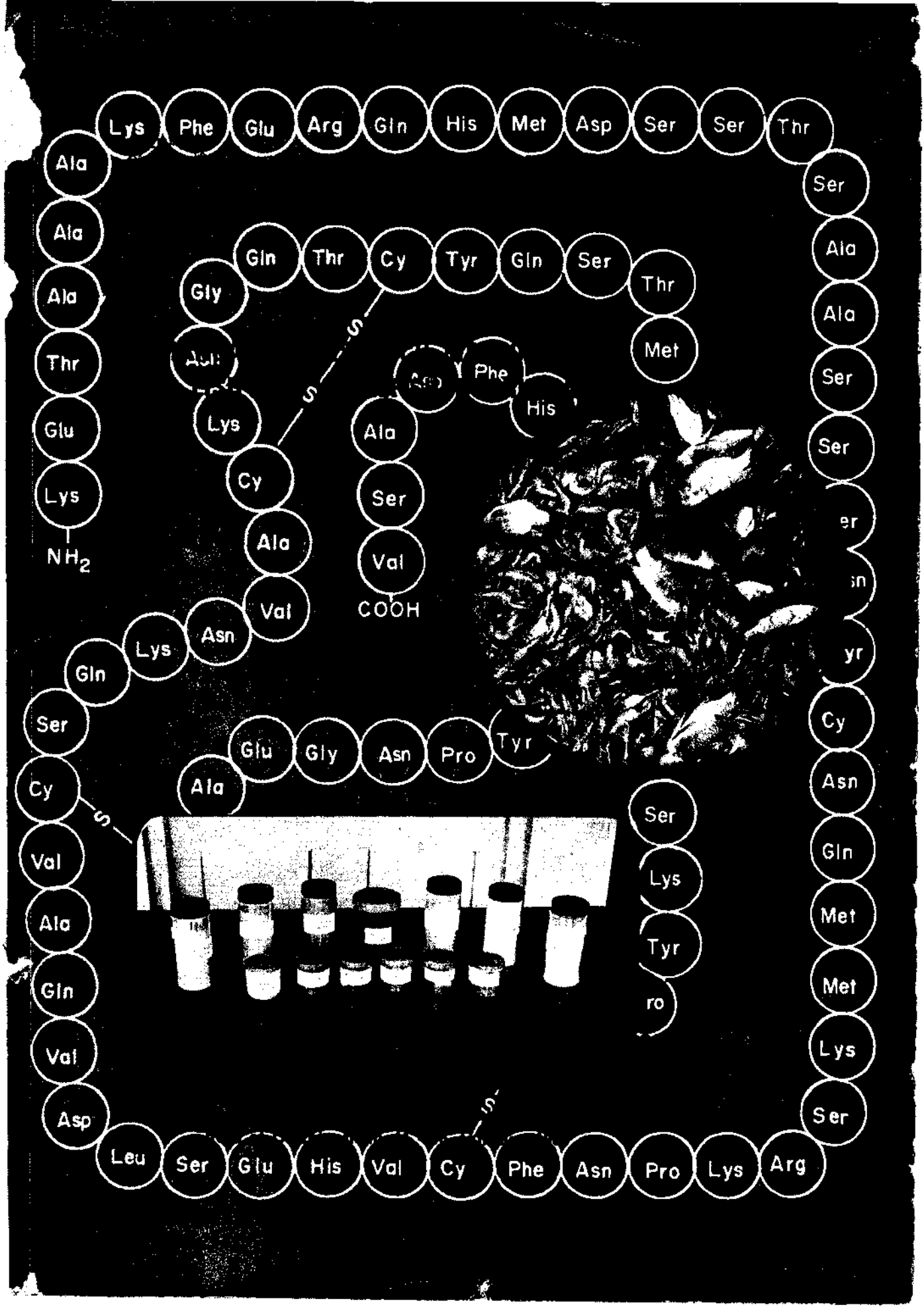

Lys Phe Glu Arg Gln His Met Asp Ser Ser Thr
Ala Ala Ala Thr Glu Lys NH2
Ser Ala Ala Ser Ser er in yr Cy Asn Gln Met Met Lys Ser
Gln Thr Cy Tyr Gln Ser Thr Met
Gly
Lys Cy Ala Val Asn Lys Gln Ser Cy Val Ala Gln Val Asp Leu Ser Glu His Val Cy Phe Asn Pro Lys Arg
Ala Ser Val COOH
Phe His
Glu Gly Asn Pro Tyr
Ala
Ser Lys Tyr ro

CMFRI SPECIAL PUBLICATION

Number 8

MANUAL OF RESEARCH METHODS FOR FISH AND SHELLFISH NUTRITION

Issued on the occasion of the Workshop on
METHODOLOGY FOR FISH AND SHELLFISH NUTRITION
organised by
The Centre of Advanced Studies in Mariculture,
Central Marine Fisheries Research Institute,
held at Cochin from 11 - 16 January 1982

Published by: **E. G. SILAS**
Director
Central Marine Fisheries Research Institute
COCHIN

PREFACE

The Centre of Advanced Studies in Mariculture established at the Central Marine Fisheries Research Institute has been conducting Workshops in Research Methodologies on specialised disciplines with a view to enhance the competence of the scientific workers specialising in researches connected with mariculture. The main emphasis in mariculture research has been directed towards the development of economically viable culture techniques for culturable species of fish and shellfish, with a view to augmenting the fish and shellfish production of the country. In order to develop low-cost technologies the essential operational inputs have to be rationally utilized.

It has been well established that feeding constitutes the major cost of production, often exceeding 50 per cent of the operating costs in intensive aquaculture operations. Two main factors affecting the cost of feeding are composition of the diet and efficiency of feed conversion. In order to develop least-cost formula diets of high conversion efficiency, knowledge of the nutritional requirements of the different species during the different phases of the life cycle and the nutritive value of the complex feed ingredients available in the country to the candidate species is a prerequisite.

The existing information on the nutritional requirements of cultivated species of fish and shellfish in India, is meagre and recently research has been intensified in this area. If researches on this field could be carried out using standardised experimental procedures, the data obtained on the nutritional requirements of the different species could be stored in a fish and shellfish nutrition data bank, from where data could be disseminated to the users such as feed manufacturers, farmers, extension workers and research workers as and when required. It is also necessary that the data collected on the chemical composition of the feed ingredients and their nutritive value for the species should be based on standard chemical methods and experimental procedures so that the data could be stored in

the data bank which eventually could become a National Fish Feed Information Centre.

To undertake studies on the above lines, especially by the technicians and research workers entering afresh into the field, the need of practical guides describing the research techniques and methods, planning of investigations, collection of data and their interpretation need not be emphasized. Keeping this in view, the present manual on Research Methods in Fish and Shellfish Nutrition is issued by the Centre of Advanced Studies in Mariculture on the occasion of the Workshop on Methodology of Fish and Shellfish Nutrition.

Dr. Akio Kanazawa, Professor of Nutritional Chemistry, University of Kagoshima, Japan and Consultant in Fish and Shellfish Nutrition at the CAS in Mariculture, has been kind enough to cooperate with the Scientists of CAS in Mariculture of the Central Marine Fisheries Research Institute in the preparation of this manual. There are chapters in this manual covering various methods on composition analysis of feeds, including growth inhibitors and toxins; determination of digestibility coefficient; protein evaluation; bioenergetics; determination of essential amino acid requirements using radioisotope method; research test diets for fishes and prawns; feed formulation methods; experimental design, etc. Methods of preparation of microparticulate diets, phytoplankton and zooplankton culture methods, etc. are also included to facilitate larval nutrition studies. Many of the methods given in the manual have been standardized for fish and shellfish nutrition studies in India and abroad. The users can also gain maximum benefit by suitable modifications of other methods which are given as guidelines.

I would like to thank all the scientific and technical staff especially Shri S. Ahamed Ali, Dr. K. Alagarswami, Shri D.C.V. Easterson, Shri C.P. Gopinathan, Shri T. Jacob, Shri M.S. Muthu, Dr. R. Paul Raj, Dr. A.G. Ponniah and

Dr. P. Vedavyasa Rao who have rendered assistance during the preparation of this manual. Thanks are also due to Shri Johnson, Librarian and Shri Kambadkar, Technical Assistant, Central Marine Fisheries Research Institute, for the help rendered by them in printing this manual.

(E.G. Silas)
Director, CMFRI,
Sub-Project Coordinator,
Centre of Advanced Studies in Mariculture

CHAPTER 7

METHODS IN LIPID ANALYSIS*

1 TOTAL LIPID EXTRACTION AND PURIFICATION
(Bligh and Dyer method)

1.1 Apparatus

(a) Tissue homogenizer or Waring Blender
(b) Buchner filtration apparatue
(c) Separating funnel
(d) Rotary evaporator

1.2 Reagents

(a) Chloroform
(b) Methanol

1.3 Procedure

To 100 g fish muscle (moisture, about 30%), add 100 ml $CHCl_3$ and 200 ml methanol and homogenize with a waring blender for 2 min. Further homogenize for 30 sec. after adding 100 ml $CHCl_3$, and then again homogenize for 30 sec. after adding 100 ml water. Filter the homogenate with a Buchner filtration apparatus, and then transfer the filtrate into separating funnel. Evaporate chloroform under reduced pressure by using a rotary evaporator. The chloroform extract so obtained is corresponding to total lipids. Report % lipids of fresh matter.

2 FREE FATTY ACID IN CRUDE AND REFINED OILS
(National Cottonseed Products Association Method)

2.1 Apparatus

(a) Conical flask, 250 ml and 150 ml
(b) Burettes
(c) Water bath

---

* Prepared by Akio Kanazawa, Professor of Nutritional Chemistry, Kagoshima University, Japan.

2.2 Reagents

(a) Alcohol
(b) Phenolphthalein
(c) Sodium hydroxide

2.3 Procedure

2.3.1 In crude oils:

Weigh 7.05g well mixed oil into 250 ml flask. Add 50 ml alcohol, previously neutralized by adding 2 ml phenolphthalein solution and enough 0.1 N NaOH to produce faint permanent pink. Titrate with 0.25 N NaOH with vigorous shaking until permanent faint pink appears and persists at least 1 min. Report as % free fatty acids expressed as oleic acid. One ml of 0.25 N NaOH used in titration corresponds to this percentage.

2.3.2 In refined oils:

Put ca 50 ml alcohol in clean, dry 150 ml flask and add few drops of the oil and 2 ml phenolphthalein. Place flask in H_2O at 60–65°C until warm, and add enough 0.1 N NaOH to produce faint permanent pink. Weigh 56.4 g oil into the neutralized alcohol and titrate, occasionally warming and violently shaking mixture until same faint permanent pink appears in supernatant alcohol. Multiply ml 0.1 N NaOH by 0.05 and report as % free fatty acids expressed as oleic acid.

3 SEPARATION OF LIPIDS BY THIN-LAYER CHROMATOGRAPHY

3.1 Apparatus

(a) Thin layer chromatography set
(b) Oven
(c) Dessicator

3.2 Reagents

(a) Silica Gel 'G' (Merck)
(b) Calcium chloride
(c) Petroleum ether

(d) Sulphuric acid
(e) Ethanol
(f) Acetic acid

3.3 Procedure

3.3.1 Preparation of TLC-Plate:

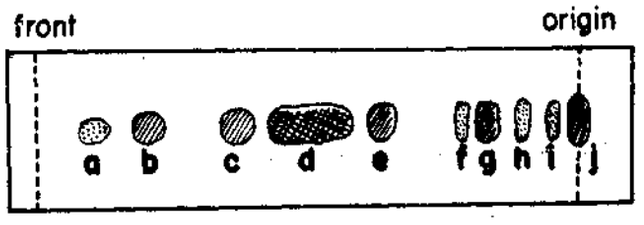
Five glass plates (20 X 20 cm) are coated to a thickness of 0.25 mm with a slurry of 30 g of silica gel G (Merck etc.) in 60 ml of distilled water, air-dried for 15 min. and activated in a oven for 40 min. at 110°C. The coated plates are stored in a dessicator over anhydrous calcium chloride.

3.3.2 Spotting and development:

Sample (total lipids from marine animals), 10-50 µg in chloroform-methanol (2 : 1), is applied about 1.5 cm from the bottom of the plate, and the plate is placed in an chromatographic tank containing the solvents;petroleum ether-ethanol-acetic acid (87.5 : 12.5 : 1). The solvent front is allowed to rise 15 cm from the origin.

3.3.3 Detection of spots:

The spots are visualised by spraying with 50% sulfuric acid or iodine vapour



a, hydrocarbons; b, steryl esters + waxester; c, glyceryl ether; d, triglycerides; e, free fatty acids; f, fatty alcohols; g, sterols; h and i, diglycerides and monoglycerides; j, phospholipids.

4 GAS LIQUID CHROMATOGRAPHY OF FATTY ACIDS AND STEROLS

4.1 Apparatus

Gas-chromatographic unit with a hydrogen flame ionisation detector

4.2 Reagents

Reference compounds and other chemicals

4.3 Sample

Cod liver oil or short-necked clam lipids

4.4 Procedure

4.4.1 Preparation of instrument:

Equilibration of column and detector: Place column in chromatograph and connect inlet to 30 lb/sq. in. N_2 supply. Do not connect effluent to detector. Bring column temperature to adequate temp. Check gas flow rate. If the gas flow rate is not suitable, adjust pressure to bring flow rate into the suitable range. Let run overnight under these conditions.

Connect column effluent to detector: Set balance current to zero and polarity to adequate position (negative or positive). Turn on recorder and set pen to zero with 'zero adjustment'. Let recorder run ca for 10 min. or more to check that baseline is steady. Adjust cell voltage to 1000 and bring pen to zero with balance current knob. Let recorder run until baseline is steady. If it does not become steady in 10 min., turn off recorder and let column and detector come to equilibrium.

Test of equipment with standards: Set relative gain to 100 or 1000. Inject 1 µl of standard solution and mark chart to show time of injection.

4.4.2 Chromatography:

Inject 1 µl standard solution and mark time of injection on chart. If the separation of peaks is not complete, adjust conditions to achieve maximum separation and sharpness of peaks.

4.4.3 Identification of peaks:

(1) Comparison of relative retention time with standards
(2) Semi-log plot of Rt (or RRT)

4.4.4 Quantitative determination:

(1) Peak area: Interglator method
(peak height) X (width in half-peak height) weight method
(2) Relative responce
Peak area/unit mol. or weight
(3) % composition of each compounds
Internal normalization method
internal standard method

4.4.5 Error in GLC:

(1) Peak area: 1% error.....5mm (width in half-peak height), difficult
1% error.....10mm (width in half-peak height), or more
(2) Sample size
(3) Temperature of injection part
(4) Tailing and leading

Note:

Date.....
Model.....
Sample..... µg/ml of solvent
Column..... _____m long X_____i.d.
Temp. _____°C
Column packing _____wt %
support_____(mesh_____ _____

Carrier gas

Flow rate _____ _____ml/min

Hydrogen flow rate_____ml/min

Air flow rate_____ml/min

Detector..... Bridge current_____mA,

Detector temp._____°C

Applied voltage_____V

Range....._____mV, V

Chart speed....._____mm/min

5 SEPARATION OF NEUTRAL LIPIDS FROM POLAR LIPIDS (BY COLUMN CHROMATOGRAPHY)

5.1 Method-1. Column Chromatography on Kieselgel 60:

Total lipids (58 mg)

↓ Kieselgel 60 (2.0 g, 13-fold)
Elution with chloroform-methanol (98 : 2) (80 ml, 500-fold) and then with methanol (55 ml, 350-fold)

Neutral lipids	Polar lipids
($CHCl_3$-MeOH fr.)	(MeOH fr.)

5.2 Method-2. Column Chromatography on silicic acid:

Total lipids

↓ Silicic acid (Malincrodt Co., 13-fold)
eluted with chloroform (500-fold) and then with methanol (350-fold)

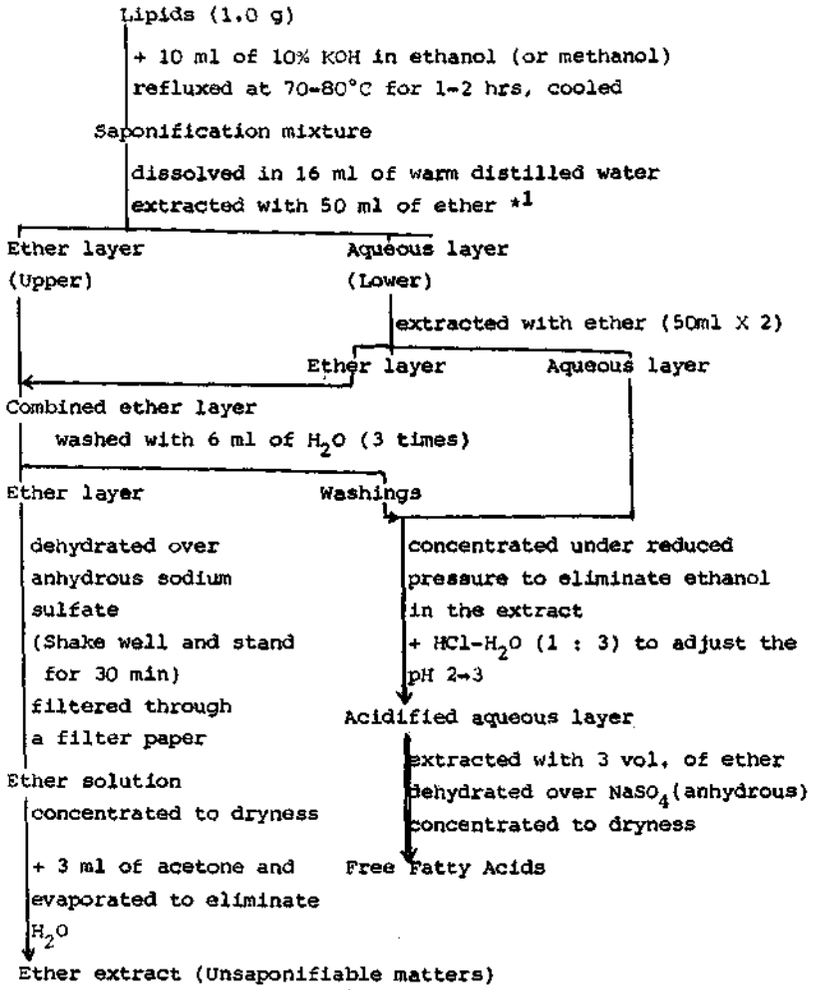
Neutral lipids	Polar lipids
(CHCl fr.)	(MeOH fr.)

---

*1 In the case of the samples which had not been analyzed previously, check the separation of neutral and polar lipids by thin-layer chromatography on Silica Gel G with petroleum ether-diethyl ether-acetic acid (90 : 10 : 1) or other appreciate solvent systems.

Notice: Samples (total lipids) are required to be completely dried prior to the column chromatography. The application of lipids containing water causes the incomplete separation of the both neutral and polar lipids.

6 UNSAPONIFIABLE MATTERS AND FATTY ACIDS (SAPONIFICATION PROCEDURE)



*1 Another method: After saponification, the saponification mixture was diluted with H_2O to contain 50% of ethanol concentration. Then, the diluted saponification mixture was extracted with 1.5 vol. of ether twice. This method will give a good result for lipids from marine invertebrates.

7 PREPARATION OF FATTY ACID METHYLESTERS (BY HYDROGEN CHLORIDE/MeOH)

Free fatty acids (about 10 mg)
+ 3% hydrogen chloride in methanol ....4 ml
(from commercial sources)
+ dry benzene .....0.5 ml
refluxed at 70-80°C for 2-3 hrs *1
cooled
Reaction mixture
+ 2 vol. of distilled water
extracted with 4 vol. of ether (or petroleum ether)*2

Petroleum ether layer — Aqueous layer
Aqueous layer: extracted with petroleum ether
Petroleum ether layer — Aqueous layer
Aqueous layer: extracted with petroleum ether
Petroleum ether layer — Aqueous layer

Combined petroleum ether layer
washed with distilled water to remove contaminating HCl
dehydrated over anhydrous Na_2SO_4
filtered
concentrated
Fatty acid methylesters (Ready for GLC)*3

---

*1 For small quantities of samples, use the reaction block For large quantities of samples, use the apparatus for refluxing.

*2 Petroleum ether or hexane is better for a solvent, because ether sometimes contains oxidative compounds

*3 To avoid the contamination and damage of column, it is better to purify the fatty acid methylester by TLC (Kieselgel G/Petroleum ether-ether-acetic acid (90:10:1)) or by column chromatography (Kieselgel 60 .... 5g; solvent, 5% ether/P.E. 50 ml).

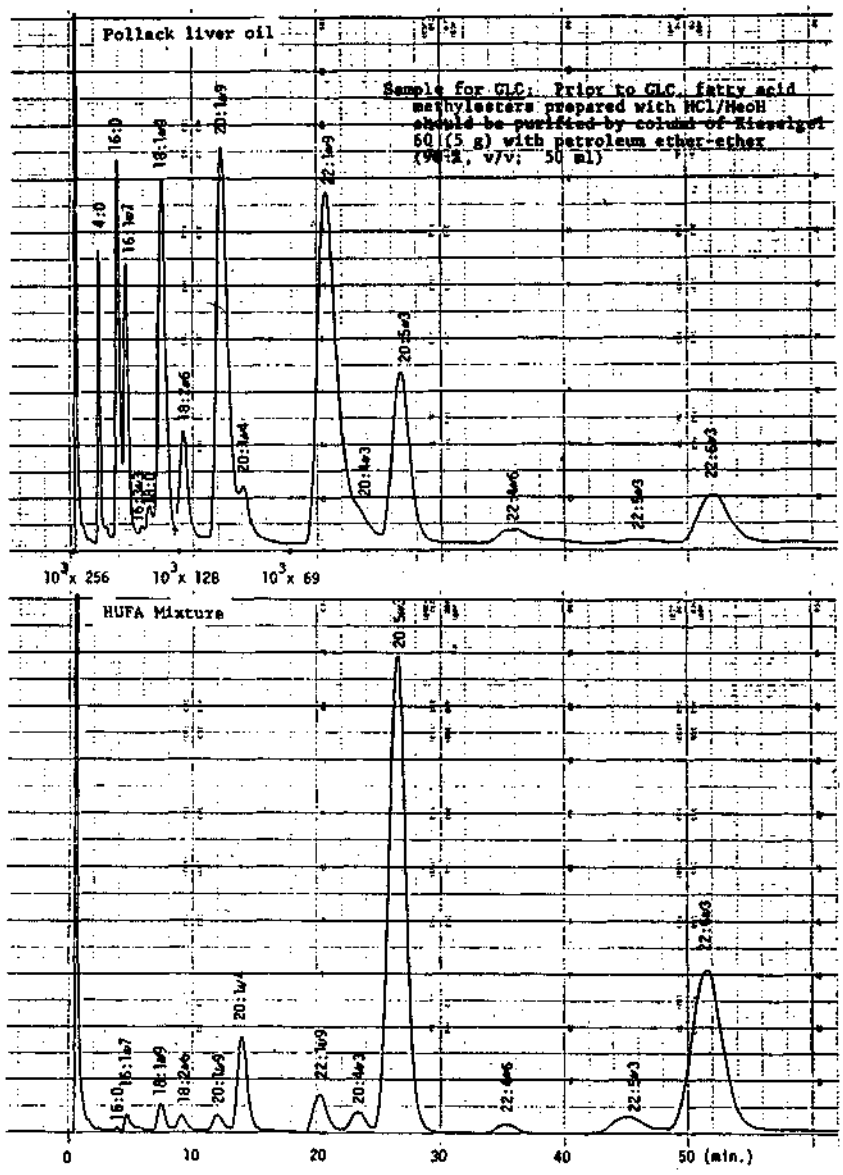
8 GAS-LIQUID CHROMATOGRAPHIC DATA OF FATTY ACID METHYLESTERS

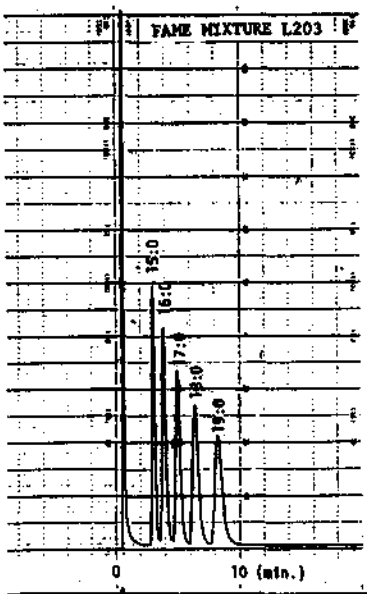
Column: 10% DEGS on 60-80 mesh Shimalite

Peak No.	Fatty acid	Rt (min.)	RRT*1	ECL*2	Ref. ECL
1	14:0	2.05	0.29		
2	15:0	2.80	0.39		
3	15:1	3.15	0.44	15.4	
4	16:0	3.83	0.54		
5	16:1W7	4.40	0.62	16.4	
6	17:0	5.25	0.73		
7	18:0	7.15	1.00	18.00	18.00
8	18:1W9	8.00	1.12	18.3	18.51
9	18:2W6	9.70	1.36	18.9	19.30
10	18:3W3	12.65	1.77	19.8	20.40
11	20:1W9	14.65	2.05	20.3	20.44
12	20:2W6	17.75	2.48	20.9	21.36
13	20:3W6 20:3W9	20.45	2.86	21.4	22.13 21.65
14	20:4W6	22.80	3.19	21.9	22.43
15	20:4W3	26.65	3.73	22.2	
16	22:1W9	27.15	3.80	22.3	22.28
17	20:5W3	29.60	4.14	22.6	23.57
18	22:4W6	40.60	5.68	23.6	24.58
19	22:5W6	46.90	6.56	24.0	24.97
20	22:5W3	53.60	7.50	24.5	25.38
21	22:6W3	60.40	8.45	24.8	26.03

*1 RRT: Relative retention time (relative to 18:0)

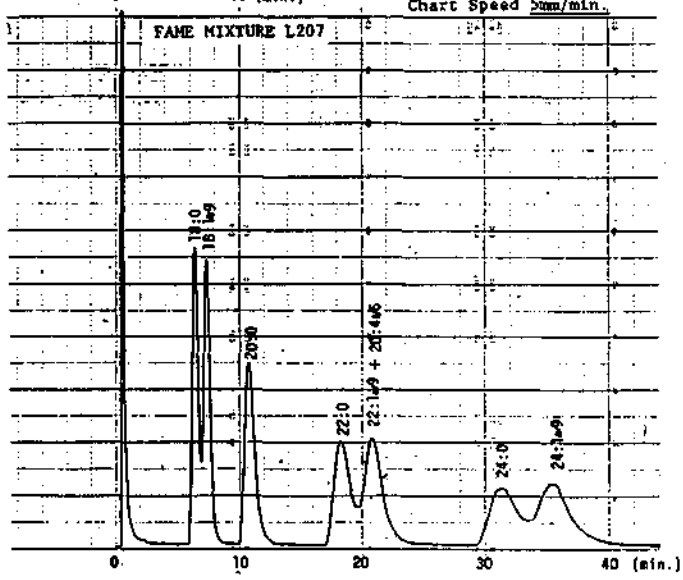
*2 ECL: Equivalent chain length


Pollack liver oil
Sample for GLC: Prior to GLC, fatty acid methylesters prepared with HCl/MeoH should be purified by column of Kieselgel 60 (5 g) with petroleum ether-ether (90:2, v/v; 50 ml)
4:0
16:0
16:1ω7
18:1ω9
20:1ω9
22:1ω9
18:2ω6
20:4ω4
20:5ω3
20:4ω3
22:4ω6
22:5ω3
22:6ω3
10³x 256
10³x 128
10³x 69
HUFA Mixture
20:5ω3
22:6ω3
0
10
20
30
40
50 (min.)



CHROMATOGRAM

Date 28th June 1981 Operator SAKAMOTO
Room Temp. 32 °C
Model Shimadzu-4BP
Sample FAME MIXTURE L203 & L207
Cod liver oil, HUFA Mix.
Size 50µg
Solvent Hexane
Column 3m ID 3mm
Temp. 180 °C
Packing 10% DEGS
Support Shimalite W
Mesh 60-80
Carrer gas N_2
Flow rate 40ml/min.atm.
Inject Press. 1.2kg/cm
H Flow Rate 50 ml/min.
Air Flow Rate 1000 ml/min.
Sensitivity 10^3 x 256
Ditector Temp. 210 °C
Injection Temp. 210 °C
Chart Speed 5mm/min.



Fatty acid composition of several samples determined by GLC on DEGS

Fatty acid	Male prawn*	Female prawn*	Rotifer (Chlorella)**	Artemia egg**	Chlorella (marine)**	Prawn (Tokiwa)
12:0	t	0.1	-	-	-	0.5
12:1	t	t	-	-	-	0.3
13:0	0.2	0.1	-	-	-	0.2
13:1	t	t	-	-	-	-
14:0	2.4	1.8	4.0	1.1	5.2	1.3
14:1	0.5	0.5	-	-	-	t
15:0	2.6	1.7	-	-	-	0.6
16:0	15.4	16.1	14.4	13.2	19.7	13.7
16:1W7	6.9	8.3	20.4	4.5	30.5	4.0
17:0	2.0	2.1	-	1.4	-	0.4
16:2(W7?)	1.7	1.3				0.5
18:0	6.5	6.2	2.2	4.0	0.7	8.2
18:1W9	9.0	11.3	10.5	27.8	2.7	21.8
19:0	0.3	0.3	-	-	-	
18:2W6	2.0	1.5	4.7	6.2	2.4	7.9
18:3W3	0.4	0.5	0.1	27.7	0.2	0.5
18:4W3	2.0	2.4	-	3.6	-	0.3
20:0	-	-				
20:1W9	7.9	5.4	1.7	-	-	8.7
20:2W6	1.2	1.2	-	-	-	0.7
20:3W9	-	-				
20:3W3	0.8	0.7	4.1	0.6	3.6	1.4
20:4W6	3.3	3.3				
20:4W3	1.8	1.5	0.2	0.3	-	-
20:5W3	13.1	12.7	27.7	1.8	27.8	17.4
22:1	-	-	1.8	-	-	-
22:3(W6?)	0.4	0.4	-	-	-	t-0.5
22:4W6	2.2	2.4	-	-	-	-
22:5W6	-	-	-	-	-	0.2
22:5W3	3.0	2.0	3.0	-	1.7	0.5
24:2(W9?)	0.8	0.8	-	-	-	-

Fatty acid	Male prawn*	Female prawn*	Rotifer (Chlorella)**	Artemia egg**	Chlorella (marine)**	Prawn (Tokiwa)
22:6W3	7.6	10.6	t	-	0.3	5.1
24:4	4.0	3.5	-	-	-	-
24:5	0.3	0.3	-	-	-	-

* J.C. Guary et al. (1974): 40, 1027 Bull. Jap. Soc. Sci. Fish
** Watanabe et al. (1978): 44, 1109 ,, ,,
Watanabe et al. (1978): 44, 1115 ,, ,,

9 PREPARATION OF ACETATE DERIVATIVES (ACETYLATION PROCEDURE)

Sterols (Free form)

↓ put in a well-dried container
+ dry pyridine-acetic anhydride (1 : 1)*1
dissolved sterols completely
refluxed at 70°C for 40-60 min. (or stood at room temperature for 24 hrs)

Reaction mixture

↓ Excess of the reagents was evaporated under reduced pressure by using a rotary evaporator (or under the stream of nitrogen gas)

(If the reaction mixture has a smell of pyridine and acetic anhydride, add ethanol and evaporate.)

Crude steryl acetates

↓ Purified by TLC or by Alumina column chromatography*2

Pure steryl acetates

Check by TLC on Kieselgel G/$CHCl_3$ whether free sterols turned to acetates completely (Sometimes, the complete formation of acetate is not accomplished due to certain reasons.)

*1 Acetic anhydride ..... Do not use an old reagent
Dry pyridine ..... Dry over sodium hydroxide

*2 TLC: Sample was applied onto the plate as a streak. After development with chloroform, the plate was dried and sprayed with Rhodamin 6G in acetone. The band corresponding to steryl acetate located under UV-light (320 mμ) was eluted with ether.
Alumina column chromatography: Sample was loaded on 10g of Alumina (II-III) and eluted with 100 ml of hexane-benzene (5 : 1) or 15% ether in hexane.

10 RELATIVE RETENTION TIME (RRT) OF STEROL IN GLC ON OV-17 AND ON QF-1

Carbon	OV-17	QF-1	Sterol
26	0.66	0.66	24-Norcholesta-5,22(E)-dienol
26	0.66	0.69	24-Norcholest-22(E)-enol
26	0.78	0.72	24-Norcholesta-7,22(E)-dienol
27	0.88	0.90	5 -Cholestan-3 -01 (Coprostanol)
27	0.88	0.88	Cholesta-5,22(Z)-dienol (ciz-22-Dehydrocholesterol)
27	0.94	0.89	Cholesta-5,22(E)-dienol (trans-22-Dehydrocholesterol)
27	1.00	1.00	Cholest-5-enol (Cholesterol)
27	1.01	1.05	5 -Cholestan-3 -01 (Cholestanol)
28	1.05	(1.07)	24-Methylcholesta-5,22(Z)-dienol
28	1.07	(1.04)	23-Methylcholesta-5,22-dienol (non-identified)
27	1.07	0.93	27-Norergosta-7,22(E)-dienol (Amuresterol)
27	1.10	0.98	Cholesta-7,22(E)-dienol
28	1.14	1.08	24-Methylcholesta-5,22(E)-dienol (24R:Brassicasterol) (24S:Clionasterol)
27	1.16	1.10	Cholest-7-enol (Lathosterol)
27	1.19	1.08	Cholesta-5,24(25)-dienol (Desmosterol)
28	1.28	1.28	24-Methylcholest-5-enol (24R: Campesterol) (24S: 22,23-Dihydrobrassicasterol)
28	1.33	1.27	24-Methylenecholest-5-enol (24 Methylene-cholesterol)
28	1.33	1.21	24-Methylcholesta-5,7,22-trienol (Ergosterol)
28	1.34	1.19	24-Methylcholesta-7,22(E)-dienol (24S: Stellasterol)
29	1.36	1.26	23,24-Dimethylcholesta-5,22-dienol
29	1.42	1.32	24-Ethylcholesta-5,22(E)-dienol (24S: Stigmasterol) (24R: Poliferasterol)
29	1.53	1.43	23,24-Dimethylcholesta-5,23-dienol
28	1.54	1.41	24-Methylcholest-7-enol
28	1.57	1.39	24-Methylenecholest-7-enol(Episterol)

Carbon	OV-17	QF-1	Sterol
29	1.59	1.54	24-Ethylcholest-5-enol (24R: -Sitosterol) (24S:Chondrillasterol)
29	1.68	1.49	(E)-24-Ethylidenecholest-5-enol (Fucosterol)
29	1.70	1.61	23-Demethylgorgost-5-enol (23-Demethylgorgosterol)
29	1.78	1.53	(Z)-24-Ethylidenecholest-5-enol (Isofucosterol
29	1.89	1.69	24-Ethylcholest-7-enol (24R: Spinasterol) (24S: Chondrillastenol)
29	2.00	1.63	(E)-24-Ethylidenecholest-7-enol
29	2.10	1.68	(Z)-24-Ethylidenecholest-7-enol
30	2.26	2.17	Gorgost-5-enol (Gorgosterol)
30	2.30	2.28	Gorgostanol
30	2.64	2.38	Gorgost-7-enol (Acanthasterol)

11 *SOLVENT SYSTEMS FOR SEPARATION OF STEROID HORMONES BY TLC ON KIESELGEL G*

Steroids	Solvent system*1								
	1	2	3	4	5	6	7	8	9-11
5α-Pregnane-3,20-dione	0.33	0.70		0.47		0.51	0.48	0.90	
'-Cholestenol	0.23			0.36			0.39	0.70	
Progesterone	0.15	0.67	0.66	0.40		0.43	0.47	0.83	
Androstenedione		0.65	0.56	0.37		0.40	0.42	0.82	
Pregnenolone	0.12	0.49	0.47	0.29	0.52 *2	0.37	0.33	0.52	
Dehydroepiandrosterone		0.50	0.41	0.27	0.43 *2	0.35	0.32	0.52	
17α-OH-progesterone		0.55		0.25			0.35	0.50	
3β-OH-5α-pregnane-20-one		0.49		0.27		0.31	0.30	0.57	
Testosterone	0.05	0.43		0.21		0.23	0.29	0.43	
5α-Pregnane-3β,20β-diol		0.32		0.19		0.23	0.23	0.33	
11-Ketotestosterone		0.21							
3β,6α-Dihydroxy-pregnane-20-one				0.04			0.08	0.23	

*1 System 1: Benzene-ethyl acetate (100 : 15). For separation of steroid hormones from sterols..... developed twice

System 2: Chloroform-methanol (97 : 3)
System 3: Ether alone
System 4: Chloroform-ethyl acetate (13 : 7)
System 5: Dichloromethane-acetone (24 : 1)
System 6: Benzene-ethyl acetate (5 : 4)
System 7: Benzene-acetone (8: 2)
System 8: Benzene-methanol (9 : 1)

The following systems are useful for separation of the mixtures

System 9: Benzene-methanol-2N NH_4OH (65 : 35 : 1).....
Cholesteryl sulfate/Pregnenolone sulfate

System 10: Benzene-ethyl acetate (3 : 1).....Progesterone/5α-Pregnane-3,20-dione

System 11: Cyclohexane-ethyl acetate (1 : 1).....5α-Pregnane-3β, 20β-diol/Testosterone

System 12: Benzene-ethyl acetate (1 : 1), twice.....,5α-Pregnane-3β, 20α-diol/5α-Pregnane-3β,20β-diol

System 13: Chloroform-ethyl acetate (100 : 15).....5α-Pregnane-3, 20-dione/Sterols/Progesterone

*[2] Rf values of acetate derivatives.