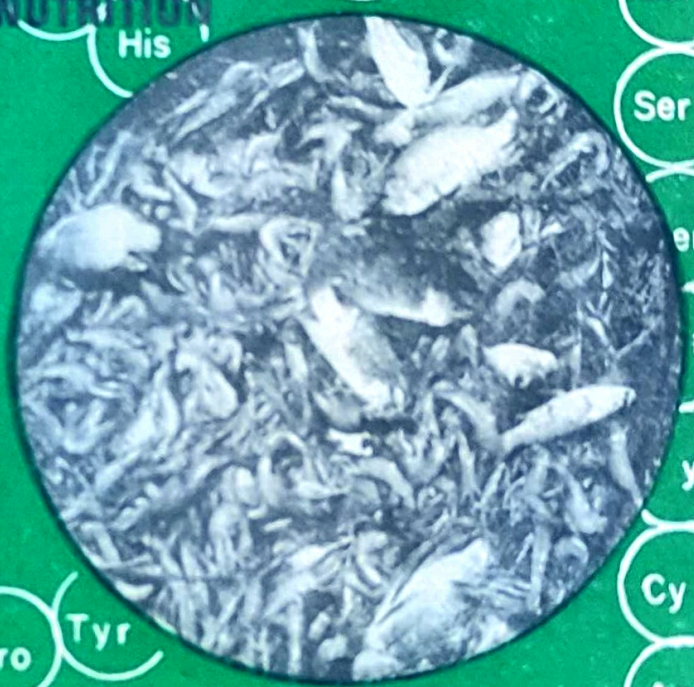
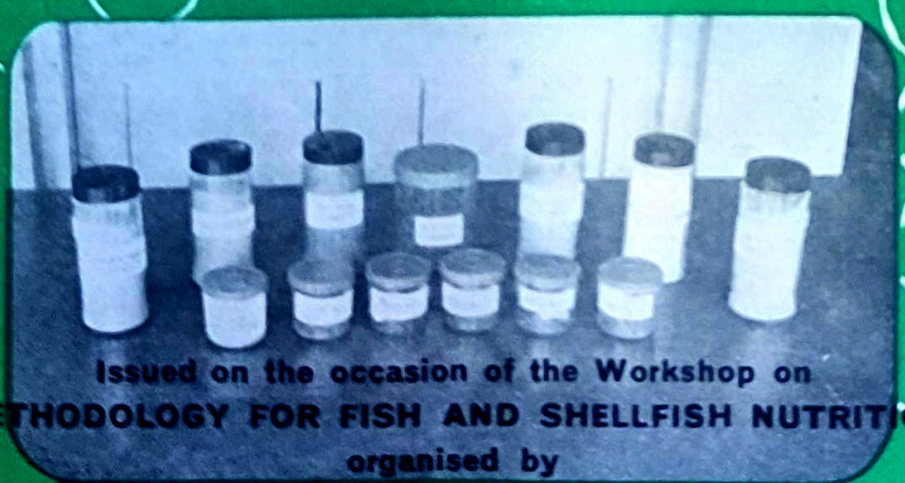
CMFRI SPECIAL PUBLICATION

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,
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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 bythem in printing this manual.

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

CONTENTS

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)

11 REFERENCES

1. AOAC - 1965 — Official Methods of Analysis of the Association of Official Agricultural Chemists. 1965, 10th Edition Published by the Association of Official Agricultural Chemists, Washington, D.C.

2. Chow, K.W. 1980 — Quality control in fish feed manufacturing. In Fish feed Technology, Aquaculture Development and Coordination Programme, FAO ADCP/Rep/80/11 p. 369-385.

3. Harris, L.E. 1970 — Nutrition research techniques for domestic and wild animals. Volume 1. Utah State University, Logan, Utah.

CHAPTER 2

DETERMINATION OF GROSS ENERGY OF FEEDS*

1 Principle

The amount of heat, measured in calories, that is released when a substance is completely oxidized in a bomb calorimeter containing 25 to 30 atmospheres of oxygen, is called the gross energy (GE) of the substance. A sample of the material to be tested is weighed into a combustion capsule. The combustion capsule is placed in an oxygen bomb containing 25 to 30 atmospheres of oxygen. The oxygen bomb is covered with 2000 g of water in an adiabatic calorimeter. After the bomb and calorimeter have been adjusted to the same temperature, the sample is ignited with a fuse wire. The temperature rise is measured under adiabatic conditions. From the hydrothermal equivalent of the calorimeter the temperature rise minus some small corrections for fuse wire oxidation and acid production, the caloric content of the sample is calculated.

2 Apparatus

(a) Parr oxygen bomb calorimeter and accessories or equivalent

(b) The calorimeter may be equipped with an automatic temperature controller. If the temperature controller is on the calorimeter, it will take less labour to run the analysis, but the controller is not necessary to obtain accurate results.

(c) Solution or trip balance with capacity to 3000 g and accurate to 0.1 g.

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* Prepared by R. Paul Raj and A.G. Ponniah, Centre of Advanced Studies in Mariculture, Central Marine Fisheries Research Institute, Cochin-18.

3 Reagents

(a) Standard sodium carbonate solution, equivalent to 1 cal/ml (3.658g Na_2 Co_3 per litre)
(b) Methyl orange indicator
(c) Benzoic acid combustion tablets or primary standard grade crystals

4 Determining the hydrothermal equivalent of the bomb

(a) Determine the hydrothermal equivalent of the bomb, bucket and water by determining the temperature rise using the same procedure as outlined above, but with a sample of known caloric content (benzoic acid combustion tablet). Make at least four determinations and use the average value. Once this value is determined, it should not change unless some parts of the bomb are replaced.

(b) Dry the benzoic acid at 105°C overnight, cool in dessicator and weigh by difference from a covered weighing bottle one tablet or approximately a 1g sample of dry calorific standard grade benzoic acid crystals. Determine the temperature rise from the benzoic acid in the bomb as with other samples.

Hydrothermal equivalent per degree (Cal)

$$\frac{\text{Wt. of benzoic acid} \times \text{Calories per gram benzoic acid} + \text{Length of fuse wire burned} \times \text{Cal/cm fuse wire} \quad X \text{ ml } Na_2 Co_3}{\text{(final temp. - initial temp.)}}$$

4.1 Example:

A 1.0622g sample of benzoic acid had a heat of combustion of 6319 cal per g. The corrected initial temperature of the bomb was 20.280°C and the final corrected temperature was 23.045°C. There were 4.8 cm of fuse wire burned with a caloric value of 2.3 cal per cm. There were 7.5 ml of Na_2Co_3 titrated (equivalent to 7.5 cal).

Hydrothermal equivalent calories per degree

$$= \frac{(1.0622 \times 6319) + (4.8 \times 2,3) + 7.5}{(23.045 - 20.280)} = 2434 \text{ small calories}$$

5 Procedure

(a) Weigh by difference approximately 1.0g of sample and place in a clean, empty combustion capsule. Samples may be pelleted, but this is usually not necessary.

(b) Attach a 10 cm length of fuse wire between the electrodes of the bomb (oxygen)and set the combustion capsule with sample in place in the loop electrode. Adjust the fuse wire so that it touches the sample.

(c) Place about 1 ml of water in the bomb cylinder and swirl it around to wet the sides. This is not necessary if the bomb is still wet from a preceeding determination.

(d) Assemble the bomb, tighten the screw cap, close the pressure release valve and fill with oxygen to 25 atmospheres gauge pressure. Place the bucket (oval) in the calorimeter, set the bomb in the bucket, and attach the clip terminal.

(e) Weigh 2000g distilled water on the solution or trip balance (use a 2000 ml volumetric flask to hold the water) and carefully pour into the calorimeter bucket. The water temperature must be within the range of the calorimeter thermometers.

(f) Close the cover, lower the thermometer and start the water circulating motor. Remove the cap from the jacket cover and fill the cover with water until it runs out of the drain hose.

(g) Adjust the temperature of the water in the outer jacket to approximately equal that of the calorimeter by adding hot or cold water, and allow one minute to

attain equilibrium. Then carefully adjust the temperature to be exactly equal and check the calorimeter temperature at one minute intervals for three minutes.

(h) Read and record the initial temperature to the nearest 0.0002° and ignite the sample. Turn in hot or cold water to keep the jacket temperature equivalent to the calorimeter temperatures during the period of rise.

(i) Compare and adjust the temperature of the outer jacket to the inner bucket of the calorimeter temperature frequently and carefully to insure adiabatic condition or that the temperatures are equal. Read and record the final temperature after the same temperature is observed in three successive one minute intervals.

(j) Raise the thermometers. Open the calorimeter, take the bomb from the calorimeter bucket, release the residual pressure of the bomb and open. Carefully remove the remaining pieces of fuse wire from the electrodes; straighten and measure the combined total length in centimeters. The calories of wire burned can be determined with the measuring scale that is supplied with the wire.

(k) Rinse all inner bomb surfaces with a stream of neutral distilled water and collect all washings in a clean beaker. Titrate the washings with the standard sodium carbonate solution using methyl orange indicator to determine the amount of acid formed from the incidental oxidation of nitrogen and sulphur compounds. A correction is made to take care of the heat liberated in the formation of the acid.

(l) Correct the initial and final temperatures from the calibration curve supplied with the thermometer.

6 Calculations

6.1 GE (cal/g) on as fed basis

$$= \frac{\left[\begin{matrix}\text{final temp.} - \\ \text{initial temp.}\end{matrix}\right] \begin{matrix}\text{Hydrothermal} \\ \text{equivalent of} \\ \text{bomb}\end{matrix} - \left[\begin{matrix}\text{Length of} \\ \text{fuse wire burned}\end{matrix} \times \begin{matrix}\text{Cal} \\ \text{per} \\ \text{cm}\end{matrix}\right] - \begin{matrix}\text{ml} \\ Na_2 \\ Co_3\end{matrix}}{\text{(weight of sample)}}$$

6.1.1 Example

A 1.0214 g sample of feed (as fed basis) was used. The initial temperature was 23.13°C and the final temperature was 25.25°C. The hydrothermal equivalent of the bomb is 2412 cal per degree C. There was 7.0 cm of fuse wire burned with a correction of 2.3 cal per cm of wire and 6.0 ml of Na_2 Co_3 titrated (equivalent of 6.0 cal).

$$\text{GE (cal/g)} = \frac{(25.25 - 23.13)\ 2412 - (7.0 \times 2.3) - 6.0}{\text{1.0214 g sample on as fed basis}}$$

$$= 3804 \text{ cal/g or } 3804 \text{ K cal/kg}$$

6.2 Adjusting to dry basis:

$$\text{GE (Kcal/kg)} = \frac{\text{GE(Kcal/kg) on as fed sample}}{\text{dry matter \% of as fed sample}} \times 100$$

7 References

1. Harris, L.E. 1970 — Nutrition Research Techniques Volume I Utah State University, Logan, Utah.

2. Parr Instrument Company 1966 — Oxygen bomb calorimetry and combustion methods. Technical Manual No.130. Parr Instrument Company, Moline, Illinois.

CHAPTER 3

DETERMINATION OF MINERALS*

1 INTRODUCTION

Mineral elements have a great diversity of uses within the animal body. The prominence of each mineral element in body tissues is closely related to its functional role. Calcium and phosphorus are too major mineral elements that must be present in adequate amounts in the feeds supplied. The amount of sodium chloride and potassium in certain feeds should also be ascertained to screen out feeds for feeding fish and shellfish.

2 DETERMINATION OF CALCIUM

2.1 Apparatus

(a) Porcelain dishes
(b) Volumetric flasks, 250 ml
(c) Beakers, 250 ml
(d) Quantitative filter paper and funnels, and
(e) Burette

2.2 Reagents

(a) Hydrochloric acid (1-3 v/v)
(b) Nitric acid (70%)
(c) Ammonium hydroxide (1-1 v/v)
(d) Methyl red indicator (dissolve 1g in 200 ml alcohol)
(e) Ammonium oxalate (4.2% solution)
(f) Sulphuric acid (98%), and
(g) Standard potassium permanganate solution (0.05N)

2.3 Procedure

Weigh 2.5 g of finely ground material into a porcelain dish and ash as above (alternatively use residue from ash

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* Prepared by R. Paul Raj and Syed Ahamed Ali, Central Marine Fisheries Research Institute, Cochin-18.

determination). Add 40 ml hydrochloric acid and a few drops of nitric acid to the residue, boil, cool, and transfer to a 250 ml volumetric flask. Dilute to volume and mix.

Pipette a suitable aliquot of the solution (100 ml for cereal feeds, 25 ml for mineral feeds) into a beaker, dilute to 100 ml and add 2 drops of methyl red. Add ammonium hydroxide one drop at a time until a brownish orange colour is obtained, then add two drops of hydrochloric acid to give a pink colour. Dilute with 50 ml water, boil, and add while stirring 10 ml of hot 4.2 percent ammonium oxalate solution. Adjust the pH with acid to bring back pink colour if necessary. Allow precipitate to settle out, and filter, washing precipitate with ammonium hydroxide solution (1+50 v/v). Place the filter paper with precipitate back in beaker and add a mixture of 125 ml water and 5 ml sulphuric acid. Heat to 70°C and titrate against the standard permanganate solution.

2.4 Calculation

Calcium (%)

$$= \frac{\text{ml permanganate solution}}{\text{wt. sample}} \times \frac{\text{aliquot used (ml)}}{250} \times 0.1$$

3 DETERMINATION OF PHOSPHORUS

3.1 Apparatus

(a) Spectrophotometer to read at 400 mμ and
(b) Graduated flasks, 100 ml.

3.2 Reagents

(a) Molybdovanadate reagent

Dissolve 40 g ammonium molybdate $4H_2O$ in 400 ml hot water and cool. Dissolve 2g ammonium metavanadate in 250 ml hot water, cool, and add 450 ml 70 percent perchloric acid. Gradually add the molybdate solution to the vanadate solution with stirring and dilute to 2 litres.

(b) Phosphorus standards

Prepare stock solution by dissolving 8.788g potassium dihydrogen orthophosphate in water and making up to 1 litre. Prepare the working solution by diluting the stock 1 in 20 (working concentrate 0.1 mgP/ml).

3.3 Procedure

Pipette an aliquot of the sample solution prepared as for the calcium determination into a 100 ml flask and add 20 ml of the molybdovanadate reagent. Make up the volume, mix, and let stand for 10 min. Transfer aliquote of the working standard containing 0.5, 0.8, 1.0 and 1.5 mg phosphorus to 100 ml flasks and treat as above. Read sample at 400 mμ setting the 0.5 mg standard at 100 percent transmission. Determine mg phosphorus in each sample aliquot from a standard curve.

4 DETERMINATION OF POTASSIUM

4.1 Apparatus

(a) Silica crucibles
(b) Flame photometer and
(c) Muffle furnace

4.2 Reagents

(a) Hydrochloric acid (concentrated)
(b) Potassium standard

To prepare stock solution (500 ppm K), dissolve 0.477g potassium chloride (Analar) and make up to 500 ml with distilled water. To prepare working standard (10 ppm), dilute 1:50.

4.3 Procedure

Dry 2g of sample in a silica crucible at 100°C to expel moisture. Add a few drops of pure olive oil and heat over flame until swelling stops. Ash at 500°C in muffle furnace for 24 h, cool, and add 2 ml concentrated hydrochloric acid to dissolve the residue. Make up to 100 ml. Take 1 ml of this solution and make a further dilution to 100 ml.

Set the flame photometer to give a reading of 100 with the 10 ppm standard, and then read sample solution. If the sample reading does not fall between 50 and 100 make a fresh dilution to give an appropriate reading.

5 DETERMINATION OF SODIUM CHLORIDE

5.1 Apparatus

(a) Conical flasks
(b) Pipettes
(c) Burettes

5.2 Reagents

(a) Standard 0.1 N silver nitrate solution
(b) Standard 0.1 N ammonium thiocyanate solution
(c) Ferric indicator - saturated aqueous solution of ferric aluminium
(d) Potassium permanganate solution - 6% w/v
(e) Urea solution - 5% w/v and
(f) Acetone (A.R. grade)

5.3 Procedure

Weigh 2g sample into a 250 ml conical flask. Moisten sample with 20 ml water and then add, by pipette, 15 ml 0.1 N silver nitrate solution and mix well. Add 20 ml concentrated nitric acid and 10 ml potassium permanganate solution and mix. Heat mixture continuously until liquid clears and nitrous fumes are evolved; then cool. Add 10 ml acetone and 5 ml ferric

indicator, and back titrate the excess silver nitrate with the 0.1 N thiocyanate solution to the red brown end point.

5.4 Calculation

Calculate results as sodium chloride,

$$\% \text{ NaCl} = \frac{(15.00 - \text{ml } 0.1 \text{ N } NH_4CNS \times 0.585)}{\text{g sample taken}}$$

6 REFERENCES

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CHAPTER 4

DETERMINATION OF VITAMINS IN FEEDS*

1 INTRODUCTION

The vitamins, though required in small amounts in the diet, play major roles in growth, physiology and metabolism of the animal. Their absence in the diet causes major deficiency syndromes. Also, the vitamins should be present in optimum levels in the diet; any excess in certain vitamins in the diet causes pathological symptoms and inhibit growth. Therefore, the determination of the amount of different vitamins present in the diet is very important.

2 DETERMINATION OF VITAMIN A

2.1 Apparatus

(a) Saponification flask
(b) Reflux condenser
(c) Water bath
(d) Separating funnel
(e) Spectrophotometer or colorimeter

2.2 Reagents

(a) Chloroform:

Wash 3 times with an equal volumes of water, dry over anhydrous sodium sulfate, distill, and store over anhydrous sodium sulfate.

(b) Antimony Trichloride Solution:

Weigh an unopened bottle of antimony chloride (25-30 g.), open it, and transfer to a glass-stoppered, wide-mouthed, amber-coloured bottle

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

containing 100 ml. of chloroform. Reweigh the opened bottle and obtain weight of antimony trichloride added to the chloroform by difference. This solution should be filtered if turbid.

(c) Alcoholic Potassium Hydroxide Solution (0.5 M):

Dissolve about 35g. of potassium hydroxide in 20 ml. of water and add sufficient alcohol to make 1000 ml. of solution.

(d) Aqueous Potassium Hydroxide Solution (0.5 M)

(e) Diethyl Ether:

Freshly distilled over sodium hydroxide pellets.

(f) Vitamin A Standard:

A chloroform solution containing 100 U.S.P. units of vitamin A per ml. This is prepared by dissolving a weighed amount of distilled vitamin A esters in chloroform.

2.3 Procedure

Weigh an amount of fat or oil containing at least 50 U.S.P. Units into a saponification flask, add the alcoholic potassium hydroxide (10 ml. per g. of sample) and attach to a reflux condenser. Heat on a water bath for 30 min. Wash condenser with ml. of water. Cool, dilute with 50-100 ml. of water, and transfer to a separatory funnel. Extract 4 times with 50-100 ml. of ether. Combine the ether extracts, pour two 50 ml. portions of water through the combined ether extracts and discard the water without shaking. Wash the ether extract with 50 ml. of the 0.5 M aqueous potassium hydroxide, shaking gently. Allow to separate, draw off aqueous layer, and discard. Wash with 50 ml. portions of water until free of alkali. Allow ether extract to stand 5 min., discard separated water, filter through 305 g. of anhydrous sodium sulfate, placed on filter paper in a funnel, into a 250 ml. flask. Rinse the separatory funnel with small portions of ether and add the rinses to the 250 ml. flask. Evaporate the ether to dryness on a water bath, removing the flask from direct heat toward end of the evaporation (viscous oily residue). Take up the residue

immediately in chloroform, adjusting the concentration to 5-15 U.S.P. units per ml.

Transfer 2 ml. of chloroform to a colorimeter tube or cuvet and add 9 ml. of the antimony trichloride reagent with the aid of a fast delivery pipette and zero the instrument (Blank). To another tube or cuvet containing 1 ml. of the unknown solution and 1 ml. of chloroform are added 9 ml. of the antimony trichloride solution. The tube is immediately stoppered, swirled, and the absorbance read with the instrument set at 620 mμ (A). The reading should be made within 3-6 sec. after the addition of the antimony trichloride solution. To another tube or cuvet, add 1 ml. of the unknown solution, 1 ml. of a known vitamin A solution in chloroform approximately equal in concentration to that of the unknown, and treat as above (B).

2.4 Calculation

$$\text{U.S.P. units per ml unknown} = \frac{A}{B-A} \times \text{concentration of standard (U.S.P. units/ml.)}$$

$$\text{Vitamin A/g. sample} = \text{U.S.P. units/ml. unknown} \times \frac{\text{final volume}}{\text{sample weight}}$$

If the unknown is colored, a blank correction is made by measuring the absorbance at 620 mμ of 1 ml. of unknown plus 10 ml. of chloroform.

3 DETERMINATION OF THIAMINE

3.1 Apparatus

(a) Steam bath
(b) Incubator (45-50°C)
(c) Glass distillation apparatus
(d) Buchner funnel
(e) Refrigerator
(f) Thiochrome tube
(g) Fluorometer

Then wash similarly 3 times with hot 25 percent potassium chloride solution. Finally wash the zeolite repeatedly with water, filter on a Buchner funnel with the aid of suction, allow to dry at 100°C, and bottle.

(h) Enzyme Solution:

Prepare a fresh 6 percent aqueous solution from a suitable source of enzyme. Mylase.P, Polidase-S, Clarase, or Takadiastase are generally suitable.

(i) Standard Thiamine Stock Solution:

Transfer about 25 mg. of U.S.P. Thiamin Hydrochloride Reference Standard, previously dried at 105° for 2 hr., and accurately weighed, to a 1000-ml. volumetric flask. Dissolve the weighed samples in 300 ml. of dilute alcohol solution (1 : 3), adjusted to a pH 3.5-4.3 with diluted hydrochloric acid, and dilute to volume with the acidified dilute alcohol. Store in a light resistant bottle in a refrigerator and renew each month.

(j) Standard Thiamine Solution:

Pipette a volume of Standard Thiamine Stock Solution, equivalent to 100 µg. of U.S.P. Thiamine Hydrochloride Reference Standard, into a 100-ml. volumetric flask, and dilute with acid potassium chloride solution to volume. Dilute 10 ml. of this solution with acid potassium chloride solution to 50 ml. Each ml. of the resulting standard preparation contains 0.2 µg. of thiamine hydrochloride.

(k) Quinine Sulfate Stock Solution:

Dissolve 0.025 of quinine sulfate $(C_{20}H_{24}N_2O_2)_2 H_2SO_4.2H_2O$, in sufficient 0.05 M sulfuric acid to make 250 ml. Store in a dark-brown bottle at a temperature below 5°C

3.2 Reagents

(a) Acid Potassium Chloride Solution:

Dissolve 250 g. of potassium chloride in sufficient water to make 1000 ml. Add 8.5 ml. of concentrated hydrochloric acid to the 1000 ml of potassium chloride solution.

(b) Sodium Hydroxide Solution, 15 percent:

Dissolve 15 g. of sodium hydroxide in sufficient water to make 100 ml.

(c) Potassium Ferricyanide Solution, 1 percent:

Dissolve 1 g. of potassium ferricyanide ($K_3Fe(CN)_6$) in sufficient water to make 100 ml. Prepare fresh on the day of use.

(d) Oxidizing Reagent:

Dilute 4.0 ml. of 1 percent potassium ferricyanide solution to 100 ml. with 15 percent sodium hydroxide solution. The solution must be used within 4 hr.

(e) Isobutyl Alcohol:

The fluorescence of the isobutyl alcohol should not exceed 10 percent of the fluorescence of the quinine standard (below). Redistill in an all-glass apparatus and collect the fraction boiling in the range 105°C-108°C.

(f) Sodium Acetate Solution:

Prepare a 2 M solution of sodium acetate by dissolving 275 g. of Na $C_2H_3O_2.3H_2O$ in water and dilute to 1000 ml.

(g) Activated Zeolite:

Place 100-500 g. of 60 to 80-mesh zeolite in a suitable beaker. Stir continuously for 15 min. each with 4 portions of hot 3 percent acetic acid. The acid should cover the material.

(l) Quinine Sulfate Standard Solution:

Dilute 10 ml. of stock quinine sulfate solution to 1 litre with 0.1 N sulfuric acid. This solution is stable for three months if stored in a brown bottle at a temperature below 5°C.

(m) Bromocresol Green pH Indicator:

Dissolve 100 mg. of bromocresol green with 7.2 ml. of 0.05 M sodium hydroxide and dilute with water to 200 ml.

3.4 Procedure

3.4.1 Preparation of the Extract:

Accurately weigh or pipette into a flask of suitable size a sample estimated to contain not more than 50 µg. of thiamine. Add 65 ml. of approximately 0.05 M sulfuric acid and digest for 30 min. at 95°-100° on a steam bath, with frequent mixing. Cool the extract to below 50°C and adjust pH to 4.0-4.5 with sodium acetate solution. Add 5 ml. of the freshly prepared enzyme solution, mix, and incubate at 45°-50°C for 2 hr. Make up to 100 ml. by the addition of water, mix thoroughly, and filter. Discard the first 10 ml. of filtrate and collect remainder.

3.4.2 Purification:

Plug the bottom of an adsorption column (Thiochrome tube) and introduce an aqueous suspension of activated zeolite to give a 6-cm. column. Allow the water to drain, keep a small layer of liquid above surface of column, and pour in 100 ml. of 3 percent acetic acid. Allow to drain as before.

Transfer 10 to 50 ml. of the original extract, containing about 5 µg. of thiamine, to the prepared chromatographic tube. Wash the column with three 10-ml. portions of boiling hot water and discard washings.

Eluate the thiamine from the zeolite by passing through the column hot acid potassium chloride solution. Collect eluate in a 25 ml. volumetric flask. Add a second 10 ml. aliquot when all of the first portion has entered the column and collect the eluate as before. Cool and dilute to volume with acid-potassium chloride solution. This is the "sample eluate". Repeat with an aliquot of the standard thiamine solution using 5.0 µg. of thiamine in place of the unknown.

3.4.3 Oxidation to Thiochrome:

In this and all subsequent stages undue exposure of the solutions to light must be avoided. Pipette 5 ml. of the sample eluate into each of two reaction vessels. To the first add quickly with mixing 5 ml. of the alkaline potassium ferricyanide solution; to the second, add 5 ml. of 15 percent sodium hydroxide solution. Add 25 ml. of water saturated isobutyl alcohol and shake the tubes vigorously for 1.5 min. Centrifuge the tubes at low speed until clear supernatant extract can be obtained from each tube. Remove the stoppers, drain off the lower layer, add approximately 2 g. of anhydrous sodium sulfate to each tube, and shake vigorously for a moment.

Add 5 ml. of the standard thiamine solution into each of two reaction vessels. Treat these tubes in the same manner as directed for tubes containing the "sample eluate".

Caution:

To avoid changes in experimental conditions the oxidation of all solutions used in a given assay should be carried out in immediate succession. Similar precautions must be taken in measurement of their fluorescence.

3.4.4 Thiochrome Fluorescence Measurement:

Filter should have a narrow transmittance range; input filter with maximum about 365 mμ, and output filter with maximum about 435 mμ. Use the quinine sulfate standard solution to govern reproducibility of fluorometer. Measure fluorescence of the isobutyl alcohol extract from the oxidized sample eluate and call this reading A. Next, measure fluorescence of the extract from sample eluate which has been treated with 5 ml. of 15 percent sodium hydroxide solution and call this reading b (sample blank). Measure fluorescence of the extract from the oxidized thiamine standard solution (S). Finally, measure fluorescence of the extract of the thiamine standard solution which has been treated with 5 ml. of 15 percent sodium hydroxide and call this reading d (standard blank).

3.5 Calculation

Micrograms of thiamine hydrochloride in 5 ml. sample eluate = (A-b)/(S-d)

4 DETERMINATION OF RIBOFLAVIN

This method is applicable to whole-grain products, grits, meal, flaked and puffed cereals, and bread.

4.1 Apparatus

(a) Dessicator
(b) Refrigerator
(c) Autoclave
(d) Fluorometer

4.2 Reagents

(a) Sulfuric Acid Solution (0.05 M)

(b) Sodium Acetate (2.5 M):

Dissolve 340 g. sodium acetate trihydrate and dilute to 1 litre.

(c) Potassium Permanganate, 4 percent:

Prepare fresh daily.

(d) Hydrogen Peroxide, 3 percent:

Dilute 30 percent hydrogen peroxide (Superoxol) 1 : 10 with water.

(e) Riboflavin Stock Solutions

Riboflavin Stock Solution I:

Dry Riboflavin Reference Standard (U.S.P.) over phosphorus pentoxide in dessicator for 24 hr. Dissolve 50 mg. in 0.02 M acetic acid in a 500 ml. volumetric flask and make up to volume. Store under toluene in an amber bottle and refrigerate. 1.0 ml. = 100 µg. riboflavin.

Riboflavin Stock Solution II:

To 100 ml. of Riboflavin Stock Solution I add 0.02 M acetic acid solution to make 1 litre. Store under toluene in amber bottle and refrigerate. 1.0 ml. = 10 µg. riboflavin.

Riboflavin Stock Solution III:

Dilute 10 ml. of Riboflavin Stock Solution II with water to make 100 ml. 1 ml. = 1 µg. riboflavin. Prepare fresh daily, and protect from light.

4.3 Procedure

Sodium Hydrosulfite:

Accurately weigh a sample into a 100 ml. volumetric flask, using the following plan:

For Sample Containing (mg./lb.)	Weight of Sample (g)
0.0 - 0.8	5
0.8 - 2.0	4
2.0 - 4.0	2

Add 75 ml. of 0.05 M sulfuric acid, mix, and either autoclave at 15 lb. for 30 min. or immerse flask in boiling

water for 30 min. Shake flask every 5 min. and cool. Add 5 ml. of 2.5 M sodium acetate solution. Mix, Let stand for 1 hr. Dilute mixture to volume and filter through medium-fast paper such as Whatman No.2 or No.4 (or equivalent), discarding first 10 to 15 ml of filtrate.

To each of four test tubes and 10 ml. of sample solution. To each of two of these tubes add 1 ml. of the standard riboflavin solution and 1 ml. of water (Solution A). To each of the two remaining tubes add 2 ml of water (Solution B). Mix. To each tube add, with mixing, 0.5 ml. of 4.0 percent potassium permanganate solution. Let stand 2 min.; then to each tube add, with mixing, 0.5 ml. of 3 percent hydrogen peroxide solution. Shake after adding peroxide to the solution.

Adjust fluorometer so that glass standard or sodium fluorescein solution gives suitable galvanometer deflection as directed for the instrument. Determine fluorescence of solutions A and B. Measure fluorescence with no more than 10 sec. of exposure in fluorometer. To dilution B add, with mixing, 20 mg. sodium hydrosulfite and determine blank fluorescence, C. (Do not use reading C after colloidal sulfur begins to form).

4.4 Calculation

$$\text{Riboflavin mg./lb} = \frac{B-C}{A-B} \times \frac{R}{S} \times \frac{V}{V_1} \times 0.454$$

where A = fluorometer reading of sample plus riboflavin standard,

B = fluorometer reading of sample plus water,

C = fluorometer reading after addition of sodium hydrosulfite,

R = standard riboflavin,

V = original volume of sample solution in ml.,

V_1 = volume of sample solution taken for measurement ml., and

S = sample weight in grains.

5 DETERMINATION OF VITAMIN C

5.1 A.O.A.C. METHOD

5.1.1 Apparatus

(a) Dessicator
(b) Refrigerator
(c) Pulverizer

5.1.2 Reagents

(a) Metaphosphoric acid-acetic acid stabilizing extracting solution: Dissolve, with shaking, 15 g glacial HPO_3 pellets or freshly pulverized stick HPO_3 in 40 ml acetic acid and 200 ml water; dilute to ca 500 ml and filter rapidly through fluted paper into glass-stoppered bottle. HPO_3 slowly changes to H_3PO_4, but if stored in a refrigerator this solution remains satisfactory for 7-10 days.

(b) Ascorbic acid standard solution: Reference ascorbic acid should be kept cool, dry, and out of sunlight.

(c) Indophenol standard solution: Dissolve 50 mg 2.6 dichloroindophenol Na salt (Eastman No.3463), that has been stored in dessicator over soda-lime, in 50 ml H_2O to which has been added 42 mg $NaHCO_3$; shake vigorously, and when dye dissolves, dilute to 200 ml with H_2O. Filter through fluted paper into amber glass-stoppered bottle. Keep stoppered, out of direct sunlight, and store in refrigerator. Decomposition products that make end point indistinct occur in some batches of dry indophenol and also develops with time in stock solution. Add 5 ml extracting solution containing excess ascorbic acid to 15 ml dye reagent. If reduced solution is not practically colorless, discard, and prepare new stock solution. If dry dye is at fault, obtain new specimen.

Weigh accurately (0.1 mg) Ca 100 mg of the reference standard ascorbic acid, transfer to 100 ml glass-stoppered volumetric flask, and dilute mark with the HPO_3-HOAC reagent. Standardize indophenol solution at once as follows: Transfer three 2.0 ml aliquots of the ascorbic acid solution to each of three 50 ml Erlenmeyers flasks containing 5.0 ml of the HPO_3-HOAC reagent. Titrate rapidly with the indophenol solution from 50 ml burette until light but distinct rose-pink colour persists at least 5 sec. (Each titration should require ca 15 ml of the indophenol solution, and titrations should check within (0.1 ml). Similarly titrate 3 blanks composed of 7.0 ml of the HPO_3-HOAC reagent plus volume H_2O ca equivalent to volume indophenol solution used in direct titrations. After substracting average blanks (usually ca 0.1 ml) from standardization titrations, calculate and express concentration of indophenol solution as mg ascorbic acid equivalent to 1.0 ml reagent. Standardize indophenol solution daily with freshly prepared standard ascorbic acid solution.

3.1.3 Preparation of sample and determination

Prepare a juice from sample as follows: Mix thoroughly by shaking to insure uniform sample, and filter through absorbent cotton or rapid paper. Prepare fresh juices by pressing well-pulped fruit and filtering. Express juice of citrus fruits by one of common devices used for squeezing oranges or lemons, and filter. Add aliquots of at least 100 ml prepared juice to equal volumes of the HPO_3-HOAC reagent. Mix, and filter rapidly through rapid folded paper (Eaton-Dikeman No.195, 18.5 cm, or equivalent). Titrate 10 ml aliquots, and make blank determinations for corrections of titrations as described previously, using proper volumes of acid reagent and H_2O. Express ascorbic acid as mg/100 ml original juice.

5.2 ALTERNATE METHOD

5.2.1 Reagents

(a) Oxalic Acid Solution, 0.4 percent

(b) Stock Ascorbic Acid Solution:

Weigh accurately 100 mg. of the reference standard ascorbic acid, transfer to a 100-ml. volumetric flask, and dilute to mark with 0.4 percent oxalic acid solution.

(c) Ascorbic Acid Standard Solutions:

Transfer 5, 10, 15, 20, and 25 ml. of the stock ascorbic acid solution to each of a series of 500-ml. volumetric flasks, and dilute to the mark with 0.4 percent oxalic acid solution. These solutions, numbered 1 to 5, contain 1, 2, 3, 4, and 5 mg. of ascorbic acid per 100 ml., respectively.

(d) Indophenol Standard Solution:

Dissolve 12 mg. of 2,6-dichlorophenolindophenol in warm water. Filter and dilute to 1 liter with water.

5.2.2 Preparation of Standard Curve

To four colorimeter tubes add the following: 10 ml. water (W); 1 ml. of 0.4 percent oxalic acid (No.1); 1 mg. of working standard No.1 plus 9 ml. of water (S); 1 ml. of working standard No.1 (No.2). Transfer tube W to a colorimeter set at 520 mμ and set instrument at zero on absorbance scale. To tube marked No.1 add 9 ml. of standard dye solution, mix, and record reading (L_1) exactly 15 sec. after adding the dye solution. Then adjust the instrument to zero with tube S in the colorimeter. To tube No.2 add 9 ml. of the standard dye solution, mix, and record reading (L_2) exactly 15 sec. after adding the dye solution. Treat each of the standard solutions in the same manner and construct standard curve by plotting absorbance of total dye minus that of the standard solutions ($L_1 - L_2$) vs. concentrations of the standard solutions (mg./100 ml.)

5.2.3 Procedure

Blend 50 g. of sample for 30 min. in a Waring Blender with 350 ml. of 0.4 percent oxalic acid solution and filter. Obtain L_1 reading as described above. To tube 8 add 1 ml. filtrate plus 9 ml. water and adjust instrument to zero. To tube No.2 add 1 ml. filtrate plus 9 ml. of dye and record L_2 reading after 15 sec. Calculate $L_1 - L_2$ and obtain the concentration of ascorbic acid from the standard curve.

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CHAPTER 5

DETERMINATION OF GROWTH INHIBITORS AND TOXINS*

1 INTRODUCTION

Certain feedstuffs contain natural toxins that, at high enough levels, are growth inhibitors and sometimes fatal to the animal consuming them. Principal among these are: (a) Urease-an enzyme found in raw soybean which produces toxicity through the hydrolysis of urea to ammonia; (b) gossypol-an endogenous toxin present in the gland of cottonseed which persists during production of the meal unless removed by a special process, or, unless, the cotton seed is a glandless variety; (c) Isothiocyanates-cyanogenic glycosides are found in linseed and cassava; (d) aflatoxin is a class of extremely potent toxins produced by the mould Aspergillus flavus. Aflatoxin may be present in any materials produced and stored under hot and humid conditions and is usually found in groundnut cake, palm cake, copra cake and maize.

2 DETERMINATION OF UREASE ACTIVITY IN SOYBEAN MEAL

2.1 Apparatus

(a) Water bath at 40°C, capable of maintaining temperature within ± 1°C, with shaking device,

(b) Conical flasks, 125 ml

(c) Volumetric flasks, 25 ml and

(d) Spectrophotometer.

2.2 Reagents

(a) Dimethylaminobenzaldehyde solution (DMAB):

Dissolve 16g DMAB in 1 litre 95% ethyl alcohol, and add 100 ml concentrated hydrochloric acid (stable for one month)

(b) Pyrophosphate buffer:

Dissolve 23.3g $Na_4P_2O_7 10H_2O$ in approximately 980 ml distilled water. Add 3 ml of concentrated

* Prepared by R. Paul Raj and Syed Ahamed Ali, Central Marine Fisheries Research Institute, Cochin-18.

HCl and then additional HCl until the pH of the buffer is 7.7 - 7.8. Dilute to 1 litre.

(c) Buffered urea solution:

Dissolve 04g urea in 1 litre pyrophosphate buffer (stable for one week).

(d) Zinc acetate solution:

Dissolve 22.0g zinc acetate $2H_2O$ in distilled water, add 3 ml of glacial acetic acid, and dilute to 100 ml.

(e) Potassium ferrocyanide solution:

Dissolve 10.6g $K_4Fe(CN)_6 3H_2O$ in distilled water, and dilute to 100 ml.

(f) Charcoal

2.3 Procedure

Accurately weigh 1 g of soybean meal into a conical flask and 50 ml of the buffered urea solution. Incubate in water bath for exactly 30 min at 40°C with shaking. Remove from water bath and quickly add 0.5 ml each of concentrated HCl, ferrocyanide solution, zinc acetate solution and 0.1g of charcoal. Shake for 15 min. and filter. If the filtrate is coloured, repeat the procedure using more charcoal. Pipette 10 ml aliquots of the filtrate and the DMAB solution into a 25 ml volumetric flask and make up to volume with distilled water. Make up also a reagent blank (10 ml DMAB made up to 25 ml with water) and a urea blank (10 ml buffered urea solution and 10 ml DMAB made upto 25ml with water). Prepare a standard curve by pipetting aliquots of buffered urea solution from 2 to 12 ml into 25 ml volumetric flasks, adding 10 ml of DMAB and make up to volume.

Mix flasks well, stand in water bath at 25°C for 10 min. and then read at 430 mμ. Calculate urease activity as mg/litre urea in urea blank less mg/litre urea in sample.

3 DETERMINATION OF FREE GOSSYPOL IN COTTONSEED MEAL

3.1 Apparatus

(a) Mechanical shaker
(b) Spectrophotometer
(c) Conical flasks, 250 ml capacity
(d) Volumetric flasks, 25 and 250 ml and
(e) Water bath (boiling)

3.2 Reagents

(a) Aqueous acetone, 7 parts acetone, 3 parts distilled water (v/v)

(b) Aqueous acetone - aniline solution:

To 700 ml acetone and 300 ml distilled water add 0.5 ml redistilled aniline. Prepare solution daily.

(c) Aqueous isopropyl alcohol solution: 8 parts isopropyl alcohol, 2 parts distilled water (v/v)

(d) Aniline:

Distill reagent grade aniline over a small quantity of zinc dust, discarding the first and last 10 percent of the distillate. Store refrigerated in a brown glass stoppered bottle. Stable for several months.

(e) Standard gossypol solution:

(i) Dissolve 25 mg of pure gossypol in aniline-free acetone and transfer to a 250 ml volumetric flask using 100 ml of acetone. Add 75 ml of distilled water, dilute to volume with acetone, and mix.

(ii) Take 50 ml of solution (a) add 100 ml pure acetone, 60 ml of distilled water, mix, and dilute to 250 ml with pure acetone. Solution (b) contains 0.02 mg gossypol/ml and is stable for 24 h in darkness.

3.3 Procedure

Grind sample to pass 1 mm screen taking care not to overheat. Take approximately 1g of the sample and add 25 ml of pure acetone. Stir for a few minutes, filter, and devide filtrate into two. To one portion add a pellet of sodium hydroxide and heat in a water bath for a few minutes. A light yellow extract which does hot change colour with sodium hydroxide indicates that the cottonseed meal is untreated and procedure (1) should be used. A deep orange red colour in the tube containing sodium hydroxide indicates the presence of dianilinogossypol and this requires that procedure (2) be used.

3.3.1 Procedure (1):

Weigh 0.5 to 1g of sample, depending on expected gossypol content, into a conical flask and add glass beads. Pipette in 100 ml of aqueous acetone solution, stopper the flask, and shake for one hour. Filter, discarding the first few ml of filtrate, and then pipette out duplicate aliquots into 25 ml volumetric flasks. (Take aliquots from 2 to 10 ml again depending on expected gossypol content). Dilute one of the aliquots to volume with aqueous isopropyl alcohol (solution a), while to the other aliquot (solution b) add 2 ml redistilled aniline, heat in a boiling water bath for 30 min together with a reagent blank containing 2 ml of aniline and a volume of aqueous acetone solution equal to the sample aliquot. Remove solution b and the blank, add sufficient aqueous isopropyl alcohol to effect homogeneous solution, and cool to room temperature in a water bath. Dilute to volume with aqueous isopropyl alcohol.

Read samples at 400 mμ. Set instrument to 0 absorbance with aqueous isopropyl alcohol, and determine absorbance of solution 'a' and reagent blank. If the reagent blank is below 0.022 absorbance proceed

as below, otherwise repeat the analysis using freshly distilled aniline.

Determine the absorbance of solution b, with the reagent blank set at 0 absorbance. Calculate the corrected absorbance of the sample aliquot: the corrected absorbance is the absorbance of solution b minus the absorbance of solution a. Determine the mg of free gossypol present in the sample solution using the calibration curve (see below).

3.3.2 Procedure (2):

Weigh 1g of sample into a conical flask, and add 50 ml aqueous acetone, shake, and filter as above. Pipette duplicate aliquots of the filtrate (from 2 to 5 ml, depending on expected free gossypol level) into 25 ml volumetric flask. Dilute one of the aliquots to volume (solution a) with aqueous isopropyl alcohol and leave for at least 30 min before reading on the spectrophotometer. Treat the other aliquot (solution b) as in procedure (1), determine the absorbances of solutions a and b as before, and calculate the apparent content of gossypol in both solutions a and b by using the calibration curve (see below).

3.3.3 Preparation of calibration curve:

Pipette duplicate 1, 2, 3, 4, 5, 7, 8 and 10 aliquots of the 0.02 mg/ml gossypol standard into 25 ml volumetric flasks. Dilute one set (solution a) to volume with aqueous isopropyl alcohol and determine absorbances as previously. To the other set (solution b) add 2 ml of redistilled aniline and proceed as previously. Prepare one reagent blank, using 2 ml aniline and 10 ml of aqueous acetone, heated together with the standards. Determine absorbances as in procedure (1) and calculate the corrected optical density for each standard solution:

Corrected absorbance = (absorbance solution b - absorbance solution a). Plot the standard curve, plotting corrected absorbance against gossypol concentrate in the 25 ml volume.

3.4 Calculation

Calculate free gossypol percent in normal meals as:

$$\text{Free gossypol \%} = \frac{5G}{WV}$$

where G - is the graph reading
W - sample weight
V - aliquot volume used

For chemically treated meals:

$$\text{Free gossypol \%} = \frac{5\ (B - A)}{WV}$$

where A - mg apparent free gossypol in sample aliquot (a)
B - mg apparent free gossypol in sample aliquot (b)
W - sample weight
V - aliquot volume used

4 THIOGLUCOSIDE DETERMINATION

The method described will give approximate thioglucoside content but does not allow the individual thioglucosides and isothiocyanates to be determined.

4.1 Apparatus and Reagents

(a) Barium chloride (5% solution)
(b) Volumetric flasks, 600 ml and
(c) Steam bath.

4.2 Procedure

To 10g meal (de-fatted by Soxhlet extraction) add 250 ml distilled water, hydrolyse at 54°C for 1 h and then

boil for 2 h, keeping volume constant. Filter, retaining filtrate, and wash residue three times with 50 ml hot water. Add washings to initial filtrate and make up volume to 600 ml. Precipitate barium sulphate by heating and adding excess barium chloride solution. Leave on a steam bath for a few hours and then filter. Ash in a muffle furnace and then weigh precipitate.

4.3 Calculation

Calculate approximate thioglucoside content as:

% thioglucoside

$$= \frac{\text{(M.wt. thioglucoside) (Wt. of Ba } SO_4)}{\text{(M. wt. } BaSO_4\text{) (Sample Wt.)}} \times 100$$

5 AFLATOXIN ANALYSIS

A method of aflatoxin analysis is outlined below which is suitable for materials such as groundnut meal, coconut meal, and palm kernel meal. For full details of the method, and for alternative procedures reference should be made to Methods of Aflatoxin Analysis by B.D. Jones (1972).

5.1 Apparatus

(a) Thin layer chromatography plates, 20 X 20 cm
(b) UV lamp, peak emission at 365 nm
(c) Bottles, wide-mouthed, 250 ml
(d) Micropipettes, and
(e) Shaking device.

5.2 Reagents

(a) Chloroform (reagent grade)
(b) Diethyl ether (reagent grade)
(c) Chloroform/methanol mixture (95/5 v/v)
(d) "Celite", diatomaceous earth
(e) Kieselgel 'G' (Merck)

(f) Qualitative standard:

Helps to distinguish aflatoxin spots from other fluorescent spots which may be present. A groundnut meal containing aflatoxins B, obtained from the Tropical Products Institute, London, can be used for this purpose.

5.3 Procedure

Weigh 10 ml of material into a wide mouthed bottle and thoroughly mix in 10 ml of water. (If high fat material is used, a prior Soxhlet extraction with petroleum ether will be necessary). Add 100 ml of chloroform, stopper with a chloroform resistant bung, and shake for 30 min. Filter the extract through "Celite", take 20 ml of filtrate and make up to 25 ml (solution a). Take another 20 ml of filtrate and concentrate to 5 ml (solution b).

Prepare thin layer plates by shaking Kieselgel 'G' (100 g) with water (220 ml) for 20 min and apply the mixture to the plates with a suitable apparatus to a depth of 500 µm. Leave for 1 h, then dry at 100°C. Spot 10 and 20 µl of solution b, and 5 and 10 µl of solution a onto a plate, together with a qualitative standard spot, in a line 2 cm from the bottom of the plate and at least 2 cm in from each side. Carry out the spot application in subdued light.

Develop the plate in diethyl ether to a height of 12 cm. Allow to dry in subdued light then redevelop the plate in chloroform methanol (95/5, v/v) to a height of 10 cm from the baseline. Examine the plate in a dark room, 30 cm from the UV source. The presence of a blue fluorescent spot at Rf 0.5 to 0.55 indicates aflatoxin B (check that the standard spot also lies in this range). The presence of a second spot at Rf 0.15 to 0 indicates aflatoxin G. The toxicity level of a sample can then be classified in terms of aflatoxins B and G according to Table 1.

Table 1. Toxicity Levels for Aflatoxins B and G

Vol. applied (µl)	Concentration of aflatoxins (µg/kg)		Toxicity level of fluorescence observed
	No fluorescence	With fluorescence	
5 µl (soln. a)	≤ 1000	> 1000	Very high
10 µl (soln. a)	< 500	500 - 1000	high
10 µl (soln. b)	≤ 100	100 - 500	medium
20 µl (soln. b)	< 50	50 - 100	low

6 REFERENCES

1. Chow, K.W. 1980 — Quality control in fish feed manufacturing. In Fish feed Technology, Aquaculture Development and Coordination Programme, FAO ADCP/Rep/80/11 p. 369-385.

2. Cockerell, I. and D. Holliday 1975 — Quality control in the animal feedstuffs manufacturing industry. Trop. Prod. Inst. Rep., (G 97)

3. Jones, B.D. 1972 — Methods of aflatoxin analysis Trop. Prod. Inst. Rep., (G 70)

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).

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* 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.

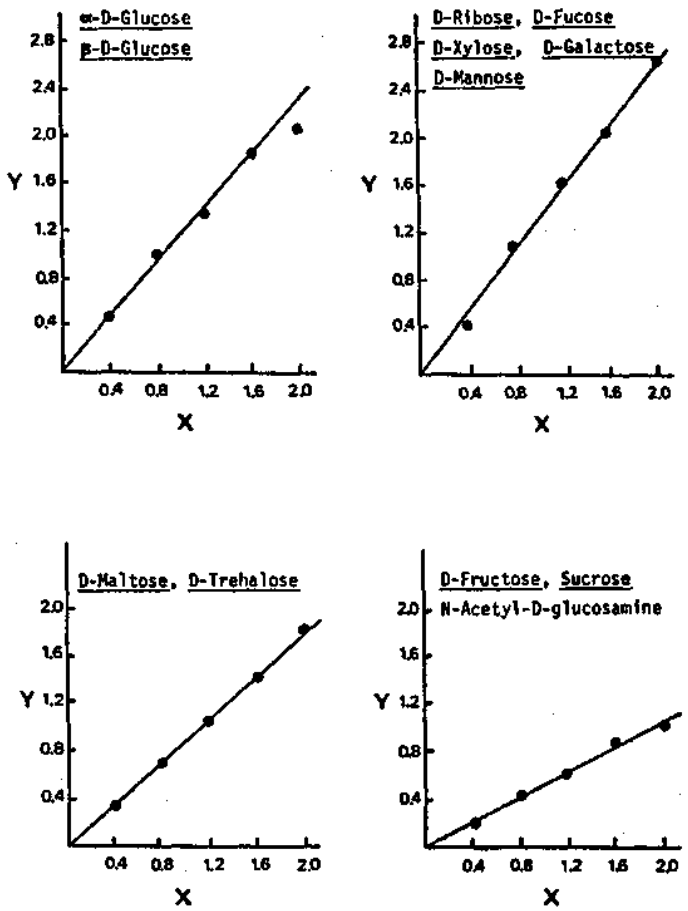

Fig. Relation between the amount of sugars injected and their area on the chromatograms.

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

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

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.

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 apparatus
(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

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* 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:

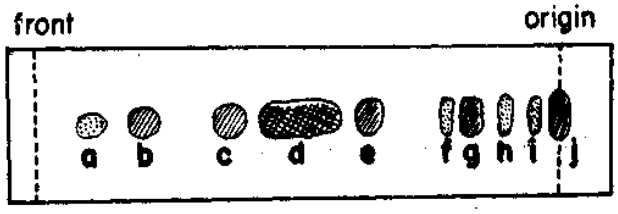
Five glass plates (20 X 20 cm) are coated to a thickness of 0.25 mm with a slurry of 30 g of silica gel G (Merck etc.) in 60 ml of distilled water, air-dried for 15 min. and activated in a oven for 40 min.at 110°C. The coated plates are stored in a dessicator over anhydrous calcium chloride.

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 tark 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)

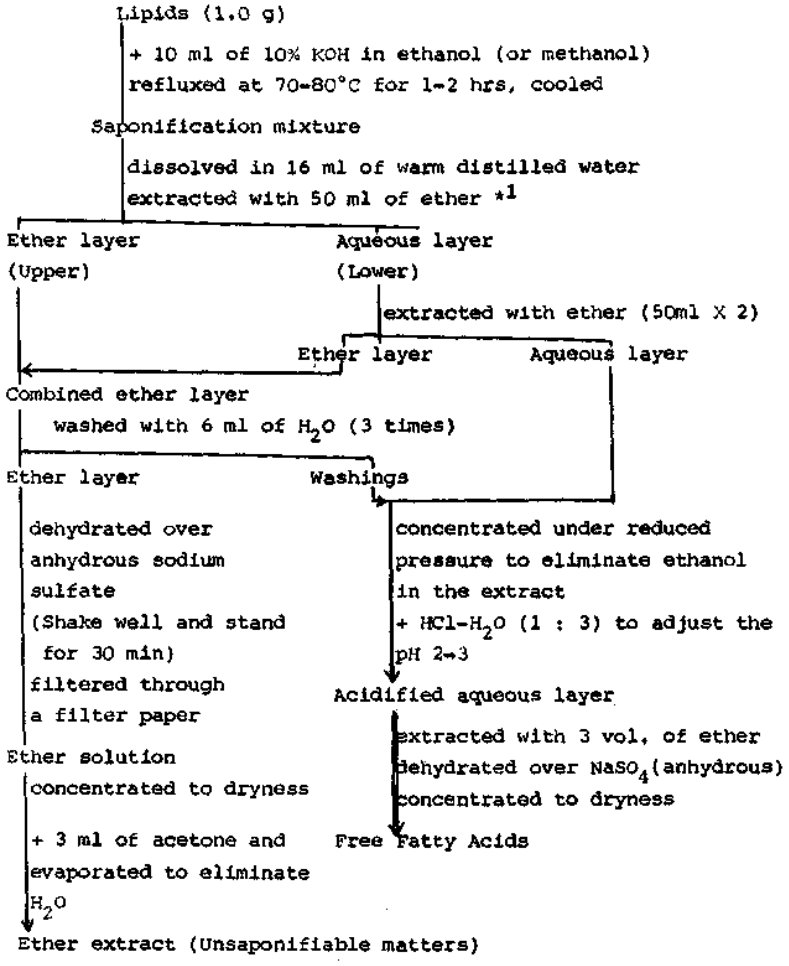
Neutral lipids	Polar lipids
(CHCl fr.)	(MeOH fr.)

---

*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.

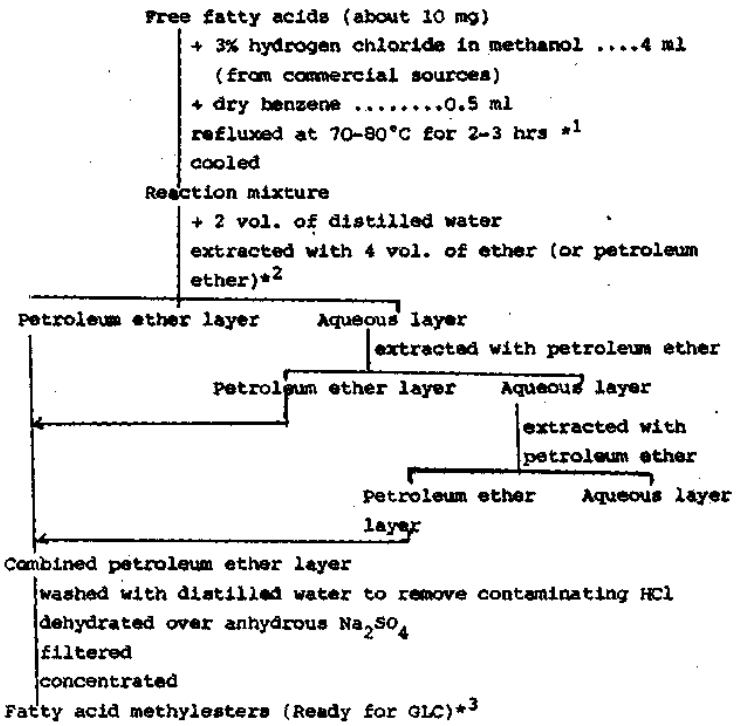
Notice: Samples (total lipids) are required to be completely dried prior to the column chromatography. The application of lipids containing water causes the incomplete separation of the both neutral and polar lipids.

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)



*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).

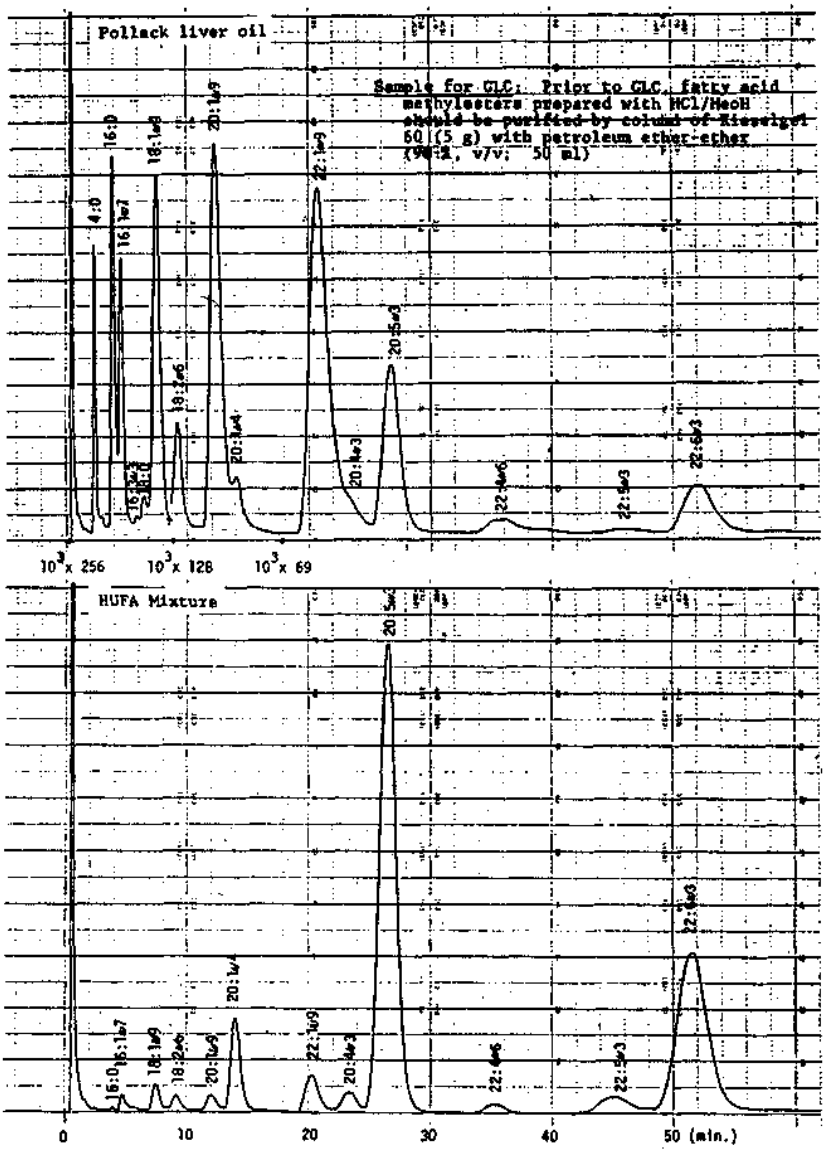
8 GAS-LIQUID CHROMATOGRAPHIC DATA OF FATTY ACID METHYLESTERS

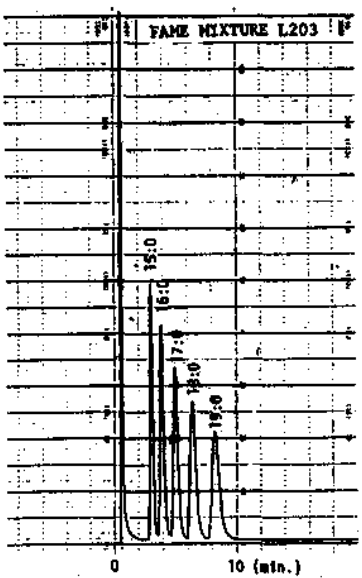
Column: 10% DEGS on 60-80 mesh Shimalite

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	20.45	2.86	21.4	22.13
	20:3W9				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)

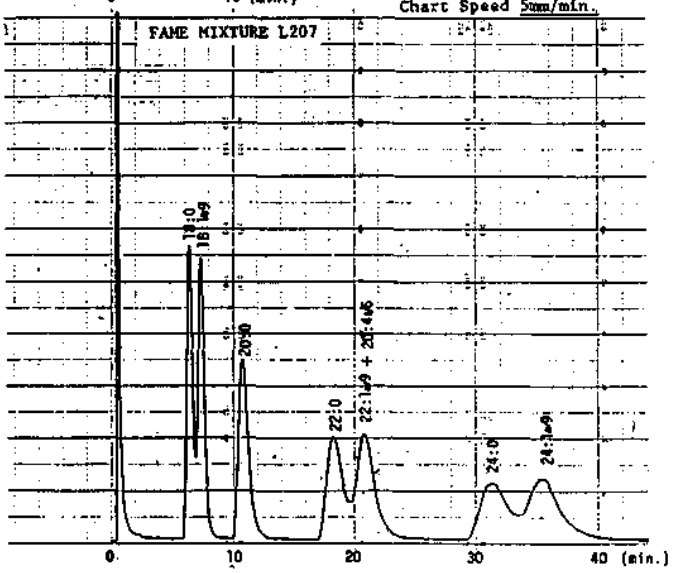
*2 ECL: Equivalent chain length


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
18:0
18:1ω9
18:2ω6
20:1ω9
20:1ω4
20:4ω3
22:1ω9
20:5ω3
22:4ω6
22:5ω3
22:6ω3
10³ x 256
10³ x 128
10³ x 69
HUFA Mixture
0
10
20
30
40
50 (min.)



CHROMATOGRAM

Date 28th June 1981 Operator SAKAMOTO
Room Temp. 32 °C
Model Shimadzu-4BF
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

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.)

Pure steryl acetates

*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(35)-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.

CHAPTER 8

METHODOLOGY OF NUTRITIONAL BIOENERGETICS - AN OUTLINE*

1 Principle

Nutritional Bioenergetics, the study of transformation and partitioning of food energy offers a conceptional framework to anabolism and catabolism. While the rate of transfer can be expressed as dB/dt, the whole process can be expressed in the form of a simplified equation, which is also known as energy budget.

$$C = F + U + M + \Delta B \qquad \ldots\ldots \quad (1)$$

where 'C' denotes the amount of food energy consumed also known as ration, 'F' that part lost as faeces, 'U' non-faecal nitrogenous loss, 'M' loss by way of metabolism and 'ΔB' change in materials of body, growth. (Fig. 1).

Food Energy (C)

- Faecal Loss (F)

Digestible (Assimilated) Energy

- Calorigenic Effect of food (SDA)
- Nonfaecal Loss (U)

Net Physiologically useful energy

- Metabolism (M)

Growth (ΔB)

- i. Tissue repair & addition
- ii. Exudates (mucos etc.,)
- iii. Exuvia
- iv. Gonadial products

Fig. 1. A diagramatic sketch showing partitioning of food energy.

* Prepared by D.C.V. Easterson and A.G. Ponniah, Central Marine Fisheries Research Institute, Cochin-18.

Since the equation is a balanced one, if one of the parameters is not known, it can be calculated. Though calorie (the measure of energy), is used as the unit of expression, units like carbon, nitrogen can also be used, when required. One gram calorie (g cal) is defined as the amount of heat required to raise the temperature of one gram of distilled water by 1°C at 15°C (1 Kcal or Cal = 1000 g cal = 3.9681 Btu = 4185 Joules).

2 Experimental Procedures

Before setting up the experiment, knowledge about the food and feeding habit of the experimental animal is necessary. For which, the study of mouth parts, gut and faecal contents would be informative. Some animals are nocturnal feeders and to these feeding should be done during the night. Preliminary studies on satiation ration, satiation time and frequency of meal are also essential.

For experimentation, healthy animals of uniform size and sexual stage are chosen and acclimatized in the chosen diet for a week. Before the starting of experiment, the animals are not fed for a fixed time to allow them to evacuate their gut. Afterwards, length and wet weight are taken and introduced into the experimental tank containing good filtered and aerated seawater. Water level, salinity and temperature are to be maintained constant. Feeding should be done at the fixed time in fixed quantities. The aeration should not be too much so as to agitate and break the faecal matter. The bottom of the tank should be of a colour in which faecal pellets can be easily recognized. If transparent containers are used, they should be covered inorder to prevent the animals getting excited. At the start of the experiment, another batch of three animals similar to the experimental ones should be rinsed with distilled water, external moisture removed, weighed and oven dried at 60-70°C for the estimation of percentage water and total calorific contents.

It is better that exuvia and faecal matter are removed soon after voided. On drying, salt from the adhering water adds up to the weight and also interferes in the chemical analysis. To avoid this, faeces and exuvia are transferred to a very fine

bolting silk fixed on the mouth of a beaker and washed gently with distilled water, transferred to a pre-weighed petridish and kept in a refrigerator or oven dried. The pooled dry matter is weighed finally. Exuvia and faecal matter need to be kept separately. Record should be maintained on moulting. One type of experimental set up is shown in Fig. 2.

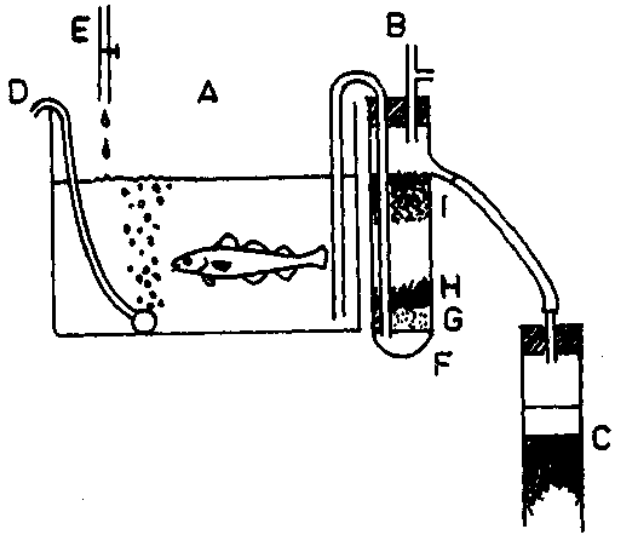


Fig. 2. The apparatus used for the separation of faecal N and urinary N.

A: A tank for maintenance of fish (36 X 20 X 27 cm).
B: A test tube for collecting faeces (3.5 X 25 cm)
C: A column of resin for adsorbing dissolved urinary nitrogenous compounds (5.5 X 60 cm)
D: Air supply
E: Water supply
F: Chloroform
G: Cupric hydroxide
H: Feces collected
I: Glass wool
(Ogino, Kakino and Chen, 1973)

3 Food Consumption

3.1 Direct method:

Food offered - food left = food consumed (C)

3.2 Indirect method:

3.2.1 $F + U + M + B = C$ ..... (2)

3.2.2 Marker methods:-

(a) Use of Radioactive isotopes:

The food is labelled with one of the isotopes viz. ^{14}C, ^{32}P, ^{45}Ca, ^{35}S. In case of phytoplankton feeders, the feed alga is grown in medium containing isotopes; by this the isotope gets incorporated into the alga. In case of formula diets the isotope is mixed with the feed. The labelled food is followed in the animal and estimated either in the animal or calculated from the decrease in the medium. ^{32}P is a very convenient isotope having a very short half-life of 14.2 days and the radiation could be easily detected and measured in small animals as a total entity or in homogenized aliquots. But due to its short half-life, it can not be used for experiments of longer duration. ^{14}C has the advantage of a half-life of 5760 years. Since it is easily metabolised, the degree of labelling might differ in different food molecules and, as different food components are not uniformly assimilated, error is likely.

(b) Use of non-assimilable markers:

The most commonly used marker is Cr_2O_3 (chromium sesquioxide) which is well mixed at 5% level with the feed and its exact quantity in the feed and faeces is chemically estimated.

4 Assimilation

Assimilation (A) is calculated as follows:

4.1 Direct method:

$$A = C - F \quad (A)$$

4.2 Indirect method:

$$A = C - \frac{\text{Concentration of indicator in food per unit weight}}{\text{Concentration of indicator in faeces per unit weight}}$$

5 Metabolism

5.1 Direct method:

By means of static or flow-through respirometers the metabolism (in terms of oxygen consumption) of animals fed with the tested diet is estimated. For this, the test animals should be acclimatized to the laboratory conditions, test diet and feeding schedule. If a diurnal rhythm in standard metabolic rate (M_s) is present, it should be taken into consideration in the schedule of feeding and oxygen consumption. After the excitment due to handling is over, hourly estimates of oxygen consumption is made for 24 hrs. This is totalled to arrive at the feeding metabolic rate (M) for a day for the tested diet. Energy equivalent of 4.63 Kcal/lit O_2 can be used to convert oxygen consumption data into calories.

5.2 Indirect method:

Metabolism (M) is calculated as the difference between sum of faecal and non-faecal loss, and growth and that of food consumed.

$$M = C - (F + U + \Delta B) \quad \ldots\ldots\ldots(3)$$

6 Specific dynamic action (SDA)

Specific dynamic action denotes the energy cost of biochemical transformation of ingested food into a metabolizable, excretable form. To estimate SDA, standard metabolism (M_s) and metabolism due to excitability and increased activity occurring in conjenction with food intake (M_e) are subtracted from total metabolism (M).

$$SDA = M - (M_s + M_e) \quad \ldots\ldots\ldots (4)$$

M - metabolic rate of fed animals;
M_s- metabolic rate of starved animals; and
M_e- the excited metabolic rate due to feeding procedure

(M and M_s are estimated in a respiration where activity is made constant by making the experimental animal to move against a steady current of water)

7 Growth

At the end of the experiment the animals are weighed, measured, and dried in the oven. It is better to determine the proximate composition, which would be of use in interpretation of the result. The weight is converted into the unit of experiment eg. Calories or Protein.

Analysis - formula:

(i) K_1 (Gross conversion efficiency) (%) $= \frac{\Delta B}{C} \cdot 100$

(ii) K_2 (Net conversion efficiency) (%) $= \frac{\Delta B}{A} \cdot 100$

(iii) Trophic coefficient $= \frac{C \text{ (in g dry wt)}}{P \text{ (in g dry wt)}}$

(iv) i. For an experiment of longer duration
Mean growth rate perday in % Body weight ($\bar{P}$) $= \frac{2\Delta B}{n(W_n + W_o)} \cdot 100$

ii. For short term experiments, especially in juveniles

$$\bar{P} = \left\{ 10^{\frac{1}{n}(\log W_n - \log W_o)} - 1 \right\} \times 100$$

8 Mean food consumption in percentage body weight per day (in ad libitum feeding studies) $\bar{C}$

$$\bar{C} = \frac{2C}{(W_n + W_o) \times n} \times 100$$

W_n = Wet weight on n day

W_o = Wet weight initial

9 Food conversion ratio (r)

Especially useful if there is any mortality to experimental animals

$$r = \frac{C}{(\bar{W}_n + D) - \bar{W}_o}$$

$\bar{W}_o$ = average initial weight (g)

$\bar{W}_n$ = average final weight (g)

D = weight of dead animals (g)

C = Total food consumed in g

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CHAPTER 9

DETERMINATION OF DIGESTIBILITY COEFFICIENT*

1 INTRODUCTION

Nutrients present in the feedstuffs are not completely available to the animal body. Large portions of the nutrients are excreted in the faeces because of being not digested in the alimentary tract. Therefore, the digestibility of the feedstuff is defined as the portion of a feed or nutrient of feed which is not recovered in faeces, i.e., the portion which has been absorbed by the animal. When the digestibility is expressed in percentage it is known as digestibility coefficient. Digestibility coefficients are calculated for dry matter, crude protein, crude fibre, ether extract and nitrogen-free extract. Digestibility of gross energy present in the food can also be determined. The digestibility coefficients normally determined are the apparent digestibility coefficients since the nutrients found in the faeces contain small proportion of nutrients from the previously utilised food in the form of mucosal debris, unspent enzymes etc.

2 DIRECT FAECES COLLECTION METHOD

2.1 Apparatus and reagents required

(a) Specially designed aquarium tanks for collection of faeces
(b) Aerators
(c) Feeding trays
(d) Polythene tubes
(e) Porcelain crucibles
(f) Hot air oven
(g) Centrifuge
(h) Single pan balance
(i) Equipment and reagents required for protein analysis
(j) Equipment and reagents required for fat analysis

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* Prepared by R. Paul Raj and D.C.V. Easterson, Central Marine Fisheries Research Institute, Cochin-18.

(k) Equipment and reagents for crude fibre analysis

(l) Equipment and reagents for determination of energy

(m) Experimental animals (fishes or crustaceans)

2.2 Procedure

(a) Take three specially designed aquarium tanks of identical size, with provision for continuous collection of faeces. Fill them with water and maintain the level, volume and temperature of water similar in all the aquaria. The water used in all the aquaria should be of the same source and salinity level. Aerate the aquaria well to maintain oxygen levels near saturation in all the tanks.

(b) Introduce 10 healthy experimental animals of the same species, age, size group, etc in each of the tanks.

(c) During the pre-experimental feeding, the animals are fed on the feed or feedstuff in question for a period of atleast 6 days to remove the effects of previous feeds. (The number of pre-experimental feeding days can be adjusted depending on the gastric evacuation time).

(d) At the end of six days the feeding trial can be started. Feed the three groups of experimental animals with a known quantity of feed, weighed accurately to 0.1g in a feeding tray and allow the animals to feed for about 12 hours overnight. The feeding time can be increased or decreased based on the actual time taken for consumption by the experimental animals.

(e) Carefully remove the feeding trays from the aquarium tanks and collect the left-over food by centrifuging or filtering. Dry the left-over food in an oven at 105°C.

(f) Collect the faeces at 2 hourly intervals using a polythene tube by siphoning out the faecal pellets if there is no specially designed aquaria for collecting faeces. Centrifuge or filter the faeces and dry in an oven at 105°C.

(g) Continue the feeding, and collection of left-over food and faeces for a period of 10 days.

(h) After drying, the representative samples are kept separately in polythene bags.

(i) At the end of the digestibility trial all the faeces samples of each aquarium tanks are composited and analysed for the dry matter, crude protein, crude fat, crude fibre, NFE and energy contents.

2.3 Calculation

Apparent digestibility coefficient of nutrients = Da = $\frac{I-F}{I}$

where I = nutrient intake and F = faecal nutrient

True digestibility coefficient = TD = $\frac{A}{I} = \frac{I-(F-Fk)}{I}$

where A = absorbed nutrient; I = nutrient intake; F = faecal nutrient and Fk = metabolic nutrient excreted with the faeces.

3 CHROMIC OXIDE INDICATOR METHOD

A breakthrough in digestibility studies of nutrients was the use of inert materials, and in particular the use of chromium oxide (Cr_2O_3).

Chromium oxide mixed with prepared diets and measured in the faeces provides a general comparison of the overall digestibility of a feed expressed as:

Percent digestibility = $100 - \frac{\%Cr_2O_3 \text{ in feed}}{\%Cr_2O_3 \text{ in faeces}}$

* When the percentage of a nutrient in the feed and faeces is analysed as well as the corresponding percentage of the indicator substance the digestibility percentage can be calculated by the formula:

Apparent digestibility coefficient

$$= 100 - \frac{\%Cr_2O_3 \text{ in feed}}{\%Cr_2O_3 \text{ in faeces}} \times \frac{\% \text{ nutrient in faeces}}{\% \text{ nutrient in feed}}$$

Caution:

The chromic oxide for commercial use has to be purified for removing the toxic chromium compounds. The toxic chromium compounds can be removed by repeated washing in N HCl using glass fibre filter (Whatman GFC) and borosilicate glassware. After repeatedly treating with hydrochloric acid the powder is washed, thoroughly in distilled water, dried at 120°C for 24 hours and stored. Thus, treated Cr_2O_3 is mixed in a definite percentage to the feed.

4 DETERMINATION OF CHROMIC OXIDE IN FAECES

4.1 TITRIMETRIC METHOD

4.1.1 Principle

The well mixed faecal matter containing organic waste and chromic oxide marker is digested with nitric acid to remove organic matter. The resultant solution is suitable for estimation of minerals in addition to chromium. On the addition of perchloric acid the chromium oxide is oxidised to dichromate which is estimated by adding an excess of ferrous ammonium sulphate and titrating the mixture.

4.1.2 Reagents

(a) Hydrochloric acid
(b) Nitric acid, Analar
(c) Perchloric acid, 60%
(d) Sulphuric acid (6N-167 ml of Analar sulphuric acid diluted to 1 litre in distilled water)

(e) Potassium dichromate (0.1 N) standard:

Dissolve 4.90g of analar grade solid previously heated to 180°C, cooled and stored in a dessicator in 1 litre of 2 N sulphuric acid. Check the normality.

(f) Ferrous ammonium sulphate (0.1 N):

Dissolve about 40g ferrous ammonium sulphate ($FeSO_4(NH_4)_2.6H_2O$) in 1 litre of 2N sulphuric acid. Check the normality each day before use.

(g) Indicator solution:

Dissolve 0.1g of N-Phenylanthranilic acid in 2 ml of 5% sodium carbonate and dilute to 100 ml with distilled water.

4.1.3 **Procedure**

(a) Weigh about 50-100 mg of faeces containing 1-3 mg of Cr_2O_3 into a 100 ml conical flask or a kjeldahl flask.

(b) Add 5 ml of conc nitric acid

(c) After 5 minutes, boil the contents gently in a hot plate for about half an hour in a fume cup board (Alternatively samples can be digested in a Tecator Kjeltec system, if available). Additional acid may be added to prevent the content becoming dry.

(d) Cool and add 3 ml of 60% perchloric acid. Heat by keeping inside a fume cupboard with plastic screen until no more fumes evolves and all nitric acid has been removed.

(e) If chromium is present the solution becomes brilliant golden in colour. Cool and wash the digest into a 50 ml volumetric flask with distilled water.

(f) To this add 5 ml of 6N-H_2SO_4, 5 ml of 0.1 N ferrous ammonium sulphate and a drop of indicator. Titrate the mixture in a 10 ml burette with standard 0.1 N potassium dichromate until the green colour of the solution turns to slate-grey, which turns to cherry colour on standing.

4.1.4 Calculation

If 't' ml of 0.1 N potassium dichromate solution is required, then the dichromate in the digest has reached with 5 't' ml of 0.1 ferrous ammonium sulphate. 1 ml of 0.1 N dichromate is equivalent to 2.53 mg of Cr_2O_3. Thus 2.53 (5-'t') mg of Cr_2O_3 can be present in the digest.

4.2 SPECTROPHOTOMETRIC METHOD

4.2.1 Reagents

As in the titrimetric method

4.2.2 Equipment

Spectrophotometer

4.2.3 Procedure

Follow procedures 'a' to 'd' as given in titrimetric method.

(e) Cool and wash the digest into a 100 ml volumetric flask and make up the volume by adding distilled water.

(f) Mix well and after allowing a minimum of 5 minutes measure the optical density at 350 mμ in a spectrophotometer. The percent chromic oxide is read from a standard curve where 'Y' is the optical density at 350 mμ and 'X' the Cr_2O_3 content of the sample in mg/100 ml.

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CHAPTER 10

SYSTEMS OF EXPRESSING THE PROTEIN VALUES OF THE FEED AND METHODS OF THEIR ESTIMATION*

1 INTRODUCTION

In chemical composition studies, proteins in the feeds are estimated by determining the nitrogen content and multiplying by a factor 6.25 and the resultant value is the crude protein. However, considerable variation in the efficiency of conversion of different dietary sources of proteins have been reported in the literature using isonitrogenous diets, and these variations have been attributed to the quality difference among proteins particularly in their amino acids profile. The following terms are widely used in the biological evaluation of proteins in fishes and crustaceans.

2 EXPERIMENTAL PROCEDURES

Experiments are conducted in specially designed aquaria with provision for collection of the different nitrogen fractions. The experimental set-up devised by Ogino, Kakino and Chen (1973) and shown in Fig.2 of Chapter 9 is an example. Experimental animals of the same species, size group and age are acclimatized in the aquaria for a stipulated period. During the acclimatization period feeding is done with the experimental protein diet. The oxygen, temperature, salinity levels are maintained similar in all the aquaria. The experimental protein diets are fed to the animals once or twice a day in fixed timings. Data on the various nitrogen fractions are collected by sampling food, water, faeces and also carcass analysis. Periodically weight of the animals are recorded to adjust and maintain the feeding level. At the end, the total protein consumed, wt. gained, various forms of nitrogen losses, carcass nitrogen, etc., are analysed and composed.

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* Prepared by R. Paul Raj, Central Marine Fisheries Research Institute, Cochin-18.

3 PROTEIN EFFICIENCY RATIO (PER)

In this case the nutritive value of the dietary protein is determined by the rate at which the animal grows. It is defined as the weight gain per unit intake of protein and may be calculated from the following formula:

$$PER = \frac{\text{gain in body weight (g)}}{\text{protein intake (g)}}$$

PER will vary with the percentage protein in the diet, with a maximum efficiency at a certain level. The determination of PER demand feeding over a longer period, and in fishes and crustaceans it is strongly related to the water temperature.

4 TRUE NET PROTEIN UTILIZATION (NPU)

This term originally proposed by Bender and Miller (1953) is one of the most used terms in relation to protein evaluation. The original formula was modified for fishes (Castell and Tiews 1980).

$$NPU = \frac{Ni-(Nf-Nm) - (NU-Nen) - (Nb-Neb)}{Ni} = \frac{Nct - Nco}{Ni}$$

where Ni = nitrogen intake
Nf = faecal nitrogen
Nm = metabolic faecal nitrogen
NU = Urinary nitrogen
Nen = endogenous urinary nitrogen
Nb = branchial nitrogen
Neb = endogenous branchial nitrogen
Nct = carcass nitrogen of test group
Nco = carcass nitrogen of group receiving a nitrogen-free diet

The first fraction in this equation require determinations of faecal and urinary nitrogen in groups on test diet as well as on non-protein diet. One of the major problems encoun-

tered is the solubility of the faecal nitrogen and collection of this part of the nitrogen is very difficult with the experimental devises developed so far.

The second fraction, however, can be more easily applied to fishes. Body nitrogen can be determined by analysis of each single fish in the case of fingerlings, whereas, careful homogenization would give values based on analysis of samples from larger fish. The method however, require adjustment of test and control diet to equal caloric contents.

5 APPARENT NET PROTEIN UTILIZATION
(PRODUCTIVE PROTEIN VALUE)

If no correction is made for endogenous nitrogen losses, the apparent net protein utilization would be determined. This function is identical with the term productive protein value (PPV).

$$\text{app NPU} = \frac{Ni-Nf-Nu-Nb}{Ni} = \frac{\text{N retained}}{\text{N consumed}}$$

where Nb is branchial nitrogen

The advantage of the method is that it does not require a control group, and the nitrogen retention can be conveniently and very precisely determined. The method is well suited for assay of different proteins, as the endogenous nitrogen may be considered equal in all groups.

6 BIOLOGICAL VALUE OF PROTEIN (BV)

It is defined as the percentage of the absorbed protein which is utilized by the body. In this case losses in digestion and metabolism are taken into consideration.

$$BV = \frac{Ni-(Nf-Nm) - (Nu-Nen) - (Nb-Neb)}{Ni - (Nf-Nm)}$$

From net protein utilization and protein digestibility, we can derive this value

$$BV = \frac{\text{Net protein utilization}}{\text{True digestibility of protein}}$$

7 REFERENCES

1. *Bender, A.E. and D.S. Miller. 1953 — Biochem. J., 52, vii 1953

2. Castell, J.D. and K. Tiews. 1980 — Report of the EIFAC, IUNS and ICES working group on standardization of methodology in fish nutrition research. FAO, EIFAC Technical Paper No.36. 24pp

3. Ogino, C., J. Kakino and M.S. Chen 1973 — Protein nutrition in fish. 1. Determination of metabolic faecal nitrogen and endogenous nitrogen excretions of carp. Bull. Japan. Soc. Sci. Fish., 39(5) : 519-523 (in Japanese with English summary)

4. Utne, F. 1978 — Standard methods and terminology in finfish nutrition. Proc. Symp. Finfish Nutrition and feed technology, Hamburg, 1978, Review Paper, R1, pp 1-14.

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CHAPTER 11

DETERMINATION OF ESSENTIAL AMINO ACID REQUIREMENT OF PRAWN*

(Radioisotopic tracer method)

1 INTRODUCTION

The requirement of essential amino acids (EAA) are generally estimated on the basis of the following parameters: (1) The weight gain in the feeding experiments using the test diets (amino acid-diets) whose protein sources were replaced entirely or partly by the mixture of amino acids; or (2) The daily increase of each EAA in the fish bodies when they were fed on the diets containing proteins with high biological values.

In the case of the prawn, however, the amino acid-diets are known to attain only poor growth as also observed in the carp. This makes it difficult for us to estimate the EAA requirements of prawn by the feeding trials. Therefore, to define the amino acids essential for the growth of the prawn, radio-isotopic methods are used.

2 PROCEDURE

2.1 Injection of ^{14}C Acetate to the prawn

Two speciments of the prawn, about 6.0g in total body weight, are each injected with 15 uCi of ^{14}C sodium acetate dissolved in 1.0 µl of aqueous solution containing carrier sodium acetate (0.7%). The radioactive acetate is injected into the muscle between the carapace and the second abdominal segment, and then the prawns are maintained on the fresh meat of short-necked clam in the aquarium (30 l) at 25°C.

2.2 Fractionation of the prawn

Six days after injection of ^{14}C acetate, the whole body of prawns is fractionated into 4 fractions by the method of

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SCHNEIDER; that is, the prawns are minced, homogenized in an ice-cold trichloroacetic acid (TCA), and separated into the TCA-soluble compound fraction and the residue. The residue is then extracted with organic solvents such as ethanol-water (4:1), ethanol, and ethanol-ether (3:1) to give the lipid fraction. The defatted residue is further separated into the protein and nucleic acid fractions by using a hot TCA solution.

2.3 Separation and Quantification of individual amino acids

The protein fraction is oxidized with performic acid as described by HIRS and then hydrolized in a sealed test tube with 5.7N hydrochloric acid by the method of MOORE and STEIN. The performic acid oxidation converts cysteine and cystine to cysteic acid and methionine to the corresponding sulphone. The amino acids of protein hydrolysate are separated and quantified by the method of MOORE et al. using column chromatography on Amberlite IR-120 (type II, Rohm and Hass co.) with sodium citrate buffers. Tryptophan is also quantified and separated in the same manner after hydrolyzing the protein fraction in 5N sodium hydroxide solution as mentioned by OELSHLEGEL et al.

2.4 Measurements of Radioactivity

The radioactivity of lipid, TCA-soluble compound, and nucleic acid fractions from the prawns and individual amino acids from the protein fraction is measured wth a Beckman Liquid Scintillation Counter LS-230 using a dioxane solution of ppo (0.6 %) and naphthalene (11.2%) as a scintillation cocktail. The radioactivity of protein fraction is determined in the similar manner after hydrolysis with hydrochloric acid.

Distribution of radioactivity in the fractions obtained from the prawns 6 days after injection of acetate (30 μCi)

Fraction	Total radioactivity recovered (μCi)
TCA-soluble compounds	0.39
Lipids	0.73
Nucleic acids	0.03
Proteins	0.15
Whole body of prawns	1.30
Sea Water	25.80
Total radioactivity recovered	27.10

Incorporation of radioactivity into the individual amino acids of protein fraction in the prawns 6 days after injection of acetate

Amino acids	Specific activity cpm/μmol)	Amino acids	Specific activity (cpm/μmol)
Aspartic acid	203	Valine	0.1
Serine	102	Methionine	2.5
Glutamic acid	196	Isoleucine	0.7
Proline	138	Leucine	0.1
Glycine	132	Phenylalanine	0.1
Alanine	187	Lysine	0.2
Cysteic acid	159	Histidine	0.7
Tyrosine	1.2	Arginine	1.2
Threonine	1.9	Tryptophan	0.4

Radioactivity is unambigously incorporated into aspartic acid, serine, glutamic acid, proline, glycine, alanine, and cysteic acid. These amino acids are suspected to be unnecessary for the prawn. On the other hand, little or no radioactivity is incorporated into valine, methionine, isoleucine, leucine, phenylalanine, lysine, histidine, arginine, threonine, and tryptophan. These 10 amino acids are not synthesized de novo and are probably essential for the growth of the prawn. Tyrosine is thought to be formed from the ingested phenylalanine.

3 REFERENCE

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

CHAPTER 12

PREPARATION OF ARTIFICIAL DIETS FOR NUTRITIONAL STUDIES*

1 TEST DIET FOR FISHES

The H 440 standard reference diet of Halver (1969) has proven satisfactory for use with a variety of species of fishes. If this exact formula does not prove satisfactory for growth and survival of the test fish, slight modifications of clearly explained ingredient changes still permit meaningful comparisons of the test fish results with other species.

Diets may be prepared as moist, semi-moist or dry diet, or as a powder, rolled pellets, extruded pellets, or compressed pellets.

1.1 Standard Reference Diet H-440

Complete test diet	(g)	Vitamin Mixture	(g)
Vitamin-free casein	38	L-cellulose	8.000
White dextrin	28	Choline chloride	0.500
Gelatin	12	Inositol	0.200
Corn oil	6	L-Ascorbic acid	0.100
Cod liver oil	3	Nicotinic acid	0.075
Vitamin mixture	9	Ca-pantothenate	0.050
Mineral Mix	4	Riboflavin	0.020
Total	100	Thiamine HCl	0.005
Water	200	Pyridoxine HCl	0.005
		Menadione (K)	0.004
Total diet as feed	300	Folic acid	0.0015
		Vitamin B_{12}	0.0011
		Biotin	0.0005
		L-tocopherol-acetate (E)	0.040

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Mineral Mix	(g)	USP XII No.2	(g)
USP XII No.2	99.50	Calcium biphosphate	13.58
$AlCl_3.6H_2O$	0.015	Calcium lactate	32.70
$ZnSO_4.H_2O$	0.300	Ferric citrate	2.97
CuCl	0.010	Magnesium sulphate	13.20
$MnSO_4.H_2O$	0.080	Potassium phosphate (dibasic)	23.98
KI	0.015	Sodium biphosphate	8.72
$CoCl_2.6H_2O$	0.100	Sodium chloride	4.35
			99.50

1.2 Preparation of Halver's diet

Gelatin

Take in the container, add cold water and heat with stirring in a water bath at 80°C to dissolve gelatin.

remove from heat

add with stirring - dextrin (first) Casein (second) minerals, oils and vitamins as temperature decrease

mix well until the temperature decreased to 40°C

Warm diet

transfer into another container put into the refrigerator at 5°C

Artificial diet

Mix L-tocopherol with oils

Mix 1 part of vitamins with 8 parts of L-cellulose and

Add vitamin B_{12} in water during final mixing.

2 TEST DIET FOR THE PRAWN, PENAEUS JAPONICUS

Ingredients	g
Casein (vitamin-free)	50.0
Glucose	5.5
Sucrose	10.0

Ingredients	g
α-Starch	4.0
Glucosamine HCl	0.8
Sodium citrate	0.3
Sodium succinate	0.3
Cholesterol	0.5
Pollack liver oil	12.0
Minerals*1	3.5
Vitamins*2	3.2
Cellulose powder	1.9
+ Water 130-135 ml	100.02

*1, *2 Minerals and vitamins for the test diet of prawn, P. japonicus

Vitamins	mg	Vitamins	mg
Thiamine HCl (B_1)	4.9	Menadione	4.0
Riboflavin (B_2)	8.0	β-Carotene	9.6
P. Aminobenzoic acid	10.0	α-Tocopherol (vitamin E)	20.0
Biotin	0.4	Calcipherol	1.2
Inositol	400.0	Cyanocobalamine (B_{12})	0.08
Niacin	40.0	Na Ascorbate (vitamin C)	2000.00
Ca Panthothenate	60.0	Folic acid	0.30
Pyridoxine HCl	12.0	Choline HCl	600.00
		Total	32000

Minerals	g
K_2HPO_4	2.0
$Ca_3(PO_4)_2$	2.72
$NaH_2PO_4 2H_2O$	0.79
$MgSO_4 7H_2O$	3.02
$MnSO_4 5H_2O$	0.004
$FeSO_4 7H_2O$	0.015
Total	8.549

2.1 Preparation of Test Diets for the Prawn

i) Vitamins are dissolved in either water or ethanol and stored in a refrigerator at 5°C.

ii) Minerals are powdered into a fine particle with a mortar and mixed well.

iii) The pH of diet is adjusted with 0.5N-NaOH (in most case, 65-70 ml. of 0.5N-NaOH solution) to pH 6.8-7.0 (optimum pH for the prawn diet).

Detailed procedures:

All the ingredients are weighed and powdered into a fine particle. Casein, glucose, sucrose, a-starch, glucosamine HCl, Na citrate, Na succinate, cholesterol, and agar are combined and mixed thoroughly. To this, cellulose powder, minerals, vitamins, and pollack liver oil are added in turn and mixed well. Finally, distilled water (130-135 ml) is added to the mixture of dry ingredients and mixed again, the pH of diet being adjusted to pH 6.8-7.0 with 0.5N-NaOH (or 1N-HCl). The mixture so obtained is heated in autoclave (without addition of pressure) at 100°C for 10 min. The container is taken out from the autoclave, and the ingredients are mixed well and again heated at 100°C for 10 min. by the same manner. The heated mixture of the ingredients is packed into the plastic tubes (Cleharon tubes) by the similar manner to that of casing of sausage-making. The cased diet is again heated at 100°C for 10 min. and then cooled.

3 REFERENCES

1. Castell, J.D. and K. Tiews 1980 Report of the EIFAC, IUNS and ICES working group on standardisation of methodology in fish nutrition research EIFAC Technical Paper No.36. 24 pp.

2. Halver, J.E. (ed:). Fish Nutrition. New York, Academic Press, 719 p.

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

CHAPTER 13

FEED FORMULATION METHODS*

1 INTRODUCTION

The requirements for protein and energy levels of the candidate species should be balanced in the formula diets. In addition, the specific amino acid levels, vitamin level, mineral level and roughage should also be balanced. Each feed ingredient in any feed formulation should serve some specific purpose, and have least cost. Usually in animal diets the protein level is adjusted first and the energy level is adjusted by addition of high energy supplements.

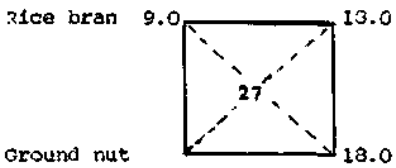
2 SQUARE METHOD

2.1 Balancing crude protein level

2.1.1. Using two ingredients:

Using rice bran (crude protein 9%) and ground nut cake (C.P. 45%) a fish diet with 27% crude protein can be prepared as follows:

A square is constructed and the two ingredients are put on the two left corners along with the protein content of each. The desired protein level of the feed is placed in the middle of the square. Next the protein level of



the feed is substracted from that of the ingredients and the answer is placed in the opposite corner. The positive or negative sign is ignored.

* Prepared by Syed Ahamed Ali, Central Marine Fisheries Research Institute, Cochin-18.

Now add the figures on the right side corner of the square 18 + 18 = 36

$$\text{Then the \% of ricebran} = \frac{18}{36} \times 100 = 50\%$$

$$\text{\% of ground nut cake} = \frac{18}{36} \times 100 = 50\%$$

To make 100 kg of feed, 50 kg of ricebran and 50 kg of groundnut cake have to be utilised.

2.1.2 Using more than two ingredients:

Using prawn waste (C.P. 35%), fish meal (C.P. 60%), wheat bran (C.P. 15%) and tapioca (C.P. 2%) a prawn diet with 32% crude protein can be prepared as follows:

Group the ingredients with less than 20% crude protein and average their protein content

Wheat bran		15%
Tapioca		2
		17
Average	=	8.5%

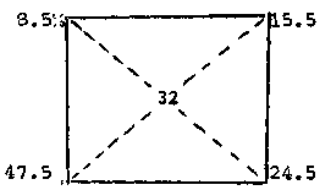
Group the ingredients with more than 20% crude protein and average their protein content

Fish meal		60%
Prawn waste		35%
		95
Average	=	47.5%

Now the averaged protein contents are put on the two left corners of the square, and the rest is same.

Wheat bran + tapioca

Prawn waste + fish meal



Now add the figures on the right hand side corners of the square.

$$15.5 + 24.5 = 40.0$$

$$\text{Wheat bran + tapioca} = \frac{15.5}{40} \times 100 = 38.75$$

$$\text{Prawn waste + fish meal} = \frac{24.5}{40} \times 100 = 61.25$$

Wheat bran	=	38.75/2 = 19.375%
Tapioca	=	38.75/2 = 19.375%
Prawn waste	=	61.25/2 = 30.625%
Fish meal	=	61.25/2 = 30.625%

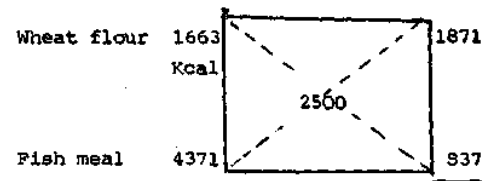
The composition of the feed is

Prawn waste	30.625%
Fish meal	30.625%
Wheat bran	19.375%
Tapioca	19.375%
Total	100.00

2.2 Balancing energy levels

The square method can also be used to calculate the proportion of feed ingredients to mix together to achieve a desired dietary energy level.

Using wheat flour 1663 Kcal Me/kg and fish meal 4371 Kcal Me/kg, a feed containing 2500 Kcal Me/kg can be prepared as follows:



$$\text{Wheat flour} = \frac{1871}{2708} \times 100 = 69.1\%$$

$$\text{Fish meal} = \frac{837}{2708} \times 100 = 30.9\%$$

To make 100 kg of the feed with 2500 Kcal of ME/kg we have to mix 69.1 kg of wheat flour and 30.9 kg of fish meal.

The square method cannot be used to balance for both crude protein level and energy level simultaneously.

3 REFERENCE

1. Hardy, R. 1980 Fish feed formulation. In Fish feed Technology, Aquaculture Development and Coordination Programme, FAO ADCP/Rep/80/11. p. 233-239

CHAPTER 14

DESIGNING FISH AND SHELLFISH NUTRITION EXPERIMENTS*

1 INTRODUCTION

Statistical method is a powerful tool in experimental investigations. While planning and implementing experimental programmes, this fact, however, has been often ignored by many investigators may be due to lack of familiarity with the subject, as the foundations of statistical science are mathematical. But the logical reasoning and principles underlying the statistical method are not difficult to comprehend and the actual application of these methods in designing experiments and analysing the resultant data are relatively straight-forward and in many cases fairly simple. The present article gives in brief the role of statistical designing in experiments with special reference to fish and shellfish nutrition. A few designs which can be used in nutrition experiments are also dealt with.

2 WHAT IS STATISTICAL DESIGNING TO AN EXPERIMENTER

The formulation and testing of hypothesis are the main features of a scientific method (Kempthorne, 1972). A researcher postulates a hypothesis which he would like to verify. This verification necessitates the collection of observations through an experiment and the designing of experiment is concerned with the pattern of observations to be collected which should be relevant to his hypothesis. Statistical designing involves the formulation of a scheme or lay-out plan where the placements of treatments in experimental units are specified to meet the objectives of the particular problem while keeping in view the statistical requirements like randomisation, replication and local control. (The term 'treatments' is used here in a general way and may mean level of feeding, doses of stimulants, stocking densities, etc.). The experimenter must have such a lay-out plan before administering the treatments so as to enable him to arrive at valid conclusions, the logic of which is acceptable to the concerned scientific community.

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3 WHY STATISTICAL DESIGNING

Variability in experimental material is an inevitable feature in any field of research. Consider for example two fish culture ponds, kept almost under identifical conditions with same species, stocking density, etc. At the harvesting time one would find that the yield of one pond is different from the other. This may be attributed to the uncontrolled or random variation inherent in the production process. Consider again two ponds kept as similar as possible except that in one pond supplementary feeding is provided. Here again at the harvesting time the yields would be found to be different. Can we straight away attribute the difference to the effect of the supplementary feeding? We cannot. May be supplementary feeding did not contribute in any way to the difference in yield and the difference could be purely due to the uncontrolled inherent variation. Differences are expected even when similarity is maintained in the two ponds. One might then say that if the difference is quite high it can be attributed to the level of feeding. But how high the difference should be to attribute it to the level of feeding? The answer becomes quite subjective. Thus variation introduces a degree of uncertainty to the conclusions that are drawn from the results. A mathematical measure of uncertainty is probability, the theory of which enables us to make numerical statements about uncertain outcomes. But this is possible only if the experiment is planned taking into consideration the statistical principles. Only a statistically designed experiment can permit a valid test of significance involving probability statements whether a particular difference is due to chance causes or can be attributed to real difference between treatments.

In practical experimentation one great difficulty is that factors extraneous to those under study mask the real treatment effects. A statistically planned experiment attempts to reduce the effect of extraneous factors from treatment comparisons and also has many desirable properties.

4 CONTROL OF EXPERIMENTAL ERROR

The extraneous variation mentioned earlier is conventionally termed as 'experimental error' where the word 'error' is not synonymous with mistakes but indicate all types of extraneous variation (Cochran et al, 1973). There are two sources of experimental error: one refers to the inherent variability in the experimental material or units to which the treatments are applied and the other type refers to the failure to standardise the experimental technique. It is desirable that the experimental error is kept as minimum as possible as otherwise only a large difference in the treatment means will be detected as significant. Reduction of experimental error automatically increases the precision. One way to reduce the error is by ensuring uniformity in the conduct of the experiment. Two other methods to effect the reduction of the error are one by providing more replications and the other by skilful grouping of units in such a way that the unit to which one treatment is applied are closely comparable with those to which another treatment is applied. Some of the general principles governing these methods and other related aspects are elaborated.

5 REPLICATION, RANDOMISATION AND LOCAL CONTROL

Two primary requisites in designing experiments are replication and randomisation. Replication or repetition of treatments provides stability to the mean but more than that makes it possible to estimate the experimental error. It also increases the precision of the estimates of both the treatment mean and the experimental error.

Randomisation which means random allocation of treatments to various experimental units, ensures that a treatment will not be unduly favoured or handicapped in successive replications. It ensures unbiassedness of the estimates of experimental error and provide for valid treatment comparisons against the experimental error (Fisher, 1949). When treatments are replicated and allocated randomly to the various units we are in a position to test the significance of observed treatment differences by

the use of test of significance procedures. Thus it is essential to provide for adequate number of replications and ensure proper randomisation at the planning stage (Panse et al, 1964).

Grouping of Units often help in reducing experimental error. Thus if the experimental units form a very heterogeneous set, try to group them so that units in the same replicate could be large. By this process, from the total variation in the observations the variation between replicates can be removed resulting in the reduction in the error variance (experimental error). The device of reducing errors through suitable groupings is called local control. Looking from another angle, if treatments are allotted to a replication with homogeneous units the observed differences would reflect the real differences between the treatment effects. The principle of local control is the basis for experimental designs such as 'randomised blocks' and 'latin squares'. When the number of treatments to be accommodated in a replicate becomes large, the homogeneity within a replicate tends to be lost and can be restored by dividing the replication into smaller blocks which is the basis of 'confounding' in factorial experiments and also various 'incomplete block designs' (Cochran et al, 1973).

6 SOME USEFUL PLANS

6.1 Randomised block

One of the most commonly used plans is the randomised block design where experimental material is divided into blocks each of which constitute a single replicate in such a way that the units within a block is as homogeneous as possible. The treatments are now randomly allotted to the experimental units within a block. This increases the comparability of treatment effects as they act under conditions which are similar except for the treatments. For instance in an experiment to select an economic supplementary feed mixture from among 4 prepared mixtures for prawn culture, 4 ponds all located by the side of the main water

body like the backwater or estuary, could be grouped as one block or replication and allot treatments at random. The next 4 could be ponds running parallel to the first set but more inside the land so that within a block salinity and associated features are likely to be similar. This arrangement takes care to a good extent salinity gradient likely to be reducing when moved away from the main water body. In the experiment if there are 5 replications there will be totally 20 ponds. If all the 20 ponds are more or less similar no blocking or stratification if required and the treatments could be randomly allotted over the entire range of the 20 ponds. Such a design is called completely randomised design. However, if heterogeneity in the features of the ponds is suspected it is desirable to provide blocks which may help in reducing the experimental error. It may be stated here that if two-way heterogeneity is suspected a latin-square design has to be followed instead of randomised block design which takes care of only one-way heterogeneity.

1.2 Factorial experiments in complete and incomplete blocks

Consider an experiment to study the effect of different levels of protein and energy on body weight of fish in culture ponds. If there are, say, 2 levels for each factor there will be in all 4 (2^2) treatment combinations. A group of treatments which contains two or more levels of two or more factors in all combination is known as the factorial arrangement. The different combinations could be allotted as in a randomised block design. The experimenter could try a one-factor-at-a-time approach. But the advantage in a factorial experiment is that not only the main effects but also the interactions between factors can be studied and tested for statistical significance.

If the number of factors and levels are large say 3 factors at 3 levels each, the number of treatment combinations will be 27. It may be difficult to get 27 experimental ponds, which are more or less homogeneous with regard to factors other than being tested so that the principle of stratification to reduce experimental error cannot be implemented. An

ingenious device to overcome this situation is called confounding where a homogeneous block will not accommodate the full replication. One replication is divided into say, 3 compact blocks such that the units in the smaller blocks are homogeneous. The 27 treatment combinations can be divided into 2 groups of 9 each and allotted to the 3 compact blocks. However some of the treatment comparisons will not be distinguishable from blockdifferences or in other words, get confounded with block differences. Thus some sacrifices have to be made. But at the planning stage this aspect can be considered and the scheme can be so formed that all major and important comparisons are kept free from block differences. Factorial set-up can be easily superimposed in polyculture experiments in pens or in ponds.

6.3 Switch-over

There are occasions in which treatments are applied in sequence over several periods on a group of individuals. Consider an experiment to study the effect of mineral supplementation of two types in lobsters kept in artificial tanks. If there are say six groups of lobsters separated and kept in tanks with sub-partitioning, then the two types of supplementations are given such that half the groups received say, type A and the other half type B in period 1. The lobsters receiving type A in period 1 will be switched over to get type B in period 2 and vise versa. Such a design is called switch-over or change-over design. (Federer, 1967). On the other hand if a time trend is expected in the character under study a switch-back or a double reversal design will have to be used. In these procedures a rest period is to be provided between two treatment periods so that there is no carry over effect or residual effect influencing the treatments during the second period. However if a reasonably long rest period is not feasible or the residual effect is itself a topic of interest the procedure is to be modified so that direct and residual effects of treatments can also be measured.

7 DISCUSSION

The need for statistical designing in scientific experimentation and some common design which can be used in fish nutrition research have been dealt with in the preceding paragraphs. There are several designs available in statistical literature accounts of which are detailed in the references cited.

Once a design is fixed the data collected should be analysed by following procedures relevant to the design. The details of the procedures of analysis corresponding to each design are available in the references mentioned.

The importance of reducing experimental error has been stressed and local control method has been mentioned as a procedure to achieve it. In addition there is a purely statistical procedure to reduce experimental error called analysis of covariance technique where information on a suitably chosen auxiliary variable is used to build up a regression relationship for adjusting the error variance (Snedecar et al, 1967). For instance in a pen culture experiment if the body weights of the fish released into the pens are initially not the same, the treatment comparisons may get vitiated and error increased. The influence if any of the differing body weights on the character under study can be assessed and if found significant necessary adjustments can be made through analysis of covariance procedures.

The cost involved in mariculture experiments will be generally high compared to experiments on land. Replication being one of the essential features of designing, a question often asked is what would be the minimum number of replications required to render test of significance sufficiently sensitive to detect real treatment differences. This depends on the magnitude of variation in the experimental units. If the magnitude is known the number of replications required for detecting a particular difference with a certain level of confidence can be worked out. A fair idea of the magnitude of variation can be obtained from available feed-back data or by conducting a uniformity trial where a particular crop is grown in several contiguous small-sized ponds with uniform treatment and studying

the variation in the yields from these units (Panse et al, 1965). In fact information from uniformity trials would facilitate preparation of contour maps and help in the formation of lay-out plans for the experiment.

There are workers who do not bother to follow a design but want to analyse the data statistically. Some others follow a design but are satisfied with some minimum analysis. It is essential in scientific experimentation to follow a suitable design and make a comprehensive analysis of the valuable resultant data through appropriate statistical procedures. The two aspects go hand in hand.

8 REFERENCES

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9 APPENDIX TO CHAPTER

9.1 Illustration

A randomised block design was employed for carrying out a nutrition experiment to study the comparative effect of

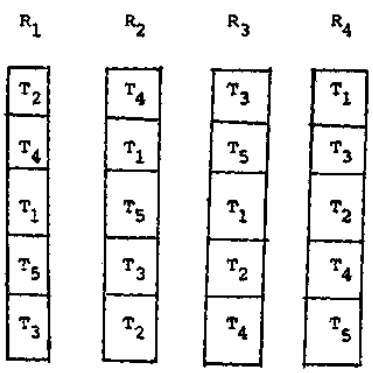
different supplementary feeds (treatments) on growth of sillago fish, keeping factors other than feed uniform.

Number of replications: Four (R_1, R_2, R_3 and R_4)

Number of treatments: Five, one control (T_1) and four supplementary feeds (T_2, T_3, T_4 and T_5)

Total number of ponds used = 4 X 5 = 20

The ponds were grouped into replications such that the five ponds in one replication were as similar as possible. In each replication the treatments were allotted at random. The lay-out plan is shown below:



The figures of the gain in weight of fish (gms./fish) for each of the ponds at the end of the experimental period are given in the following table.

Replication / Treatment	1	2	3	4	Total
1	24	25	23	21	93
2	25	22	24	19	90
3	24	26	21	22	93
4	33	36	32	31	132
5	34	33	31	29	127
Total	140	142	131	122	535

Analyse the data and draw conclusions.

9.1.1 Step-by-step procedure

9.1.1.1 Totals:

(a) The replication totals are:

$R_1 = 24 + 25 + 24 + 33 + 34 = 140$

$R_2 = 25 + 22 + 26 + 36 + 33 = 142$

$R_3 = 23 + 24 + 21 + 32 + 31 = 131$

$R_4 = 21 + 19 + 22 + 31 + 29 = 122$

(b) The treatment totals are:

$T_1 = 24 + 25 + 23 + 21 = 93$

$T_2 = 25 + 22 + 24 + 19 = 90$

$T_3 = 24 + 26 + 21 + 22 = 93$

$T_4 = 33 + 36 + 32 + 31 = 132$

$T_5 = 34 + 33 + 31 + 29 = 127$

(c) The grand total is

$G.T = 140 + 142 + 131 + 122 = 535$

9.1.1.2 Sums of squares (S.S.):

(a) Total:

Crude S.S. $= 24^2 + 25^2 + \ldots + 29^2 = 14811.00$ (20 terms)

Correction factor $= \frac{(535)^2}{20} = 14311.25$

Corrected S.S. = crude S.S. - C.F. = 499.75

(b) Replication:

Crude S.S. $= \frac{1}{5}(140^2+142^2+131^2+122^2) = 14361.80$

C.F. $= 14311.25$

Corrected S.S. $= 50.55$

(c) Treatments:

Crude S.S. = $\frac{1}{4}$ $(93^2+90^2+93^2+132^2+137^2)$ = 14737.75

C.F. = 14311.25

Corrected S.S. = 426.50

(d) Error S.S. is obtained by substraction of corrected S.S. for replication and treatment from the corrected total S.S.

Error S.S. = 499.75 - 50.55 - 426.50 = 22.70

9.1.1.3 Formation of analysis of variance table:

A N O V A

Source of variation	D.F.	S.S.	M.S.S.	F
Replication	3	50.55	16.85	8.94**
Treatments	4	426.50	106.63	56.42**
Error	12	22.70	1.89	
Total	19	499.75		

** Significant at 1% level

It can be seen from the table that the treatment effect is highly significant (1% level). Also the replication effect is highly significant indicating that the grouping of ponds has been effective in reducing the error.

9.1.1.4 Calculation of standard errors (S.E.) for the comparison of two different means:

(a) S.E. of any treatment mean,

$$S.E._{\bar{x}} = \sqrt{\frac{\text{Error M.S.}}{\text{No. of replications}}} = \sqrt{\frac{1.89}{4}} = 0.69$$

(b) S.E. of difference between any two treatment means.

$$S.E._d = \sqrt{\frac{2(\text{Error M.S.})}{\text{No. of replications}}} = \sqrt{\frac{3.78}{4}} = 0.97$$

(c) Critical difference (C.D.) at 1% level for comparing two means.

$= S.E._d \times t_{0.01}$, where $t_{0.01}$ refers to the 't' value at 1% level for 12 d.f. $= 0.97 \times 3.05 = 2.96$

Note: Comparing two individual means is to be resorted to only, if F test, which is an overall test, is found to be significant.

9.1.1.5 Summary table of means:

Mean gain in weight (gm/fish) for different feeds

T_4	T_5	T_3	T_1	T_2
33.00	31.75	23.25	23.25	22.50

Note: Feeds which do not differ significantly as can be found out with the help of C.D. value are underlined by a bar. Thus T_4 and T_5 are not significantly different as also T_1, T_2 and T_3

9.1.1.6 Conclusions:

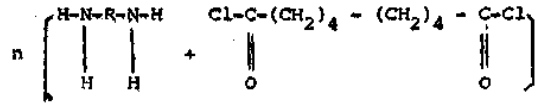
The supplementary feeds T_4 and T_5 gave significantly higher gain in weight than rest of the feeds.

Note: If economics is the consideration the cost for unit gain in weight of fish can be computed for each pond and then analysis of variance can be conducted and conclusions drawn by an identical procedure as above.

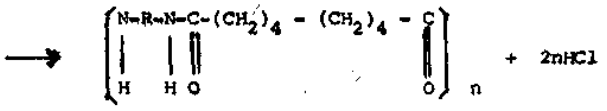
CHAPTER 15

PREPARATION OF MICROENCAPSULATED DIET*

1 Principle



Diamine Sebacoyl chloride



Wall structure

2 Reagent

(a) Diet ....e.g. chicken egg

(b) Diaminohexane solution ....0.92g of
1, 6-diaminohexane + 20 ml of 0.45 M
$NaHCO_3$ - $NaCO_3$ buffer (pH 9.8)

(c) Mixed solvent solution .....
chloroform - cyclohexane (1 : 4)

(d) Span 85

(e) Sebacoyl chloride

(f) Sucrose monolaurate

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

3 **Procedure**

Mixed solvent solution (25 ml) + Span 85 (0.5 ml)

↓

+ Diamine soln. (0.5 ml) + Diet Soln. (2.5 ml)
Emulsification for 3 min. by homogenizer
+ Mixed solvent soln. (10 ml) + Sebacoyl chloride (0.2 ml)
+ Mixed solvent soln. (30 ml)

Precipitate (Microencapsulated diet)

↓

Washed with mixed solvent soln. (100 ml)
2-3 times by decantation

Precipitate

↓

+ 7 ml sucrose monolaurate
stirring for 10 min.
Washing for 24 hr. in water (2 litre)
Filtration with cloth sack of 10 μm mesh
Wash with water (2 litre)
2 times by filtration

↓

Microencapsulated diet

store in 1 mol NaCl

CHAPTER 16

METHODS OF CULTURING PHYTOPLANKTON*

1 INTRODUCTION

It is an established fact that the success of any hatchery operation will depend mainly on the availability of the basic food, the phytoplankton. The maintenance and supply of the required species at appropriate time form a major problem facing the algal culturists. The procedure for the phytoplankton culture involves aspects such as the isolation of the required species, preparation of the suitable culture media, maintenance of the culture in the laboratory scale, as well as large scale under controlled conditions of light, temperature and aeration and their constant supply in different phases of growth.

2 METHODS OF ISOLATION AND CULTURE

2.1 Methods of isolation

The isolation of the required species can be done by one of the following methods:

a. Pipette method:

Larger organisms can be pipetted out using micro-pipettes under a microscope.

b. Washing method or centrifugation:

Repeated washing or centrifuging the water samples results in the isolation of larger organisms.

c. By exploiting the phototactic movement:

By this method, the phytoflagellates, will move to one direction and with a micro-pipette can be isolated.

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* Prepared by C.P. Gopinathan, Central Marine Fisheries Research Institute, Cochin-18.

1. By agar-plating method:

For preparing the agar medium, 1.5% agar is added to 1 litre of suitable medium or even natural seawater and this agar solution is sterilised in an autoclave for 15 minutes under 150 lbs pressure and 120°C temperature. Now this medium is poured in sterilised petri-dishes and left for 24 hrs. In case of culture tubes, the medium is poured in 1/3 part in tubes and properly plugged with cotton before autoclaving.

The isolated species can be picked up by platinum needle or platinum loop under microscope and streaked on the surface of agar plates. After innoculation, these petri-dishes are placed in an incubation chamber for 10-15 days. Light intensity and temperature should be maintained constant. Within 15 days if the required species forms a colony, remove it by platinum loop and transfer to culture tubes half filled with suitable culture media, and subsequently from the culture tubes to culture flasks and then to 20 l glass carbuoys if the species could be cultured on a mass scale.

2.2 Culture apparatus

Vessels made of 'Pyrex' or 'corning' glass are usually used for phytoplankton culturing. But from test tube to concrete tanks may be used, depending on the quantity of the culture required. For small scale experiments Erlenmeyer flasks equipped with inlet and outlet tubes for aeration are used. Glass tubes or flasks plugged with cotton provides enough aeration. The vessels should be cleaned well and sterilized in a hot air oven.

2.3 Selection of the culture medium

On securing the desired organism, transfer the sample into a series of petri-dishes, each containing different enriched media. Keep them exposed to sunlight or artificial

light. This preparatory culture is used to select the suitable medium for the particular species. During this time, the organism multiply in one of the media and provide enough material for further process of culturing. Pure cultures are sometime obtained only after several attempts. The preparatory cultures may be maintained till pure cultures are obtained.

2.3.1 Culture media or solutions:

The following are some of the culture media found suitable to most planktonic algae:

2.3.1.1 Schreiber's solution:

Sodium nitrate 0.1 gm
Sodium acid phosphate 0.02 gm
Soil extract 50 cc
Filtered water 1 litre

The soil extract is prepared by boiling 1 kg good garden soil with 1 litre of distilled water in the autoclave for one hour. After 2-3 days, the supernatant liquid is separated into a flask and sterilised in an autoclave at 120°C for 20 minutes. It is advisable to keep the soil extract in a refrigerator.

2.3.1.2 Miquel's solution:

A	B
20.2 gm of Potassium nitrate dissolved in 100 ml of distilled water	Sodium phosphate -- 4 gm Calcium chloride -- 4 gm Ferric chloride -- 2.0 gm Conc. HCl -- 2 ml Dissolved in 100 ml of distilled water

To each litre of filtered seawater, 0.55 ml of A and 0.5 ml of B are added.

2.3.1.3 Convey or Walne's medium:

This medium is mainly used for the mass culture of phytoflagellates such as Isochrysis, Tetraselmis, Monochrysis and Dicrateria species.

a. Sodium nitrate or potassium nitrate .... 100 gm
Sodium phosphate .... 20 gm
Ferric chloride .... 1.3 gm
Manganese chloride .... 0.36 gm
Bromic acid (H_3BO_3) .... 33.4 gm
EDTA .... 45 gm

Dissolve all the chemicals in one litre of distilled water. One ml is added to each litre of filtered seawater.

b. Trace metal solution:

Zinc chloride .... 2.1 gm
Calcium chloride .... 2.0 gm
Ammonium molybdate .... 2.0 gm
Copper sulphate .... 2.0 gm
Distilled water .... 100 ml

One ml is added to each litre of the seawater.

c. Vitamin stock solution:

B_{12} .... 5 mg
B_1 .... 100 mg

Dissolve in 100 ml of distilled water. 0.1 ml is added to each litre of seawater.

2.3.1.4 TMRL medium:

This solution is mainly used for the mass culture of diatoms, such as Skeletonema costatum and Chaetoceros spp.

Pottassium nitrate	....	10 gm
Sodium phosphate	....	1 gm
Ferric chloride	....	0.3 gm
Sodium silicate	....	0.2 gm

Prepare each chemical in 100 ml distilled water in separate bottles. Add 1 ml of the solution to 1 litre of filtered seawater.

2.3.1.5 P M solution:

This is another culture solution used mainly for the mass culture of diatoms, especially in 20 l glass carbuoys as a starter for the large-scale culture in bigger tanks.

Sodium nitrate	....	10 gm
Potassium phosphate	....	1 gm
Ferric chloride	....	0.2 gm
Sodium silicate	....	0.2 gm
Agrimin	....	0.1 gm
EDTA	....	0.2 gm
Thiamine (B_1)	....	0.005 gm
Cobalamine (B_{12})	....	0.005 gm

Prepare the solution in separate 100 ml reagent bottles and added to the filtered seawater 1 ml/l.

2.3.1.6 In open system (100 ton or more) the mass production of diatoms is conducted by using Commercial fertilizers:

Urea	....	100 mg/l
Agrimin	....	1 mg/l
Ferric chloride	....	2 mg/l
16-20-0	....	5 mg/l
Sodium silicate	....	2 mg/l
Potassium phosphate	....	5 mg/l

2.3.1.7 For raising a mixed culture of phytoplankters, the following chemicals can be used.

Potassium nitrate	....	1.32 gm
Sodium phosphate	....	0.66 gm
Sodium silicate	....	0.66 gm
EDTA	....	0.66 gm

Mix the chemicals in a one litre beaker, diluting with distilled water and pour to 100 l of unifiltered seawater. Within 3 days, a bloom of diatoms could be expected.

3 GROWTH PHASES OF THE CULTURE AND HARVESTING

The usual way of the laboratory culture of the microalgae is one in which a limited volume of medium containing the necessary inorganic and organic nutrients is inoculated with a relatively small number of cells and these are exposed to suitable conditions of light, temperature and aeration. Increase in cell numbers in such a culture follows a characteristic pattern in which the following phases can be noted.

(a) Lag phase - in which no increase in cell numbers

(b) Exponential phase - in which cell multiplication is rapid

(c) Declining phase - in which the cell numbers remain constant or no more growth

(d) Stationary phase - in which the cells are stationary

(e) Death phase - in which decay may start

Harvesting of the culture is done at the exponential phase of growth. Cultures can be maintained by occasional replacement with nutrients or by regular sub-culturing.

CHAPTER 17

METHODS OF CULTURING ZOOPLANKTON*

1 INTRODUCTION

Successful hatchery production of the fry of fish and crustaceans for aquaculture purposes depends, among other things, on theavailability of zooplanktonic organisms of appropriate size for feeding the larvae. Freshly hatched Artemia nauplii have been the popular larval feed used by scientists and aquaculturists for a long time. But the high cost of Artemia cysts has lead the aquaculturists to search for other suitable zooplankters which could be easily cultured on a large scale. The rotifer Brachionus Plicatilis, the cladoceran Moina sp. the harpacticoid copepods such as Tigriopus spp and Tisbe spp, the nematode, Panagrellus sp and the cillate Fabrea salina, all of which have a high reproductive rate, short generation time, and the ability to live and grow in crowded culture conditions have been found to be useful as live feed organisms for larval rearing of cultivable species of fish and crustaceans. Among them Brachionus plicatilis and Moina sp have been most successfully used in larval rearing work in many countries and hence, in this paper, the methods of culturing these animals on a large scale are discussed in the light of experience gained in mass culturing them at the Narakkal Prawn Culture Laboratory of the Central Marine Fisheries Research Institute.

2 BRACHIONUS PLICATILIS

This filter feeding, planktonic, euryhaline, rotifer which multiplies at a very fast rate by parthenogenesis under ideal conditions has been found to be an excellent feed for rearing marine fish fry especially during the early larval stages. It is also a good food for late mysis and early post-larval stages of penaeid prawns.

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* Prepared by M.S. Muthu, Central Marine Fisheries Research Institute, Cochin-18.

2.1 Isolation

Brachionus plicatilis grows naturally in brackishwater ponds and can be easily isolated under a binocular microscope with a fine pipette. As the rotifer is parthenogenetic, it is easy to isolate a few egg-bearing females in a petri-dish containing filtered brackishwater and by feeding them with a suitable algal food to build up a stock culture within a few days.

2.2 Culture medium

B. plicatilis is euryhaline and can be acclimatised to grow in waters with a salinity of 10 to 40 ppt. It is likely that there are geographical races which may grow better in certain salinity ranges, which will have to be determined experimentally. Generally a salinity range of 20-30 ppt is good enough. Filtered sea water can be diluted with good tap water or well water to get the desired salinity.

2.3 Culture containers

Containers ranging from 20 litre glass carbouys to 360 ton concrete tanks have been used for culturing *B. plicatilis* depending on the quantity of rotifers needed. For a hatchery, large plastic containers, fibreglass tanks or concrete pools will be necessary. It is essential that the containers are provided with good aeration facilities as the dense concentration of algae and rotifers consume a lot of oxygen especially during night time.

2.4 Feed for the rotifers

B. plicatilis has been grown on pure cultures of unicellular algae such as *Chlorella* sp., *Tetraselmis* sp., *Dunaliella* sp., bakers' yeast, marine yeast and methanol-grown yeast, and freeze-dried or spray dried *Chlorella*,

Spiruline or Platymonas, or even on powdered formulated diets. If fresh algae are used they are cultured separately and added to the rotifer cultures every day. To reduce the cost of culturing algae various types of agricultural fertilizers such as ammonium phosphate, ammonium sulphate and urea are used; organic manures such as extracts of chicken dung, pig manure, and groundnut oil cake have also been tried. These organic manures induced a good growth of Chlorella and bacteria which are utilized by the rotifer as food.

2.5 Culture methods

It is advisable to grow the rotifers and the algal feed in separate containers. Desired amount of algal suspension is added to the rotifer tank to maintain a concentration of 10^6 cells/ml in the case of Tetraselmis and Dunaliella or 5-10 X 10^6 cells/ml of Chlorella. Yeast, algal powders and formulated diets are given at the rate of 200-300 mg/litre per day in 3 divided doses. The rotifer population grows rapidly and attains a concentration of 200-300 nos/ml in 4-5 days after starting a culture at a temperature of 28-30°C. If the rotifer culture is not harvested, a density of upto 600 nos/ml is reached in 6-7 days and then the culture declines rapidly. It is better to harvest daily 1/3 to 1/4 of the culture and replace the volume harvested with fresh algal culture or rotifer medium. Harvests of 30-50 nos/ml per day could be made every day. Regular harvesting helps to maintain the culture in good condition for a longer period. Since the culture are not maintained under sterile conditions they are likely to be contaminated with the growth of undesirable filamentous algae and ciliates. Under such conditions the cultures should be discarded, the pools cleaned thoroughly and a fresh culture started again.

At the Narakkal Prawn Culture Laboratory of the CMFRI B. plicatilis is cultured in 24' dia. outdoor plastic pools using 20-30 ppt. brackishwater fertilized with groundnut oil cake (250 gm/ton) urea (8 gm/ton) and superphosphate

(4 gm/ton) to induce a bloom of Chlorella on which the rotifers feed.

2.6 Harvesting

Harvesting of rotifers is done by filtering through a nylon bolting silk cloth of 40 micron mesh size. The concentrated rotifers are washed in clean sea water and used for feeding larvae either in fresh or in frozen condition. Rotifers that are to be frozen should not be washed in freshwater as it leads to osmotic breakdown of cell membranes and at the time of thawing a very poor product is obtained. Harvesting should be done in the exponential growth phase when the rotifers are seen with 3 or more eggs attached to the body. The organic matter content of the body is maximum at this stage. In the senescent stage, the rotifers do not carry eggs, their body tissues are depleted, the test being practically empty and the animals swim feebly around. The nutritive value of such rotifers will be very poor indeed.

2.7 Nutritive value

It has been reported by Japanese workers that the nutritive value of the rotifers to the fish larvae depends on the diet on which the rotifers were reared. Fish larvae fed with rotifers grow on a diet of fresh algal cultures appear to be well nourished while those fed with rotifers reared on a diet of yeast or commercial single cell proteins are weak and their survival is low. This is attributed to the difference in the fatty acid composition of the rotifers fed with yeast and algae respectively. The latter are found to be rich in w3 long-chain unsaturated fatty acids (LUFA). Marine fish and crustacean larvae seem to have a specific requirement for w3 LUFA which can be satisfied only through the diet.

2.8 Maintenance of cultures

New cultures can be started from resting eggs which can be stored for long periods. B. plicatilis which normally reproduces by parthenogenesis, produces resistant

resting eggs under unfavourable conditions or when the density of the rotifer population becomes too high. B. plicatilis which have been growing in normal sea water have been induced to produce resting eggs when they are transferred to 25% seawater. By this method resting eggs could be collected and stored in a deep freeze at -14°C for 3 months and for 3 weeks in a desiccator at room temperature without loss of viability. It is reported that the shelf life of desiccated eggs can be increased to 25 weeks by a process known as sonification at low energies.

3 MOINA SP.

The freshwater cladoceran Moina is frequently found in temporary ponds. It is readily eaten by bigger fish fry and by older postlarvae of penaeid prawns. Moina also reproduces by parthenogenesis under favourable conditions and forms resting eggs through sexual reproduction under unfavourable conditions. The embryos develop inside the dorsal brood pouch and the young ones hatch out fully formed.

3.1 Isolation

Moina can be collected from ponds and a stock built up starting from a single parthenogenetic female. From a single female kept in a 2 litre beaker containing Chlorella water, it has been possible to obtain 42,000 Moina within 12 days.

3.2 Culture medium

Good tap water or well water can be used for growing Moina. Salinities above 3 ppt are not tolerated by them.

3.3 Culture containers

3' - 12' dia. plastic pools or concrete tanks can be used for Moina culture. Good aeration should be provided. The tanks are kept outdoors.

3.4 Feed for Moina

It is a filter feeder living on a variety of unicellular fresh water algae. Chlorella appears to be the best feed.

3.5 Culture methods

The culture tank is filled with freshwater, innoculated with a culture of Chlorella, fertilized with groundnut oil cake (250 gm/ton), urea (8 gm/litre) and superphosphate (4 gm/ton) and well aerated. On the second day after the water becomes slightly greenish the culture is innoculated with a pure culture of Moina which multiplies rapidly and attains a concentration of 20 to 30 thousand individuals per litre in 6-7 days. At this stage 1/3 - 1/2 the volume can be harvested and replaced by freshwater along with the proportional amounts of the above fertilizers, or by Chlorella water cultured in a separate tank using the same fertilizers. If the latter method is followed 1/3 of the culture volume can be harvested everyday. The groundnut oil cake sustains the algal bloom for a longer period and in the finely divided state may be eaten directly by the cladocerans. Chlorella blooms can be maintained by fertilizing with chicken dung or pig manure also.

Appearance of males in the culture, heralds the decline of the population by formation of resting eggs. When this happens it is better to remove all the water leaving only the sediments at the bottom and filling up the tanks with Chlorella water again. Moina culture revives in a few days.

3.6 Harvesting

Harvesting is done with a plankton net in the exponential growth phase when the females are reproducing actively by parthenogenesis. Parthenogenetic females containing 8-12 embryos in the brood pouch are rich in organic matter and are evidently more nutritive than females with resting eggs or the males. Harvested Moina are washed in water and frozen into small blocks for future use.

3.7 Nutritive value

Moina which can also be grown on yeast or commercial single cell proteins, are said to be deficient in w3 LUFA and are therefore inferior as feed for the fish and crustacean larvae. Moina fed with fresh algal cultures are nutritionally adequate.

3.8 Maintenance of cultures

Resting eggs can be collected from the bottom of the culture containers and stored in dry conditions at room temperature for 2-3 weeks without loss of viability. Fresh cultures can be started by keeping the dry resting eggs in well aerated Chlorella water; parthenogenetic females hatch out from the resting eggs within 48 hours.

4 ARTEMIA SALINA

Artemia saline nauplii are usually hatched out from cysts stored in sealed cans. Cysts hydrated in seawater hatch out in 24-30 hrs. They are isolated from the floating empty cysts by keeping a light at the transparent bottom of the conical containers used for hatching the cysts. The nauplii which congregate near the light are siphoned out and used for feeding the larvae or for starting batch cultures of adult Artemia to feed the juveniles and adults of fish or prawns. Freshly hatched Artemia nauplii are said to be nutritionally superior to one day old nauplii.

Dense batch cultures are grown in large containers using well aerated, high saline water (40-60 ppt). Suspensions of yeast, dry Chlorella or Spirulina powder, rice bran or groundnut-oil-cake-milk have been used to feed the Artemia. They become egg bearing adults in 12-14 days. The nutritional quality of the adults grown on different diets will be different and will have to be assessed in relation to the purpose for which they are raised.

Maintaining continuous cultures of Artemia under controlled conditions for cyst production is possible only on a small scale for experimental purposes. However Artemia is being cultured in salt pans for commercial production of cysts.