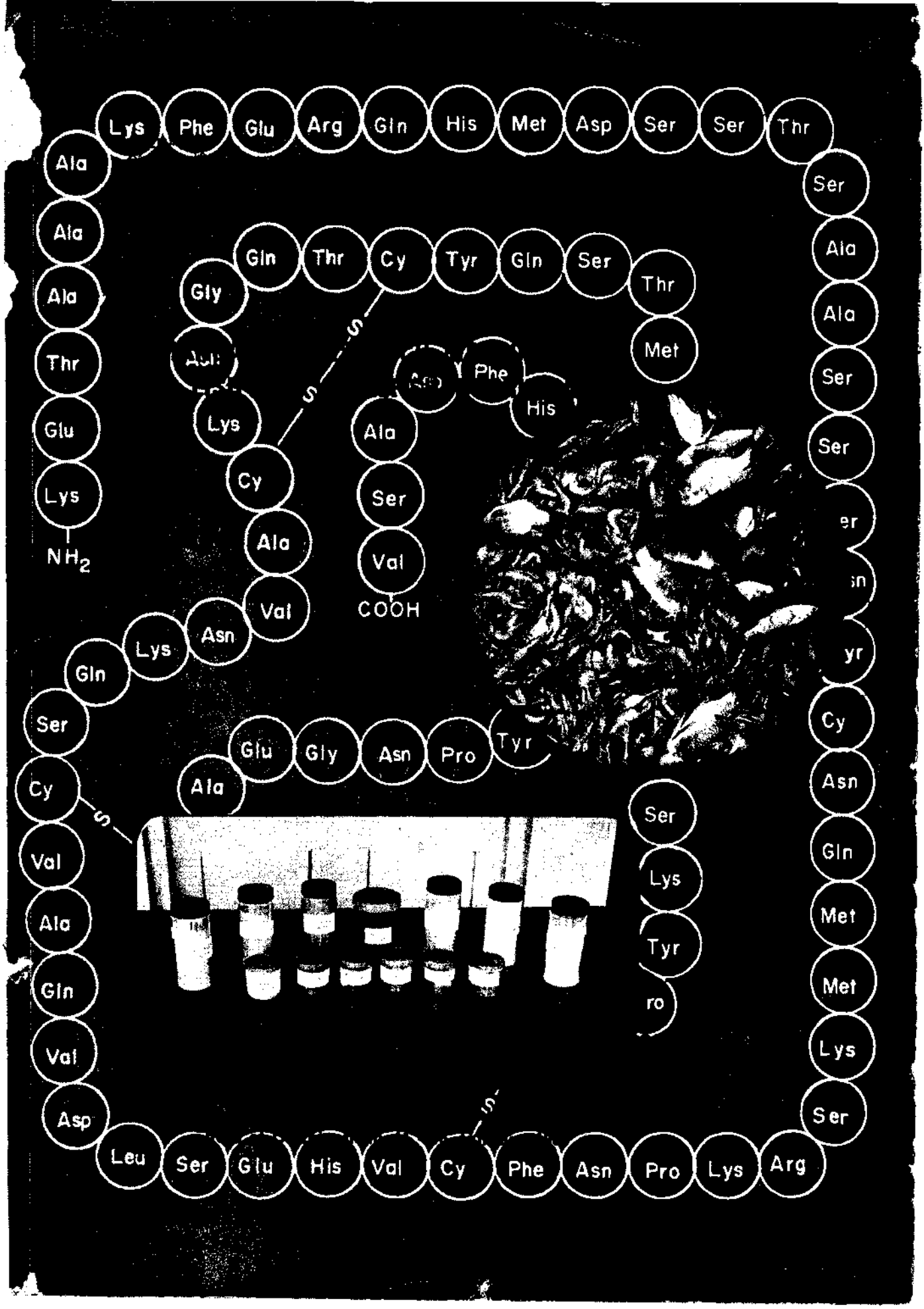
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Gln Thr Cy Tyr Gln Ser Thr Met
Gly
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Phe His
Glu Gly Asn Pro Tyr
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Ser Lys Tyr ro

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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,
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Published by: **E. G. SILAS**
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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

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CHAPTER 6

METHODS IN SUGAR ANALYSIS*

1 DETERMINATION OF TOTAL SUGARS IN MOLASSES

1.1 Apparatus

(a) Electric heater, and
(b) Conical flasks, 300 ml.

1.2 Reagents

(a) Fehling's solution (Soxhlet modification):

(i) Dissolve 34.639 g of copper sulphate $5H_2O$ in water and make up to 500 ml. Filter, and

(ii) Dissolve 173g of potassium sodium tartrate $4H_2O$ and 50g sodium hydroxide in water, dilute to 500 ml, stand for two days, and filter through prepared asbestos.

(b) Invert sugar standards:

Prepare stock solution by adding 5 ml of hydrochloric acid (sp.g 1.18) to 9.5g of sucrose in solution and dilute to about 100 ml. After storing for two days at room temperature, dilute to 1 litre. Prepare working solutions (5 mg/ml) by pipetting 100 ml of the stock solution into a 200 ml volumetric flask, and neutralising with 20 percent sodium hydroxide using phenolphthalein as the indicator. Dilute to mark and mix.

(c) Hydrochloric acid (sp. g 1.18),

(d) Hydrochloric acid (0.5N),

(e) Sodium hydroxide (20%),

(f) Phenolphthalein indicator (1% solution in alcohol),

(g) Methylene blue indicator (1% aqueous solution).

* Prepared by Akio Kanazawa, Professor of Nutritional Chemistry, Kagoshima University, Japan and R. Paul Raj, Central Marine Fisheries Research Institute, Cochin-18.

1.3 Procedure

Dissolve 8g of liquid molasses and make up to 500 ml. Carry out an acid hydrolysis on 100 ml of the filtrate by adding 5 ml of hydrochloric acid (sp. g 1.18) and allow to stand for 24 h. Neutralise with sodium hydroxide (20 percent) using phenolphthalein as indicator, and then dilute to 200 ml.

1.3.1 Standardisation of Soxhlet solution:

Pipette 10 ml of Soxhlet solutions (i) and (ii) into a conical flask, mix, and add 30 ml of water. Add from a burette a volume of working standard that is almost sufficient to reduce the copper in the Soxhlet solution. Bring to boiling and continue boiling for two minutes. Add four drops of methylene blue and rapidly complete the titration, while still boiling, until a bright orange colour is resumed. Repeat several times and determine the volume of solution required to completely reduce 20 ml of the Soxhlet solution.

1.3.2 Titration of sample:

First, carry out an approximate titration: Pipette 10 ml of solutions (i) and (ii) into a flask and add 10 ml aliquot of the sample solution. Add 40 ml of water and bring to boil. If blue colour persists, titrate with a standard working solution and calculate the approximate sugar content of the sample.

To accurately determine the sugar content, pipette 10 ml of Soxhlet solution (i) and (ii) into a flask and add an aliquot of the sample solution. The volume of sample used will depend on the sugar content of the sample (see Table 1).

Table 1. Sample Volumes Used in Soxhlet Titration

ml H_2O	ml sample	g sample in aliquot	Total sugar as invert, %
40	10	0.08	73
35	15	0.12	82-58
30	20	0.16	61-41
25	25	0.20	49-35
20	30	0.24	41-29

Add water as indicated in the table, mix, and boil. During boiling, add a quantity of working standard from a burette so that the titration is nearly complete. Add methylene blue and complete the titration.

1.3 Calculation

Calculate the percentage sugar (as invert) by the formula:

$$\% \text{ sugar} = (F-M) \times l \times 100/W$$

where F = is the volume of standard needed to reduce 20 ml of Soxhlet solution.

M = is the volume of standard sugar solution required to complete the back titration.

l = is the weight of invert sugar in 1 ml of working standard, and

W = is the weight of sample in aliquot used.

2 GAS-LIQUID CHROMATOGRAPHY (GLC) OF SUGARS

The trimethylsilyl derivatives (TMS) of charbohydrates were shown to be the most satisfactory form for general analytical studies in gas liquid chromatography. The procedure

given in this Chapter has been routinely used for the GLC of Sugars at the University of Kagoshima.

2.1 Preparation of TMS derivatives

TMS reagents:

Anhydrous pyridine, 5.0 ml
(dried over KOH)

Hexamethyldisilane, 1.0 ml
(Kodak Eastman)

Trimethylchlorosilane, 0.5 ml
(Kodak Eastman)

A mixture of the above compounds, in the proportion given is used for the trimethylsilylation reaction. The reagent should not be more than slightly turbid at first; if it is very cloudy, the pyridine is not sufficiently dry. The reagent mixture should be stable for at least 7 days at room temperature, provided moisture is carefully excluded.

2.2 Procedure

Weigh a known amount of sugar (about 10 mg) into a plastic-stoppered vial and add 1 ml of the above pyridinesilanes mixture. The mixture is shaken at intervals until the sugar dissolves completely. Occasionally, difficulty may arise from the low solubility of the crystalline forms of particular sugars such as sucrose and trehalose. Heating of the mixture at 70°C for 3-4 min. was found useful in dissolving these sugars without interfering with the trimethylsilylation process. After which the mixture is allowed to stand for at least 25 min. at room temperature before injections are made into the gas chromatograph.

2.2.1 Analysis of monosaccharides and disaccharides:

Standard Sugars: The standard sugars purchased from Applied Science Laboratories, State College Penna, U.S.A. can be used.

2.2.1.1 Qualitative analysis:

Gas liquid chromatography is carried out under the conditions as shown in Table 2 using two types of column packing.

Table 2. Conditions used in gas liquid chromatography

Instrument	Shimadzu gas chromatograph GC-4BP
Column	Stainless steel, 3 mm i.d. X 3 m long
Column temp.	Programm temp. 140 - 260°C, rate 4°C/min
Injection temp.	230°C
Detection temp.	290°C
Carrier gas	Nitrogen 44 ml/min
Detector	FID
Column packing	1.5% SE-30 or 1.5% OV-17 (Shimalite)

Table 3 and 4 show the retention times of standard sugars. The examined reference sugars are well separated each other by GLC on 1.5% OV-17. The separation of maltose and trehalose is not achieved by GLC on 1.5% SE-30, however, this packing has the advantage that the contaminant amino acid do not interfere in the analysis of sugars. Some samples of sugars show more than one peak owing to the presence of various forms.

2.2.1.2 Quantitative analysis:

Sorbose is used as an internal standard for quantitative analysis of sugars. For calibration with an internal standard, injections are carried out with varying amounts of a standard solution of sugar and the sorbose. Since the relative responses (peak areas) of unit weight of sugars varies with the types of sugars, a standard curve is made for each sugar (see Fig.).

Table 3. Relative Retention Times (RRT) of standard sugar derivatives

(SE-30, Programm Temp. 100 260°C, rate 4°C/min.)

Sugar	Retention Time (minutes)	RRT*
D-Ribose	13.00	0.699
D-Fucose	13.40	0.720
D-Xylose	14.00	0.753
D-Fructose	17.00	0.914
D-Mannose	17.30	0.930
D-Sorbose (I.S.)	17.90	0.962
α-D-Galactose	18.20	0.978
α-D-Glucose	18.60	1.00
β-D-Glucose	21.00	1.129
N-Acetyl-D-glucosamine	23.00	1.236
Sucrose	34.10	1.833
D-Maltose**	35.00	1.882
	36.2	1.946
D-Trehalose	35.00	1.882

* Relative to α-D-Glucose (18.6 minutes)

** Two anomers

Table 4. Relative Retention Times (RRT) Of standard sugar derivatives

(OV-17, Programm Temp. 140→260°C, rate 4°C/min)

Sugar	Retention Time (minutes)	RRT*
D-Ribose	10.58	0.645
D-Fucose	11.07	0.675
D-Xylose	12.00	0.732
D-Fructose	13.75	0.838
D-Mannose	14.00	0.853
D-Sorbose (I.S.)	15.40	0.939
α-D-Galactose	15.60	0.951
α-D-Glucose	16.40	1.000
β-D-Glucose	18.05	1.100
N-Acetyl-D-glucosamine	22.50	1.372
Sucrose	31.40	1.914
D-Maltose**	33.20	2.024
	33.90	2.067
D-Trehalose	34.50	2.103

* Relative to α-D-Glucose (16.4 minutes)

** Two anomers.

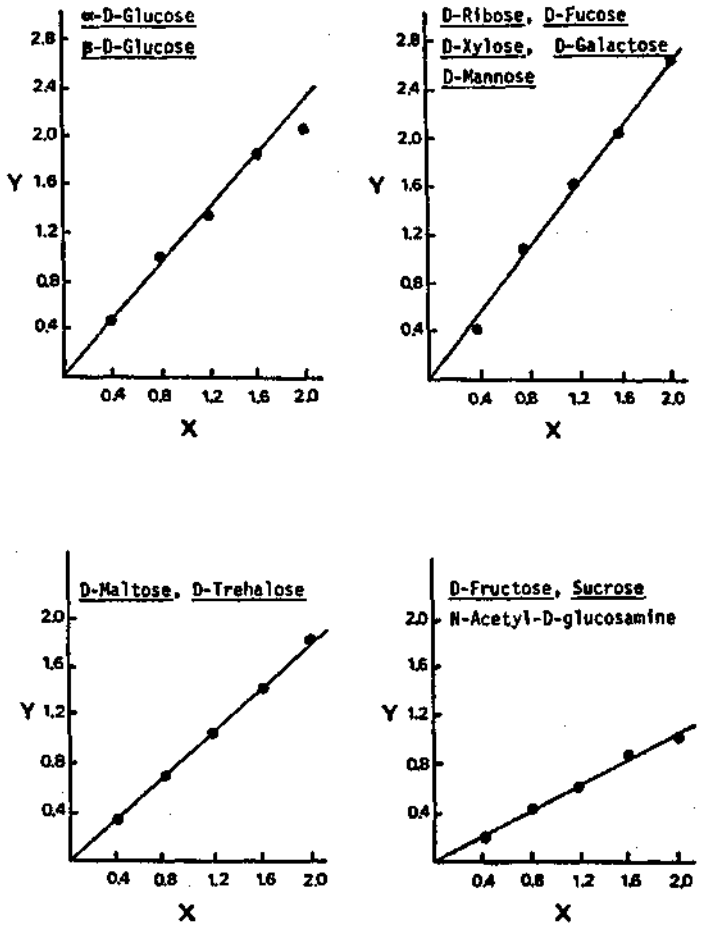

Fig. Relation between the amount of sugars injected and their area on the chromatograms.

$$X = \frac{\text{Amount of sugar injected in GLC}}{\text{Amount of internal standard (Sorbose) injected}}$$

$$Y = \frac{\text{Area of the sugar on the chromatogram}}{\text{Area of internal standard (Sorbose) injected}}$$

2.2.2 Haemolymph sugars:

Haemolymph samples are taken by bleeding from a cut near the end of the uropod into a calibrated tube. During sampling the prawn is held softly with the fingers to prevent its struggling. Haemolymph sample (2 ml) is deproteinized immediately with somogyi reagent (5 ml of 2% $ZnSO_4.7H_2O$ and then 5 ml of 1.8% $Ba(OH)_2.8H_2O$), diluted to 20 ml with water, and then centrifuged at 3000 r.p.m. for 15 min. The supernatant is removed and the precipitate is washed again with 20 ml of water. The combined supernatant is then concentrated to small volume, passed through ion-exchange column (MB-3 resin) and the effluent concentrated to dryness with rotary evaporator. Trimethylsilyl reagent is then added, and the sample is treated as described above.

2.2.3 Muscle sugars:

Muscle is homogenized (18000 r.p.m. at 0°C) with 19 volumes of chloroform-methanol (2:1, v/v) to extract lipids, the lipids are then washed according to the method of Folch et al. (1957). The fat-free residue thus obtained is suspended in water overnight at 4°C, and then the suspension is filtered. The filtrate is combined with the aqueous washes of the lipid fraction and concentrated to a smaller volume. The aqueous sample is passed through ion exchange column (MB-3 resin) and the effluent is concentrated to dryness. The dry sample is trimethylsilylated as described above.

3 REFERENCES

1. Chow, K.W. Quality control in fish feed manufacturing. In Fish feed Technology, Aquaculture Development and Coordination Programme, FAO/Rep/80/11 p. 369-385.

2. Manual Book of Experiments in Laboratory of Fisheries Chemistry, University of Kagoshima, Japan. 1980-81.