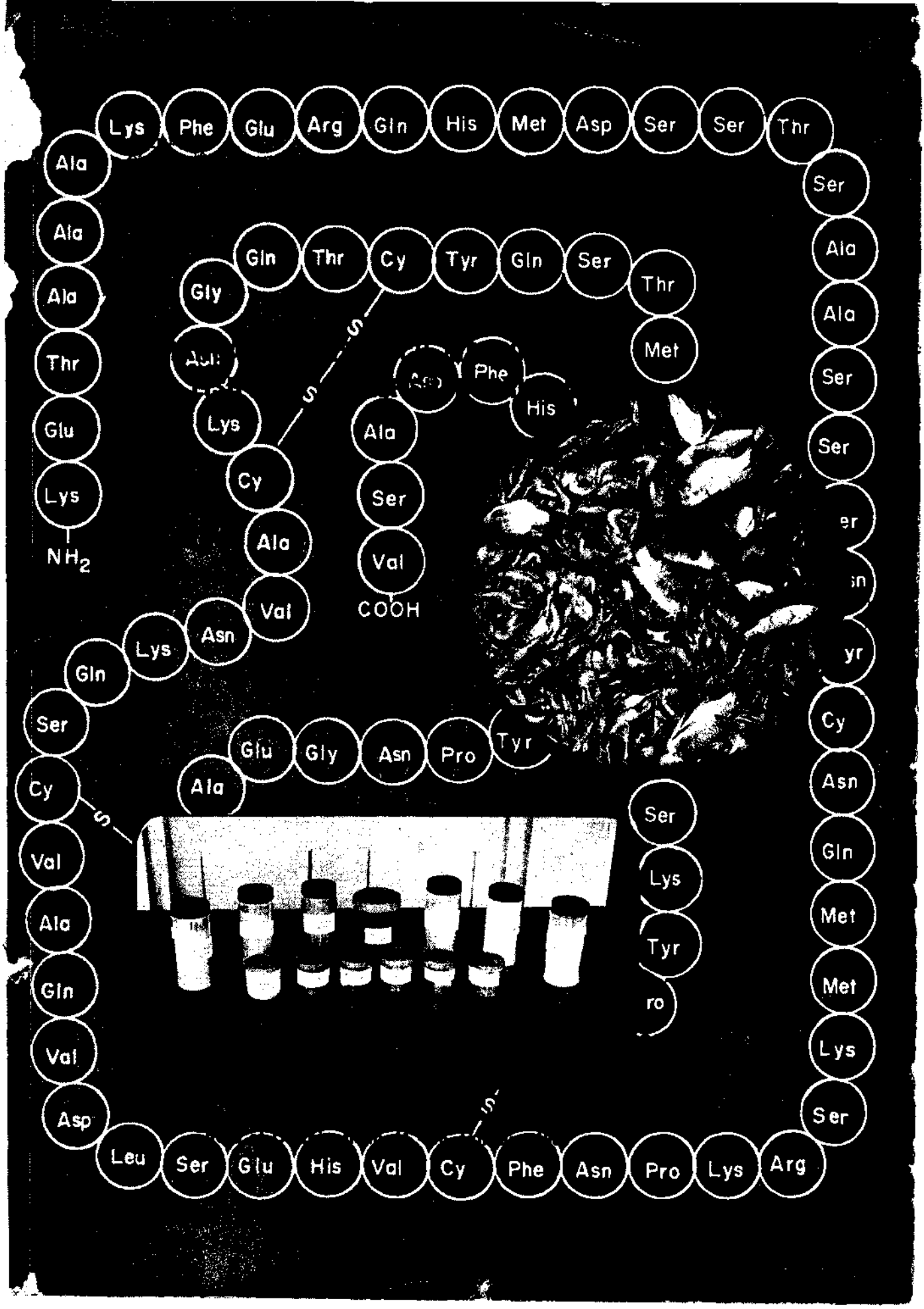
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MANUAL OF RESEARCH METHODS FOR FISH AND SHELLFISH NUTRITION

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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(E.G. Silas)
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CHAPTER 1

PROXIMATE COMPOSITION ANALYSIS OF FEEDS*

1 INTRODUCTION

The chemical composition of the feed gives its potential nutritive value and hence, in the assessment of quality of a feed the proximate principles are first determined. The Weende proximate analysis system for analyzing animal feed includes the determination of moisture (dry matter) ether extract (crude fat) crude protein, ash, crude fibre and nitrogen free extract (NFE).

2 COLLECTING AND LABELLING SAMPLES FOR ANALYSIS

The method used to collect samples for chemical analysis affects the values of the parameters obtained. The aim is to take a sample of the material which represents the total and to analyse it in such a way that it represents the material which is consumed by the animal or to sample other material which affects the objectives of the experiment.

Sampling of bagged ingredients is done with a spear probe. The probe is inserted diagonally and as horizontally as possible, from one corner of the bag to the other. In lots of 1-10 bags, all bags are sampled. In larger lots, 10 percent of all bags are sampled. Materials received in bulk are sampled using a scoop, according to the size of the consignment. For smaller than 10 tonnes consignments, two samples per tonne are taken. Larger consignments, up to 100 tonnes, require one sample per tonne or one sample for every two tonnes depending on the size of the consignment.

Oil cakes and other coarse materials are sampled by random selection of pieces from different parts of the entire consignment.

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* Prepared by R. Paul Raj and Syed Ahamed Ali, Centre of Advanced Studies in Mariculture, Central Marine Fisheries Research Institute, Cochin-18.

2.1 Grinding and sub-sampling

Samples taken in the above manner should be pooled, thoroughly mixed, and ground to pass through 1 mm screen in Wiley Mill (large samples may be ground through hammer mill (3/8" screen), sub sampled, reground through 4 mm screen, sub sampled and ground through 1 mm screen). Combine all the materials left in mills with the ground portion before subsampling; take precautions to mix ground samples well before subsampling in a waring blender.

Place sample in airtight containers.

If possible preserve all samples high in moisture content by freezing.

2.2 Method of submitting samples for chemical analysis

At the time a sample is collected, a tag is attached. The person collecting the sample should fill in the project number, experiment number, date taken, a brief description of the sample and the name of the person doing the sampling.

2.3 Laboratory sample numbers

At the time the sample comes to the laboratory, it is given a laboratory sample number.

3 DETERMINATION OF DRY MATTER

3.1 Principle

The moisture of the sample is lost by volatilization caused by heat. The amount of material left after the removal of the moisture is the dry matter.

3.2 Apparatus

(a) Oven, 105°C
(b) Covered aluminium dishes, 50 mm diameter
(c) Dessicators

3.3 Procedure

(a) Wash the dishes with a detergent. Dry the dishes in 105°C oven overnight. Place in dessicator, cool, and weigh. Handle dishes with metal tongs.

(b) Weigh by different 2.0 g. of sample into a weighed dish. Place it in 105°C oven overnight. Remove dishes, put cover on top and place in dessicator and cool. Remove from dessicator and weigh as quickly as possible.

3.4 Calculation

Dry matter (%)

$$= \frac{\text{(Wt. of dish + Wt. of dried sample) - Wt. of dish}}{\text{Wt. of sample before drying}} \times 100$$

$$= \frac{\text{Wt. of dry sample} \times 100}{\text{Wt. of sample before drying}}$$

Moisture content (%)

$$= \frac{\text{Wt. of fresh sample - Wt. of dry sample}}{\text{Wt. of fresh sample}} \times 100$$

4 DETERMINATION OF ASH

4.1 Principle

The sample is ignited at 600°C to burn off all organic material. The inorganic material which does not volatilize at that temperature is called ash.

4.2 Apparatus

(a) Muffle furnace
(b) Silica crucibles
(c) Dessicator, with magnesium perchlorate dessicant

4.3 Procedure

(a) Place clean crucibles in a muffle furnace at 600°C for one hour. Transfer crucibles from furnace to a dessicator and cool to room temperature. Weigh as quickly as possible to prevent moisture absorption. Use metal tongs to move the crucibles after they are ashed or dried.

(b) Weigh by difference 2.0 g. of sample into tared silica crucibles. Place in a muffle furnace and hold the temperature at 600°C for 6 h.

(c) Transfer the crucibles to a dessicator and cool to room temperature. When cool, weigh the crucibles as quickly as possible to prevent moisture absorption.

(d) Save the ash sample if mineral determinations are to be made.

4.4 Calculation

Ash (%) on partial dry or as fed basis

$$= \frac{\text{Wt. of ash}}{\text{Wt. of sample}} \times 100$$

Adjusting to dry basis

$$\frac{\text{ash \% on as fed sample}}{\text{dry matter \% of as fed sample}} \times 100$$

5 ACID SOLUBLE AND INSOLUBLE ASH

5.1 Apparatus and reagents

(a) Hydrochloric acid (1-2.5 v/v)
(b) Filter paper, ashless, and
(c) Dishes, porcelain

5.2 Procedure

Use the residue obtained from the ash determination. Boil with 25 ml HCl, taking care to avoid spattering, filter through ashless filter paper, and wash with hot water until acid-free. Place filter paper and residue into a dry, tared porcelain dish and place in a muffle furnace at 600°C for 2 h. or until carbon-free.

5.3 Calculation

$$\text{Acid insoluble ash (\%)} = \frac{\text{Wt. of acid-treated ash}}{\text{Wt. of sample}} \times 100$$

6 DETERMINATION OF CRUDE FIBRE

6.1 Principle

A moisture-free and ether extracted sample is digested first with a weak acid solution, then a weak base solution. The organic residue is collected in a filter crucible. The loss of weight on ingition is called crude fibre.

6.2 Apparatus

(a) Beakers, 600 ml tall-sided
(b) Round-bottom flask condenser unit
(c) Buchner flasks, 1 litre
(d) Buchner funnels, Hartley 3 section pattern
(e) Crucibles, silica with porous base, and
(f) Rubber cones to fit above.

6.3 Reagents

(a) Sulphuric acid solution (0.25 N)
(b) Sodium hydroxide solution (0.313 N)
(c) Antifoam reagent (Octyl alcohol)
(d) Ethyl alcohol
(e) Hydrochloric acid, 1% v/v

6.4 Procedure

Weigh about 2 g. of the dried, fat-free sample into a 600 ml beaker. Add 200 ml of hot sulphuric acid, place the beaker under the condenser, and bring to boiling within 1 min. Boil gently for exactly 30 min., using distilled water to maintain volume and to wash down particles adhering to the sides. Use antifoam if necessary. Filter through Whatman No.541 paper in a Buchner funnel, using suction, and wash well with boiling water. Transfer residue back to beaker and add 200 ml hot sodium hydroxide solution. Replace under the condenser and again bring to boiling within 1 min. After boiling for exactly 30 min., filter through porous crucible and wash with boiling water; 1% hydrochloric acid and then again with boiling water. Wash twice with alcohol or acetone, dry overnight at 100°C, cool, and weigh. Ash at 500°C for 3 h., cool, and weigh. Calculate the weight of fibre by difference.

6.5 Calculation

Crude fibre (% of fat-free DM)

$$= \frac{\text{(Wt. of crucible + dried residue)} - \text{(Wt. of crucible + ash residue)}}{\text{(Wt. of sample)}} \times 100$$

7 DETERMINATION OF CRUDE FAT
(Soxhlet Method)

7.1 Principle

Ether is continuously volatilized, then condensed and allowed to pass through the sample, extracting ether soluble materials. The extract is collected in flask. When the process is completed, the ether is distilled and collected in another container and the remaining crude fat is dried and weighed.

7.2 Apparatus and reagents

(a) Soxhlet extraction apparatus
(b) Extraction thimbles
(c) Petroleum ether (b.p. 40-60°C) or ethyl ether

7.3 Procedure

Weigh by difference 2 to 3 g. of the dried sample (residue from dry matter determination can be used). Place the thimble inside the soxhlet apparatus. Connect a dry pre-weighed solvent flask beneath the apparatus and add the required quantity of solvent and connect to condenser. Adjust the heating rate to give a condensation rate of 2 to 3 drops and extract for 16 h. On completion, remove the thimble and reclaim ether using the apparatus. Complete the removal of ether on a boiling water bath and dry flask at 105°C for 30 minutes. Cool in dessicator and weigh.

7.4 Calculation

$$\text{Crude fat (\% of Dry matter)} = \frac{\text{Wt. of fat}}{\text{Wt. of sample}} \times \frac{100}{1}$$

8 DETERMINATION OF FREE FATTY ACIDS

8.1 Apparatus and reagents

(a) Ethyl alcohol
(b) Phenolphthalein (1% solution in alcohol)
(c) Sodium hydroxide (0.25N)
(d) Stoppered flasks, 250 ml.

8.2 Procedure

Weigh oil or fat into a stoppered flask and add 50 ml. alcohol previously neutralised by adding sufficient 0.25N sodium hydroxide to give faint pinkish colour with phenolphthalein (2 ml.). Titrate with sodium hydroxide and vigorously shake until a permanant faint pink colour appears.

8.3 Calculation

Free fatty acids % (as oleic acid)

$$= \frac{\text{g. oil or fat}}{7.05} \times \text{volume of 0.25N NaOH used in titration}$$

9 DETERMINATION OF CRUDE PROTEIN
(Kjeldahl Method)

9.1 Apparatus

(a) Macro kjeldahl digestion and distillation units or micro kjeldahl apparatus
(b) Kjeldahl flasks (500 ml. capacity or larger), and
(c) Conical flasks, 250 ml.

9.2 Reagents

(a) Sulphuric acid (98%), nitrogen free,
(b) Potassium sulphate, reagent grade,
(c) Mercuric oxide, reagent grade,
(d) Paraffin wax,
(e) Sodium hydroxide, 40% solution
(f) Sodium sulphide, 4% solution
(g) Pumice chips,
(h) Boric acid/indicator solution. Add 5 ml of indicator solution (0.1% methyl red and 9.2% bromocresol green in alcohol) to 1 litre saturated boric acid solution,
(i) Hydrochloric acid standard solution (0.1N)

9.3 Procedure

Accurately weigh 1 g. of sample into a digestion flask. Add 10 g. potassium sulphate, 0.7 g. mercuric oxide and 20 ml sulphuric acid. Heat the flask gently at an inclined angle until frothing subsides and then boil until the solution clears. Continue boiling for an additional half hour. If the frothing is excessive, a small amount of paraffin was may be added.

On cooling, add about 90 ml. distilled water, recool, add 25 ml. sulphide solution, and mix. Add a small piece of boiling chip to prevent bumping and 80 ml. of sodium hydroxide solution while tilting the flask so that two layers are formed. Connect rapidly to the condenser unit, heat, and collect distilled ammonia in 50 ml. boric acid/indicator solution. Collect 50 ml of distillate. On completion of distillation, remove the receiver (wash condenser tip) and titrate against standard acid solution.

9.4 Calculation

Nitrogen content of sample (%)

$$= \frac{(\text{ml. acid} \times \text{normality of standard acid})}{\text{Wt. of sample (g)}} \times 0.014 \times 100$$

Crude protein content (%) = nitrogen content X 6.25

10 DETERMINATION OF NITROGEN FREE EXTRACT

10.1 Procedure

Nitrogen free extract (NFE) of a feed is determined by difference after the analyses have been completed for ash, crude fibre, crude fat and crude protein.

10.2 Calculation

NFE (%) on dry basis
= 100% - (% ash on dry basis + % crude fibre on dry basis + % crude fat on dry basis + % protein on dry basis)

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