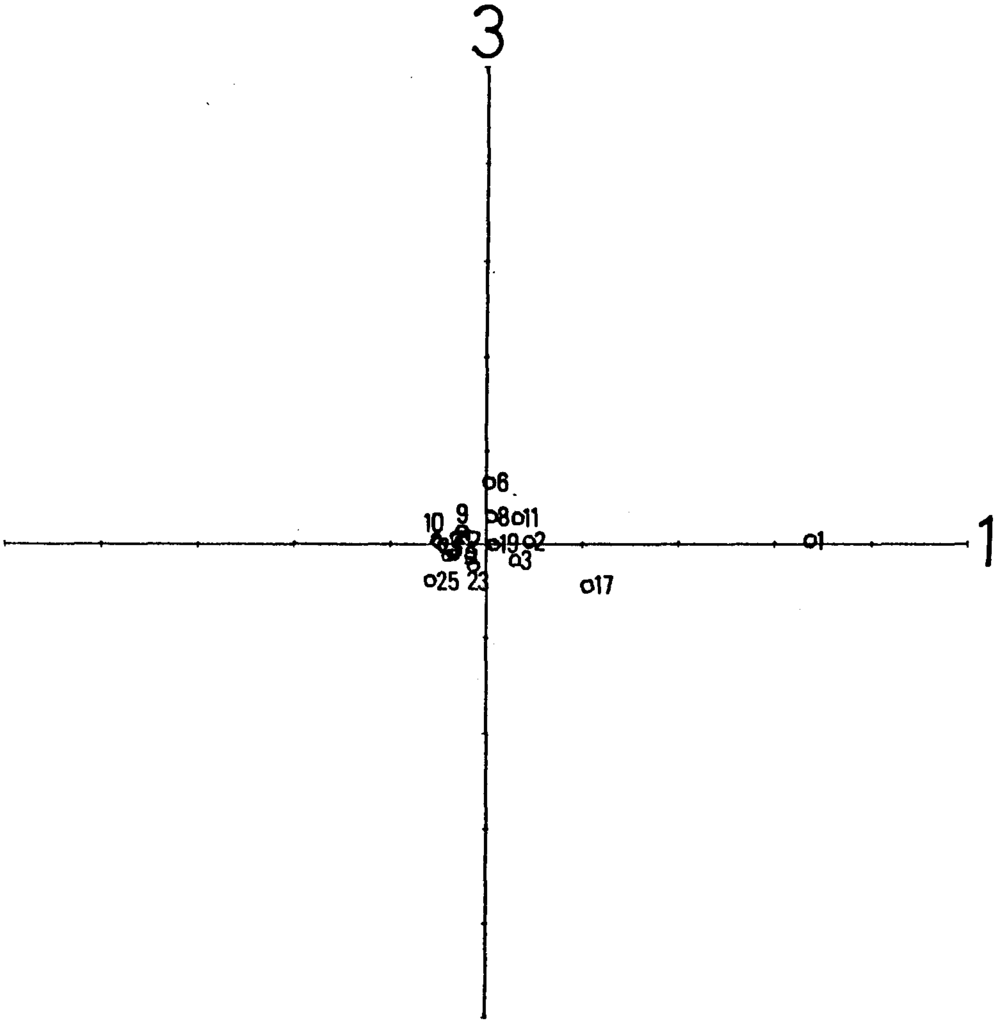
Line A: SARDINE PC (Dark muscle)
Line B: CHUM SALMON PC, MALE
Line C: RAINBOW TROUT PC

Fig. IV-42. Movement of the plots between the two different seasons on the same plane in Fig. IV-39.



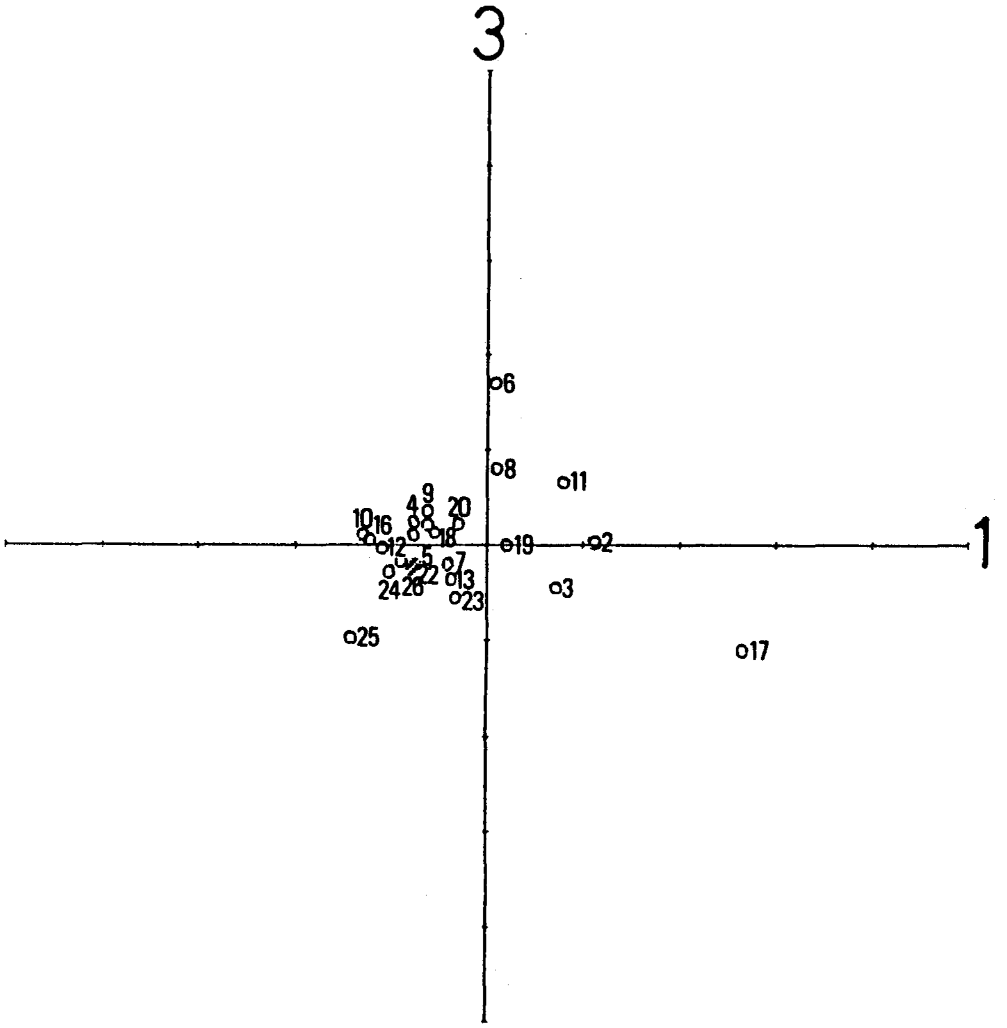
Line A: SARDINE PC (Dark muscle)
Line B: CHUM SALMON PC, MALE
Line C: RAINBOW TROUT PC

Fig. IV-43. Movement of the plots between the two different seasons on the same plane in Fig. IV-40.



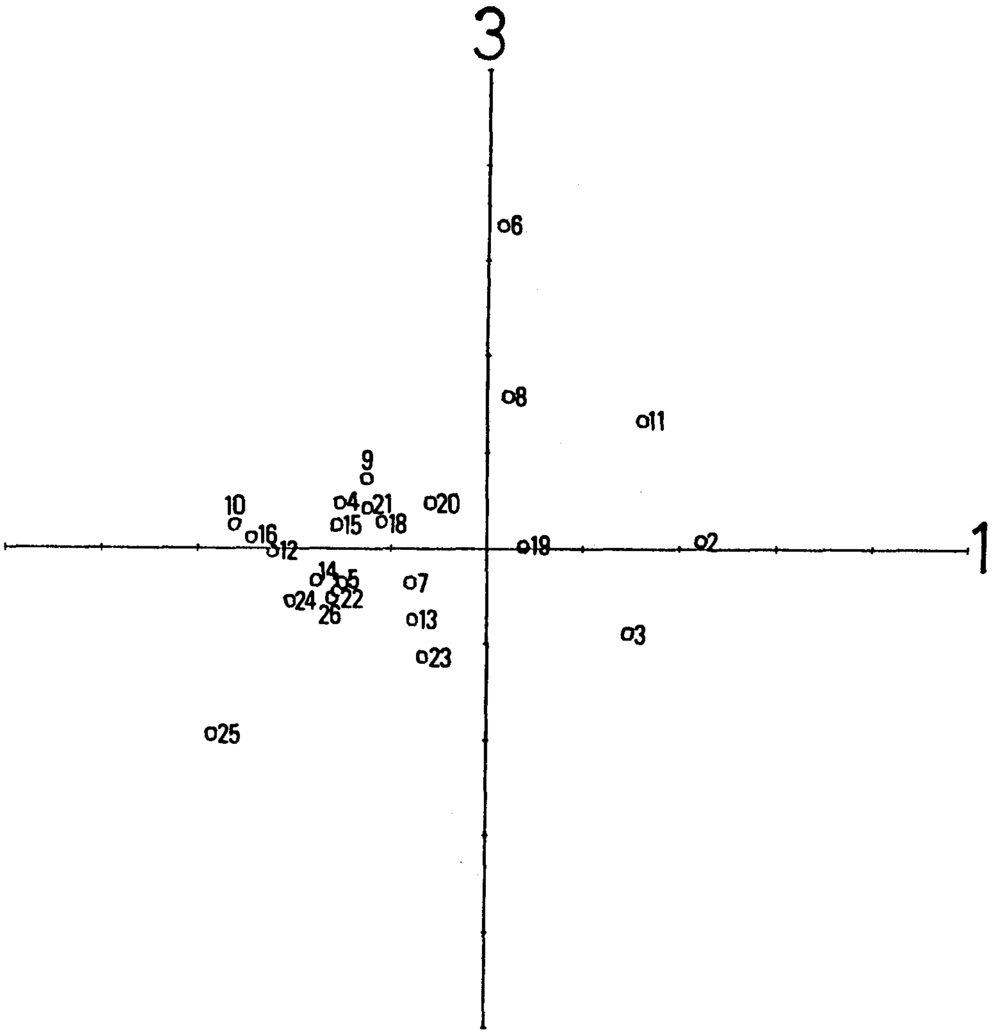
1. SARDINE PC (Dark muscle), July 1982
2. SARDINE PC (Dark muscle), Oct. 1983
3. SARDINE PC (White muscle), July 1982
4. SARDINE PC (White muscle), Oct. 1983
5. MACKEREL PC (Dark muscle), July 1982
6. MACKEREL PC (Dark muscle), Oct. 1983
7. MACKEREL PC (White muscle), July 1982
8. MACKEREL PC (White muscle), Oct. 1983
9. BIG-EYED TUNA PC, Frozen 1981
10. BIG-EYED TUNA PC, Frozen 1983
11. BROWN SOLE PC, Oct. 1982
12. BROWN SOLE PC, May 1983
13. SAND FLOUNDER PC, Dec. 1982
14. SAND FLOUNDER PC, May 1983
15. ROCK FISH PC, Oct. 1982
16. ROCK FISH PC, July 1983
17. ALASKA POLLACK PC, Dec. 1981
18. ALASKA POLLACK PC, Jan. 1984
19. CHUM SALMON PC, MALE, June 1980
20. CHUM SALMON PC, MALE, Aug. 1983
21. CHUM SALMON PC, FEMALE, Aug. 1983
22. BLUE SHARK PC, June 1982
23. MACKEREL SHARK PC, July 1982
24. CARP PC, Sep. 1980
25. RAINBOW TROUT PC, Sep. 1982
26. RAINBOW TROUT PC, May 1983

Fig. IV-44. Plots of principal loading on the first and third principal component plane on PCA.
Refer to the eigenvectors in Fig. IV-37 as background of this plane.



1. SARDINE PC (Dark muscle), July 1982
2. SARDINE PC (Dark muscle), Oct. 1983
3. SARDINE PC (White muscle), July 1982
4. SARDINE PC (White muscle), Oct. 1983
5. MACKEREL PC (Dark muscle), July 1982
6. MACKEREL PC (Dark muscle), Oct. 1983
7. MACKEREL PC (White muscle), July 1982
8. MACKEREL PC (White muscle), Oct. 1983
9. BIG-EYED TUNA PC, Frozen 1981
10. BIG-EYED TUNA PC, Frozen 1983
11. BROWN SOLE PC, Oct. 1982
12. BROWN SOLE PC, May 1983
13. SAND FLOUNDER PC, Dec. 1982
14. SAND FLOUNDER PC, May 1983
15. ROCK FISH PC, Oct. 1982
16. ROCK FISH PC, July 1983
17. ALASKA POLLACK PC, Dec. 1981
18. ALASKA POLLACK PC, Jan. 1984
19. CHUM SALMON PC, MALE, June 1980
20. CHUM SALMON PC, MALE, Aug. 1983
21. CHUM SALMON PC, FEMALE, Aug. 1983
22. BLUE SHARK PC, June 1982
23. MACKEREL SHARK PC, July 1982
24. CARP PC, Sep. 1980
25. RAINBOW TROUT PC, Sep. 1982
26. RAINBOW TROUT PC, May 1983

Fig. IV-45. Plots of principal loading on the magnified first and third principal component plane on PCA.
Refer to the eigenvectors in Fig. IV-37 as background of this plane.



1. SARDINE PC (Dark muscle), July 1982
2. SARDINE PC (Dark muscle), Oct. 1983
3. SARDINE PC (White muscle), July 1982
4. SARDINE PC (White muscle), Oct. 1983
5. MACKEREL PC (Dark muscle), July 1982
6. MACKEREL PC (Dark muscle), Oct. 1983
7. MACKEREL PC (White muscle), July 1982
8. MACKEREL PC (White muscle), Oct. 1983
9. BIG-EYED TUNA PC, Frozen 1981
10. BIG-EYED TUNA PC, Frozen 1983
11. BROWN SOLE PC, Oct. 1982
12. BROWN SOLE PC, May 1983
13. SAND FLOUNDER PC, Dec. 1982
14. SAND FLOUNDER PC, May 1983
15. ROCK FISH PC, Oct. 1982
16. ROCK FISH PC, July 1983
17. ALASKA POLLACK PC, Dec. 1981
18. ALASKA POLLACK PC, Jan. 1984
19. CHUM SALMON PC, MALE, June 1980
20. CHUM SALMON PC, MALE, Aug. 1983
21. CHUM SALMON PC, FEMALE, Aug. 1983
22. BLUE SHARK PC, June 1982
23. MACKEREL SHARK PC, July 1982
24. CARP PC, Sep. 1980
25. RAINBOW TROUT PC, Sep. 1982
26. RAINBOW TROUT PC, May 1983

Fig. IV-46. Plots of principal loading on the highly magnified first and third principal component plane on PCA. Refer to the eigenvectors in Fig. IV-37 as background of this plane.

eigenvector number 4 that shows the direction of (20:5)(18:1)+(18:1)(20:5) combinations. So we might say that sardine dark muscle has a large seasonal variation in the molecular species composed of 20:5 and 18:1.

Fig. IV-39 is the magnified figure of Fig. IV-38. And Fig. IV-40 is the further magnified figure of Fig. IV-39. From these two Figures i.e. Fig. IV-39 and Fig. IV-40, generally speaking, there seems to be a direction on the axis of oval shape shown in the movement between the same fish except in the cases of sardine dark muscle, chum salmon and rainbow trout. This direction coincide with eigenvector of (20:5)(20:5). Though, further supplementary studies should be done to get a conclusion, it is assumed that (20:5)(20:5) is the most reflectable molecular species against seasonal variations among the majority of fish. This is more evidently shown in Figs. IV-41~43 in three different scales, and only sardine dark muscle (shown as A), chum salmon (shown as B) and rainbow trout (shown as C) have different directions. As it is well known, the general characteristics of these three fish are as follows:

Sardine dark muscle : Drastic change in lipid content.
Chum salmon : Migration from sea to river.
Rainbow trout : Fresh water fish.

So, these characteristics might affect the molecular species of muscle lecithin.

Through Fig. IV-44 to Fig. IV-46 show the distribution of each fish on the first and third principal component plane. The outstanding deviated points are those of sardine

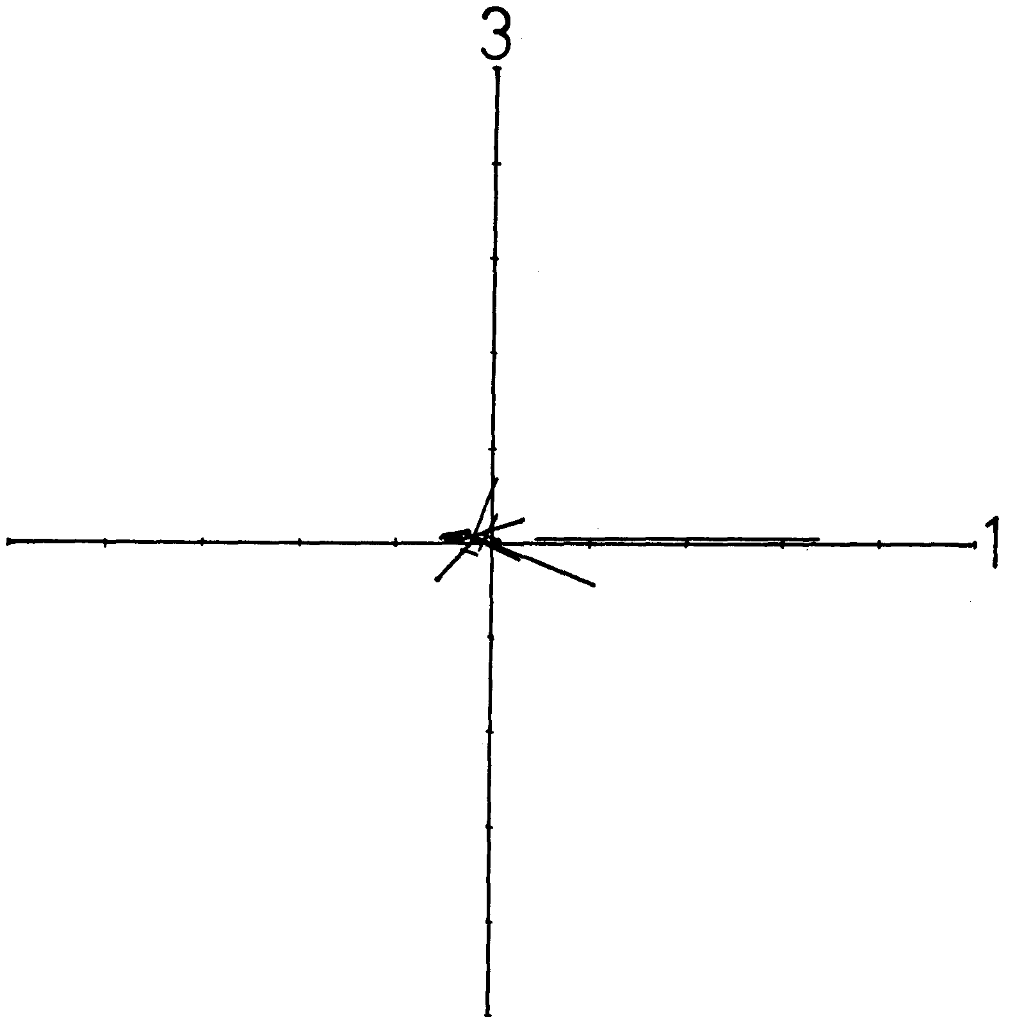


Fig. IV-47. Movement of the plots between the two different seasons on the same plane in Fig. IV-44.

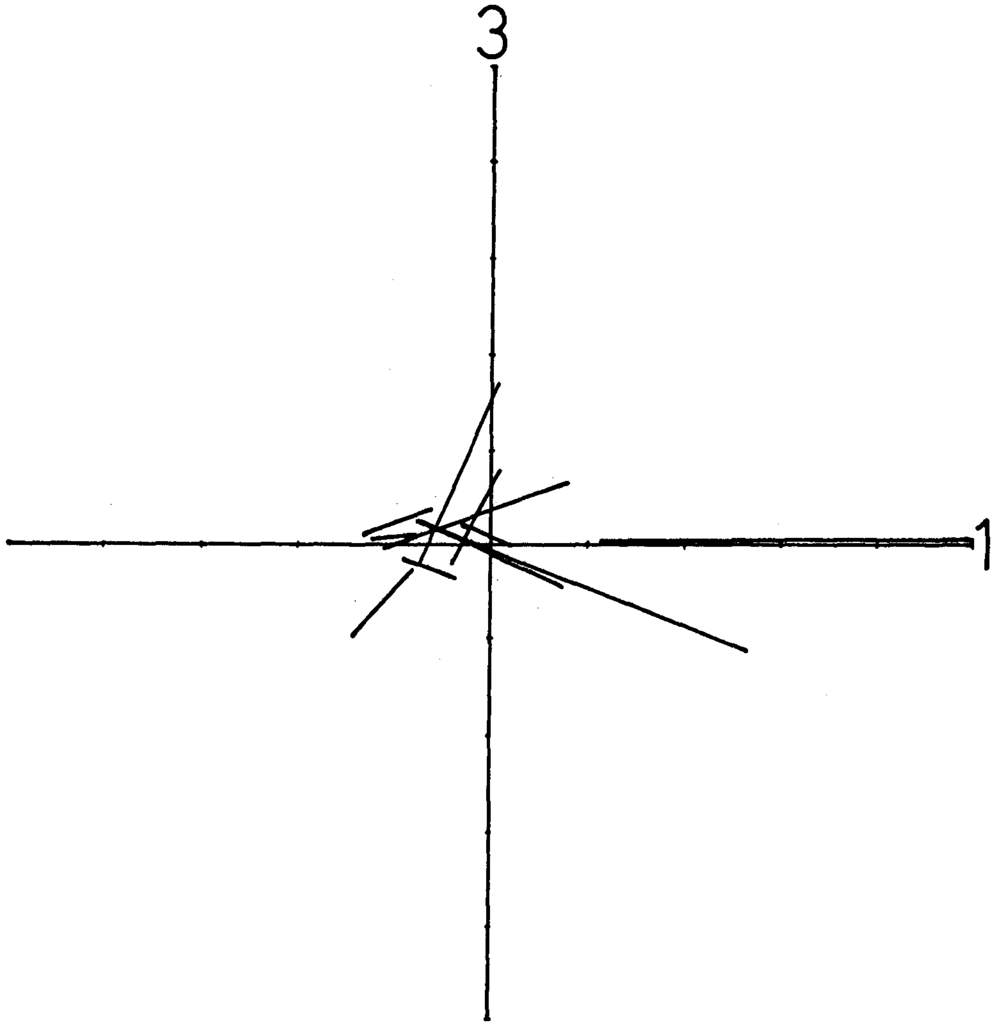


Fig. IV-48. Movement of the plots between the two different seasons on the same plane in Fig. IV-45.

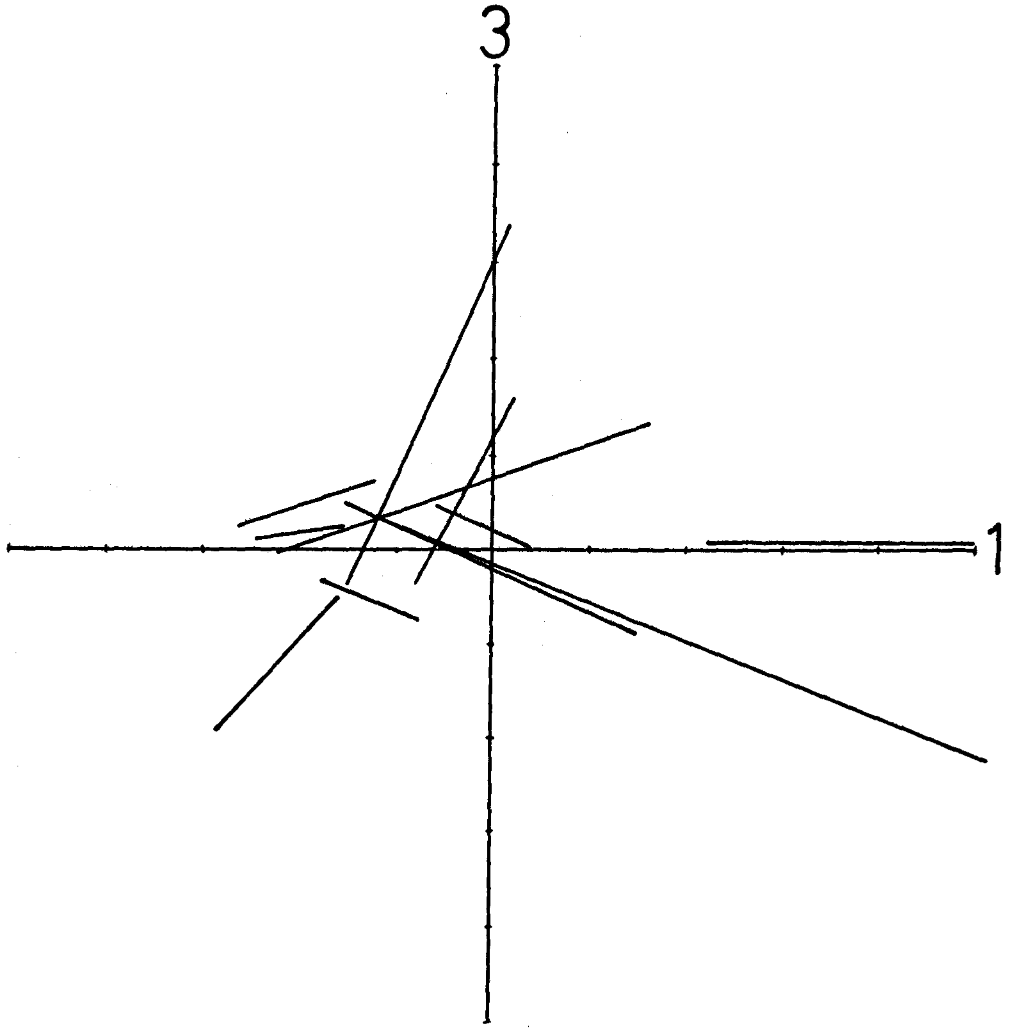


Fig. IV-49. Movement of the plots between the two different seasons on the same plane in Fig. IV-46.

dark muscle (point number 1) and Alaska pollack (point number 17) as it is seen in the first and second principal component plane. Those of rainbow trout and mackerel dark muscle also have a deviation in this plane, but not so large as it is in the case of sardine dark muscle and Alaska pollack. Figure IV-47 through Fig. IV-49 show the seasonal variations by lines on the first and third principal component plane. It is hard to find a general movement of molecular species in this plane according to the seasonal variation unlike it is observed in the first and second principal component plane.

Section 4. Changes in Fish Muscle Lecithin of Chum Salmon during Migration

Total lipids were obtained in the same way as described in section 1 from the fish muscle of chum salmon tabulated in Table IV-8. Lipid composition analysis as well as molecular species analysis were also done in the same manner as described in section 1. PCA analysis was done in the same way as described in section 1.

Table IV-9 shows the yield of total lipid and the percentage of each lipid class against total lipid. And on the right end of this table, the lecithin amounts/100g muscle are shown. Though there is a drastic drop in the amount of total lipid and triglycerides between the stage of feeding migration and spawning migration, the amount of lecithin ranges between 161~230mg/100g muscle.

In Table IV-10, changes in fatty acid composition of diglyceride acetates (this represents the fatty acid composition of lecithin) during migration are shown. At the level of this analysis, not any significant differences are observed amoung the four stages.

HPLC chromatograms of each stage of migration are shown in Fig. IV-50 and the detected molecular species of lecithin in sequence of elution on HPLC with the percentage data are shown in Figs. IV-51~54. As it is obviously seen in these figures chum salmon at the stage of feeding migration has (16:0)(22:6) as the most outstandingly predominant peak (shown by arrows in Fig. IV-50) compared with other three stages. At the stage of spawning migration, this peak i.e.

Table IV-8. Chum salmon (*Oncorhyncus keta*) examined

Stage of migration and sex	Mean body length and weight Locality of catch	Date of catch
Feeding migration, male	42.7cm, 1367g, 45°59'-49°29'N, 167°07'-175°30'E,	Aug. 1983
Feeding migration, female	46.8cm, 1593g, 45°59'-49°29'N, 167°07'-175°30'E,	Aug. 1983
Spawning migration,male Sea run	63.0cm, 3267g, Yakumo-cho, Hokkaido, Japan	Oct. 1983
Spawning migration,female Sea run	64.3cm, 3573g, Yakumo-cho, Hokkaido, Japan	Oct. 1983
Spawning migration,male River	73.0cm, 5000g, Yakumo-cho, Hokkaido, Japan	Nov. 1983
Spawning migration,female River	70.0cm, 4740g, Yakumo-cho, Hokkaido, Japan	Nov. 1983
Spent, male	74.2cm, 5140g, Yakumo-cho, Hokkaido, Japan	Nov. 1983
Spent, female	72.7cm, 4273g, Yakumo-cho, Hokkaido, Japan	Nov. 1983

Table IV-9. Lipid composition of Chum Salmon examined

Lipid / Sample	Yield*	Non-phospholipid**				Phospholipid**			PC mg/ 100g muscle
		TG	FFA	ST	NP others	PC	PS+PE	PL others	
Feeding migration, Male	10.4	92.1	0.4	0.7	0.2	2.3	3.6	0.7	230
Feeding migration, Female	12.4	94.1	trace	0.7	0.7	1.3	2.9	0.3	161
Spawning migration, Male (Sea)	1.1	40.7	0.8	4.7	1.1	19.3	29.8	3.6	212
Spawning migration, Female (Sea)	1.4	49.9	trace	2.6	0.3	17.3	26.7	3.2	242
Spawning migration, Male (River)	1.3	40.7	5.7	6.2	trace	12.7	32.9	1.8	165
Spawning migration, Female (River)	1.6	41.5	2.6	15.5	0.1	9.2	28.1	3.0	147
Spent, Male	1.1	32.7	22.5	4.3	5.2	12.2	11.2	11.9	132
Spent, Female	0.9	11.6	19.9	7.8	5.5	19.1	15.9	20.2	172

See the abbreviations in the opening.
*g/100g muscle. **g/100g total lipid.

Table IV-10. Fatty acid composition of diglyceride acetate derived from lecithin of chum salmon muscle

Sample / Fatty acid	Feeding migration		Spawning migration				Spent	
	Male	Female	Male*	Female*	Male**	Female**	Male	Female
12:0	0.09			0.03			trace	0.06
14:0	4.58	3.10	1.98	2.78	3.34	3.74	3.49	3.53
15:0	0.82	0.68	0.37	0.42	0.42	0.44	0.37	0.32
16:0	29.02	32.26	30.02	26.84	23.95	28.61	29.51	26.15
16:1	1.79	1.38	1.66	2.19	2.60	2.56	2.41	1.83
17:0	1.14	0.92	0.43	0.38	0.61	0.50	0.35	0.48
17:1	0.44	0.37	0.25	0.16	0.36	0.25	0.42	0.27
18:0	1.03	1.11	0.72	0.59	1.23	0.52	1.45	0.83
18:1	8.43	8.27	6.40	4.68	5.04	7.25	9.65	6.64
18:2	0.51	0.52	0.37	0.51	0.94	0.64	0.60	0.46
19:1 or 18:4			0.46	0.63	0.97	0.97	0.34	0.39
19:2	0.69	0.56			1.36	1.19	0.33	0.49
20:0	1.01	0.77	0.67	0.79	3.27	trace	trace	0.79
20:1	0.10	0.10	0.39	0.29	2.29	0.52	1.11	0.05
20:2	0.11	0.09	0.06	0.04			0.33	0.14
20:3	0.65	0.58	0.13	0.13	0.06		0.93	0.88
20:4	0.79	0.67	1.54	1.83	3.00	1.80	0.41	0.51
20:5	11.49	10.18	15.00	16.22	19.00	23.66	15.87	16.96
22:3	0.07							
22:4	0.42	0.34	0.18	0.19				0.12
22:5	1.05	0.98	1.16	1.87	2.48	1.93	1.75	2.37
22:6	35.00	36.40	38.20	39.30	28.77	25.43	30.67	36.70
others	0.77	0.73	0.01	0.13	0.31		0.01	0.03

% in muscle lecithin.

* Sea run. ** River.

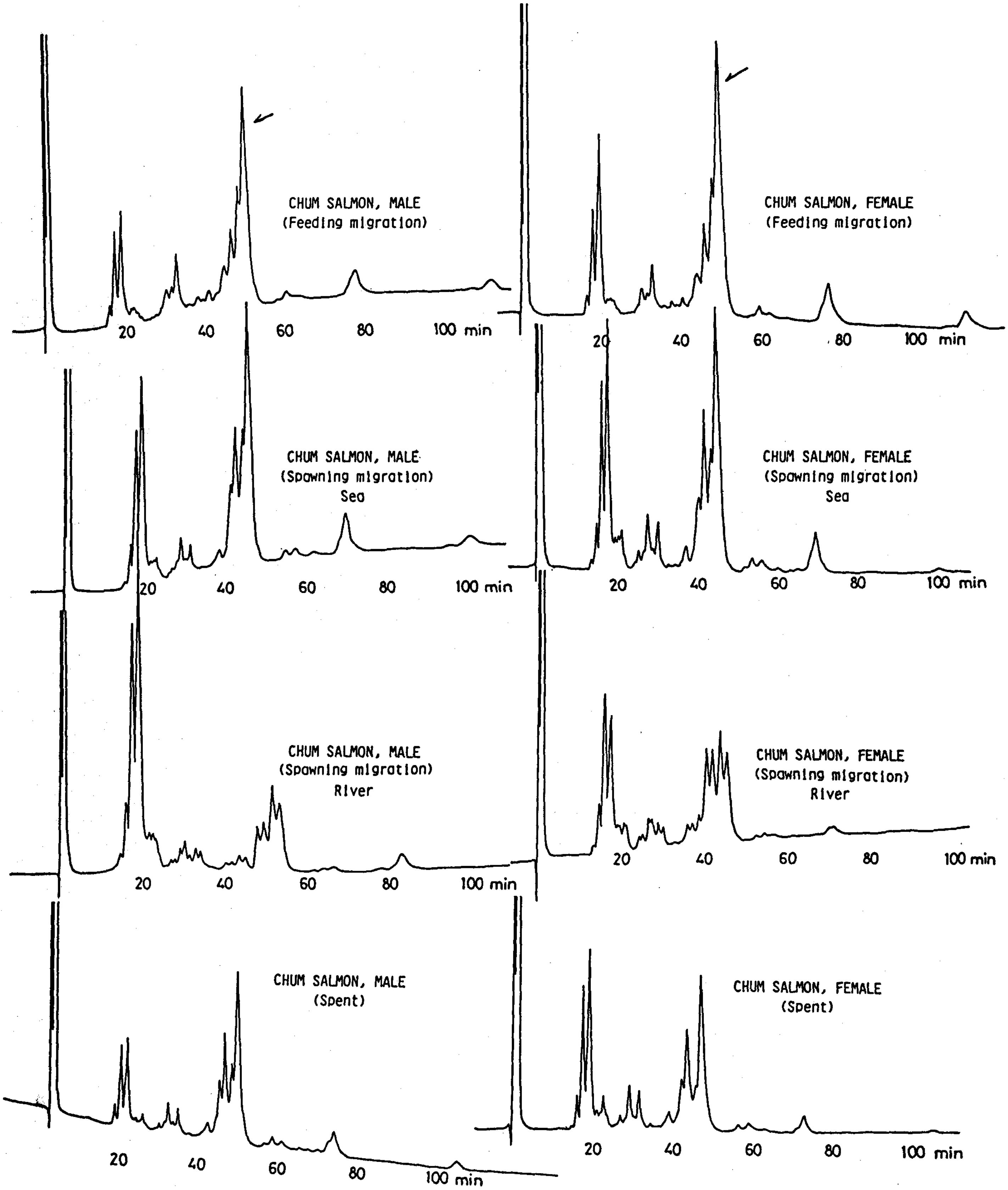


Fig. IV-50. HPLC chromatograms of chum salmon muscle lecithin on each stage of migration.

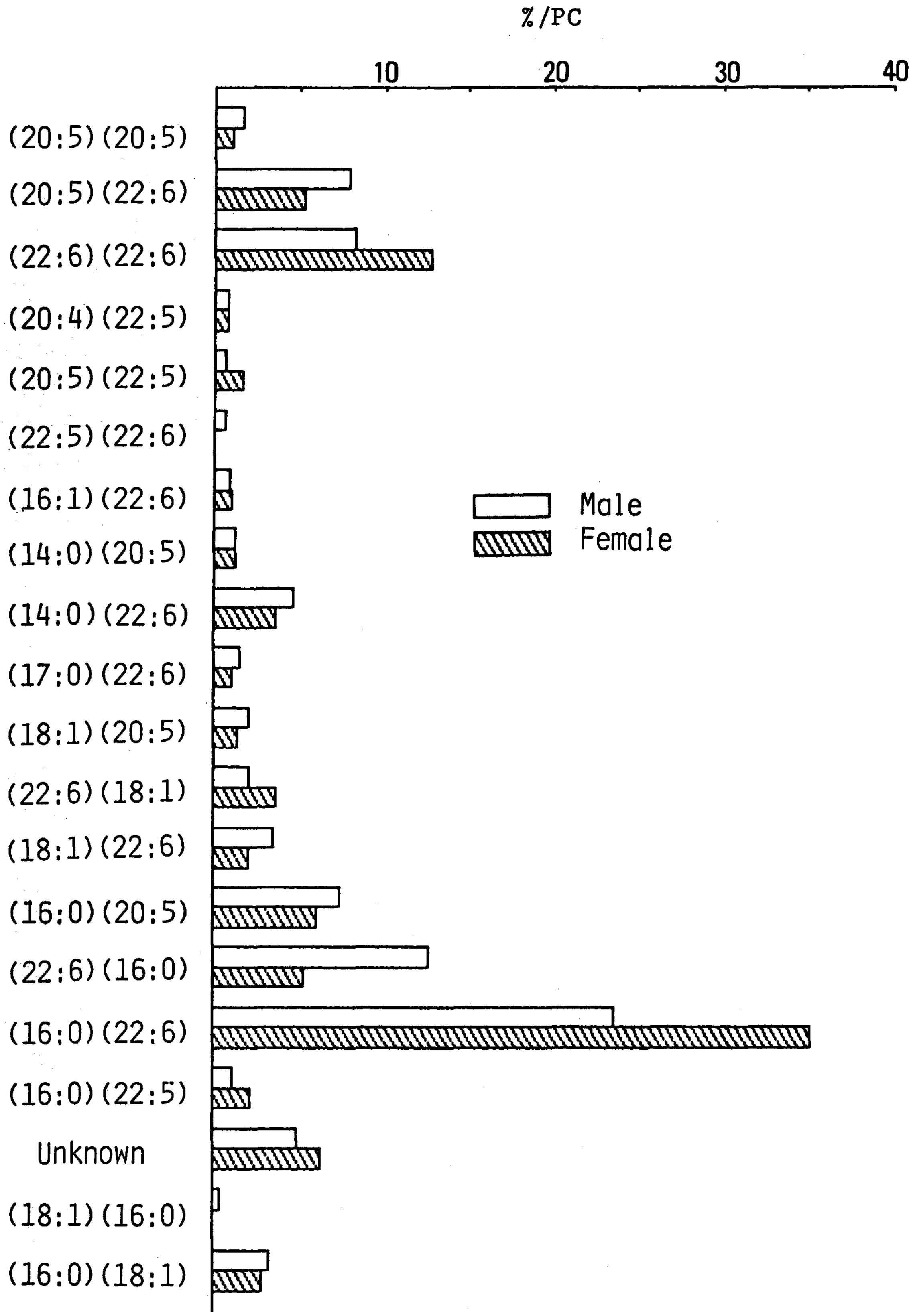
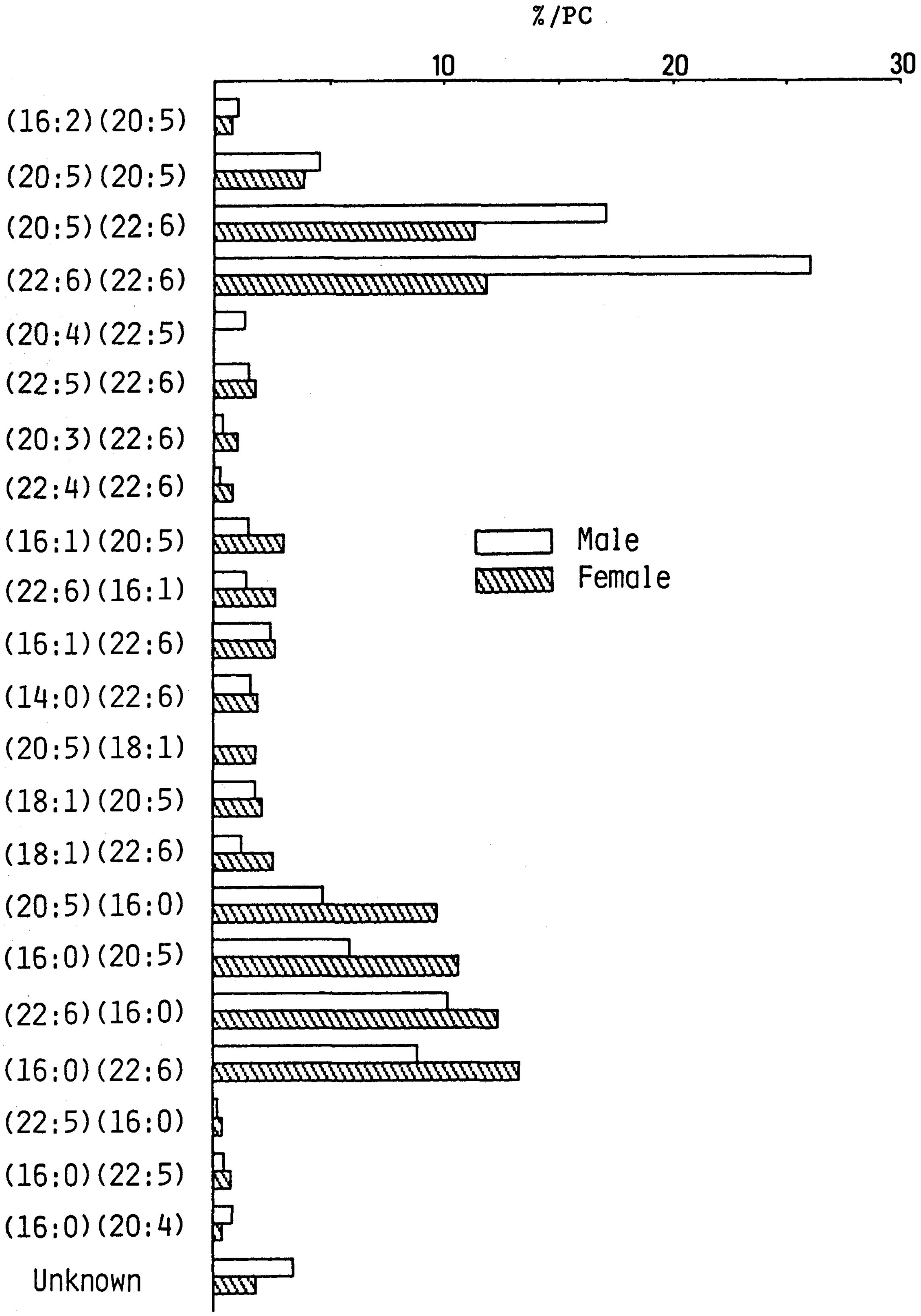


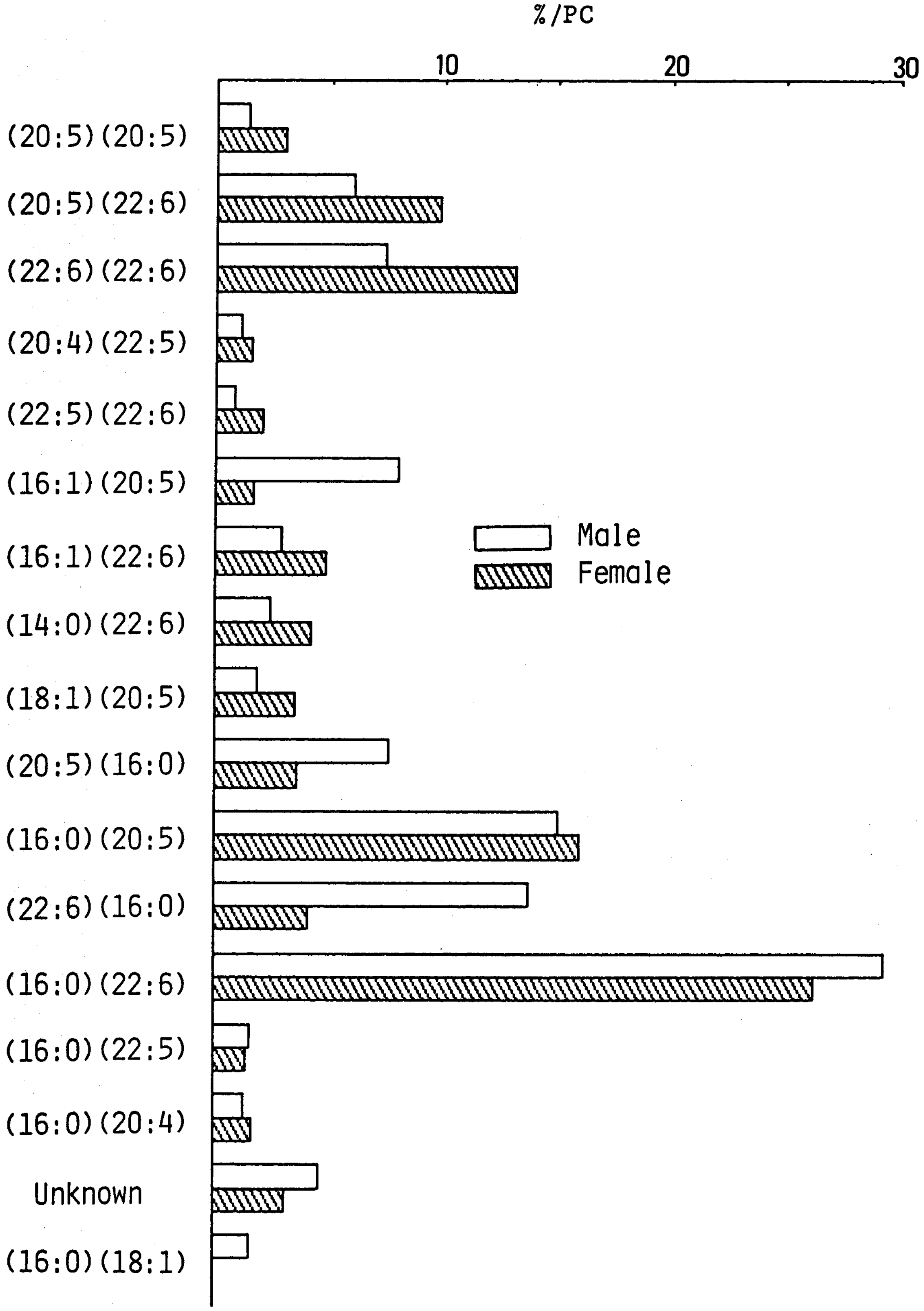
Fig. IV -51. Molecular species of chum salmon muscle lecithin at the stage of feeding migration.

(14:0)(20:5), (17:0)(22:6) and (22:6)(18:1) are trace amount.
Fig. IV-52. Molecular species of chum salmon muscle lecithin at the stage of spawning migration (sea).



(14:0)(20:5), (17:0)(22:6) and (22:6)(18:1) are trace amount.

Fig. IV-53. Molecular species of chum salmon muscle lecithin at the stage of spawning migration (river).



(14:0)(20:5), (17:0)(22:6) and (22:6)(18:1) are trace amount.

Fig. IV-54. Molecular species of chum salmon muscle lecithin. at the stage of spent.

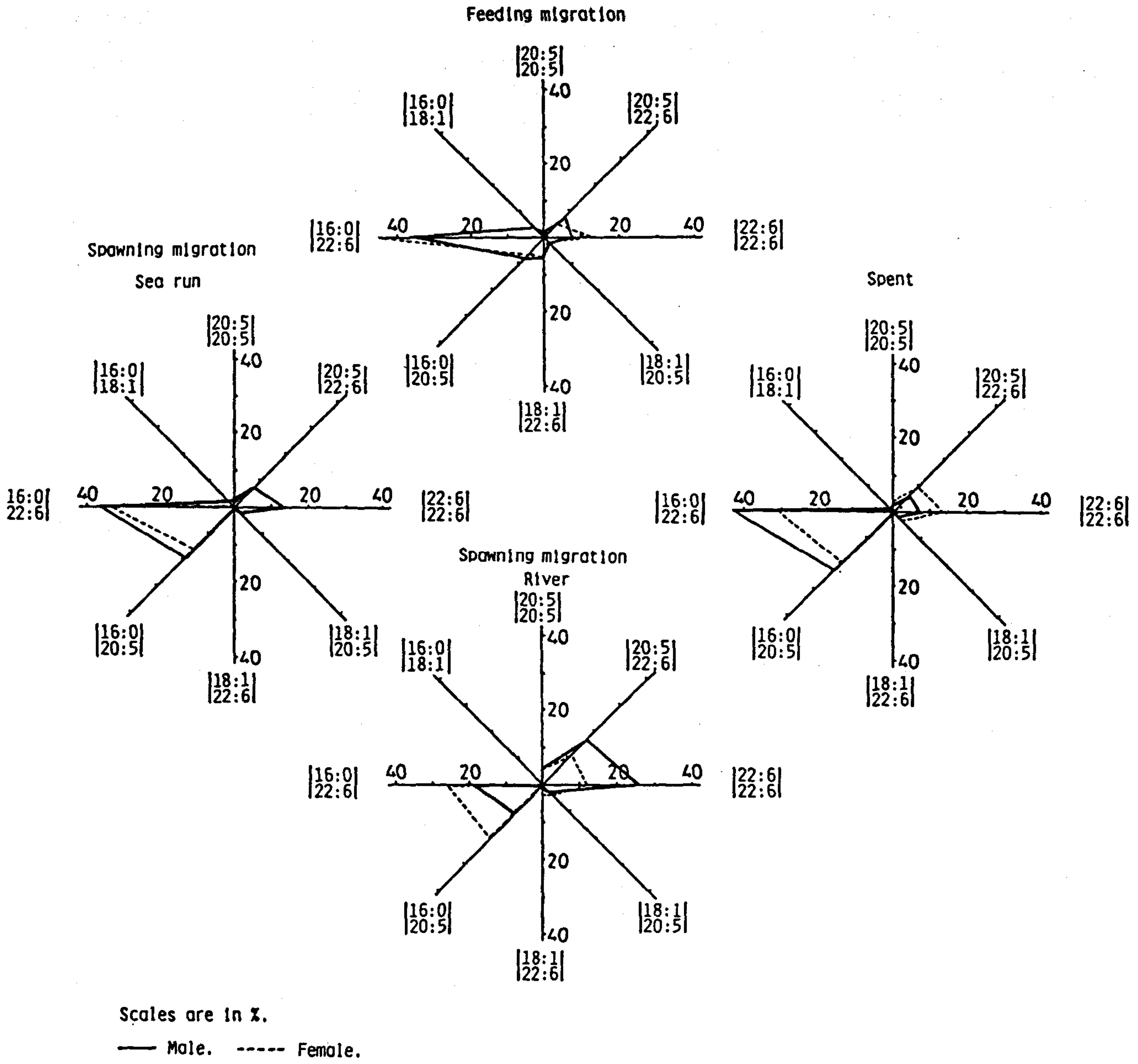


Fig. IV-55. Radar charts of main molecular species of chum salmon muscle lecithin on each stage of migration.

Table IV-11. Print out of the mean and the standard deviation of the amount of chum salmon muscle lecithin throughout all migration stages

```
VA***** PRINCIPAL COMPONENT ANALYSIS *****
Number of Variables * 31

Number of Sample* 8

VARIABLE                 MEAN          S.D.
  1                      0.474         0.681
  2                      4.163         1.861
  3                     16.050         6.058
  4                     23.849        10.126
  5                      1.563         1.148
  6                      0.640         1.186
  7                      0.531         0.831
  8                      0.515         1.020
  9                      0.628         1.182
 10                      1.549         1.568
 11                      0.227         0.475
 12                      3.076         4.453
 13                      0.785         1.519
 14                      4.548         2.561
 15                      0.635         1.201
 16                      5.148         2.699
 17                      0.633         1.270
 18                      0.331         0.936
 19                      3.678         0.907
 20                      1.279         2.385
 21                      2.105         2.793
 22                      7.587         5.567
 23                     19.627         7.958
 24                     16.170         7.630
 25                     45.032        19.780
 26                      0.115         0.223
 27                      2.051         0.879
 28                      1.387         1.039
 29                      8.357         3.925
 30                      0.195         0.368
 31                      2.369         2.691
```

* Variable numbers are shown in the footnote of Fig. IV-56 and samples are shown in the footnote of Table IV-15.

(16:0)(22:6) becomes relatively smaller. Up to these two stages, the chromatographic patterns are analogous between male and female. When the fish starts ascending the river, drastic decrease in the amount of group III (see the footnote of Fig. IV-1 in section 2) especially the molecular species of (16:0)(22:6) and (16:0)(20:5) are observed. Unlike the chromatographic patterns at the stage of sea, outstanding differences are observed between the male and female. The content of (22:6)(22:6) is extremely high followed by (20:5)(22:6) in male. Finally at the stage of spent salmon, (16:0)(22:6) as well as (16:0)(20:5) relatively increase compared with the former stage in the river. These are more concretely seen in radar charts of the main molecular species as illustrated in Fig. IV-55. The axes of these charts are the same with Fig. IV-33 in section 2. These charts are arranged in sequence of migration movement (anticlockwise). It is clearly seen from this figure that at the stage of sea run, slight differences in patterns of radar charts can be observed between the male and female while that of river, outstanding differences are observed especially at the spawning stage. It is obviously seen from this Fig. IV-55 that the amount of (16:0)(20:5) is relatively very small in feeding migration stage compared with those of other three stages. The amount of (20:5)(22:6) and (22:6)(22:6) is surprisingly high at the river spawning stage.

The author has employed PCA again as discussed in the previous section. As it is shown in Table IV-11, number of variables which correspond to the number of molecular species

Table IV-12. Print out of the correlation matrix among all molecular species detected.*

```
===============================================================================

 < Correlation Matrix >

          1        2        3        4        5        6        7        8
  1     1.000
  2     0.214    1.000
  3     0.772    0.904    1.000
  4     0.625    0.651    0.603    1.000
  5     0.162   -0.077    0.193    0.309    1.000
  6     0.843    0.218    0.628    0.356   -0.283    1.000
  7     0.203    0.479    0.255    0.200   -0.763    0.406    1.000
  8    -0.402   -0.576   -0.418   -0.230   -0.099   -0.311   -0.369    1.000
  9     0.076   -0.015    0.096    0.426    0.209   -0.328    0.266   -0.307
 10    -0.222   -0.222   -0.111    0.081    0.533   -0.609   -0.179   -0.399
 11     0.657    0.609    0.335    0.025   -0.466    0.902    0.472   -0.276
 12    -0.100   -0.213   -0.266   -0.349    0.163   -0.071   -0.205   -0.398
 13     0.760    0.715    0.471    0.169   -0.398    0.967    0.455   -0.298
 14     0.294    0.103    0.110    0.259    0.642   -0.121   -0.210   -0.627
 15    -0.421   -0.434   -0.191   -0.263   -0.009   -0.326   -0.387    0.845
 16    -0.415   -0.464   -0.168   -0.258    0.465   -0.555   -0.649    0.481
 17    -0.397   -0.349   -0.094   -0.255    0.025   -0.307   -0.364    0.724
 18     0.417    0.341    0.037   -0.259   -0.550    0.683    0.456   -0.204
 19    -0.225    0.017    0.127   -0.009    0.398   -0.441   -0.113   -0.406
 20    -0.427   -0.535   -0.331   -0.255   -0.062   -0.331   -0.392    0.770
 21    -0.017    0.022    0.153   -0.190   -0.181    0.183   -0.124    0.549
 22     0.365    0.355    0.119    0.037   -0.275    0.391    0.573   -0.786
 23    -0.316   -0.237   -0.224   -0.115    0.150   -0.532    0.162   -0.551
 24     0.067    0.074    0.260   -0.131    0.069    0.106   -0.082   -0.073
 25    -0.646   -0.723   -0.503   -0.158    0.132   -0.870   -0.131    0.314
 26     0.752    0.706    0.459    0.156   -0.405    0.962    0.458   -0.296
 27    -0.690   -0.934   -0.733   -0.538    0.213   -0.732   -0.644    0.736
 28     0.045    0.114    0.060    0.228    0.361   -0.217    0.074   -0.770
 29    -0.258   -0.437   -0.118    0.218    0.221   -0.672   -0.062    0.291
 30    -0.421   -0.054    0.127   -0.075   -0.250   -0.326    0.244    0.178
 31    -0.646   -0.544   -0.293   -0.323   -0.154   -0.543   -0.155    0.663

          9       10       11       12       13       14       15       16
  9     1.000
 10     0.757    1.000
 11    -0.290   -0.539    1.000
 12    -0.162    0.050   -0.055    1.000
 13    -0.314   -0.583    0.982   -0.065    1.000
 14     0.609    0.796   -0.114    0.237   -0.129    1.000
 15    -0.321   -0.254   -0.269   -0.418   -0.312   -0.630    1.000
 16    -0.015    0.297   -0.465   -0.259   -0.527   -0.041    0.776    1.000
 17    -0.302   -0.220   -0.272   -0.393   -0.294   -0.583    0.981    0.824
 18    -0.215   -0.399    0.931   -0.039    0.847   -0.091   -0.214   -0.353
 19     0.455    0.811   -0.375   -0.150   -0.413    0.519   -0.029    0.504
 20    -0.326   -0.363   -0.293   -0.423   -0.317   -0.654    0.950    0.635
 21    -0.456   -0.499    0.238   -0.423    0.220   -0.637    0.827    0.580
 22     0.269    0.109    0.481    0.550    0.454    0.390   -0.824   -0.714
 23     0.719    0.539   -0.387    0.356   -0.460    0.614   -0.433    0.011
 24    -0.151   -0.147    0.112    0.341    0.112   -0.199    0.332    0.365
 25     0.616    0.583   -0.742   -0.033   -0.816    0.110    0.309    0.452
 26    -0.312   -0.580    0.786   -0.065    1.000   -0.120   -0.311   -0.524
 27    -0.114    0.076   -0.603    0.130   -0.674   -0.223    0.664    0.646
 28     0.530    0.701   -0.292    0.464   -0.266    0.776   -0.307   -0.369
 29     0.653    0.496   -0.653   -0.178   -0.695    0.078    0.346    0.437
 30     0.122    0.085   -0.259   -0.358   -0.312   -0.436    0.570    0.500
 31    -0.097   -0.114   -0.461   -0.177   -0.520   -0.621    0.858    0.651

         17       18       19       20       21       22       23       24
 17     1.000
 18    -0.201    1.000
 19     0.110   -0.263    1.000
 20     0.571   -0.217   -0.250    1.000
 21     0.866    0.246   -0.059    0.699    1.000
 22    -0.775    0.434   -0.064   -0.835   -0.532    1.000
 23    -0.358   -0.295    0.637   -0.520   -0.566    0.490    1.000
 24     0.455    0.101    0.122    0.100    0.549    0.114    0.058    1.000
 25     0.293   -0.521    0.334    0.324   -0.129   -0.205    0.600    0.022
 26    -0.292    0.856   -0.410   -0.315    0.222    0.457   -0.453    0.112
 27     0.587   -0.402   -0.074    0.733    0.236   -0.560    0.007    0.112
 28    -0.760   -0.311    0.419   -0.818   -0.694    0.558    0.747   -0.256
 29     0.340   -0.568    0.298    0.329   -0.014   -0.243    0.462    0.158
 30     0.669   -0.214    0.458    0.365    0.545   -0.306    0.175    0.464
 31     0.264   -0.356    0.063    0.779    0.612   -0.563   -0.044    0.459

         25       26       27       28       29       30       31
 25     1.000
 26    -0.810    1.000
 27     0.647   -0.662    1.000
 28     0.139   -0.269   -0.330    1.000
 29     0.905   -0.695    0.465    0.077    1.000
 30     0.413   -0.310    0.129   -0.302    0.505    1.000
 31     0.593   -0.517    0.676   -0.548    0.598    0.776    1.000

TRACE =  31
```

* Variable numbers are the same with those in Table IV-11.

Table IV-13. Print out of the eigenvalue and contributions on PCA.

```
=============================================================================

COMPONENT        EIGENVALUE*         %**             ACC.%***
    1              11.534           37.206            37.206
    2               8.219           26.513            63.719
    3               3.589           11.577            75.296
    4               2.851            9.196            84.492
    5               2.189            7.061            91.553
    6               1.410            4.547            96.100
    7               1.209            3.900           100.000
    8               0.000            0.000           100.000
    9               0.000            0.000           100.000
   10               0.000            0.000           100.000
   11               0.000            0.000           100.000
   12               0.000            0.000           100.000
   13               0.000            0.000           100.000
   14               0.000            0.000           100.000
   15               0.000            0.000           100.000
   16               0.000            0.000           100.000
   17               0.000            0.000           100.000
```

* Eigenvalues were calculated by the Jacobi method.

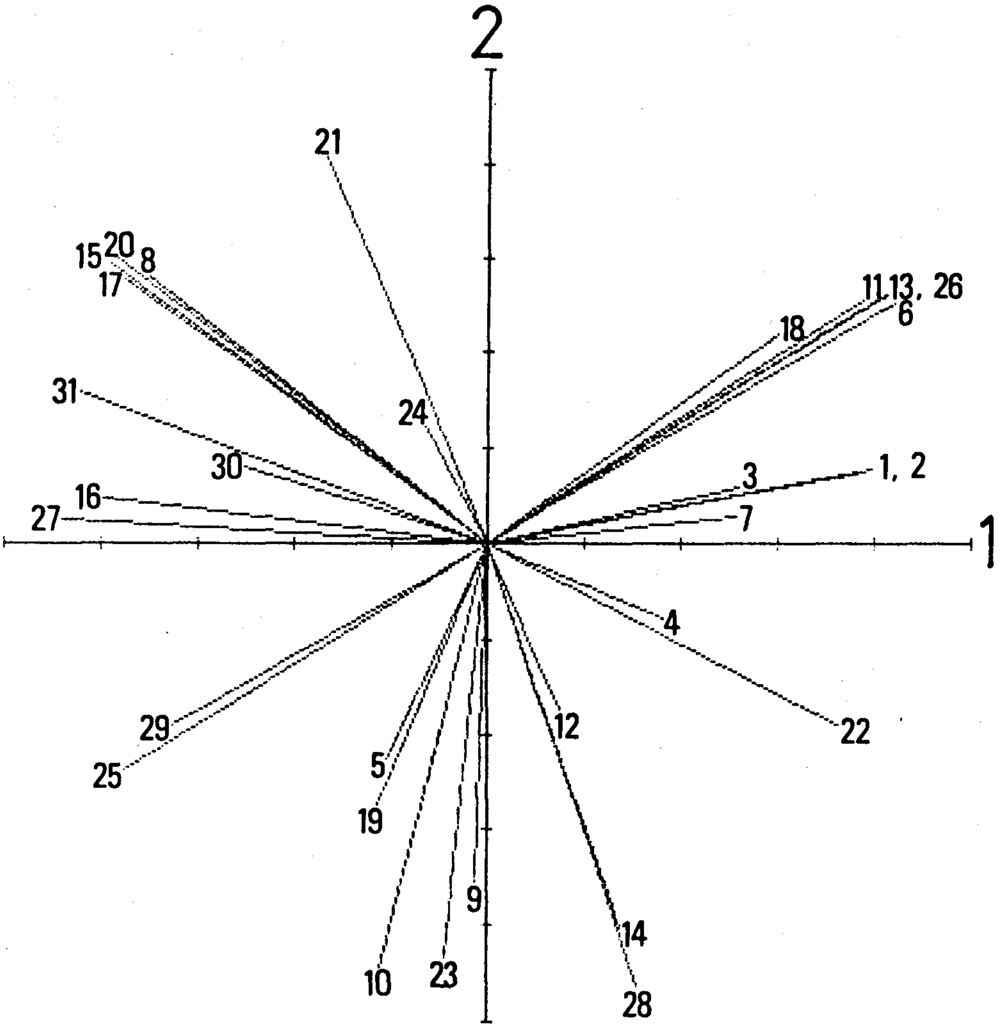
** % is the contribution.

*** ACC.% stands for accumulated contribution.

Table IV-14. Print out of the component loading which corresponds to the scalar of the eigenvector on PCA.*

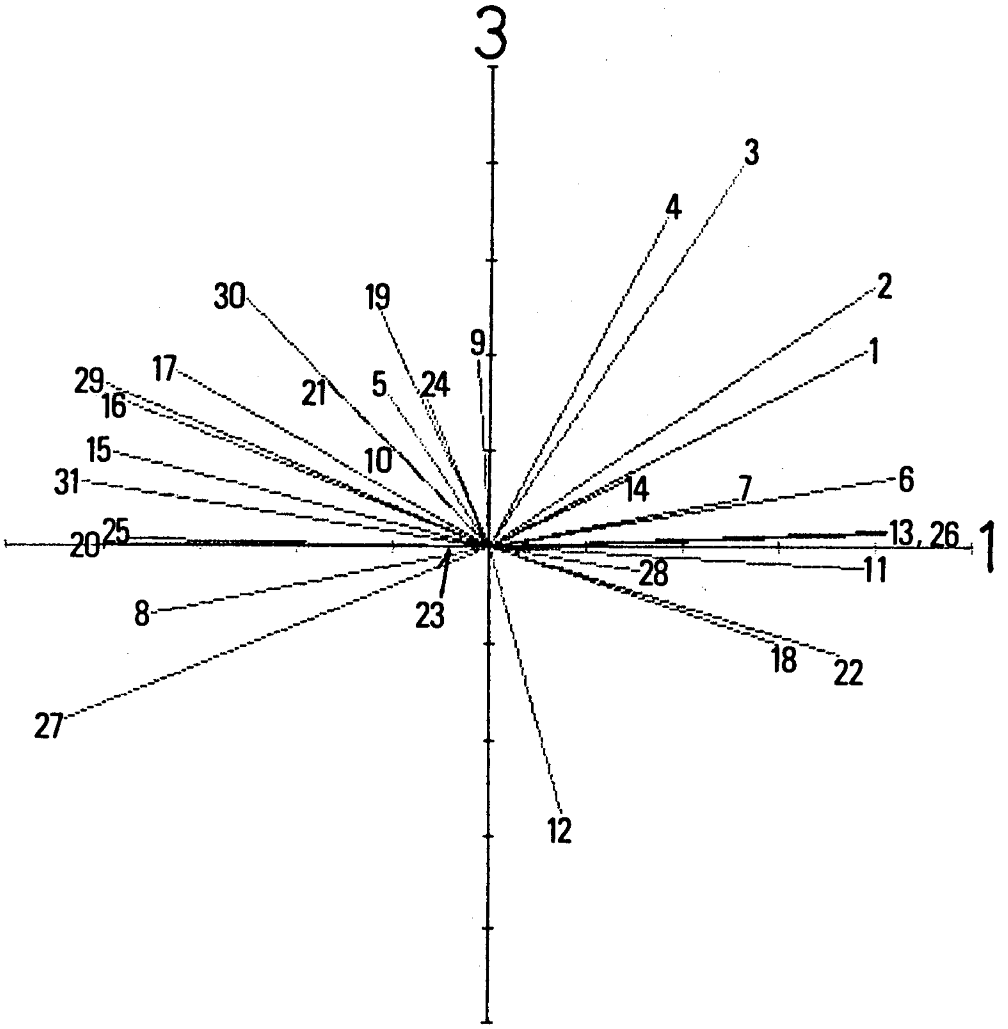
```
<COMPONENT LOADING >

       C O M P O N E N T
         1        2        3        4        5        6        7        8
  1    0.779    0.151    0.410    0.300    0.000   -0.044    0.331    0.000
  2    0.797    0.157    0.544    0.022   -0.016   -0.085   -0.193    0.000
  3    0.521    0.118    0.793    0.137    0.016   -0.243   -0.086    0.000
  4    0.364   -0.154    0.691    0.276   -0.445   -0.268    0.139    0.000
  5   -0.211   -0.453    0.315    0.735    0.304   -0.044    0.129   -0.000
  6    0.342    0.503    0.145    0.070    0.040   -0.049    0.085   -0.000
  7    0.517    0.058    0.099   -0.759   -0.371   -0.021   -0.068    0.000
  8   -0.678    0.563   -0.141    0.176   -0.326    0.047    0.194   -0.000
  9   -0.027   -0.712    0.368   -0.273   -0.263    0.147    0.406   -0.000
 10   -0.223   -0.886    0.236   -0.022    0.121    0.306    0.027    0.000
 11    0.774    0.511   -0.046   -0.160    0.152    0.228    0.192   -0.000
 12    0.151   -0.345   -0.549    0.022    0.529   -0.518    0.068   -0.000
 13    0.823    0.520    0.036   -0.064    0.107    0.114    0.151   -0.000
 14    0.276   -0.908    0.133    0.292    0.208    0.282    0.215    0.000
 15   -0.779    0.570    0.195    0.019    0.052    0.040    0.040   -0.000
 16   -0.795    0.094    0.341    0.165    0.366    0.254    0.037   -0.000
 17   -0.750    0.556    0.302   -0.040    0.185    0.034   -0.019   -0.000
 18    0.599    0.440   -0.201   -0.331    0.225    0.428    0.254    0.000
 19   -0.231   -0.550    0.494   -0.149    0.368    0.362   -0.312    0.000
 20   -0.763    0.598    0.007    0.111   -0.166    0.046    0.131   -0.000
 21   -0.333    0.817    0.339   -0.103    0.284    0.108    0.049   -0.000
 22    0.723   -0.375   -0.223   -0.427    0.187   -0.154    0.214    0.000
 23   -0.091   -0.866    0.001   -0.444    0.197    0.054    0.063    0.000
 24   -0.132    0.247    0.305   -0.285    0.721   -0.422    0.225   -0.000
 25   -0.751   -0.470    0.019   -0.315   -0.170   -0.001    0.294   -0.000
 26    0.819    0.520    0.028   -0.073    0.112    0.125    0.155   -0.000
 27   -0.881    0.051   -0.358    0.139    0.092   -0.019    0.255    0.001
 28    0.309   -0.927   -0.052    0.038    0.032   -0.125   -0.157    0.000
 29   -0.652   -0.378    0.367   -0.249   -0.249   -0.185    0.373   -0.000
 30   -0.505    0.161    0.516   -0.607    0.097   -0.083   -0.256    0.000
 31   -0.844    0.317    0.131   -0.348    0.049   -0.217    0.014    0.000
         9       10       11       12       13       14       15       16
  1   -0.000   -0.000   -0.000   -0.000   -0.000    0.000    0.000    0.000
  2    0.000    0.000   -0.000   -0.000    0.000    0.000    0.000    0.000
  3    0.000   -0.000   -0.000   -0.000    0.000    0.000   -0.000    0.000
  4    0.000    0.000   -0.000    0.000    0.000    0.000   -0.000    0.000
  5   -0.000   -0.000   -0.000   -0.000    0.000    0.000    0.000    0.000
  6   -0.000   -0.000    0.000   -0.000   -0.000   -0.000    0.000   -0.000
  7    0.000    0.000    0.000    0.000   -0.000   -0.000   -0.000   -0.000
  8   -0.000    0.000   -0.000    0.000    0.000    0.000   -0.000    0.000
  9    0.000   -0.000   -0.000    0.000   -0.000   -0.000    0.000   -0.000
 10    0.000   -0.000    0.000   -0.000   -0.000   -0.000    0.000   -0.000
 11   -0.000   -0.000    0.000    0.000   -0.000   -0.000    0.000   -0.000
 12   -0.000    0.000   -0.000   -0.000   -0.000   -0.000   -0.000    0.000
 13   -0.000   -0.000    0.000    0.000   -0.000   -0.000    0.000   -0.000
 14   -0.000    0.001    0.000   -0.000   -0.000    0.000    0.000   -0.000
 15   -0.000    0.000   -0.000    0.000    0.000    0.000   -0.000   -0.000
 16    0.001   -0.000    0.000   -0.000    0.000   -0.000    0.000   -0.000
 17   -0.000   -0.000   -0.000   -0.000    0.000   -0.000   -0.000   -0.000
 18   -0.000   -0.000    0.000    0.000   -0.000   -0.000    0.000    0.000
 19   -0.000   -0.000    0.000   -0.000    0.000   -0.000   -0.000   -0.000
 20   -0.000    0.000   -0.000    0.000    0.000    0.000   -0.000   -0.000
 21   -0.000    0.000   -0.000    0.000    0.000    0.000   -0.000   -0.000
 22    0.000   -0.000   -0.000   -0.000    0.000   -0.000   -0.000   -0.000
 23    0.000   -0.000    0.000   -0.000   -0.000    0.000    0.000   -0.000
 24   -0.000    0.000   -0.000   -0.000   -0.000   -0.000   -0.000    0.000
 25   -0.000   -0.000   -0.000    0.000   -0.000   -0.000   -0.000   -0.000
 26   -0.000   -0.000    0.000    0.000   -0.000   -0.000    0.000   -0.000
 27   -0.000   -0.000   -0.000    0.000   -0.000   -0.000   -0.000   -0.000
 28    0.000   -0.000    0.000    0.000   -0.000   -0.000    0.000    0.000
 29   -0.000   -0.000    0.001    0.000   -0.000   -0.000   -0.000   -0.000
 30    0.000    0.000   -0.000   -0.000   -0.000   -0.000   -0.000   -0.000
 31   -0.000    0.000   -0.000   -0.000    0.000   -0.000    0.000    0.000

        17
  1    0.000
  2    0.000
  3   -0.000
  4   -0.000
  5   -0.000
  6   -0.000
  7    0.000
  8    0.000
  9   -0.000
 10    0.000
 11   -0.000
 12   -0.000
 13   -0.000
 14   -0.000
 15   -0.000
 16   -0.000
 17    0.000
 18   -0.000
 19    0.000
 20   -0.000
 21   -0.000
 22    0.000
 23   -0.000
 24    0.000
 25   -0.000
 26   -0.000
 27   -0.000
 28    0.000
 29    0.000
 30    0.000
 31    0.000
```

* The row and the column are variable numbers as same with those in Table IV-11.



1. (16:2)(20:5)
2. (20:5)(20:5)
3. (20:5)(22:6)
4. (22:6)(22:6)
5. (20:4)(22:5)
6. (22:5)(22:6)
7. (20:3)(22:6)
8. (20:5)(22:5)
9. (20:4)(22:6)
10. (22:5)(22:6)
11. (22:4)(22:6)
12. (16:1)(20:5)
13. (22:6)(16:1)
14. (16:1)(22:6)
15. (14:0)(20:5)
16. (14:0)(22:6)
17. (17:0)(22:6)
18. (20:5)(18:1)
19. (18:1)(20:5)
20. (22:6)(18:1)
21. (18:1)(22:6)
22. (20:5)(16:0)
23. (16:0)(20:5)
24. (22:6)(16:0)
25. (16:0)(22:6)
26. (22:5)(16:0)
27. (16:0)(22:5)
28. (16:0)(20:4)
29. Unknown
30. (18:1)(16:0)
31. (16:0)(18:1)

Fig. IV-56. Eigenvectors of all detected molecular species on the first and second principal component plane on PCA.



1. (16:2)(20:5)
2. (20:5)(20:5)
3. (20:5)(22:6)
4. (22:6)(22:6)
5. (20:4)(22:5)
6. (22:5)(22:6)
7. (20:3)(22:6)
8. (20:5)(22:5)
9. (20:4)(22:6)
10. (22:5)(22:6)
11. (22:4)(22:6)
12. (16:1)(20:5)
13. (22:6)(16:1)
14. (16:1)(22:6)
15. (14:0)(20:5)
16. (14:0)(22:6)
17. (17:0)(22:6)
18. (20:5)(18:1)
19. (18:1)(20:5)
20. (22:6)(18:1)
21. (18:1)(22:6)
22. (20:5)(16:0)
23. (16:0)(20:5)
24. (22:6)(16:0)
25. (16:0)(22:6)
26. (22:5)(16:0)
27. (16:0)(22:5)
28. (16:0)(20:4)
29. Unknown
30. (18:1)(16:0)
31. (16:0)(18:1)

Fig. IV-57. Eigenvectors of all the detected molecular species on the first and third principal component plane on PCA.

Table IV-15. Print out of the principal loading (component score) on PCA

```
<PRINCIPAL LOADING>

      P R I N C I P A L
        1         2        3        4        5        6        7        8
1  -16.352     8.027    4.458   -1.180    2.503    0.053   -0.449   -0.000
2  -12.737     8.015   -3.564    1.902   -3.121    0.148    0.837   -0.000
3    0.044    -6.245    1.077   -5.306   -2.760   -0.548   -0.606    0.000
4   -0.927   -12.116    3.177    1.654    0.248    0.991    1.841    0.000
5   14.315     4.307    3.658    3.165   -1.011   -1.825   -0.466   -0.000
6   17.100     8.946   -1.784   -2.333    1.217    1.493    0.760   -0.000
7    0.368    -5.526   -1.912    2.337    0.221    1.715   -2.172    0.000
8   -1.812    -5.408   -5.110   -0.239    2.702   -2.025    0.255    0.000

        9        10       11       12       13       14       15       16
1    0.000    -0.000   -0.000   -0.000    0.000   -0.000   -0.000   -0.000
2    0.000    -0.000    0.000    0.000   -0.000   -0.000    0.000   -0.000
3    0.000     0.000   -0.000   -0.000    0.000    0.000    0.000    0.000
4   -0.000    -0.000    0.000    0.000   -0.000    0.000   -0.000   -0.000
5   -0.000     0.000   -0.000   -0.000   -0.000    0.000    0.000    0.000
6   -0.000     0.000    0.000    0.000    0.000   -0.000   -0.000    0.000
7   -0.000     0.000   -0.000    0.000   -0.000    0.000   -0.000   -0.000
8    0.000     0.000    0.000   -0.000    0.000    0.000   -0.000    0.000

       17
1    0.000
2    0.000
3   -0.000
4    0.000
5   -0.000
6    0.000
7    0.000
8   -0.000
```

Row

1 : Feeding migration, Male.
2 : Feeding migration, Female.
3 : Spawning migration, Male (Sea).
4 : Spawning migration, Female (Sea).
5 : Spawning migration, Male (River).
6 : Spawning migration, Female (River).
7 : Spent, Male.
8 : Spent, Female.

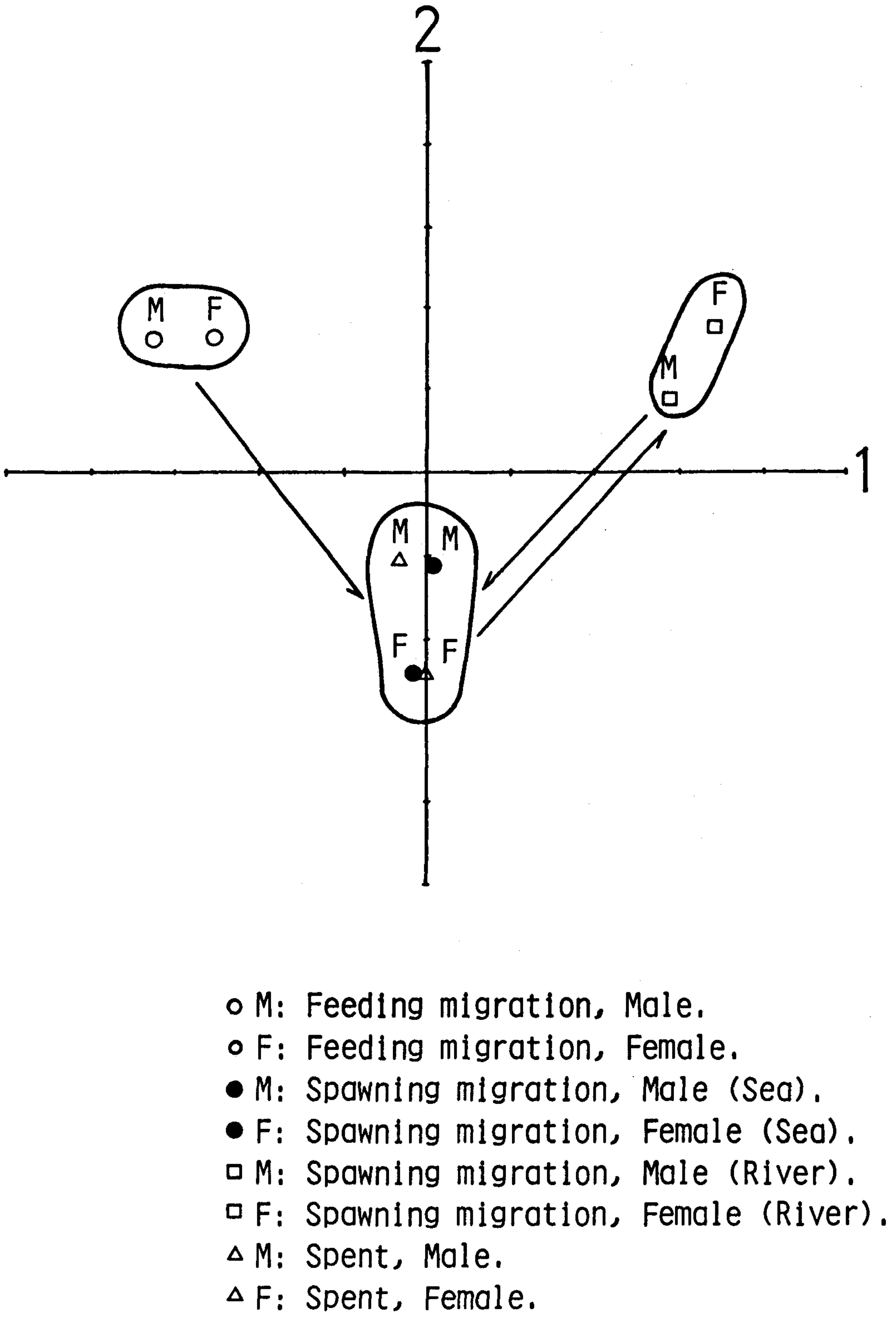


Fig. IV-58. Plots of principal loading on the first and second principal component plane on PCA.
Refer to the eigenvectors in Fig. IV-56 as background of this plane.

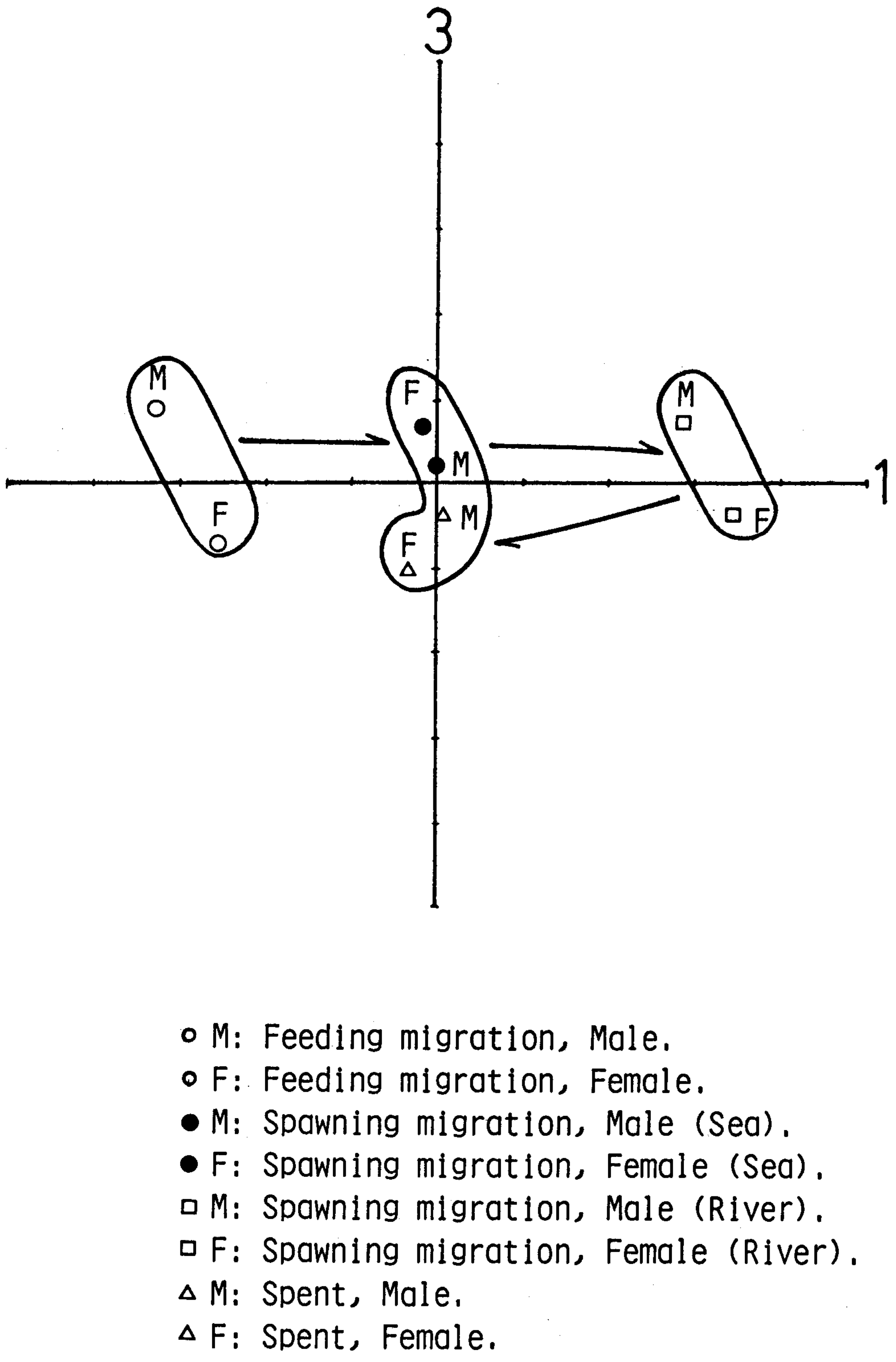


Fig. IV-59 Plots of principal loading on the first and third principal component plane on PCA.
Refer to the eigenvectors in Fig. IV-57 as background of this plane.

are 31 (see the footnote of Fig. IV-56). And number of samples correspond to the four different migratory stages of two different sexes (see the footnote of Table IV-15). "MEAN" is the average amount of each molecular species of the four stages and "S.D." is standard deviation. Correlations among all detected molecular species are shown in Table IV-12 and eigenvalues calculated by the Jacobi method from Table IV-12 are shown in Table IV-13. Values of contributions are also shown in this Table. Though variables were up to 31, the accumulated contribution was 63.7% when employed to the second principal component.

In Fig. IV-56, eigenvectors of all detected molecular species are shown as small numbers on the first and second principal component plane. These are drawn up from the print out of component loading in columns 1 and 2 in Table IV-14. And in Fig. IV-57, eigenvectors of the same molecular species are generated on the first and third principal component plane. And these are drawn up from the print out of component loading in columns 1 and 3 in Table IV-14 as well.

Principal loading in Table IV-15 was plotted on the first and second principal component plane as shown in Fig. IV-58 and on the first and third principal component plane as shown in Fig. IV-59. The movement according to the stage of migrations are shown by arrows. Very interesting movements are observed in this figure. By refering the eigenvectors in Fig. IV-56 at the same time, it can be observed that the movement direction from feeding migration stage to spawning migration stage (sea) is nearly parallel to the eigenvectors numbered 8, 15, 17, and 20 while it makes a right angle against eigenvectors 25 and 29. This shows that although the amounts of number 8 ((20:5)(22:5)), number 15 ((14:0)(20:5)), number 17 ((17:0)(22:6)) and number 20 ((22:6)(18:1)) are small as have seen in Fig. IV-51 and Fig. IV-52, drastic decrease of these molecular species occurs between these two stages, making good contrasts to number 25 ((16:0)(22:6)) and 29 (Unknown) which keep the constant amount. On the other hand, when the migratory stage proceeds to the spawning stage in the river, the movement of the arrow in this figure becomes almost parallel to the eigenvectors of number 25, 29 and also 6, 11, 13, 18 and 26. Number 25 is (16:0)(22:6), and the rest 6, 11, 13, 18 and 26 are small peaks. So it might be possible to conclude that (16:0)(22:6) is almost constant at the sea stage and becomes changeable at the stage of river.

Spent salmon showed an analogous position with that of spawning migration stage in the sea in PCA planes as it is shown in Fig. IV-58 and Fig. IV-59. And from these two figures, it is clearly seen that the differences between male and female are considerablely small compared with the differences among the migratory stages.

CHAPTER V

GENERAL SUMMARY AND CONCLUSIONS

Superiority of the reversed-phase type HPLC was emphasized.

In chapter I, it was suggested that dephosphorylation followed by acetylation is effective in separating the critical pairs of the lecithin molecular species on HPLC. The positional isomers were also separated to some extent unlike the triglycerides. And a new matrix model was proposed for the prediction of molecular species of lecithin. The matrix model was as follows:

$$CN= P_1 \cdot \log(RRT)+q_1 \qquad CN=\begin{vmatrix} x & d_1 \\ c_2 & d_2 \end{vmatrix}$$

$$DB= P_2 \cdot \log(RRT)+q_2 \qquad DB=\begin{vmatrix} c_1 & y \\ c_2 & d_2 \end{vmatrix}$$

where c_1, c_2 and d_1, d_2 are acyl carbon number and the number of double bonds in each acyl group line up in a similar way. x and y are variables of acyl carbon number and number of double bonds, respectively. P_1 and P_2 are the slopes and q_1 and q_2 are the intercepts on the ordinate of the semilogarithmic plots of the RRTs of molecular species against CN or DB. RRT is the relative retention time (in general, it should be relative retention).

In chapter II, the matrix model proposed in chapter I was developed into the rules for molecular species of triglycerides. This idea was based on the expansion of matrix of (2,2) type model for the lecithin molecular species to the matrix of (3,2) type model for the triglyceride molecular species. The matrix model for the triglyceride was as follows:

$$CN = P_1 \cdot \log(RRT) + q_1 \qquad CN = \begin{vmatrix} x & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$$

$$DB = P_2 \cdot \log(RRT) + q_2 \qquad DB = \begin{vmatrix} c_1 & y \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$$

where c_1, c_2, c_3 and d_1, d_2, d_3 are acyl carbon number and the number of double bonds in each acyl group line up in a similar way. x and y are variables of acyl carbon number and number of double bonds, respectively. P_1 and P_2 are the slops and q_1 and q_2 are the intercepts on the ordinate of the semilogarithmic plots of the RRTs of molecular species against CN or DB. RRT is the relative retention time. This matrix model was verified by the actual analysis of triglycerides from natural sources and from the chromatographic results presented by other investigators.11,38,39)

The theoritical background of the proposed new model was considered by introducing the theory of Martin.42) He formulated the equation:

$$\frac{\Delta\mu_B}{RT} = \frac{\Delta\mu_A}{RT} + \frac{\Delta\mu_X}{RT} \quad , \qquad \ln\left(\frac{\alpha_B}{\alpha_A}\right) = \frac{\Delta\mu_X}{RT}$$

where A, B are members of a homologous series, differing by the functional group X, α is the partition coefficient and $\Delta\mu$ is the differences in chemical potential of the group X in polar or non-polar phase of the chromatographic system. It follows that each group in the solute molecule contributes more or less independently to the differences in standard free energy of the solute between the two different phases. Thus, in general, there is a linear relationship between ln α or log α and the number of functional groups in a homologous series. By substituting A in Martin's theory42) as triglyceride species $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$ and B as $\begin{vmatrix} c_1+X & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$, X will become a functional group. Or we could also substitute A as $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$ and B as $\begin{vmatrix} c_1 & d_1+Y \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$, then Y will also become a functional group. In this case, X corresponds to $-CH_2-$ unit and Y corresponds to $-CH{=}CH-$ unit. The chemical potential of the triglyceride molecule is principally affected by these X or Y, since in accordance with the elongation of the hydrocarbon chain or increase in the number of double bonds in the acyl group, the plots of RRT of each molecular species draw an ascending or a descending straight line.

Thus the physicochemical background of the matrix model has been demonstrated from the theory of Martin.42) A more detailed considerations were also done in this chapter II. Strictly speaking, in addition to the physicochemical functional groups, that is, $-CH_2-$ and $-CH{=}CH-$, the differences in arrangement of these units might slightly affect the total chemical potential of the triglyceride molecule (it was called Ω factor). And from the view point of stereospecific structure, we should consider about the degree in bias between the positional isomers. So the author has concluded that the chemical potential of the triglyceride molecule is the resultant of all functional groups in the molecule. The sum upped chemical potential (μ) of triglyceride $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$ was written as follows:

$$\mu = g\{ f(c_1,\ d_1,\ \Omega_1\ ),\ f(c_2,\ d_2,\ \Omega_2\ ),\ f(c_3,\ d_3,\ \Omega_3\ )\}$$

where Ω is an Ω factor, f is the chemical potential given by the hydrocarbon chain, and g is the function of chemical potential given by the differences in positional isomers. In order to give shape to this function, it was concluded that a more precised instruments (column, pump and detector of the HPLC) that gives a perfect reproductive data is required. Namely, for example, if the differences of RRT between $\begin{vmatrix} c_1 & d_1 \\ c_2 & d_2 \\ c_3 & d_3 \end{vmatrix}$ and $\begin{vmatrix} c_2 & d_2 \\ c_1 & d_1 \\ c_3 & d_3 \end{vmatrix}$ can be measured perfectly, then the relation between $f(c_1, d_1, \Omega_1)$ and $f(c_2, d_2, \Omega_2)$ can be formularized by analyzing the multiple data. The 3-μm type column packing is expected for this purpose.

The theoritical relationship between the presented matrix model demonstrated in this study and the conventional ECN theory was also discussed in chapter II. It was proved mathematically that ECN is the degradated form of the presented matrix model.

In chapter III, the computer software for the identification of lecithin molecular species was designated.

The order of priority in the computer program for the identification of lecithin molecular species was as follows:

1. When there is a same molecular species in the data file, the RRT of this molecular species is printed out.
2. When there are molecular species that belong to the $\begin{vmatrix} x & d_1 \\ c_2 & d_2 \end{vmatrix}$ group (or $\begin{vmatrix} c_1 & d_1 \\ x & d_2 \end{vmatrix}$), a regression line is generated and the RRT of the predicted molecular species is printed out.
3. When there are two parallel line groups that slightly differ in the slope due to the arrangement of $-CH_2-$ and $-CH{=}CH-$ units in the molecule, parallelogram is generated. And the RRT of the unknown vertex of this parallelogram is printed out.
4. When there are molecular species that belong to the $\begin{vmatrix} d_1 \\ d_2 \end{vmatrix}$ group (when the combination of the acyl carbon

number is unknown), the slope of the generalized regression line is introduced. And the RRT of the predicted species is printed out.

Though there is still a limitation for the perfect identification of all the molecular species contained in the samples from natural sources due to the incomplete reproducibility of RRT, this computer software might be convenient in predicting the generally found molecular species.

In chapter IV, from the view point of muscle lecithin molecular species, sardine, mackerel, big-eyed tuna, brown sole, sand flounder, rock fish, Alaska pollack, chum salmon, blue shark, mackerel shark, carp and rainbow trout were examined on HPLC. Amoung the fish examined, flat fish such as brown sole and sand flounder were extremely characteristic since these fish contained (16:0)(20:5) as the most prominent species unlike the rest of the fish that had (16:0)(22:6) as the most prominent species. Cartilaginous fish such as blue shark and mackerel shark had a very few amount of molecular species that have the combination of highly unsaturated fatty acids such as (20:5)(20:5), (20:5)(22:6) and (22:6)(22:6). This was also observed in fish from fresh water such as carp and rainbow trout. The main differences between the cartilaginous fish and the fresh water fish were in the contents of molecular species composed of 20:5 represented by (16:0)(20:5). These were rich in the cartilaginous fish.

Principal component analysis (PCA) was done in order to characterize each fish in relation to all other fish examined. Except sardine dark muscle, chum salmon (feeding migration) and rainbow trout, a reflectable movement in the content of (20:5)(20:5) was observed in the majority kind of fish examined.

Chum salmon of four different migratory stages were analyzed in the same manner. By introducing PCA again for the interpretation of muscle lecithin molecular species analysis amoung the migratory stages, interesting movement were observed on the first and second, as well as the first and third principal component plane in accordance with the advance in migration. The plots of male and female moved almost together on these plane. This implies that although there are some differences between male and female especially at the stage of spawning migration in the river, these differences are not so large as the differences between the migratory stages. It was clearly seen that at the stage of spawning migration in the river, molecular species of (16:0)(22:6) drastically decreased.

The study in molecular species of fish lipid is at the dawn of a new age. Especially for the analysis of triglyceride molecular species, an effective high performance column that has a thorough theoritical plate to separate individual molecular species is principally required. And a conbination with the flame ionization detector (FID) or with the mass spectrometer is expected to back up a more precised determination. Attempt on the analysis using (FID) on HPLC has done by Privett et al.49~54) And that on the analysis using mass spectrometer combined with HPLC has done by Kuksis et al.55) Matsushita et al.56) have used a mass spectrometer on the fractions obtained by HPLC manually. There is also an attempt done on a mass spectrometer without using HPLC for the fractionation[57~63] or by high a field ^{13}C nuclear magnetic resonance spectrum (NMR) alone[64]. But from the author's point of view, at least the HPLC fractionation might be inevitable prior to the mass spectrometer or NMR analysis. And these should be used only when it is necessary to analyze the overlapped peak on HPLC since the analytical cost using the mass spectrometer or NMR might be wasteful.

In this study, principal consideration for the complex lipid molecular species analysis was on lecithin. But the author is quite sure that the matrix model presented for the lecithin HPLC analysis well fits to the other complex lipid molecular species analysis as well. Nakagawa et al.[65,66] worked on the phosphatidylethanolamine molecular species of bovine brain and Un Hoi Do et al.,[16] as well as Smith et al.[13] worked on the ceramides of bovine brain in addition to the studies on lecithin molecular species.[67] In accordance with the data accumulation in these field, the invariability of the matrix model for all kinds of lipid molecular species is sure to be verified.

REFERENCES

1. K. Takahashi, K. Zama and T. Matsuoka: Molecular Species of Fish Muscle Lipids. I. Molecular Species of Triglyceride and Phosphatidylcholine of Sardine and Rainbow Trout. Bull. Fac. Fish. Hokkaido Univ., 29, 378~385 (1978).
2. A. H. El-Hamdy and E. G. Perkins: High Performanced Reversed-Phase Chromatography of Natural Triglyceride Mixtures. J. Am. Oil Chem. Soc., 58, 49~53 (1981).
3. H. Hatano: "Saishin no Ekitai Kuromatografī." J. Jap. Chem., 35, 283~292 (1981). (In Japanese).
4. R. E. Pauls: A Time Normalization Study of the Separation of Olive Oil Triglycerides. J. Am. Oil Chem. Soc., 60, 819~822 (1983).
5. Waters Associates Inc. LC seminar, held in Sapporo, Japan, in June 6th, 1980.
6. S. Wada, C. Koizumi, and J. Nonaka: Analysis of Triglycerides of Soybean Oil by High-Performance Liquid Chromatography in Combination with Gas Liquid Chromatography. Yukagaku, 26, 95~99 (1977).
7. S. Wada, C. Koizumi, A. Takiguchi and J. Nonaka: A Study on Triglyceride Composition of Lipid from Commercial Beef by High-Performance Liquid Chromatography. ibid., 27, 579~584 (1978).
8. N. A. Porter, R. A. Wolf and J. R. Nixon: Separation and Purification of Lecithins by High Pressure Liquid Chromatography. Lipids, 14, 20~24 (1979).
9. R. D. Plattner, G. F. Spencer and R. Kleiman:

Triglyceride Separation by Reverse Phase High Performance Liquid Chromatography. J. Am. Oil Chem. Soc. 54, 511~515 (1977).

10. R. D. Plattner: High Performance Liquid Chromatography of Triglycerides: Controlling Selectivity with Reverse Phase Columns. ibid., 58, 638~642 (1981).

11. A. H. El-Hamdy and E. G. Perkins: High Performance Reversed Phase Chromatography of Natural Triglyceride Mixtures: Critical Pair Separation. ibid., 58, 867~872 (1981).

12. B. J. Compton and W. C. Purdy: The High-Performance Liquid Chromatography and Detection of Phospholipids and Triglycerides. Part 1. Nonpolar Stationary Phase Chromatographic Behavior in Ultraviolet Transparent Mobile Phases. Analytica Chim. Acta, 141, 405~410 (1982).

13. M. Smith, P. Monchamp and F. B. Jungalwala: Separation of Molecular Species of Sphingomyelin and Ceramide by Argentation and Reversed-Phase HPLC. J. Lipid Res., 22, 714~719 (1981).

14. J. Y-K. Hsieh, D. K. Welch and J. G. Turcotte: High Pressure Liquid Chromatographic Separation of Molecular Species of Phosphatidic Acid Dimethyl Esters Derived from Phosphatidylcholine. Lipids, 16, 761~763 (1981).

15. N. A. Porter, R. A. Wolf and H. Weenen: The Free Radical Oxidation of Polyunsaturated Lecithins. ibid., 15, 163~167 (1980).

16. C. G. Crawford, R. D. Plattner, D. J. Sessa and J. J. Rackis: Separation of Oxidized and Unoxidized Molecular

Species of Phosphatidylcholine by High Pressure Liquid Chromatography. ibid., 15, 91~94 (1980).

17. Un Hoi Do and P. T. Pei: Separation of Molecular Species of Ceramides as Benzoyl and *p*-Nitrobenzoyl Derivatives by High Performance Liquid Chromatography. ibid., 16, 855~862 (1981).

18. J. Y-K. Hsieh, D. K. Welch and J. G. Turcotte: General Method for the Analysis of Phosphatidylcholines by High-Performance Liquid Chromatography. J. Chromat., 208, 398~403 (1981).

19. M. Smith and F. B. Jungalwala: Reversed Phase High Performance Liquid Chromatography of Phosphatidylcholine : A Simple Method for Determining Relative Hydrophobic Interaction of Various Molecular Species. J. Lipid Res., 22, 697~704 (1981).

20. G. M. Patton, J. M. Fasulo and S. J. Robins: Separation of Phospholipids and Individual Molecular Species of Phospholipids by High-Performance Liquid Chromatography. ibid., 23, 190~196 (1982).

21. T. Ohshima, S. Wada and C. Koizumi: Estimation of Possible Fatty Acid Combinations in Phosphatidylcholine and Phosphatidylethanolamine of Cod. Bull. Japan. Soc. Sci. Fish., 49, 123~130 (1983).

22. T. Ohshima and C. Koizumi: Accumulation of Lysophosphatidylcholine and Lysophosphatidylethanolamine in Muscle of Fresh Skipjack. ibid., 49, 1205~1212 (1983). (In Japanese).

23. T. Ohshima, S. Wada and C. Koizumi: Deterioration of Phospholipids of Skipjack Muscle during Ice Storage:

Mainly Concerning to Enzymatic Hydrolysis of Phosphatidylcholine. ibid., 49, 1213~1219 (1983). (In Japanese).

24. T. Ohshima, S. Wada and C. Koizumi: Enzymatic Hydrolysis of Phospholipids in Cod Flesh during Cold Storage. ibid., 49, 1397~1404 (1983). (In Japanese).

25. T. Ohshima, S. Wada and C. Koizumi: Enzymatic Hydrolysis of Phospholipid in Cod Fresh during Storage in Ice. ibid., 50, 107~114 (1984). (In Japanese).

26. W. E. M. Lands and P. Hart: The Control of Fatty Acid Composition in Glycerolipids. J. Am. Oil Chem. Soc., 43, 290~295 (1966).

27. O. Renkonen: Individual Molecular Species of Different Phospholipid Classes. Part II. A Method of Analysis. ibid., 42, 298~304 (1965).

28. O. S. Privett and L. J. Nutter: Determination of the Structure of Lecithins Via the Formation of Acetylated 1, 2-Diglyceride. Lipids, 2, 149~154 (1966).

29. A. F. Prevot and F. X. Mordret: "Utilisation des Colonnes Capillaires de Verre pour l'Analyse des Corps Gras par Chromatographie en Phase Gazeuse." Rev. Fse Corps Gras, 23, 409~423 (1976). (In French).

30. S. Funahashi, I. Hara and T. Yamakawa: in "Lipid 1." Published by Kyōritsu-Syuppan, Tokyo, 1970, 531 pp. (In Japanese).

31. H. Kosugi and N. Ueda: "Hito Hishi no Bunseki-tokuni Toriguriserido no Kōzō." Proc. J. C. B. L., 18, 9~12 (1976). (In Japanese).

32. K. Zama, T. Maruyama and K. Takahashi: Lipids of the

Crustacea. I. Lipids of the Muscle and the Egg of the Prawn (Pandalopsis japonica). Bull. Fac. Fish. Hokkaido Univ., 27, 181~190 (1976).

33. K.Takahashi, F. Cabling Jr and K. Zama: Molecular Species of Fish Muscle Lecithin. II. Changes in Triglyceride and Phosphatidylcholine Molecular Species of Sardine after Frozen Storage. ibid., 29, 386~391 (1978).

34. A. Kuksis: in "Progress in the Chemistry of Fats and Other Lipids" (ed. by R. T. Holman), Vol. 12, Pergamon Press, Oxford, New York, Toronto, 1972, pp. 105~111.

35. R. Wood and F. Snyder: Tumor Lipids: Metabolic Relationships Derived from Structural Analyses of Acyl, Alkyl, and Alk-1-enyl Moieties of Neutral Glycerides and Phosphoglycerides. Arch. Biochem. Biophys., 131, 478~494 (1969).

36. D. B. Menzel and H. S. Olcott: Positional Distribution of Fatty Acids in Fish and Other Animal Lecithins. Biochim. Biophys. Acta, 84, 133~139 (1964).

37. S. Wada: User's Communications. Jasco Report, 18, 18~20 (1983). (In Japanese).

38. E. G. Perkins, D. J. Hendren, N. Pelick and J. E. Bauer: High Performance Reversed-Phase Chromatography of the Triglycerides from Human Plasma Lipoproteins. Lipids, 17, 460~463 (1982).

39. C. Merritt, M. Vajdi, S. G. Kayser, J. W. Halliday and M. L. Bazinet: Validation of Computational Method for Triglyceride Composition of Fats and Oils by Liquid Chromatography and Mass Spectrometry. J. Am. Oil Chem.

Soc., 59, 422~432 (1982).

40. M. W. Dong and J. L. Dicesare: Improved Separation of Natural Oil Triglyceride by Liquid Chromatography Using Columns Packed with 3-μm Particles. ibid., 60, 788~791 (1983).

41. M. Watari and M. Kishi: in "Personal Computer Library Vol. 3." Published by Kōgaku Tosho, Tokyo, 1982, pp. 4-1~4-11 (In Japanese).

42. A. J. P. Martin: Some Theoretical Aspects of Partition Chromatography. Biochem. Soc. Symposia, (Cambridge, England), 3, 4~20 (1950).

43. Y. Toya, M. Umezawa, T. Takatori and S. Hara: "Toriguriserido no HPLC Bunseki." Sammary book of the 22th anual meeting of Japan Oil Chem. Soc., Osaka, 1983, 144 pp. (In Japanese).

44. Z. Ishii: "Iatorosukyan (Shinkurogurafu)." Med. Technol., 8, 1196~1202 (1980). (In Japanese).

45. R. T. Crane. S. C. Goheen, E. C. Larkin and G. A. Rao: Complexities in Lipid Quantitation Using Thin Layer Chromatography for Separation and Flame Ionization for Detection. Lipids, 18, 74~80 (1983).

46. J. K. Kaitaranta: TLC-FID Assessment of Lipid Oxidation As Applied to Fish Lipids Rich in Triglycerides. J. Am. Oil Chem. Soc., 58, 710~713 (1981).

47. C. C. Parrish and R. G. Ackman: The Effect of Developing Solvents on Lipid Class Quantification in Chromarod Thin Layer Chromatography / Flame Ionization Detector. Lipids, 18, 563~565 (1983).

48. M. Watari and M. Kishi: in "Personal Computer Library

Vol. 3." Published by Kōgaku Tosho, Tokyo, 1982, pp. 9-1~9-12 (In Japanese).

49. A. Stolyhwo and O. S. Prevett: Studies on the Analysis of Lipid Class by Gradient Elution Adsorption Chromatography. J. Chromatogr. Sci., 11, 20~25 (1973).

50. W. L. Erdahl, A. Stolyhwo and O. S. Privett: Analysis of Soybean Lecithin by Thin Layer and Analytical Liquid Chromatography. J. Am. Oil Chem. Soc., 50, 513~515 (1973).

51. O. S. Privett, K. A. Dougherty, W. L. Erdahl and A. Stolyhwo: Studies on the Lipid Composition of Developing Soybeans. ibid., 50, 516~520 (1973).

52. F. C. Phillips, W. L. Erdahl and O. S. Privett: Quantitative Analysis of Lipid Classes by Liquid Chromatography Via Flame Ionization Detector. Lipids, 17, 992~997 (1982).

53. F. C. Phillips and O. S. Privett: Analysis of Lipid Classes and Lipofuscin Sabstances by High Performance Liquid Chromatography. J. Am. Oil Chem. Soc., 58, 590~594 (1981).

54. F. C. Phillips, W. L. Erdahl, J. D. Nadenicek, L. J. Nutter, J. A. Schmit and O. S. Privett: Analysis of Triglyceride Species by High-Performance Liquid Chromatography Via Flame Ionization Detector. Lipids, 19, 142~150 (1984).

55. A. Kuksis, L. Marai and J. J. Myher: Strategy of Glycerolipid Separation and Quantitation by Complementary Analytical Techniques. J. Chromat., 273,

43~66 (1984).

56. S. Matsushita, Y. Tada, N. Kawamura, E. Ohnishi, Y. Maeda and T. Ikushige: Rapid Analysis of Triglyceride of Oils and Fats by High Speed Liquid Chromatography. "Tōyō Sōda Kenkyū Hōkoku," 24, 29~33 (1980). (In Japanese).

57. M. Fukatsu, I. Watanabe and T. Tamura: GC-MS Analysis of Triglyceride I. Total Fatty Acid Composition. Yukagaku, 31, 215~217 (1982). (In Japanese).

58. M. Fukatsu, K.Tanaka and T. Tamura: GC-MS Analysis of Triglyceride II. The Influence of Low Ionization Voltage on Fragment Ion Intensity. ibid., 31, 461~463 (1982). (In Japanese).

59. M. Fukatsu and T. Tamura: GC-MS Analysis of Triglyceride III. Relationship Between the Fatty Acid Distribution and Intensity Ratios of Specific Fragment Ions. ibid., 32, 92~95 (1983). (In Japanese).

60. M. Fukatsu and T. Tamura: GC-MS Analysis of Triglyceride IV. Correction of Fatty Acid Composition. ibid., 33, 144~147 (1984). (In Japanese).

61. J. Ahlberg, T. Curstedt, K. Einarsson and J. Sjovall: Molecular Species of Biliary Phosphatidylcholines in Gallstone Patients: The Influence of Treatment with Cholic Acids and Chenodeoxycholic Acid. J. Lipid Res., 22, 404~409 (1981).

62. M. Kino, T. Matsumura, M. Gamo and K. Saito: Studies on Molecular Species of Choline and Ethanolamine Glycerophospholipids Obtained from Rat Brain Myelin and Synaptosomes by Gas-Liquid Chromatography Mass Spectrometry. Biomed. Mass Spectrom., 9, 363~369

(1982).

63. C. G. Crawford and R. D. Plattner: Ammonia Chemical Ionization Mass Spectrometry of Intact Diacyl Phosphatidylcholine. J. Lipid Res., 24, 456~460 (1983).

64. J. Y-K. Hsieh. D. K. Welch and J. G. Turcotte: General Method for the Analysis of Phosphatidylcholines by High-Performance Liquid Chromatography. J. Chromat., 208, 398~403 (1981).

65. Y. Nakagawa and L. A. Horrocks: Kōsoku Ekitai Kuromatografī ni yoru Nō Etanoruamin Bunshishu no Bunseki oyobi Taisha e no Ōyō." Proc. J. C. B. L. 25, 356~359 (1983). (In Japanese).

66. Y. Nakagawa and L. A. Horrocks: Separation of Alkenylacyl, Alkylacyl, and Diacyl Analogues and Their Molecular Species by High Performance Liquid Chromatography. J. Lipid Res., 24, 1268~1275 (1983).

67. M. Smith, P. Monchamp and F. B. Jungalwala: Separation of Molecular Species of Sphingomyelin and Ceramide by Argentation and Reversed-Phase HPLC. J. Lipid Res., 22, 714~719 (1981).