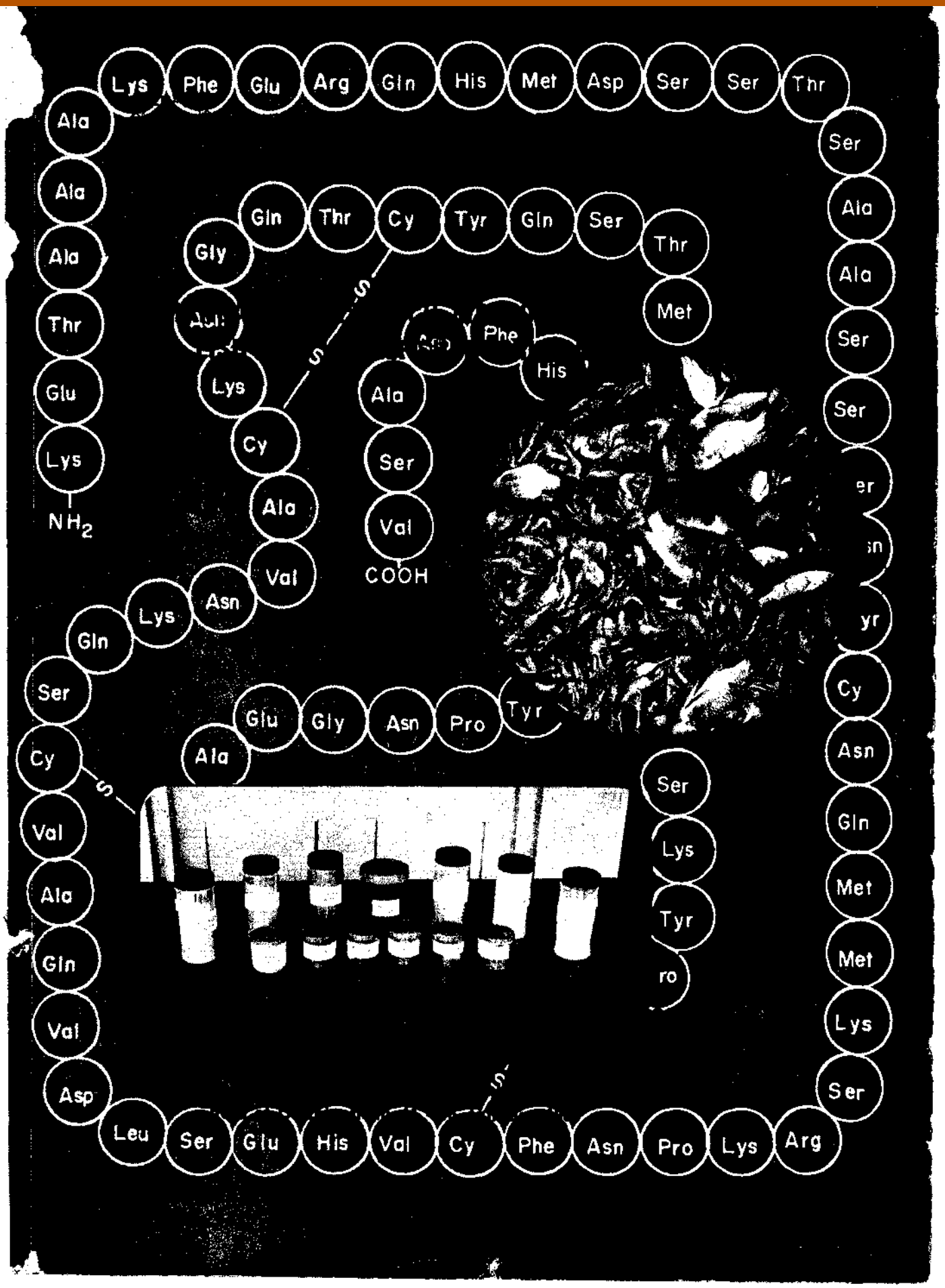
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His Phe
Glu Gly Asn Pro Tyr
Ala
Ser Lys Tyr
Ser Ala Ala Ser Ser Cy Asn Gln Met Met Lys Ser
Ser Glu His Val Cy Phe Asn Pro Lys Arg

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MANUAL OF RESEARCH METHODS FOR FISH AND SHELLFISH NUTRITION

Issued on the occasion of the Workshop on
METHODOLOGY FOR FISH AND SHELLFISH NUTRITION
organised by
The Centre of Advanced Studies in Mariculture,
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Published by: **E. G. SILAS**
Director
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COCHIN

PREFACE

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

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

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

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

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

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

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

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

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

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

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

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 oxidised 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 oxidised 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 <u>Preparation of sample and determination</u>

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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