

Lab. Manual 6

**MANUAL OF METHODS
OF
ANALYSIS OF FOODS**

MEAT AND MEAT PRODUCTS

AND

FISH AND FISH PRODUCTS



**DIRECTORATE GENERAL OF HEALTH SERVICES
MINISTRY OF HEALTH AND FAMILY WELFARE
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MANUAL FOR ANALYSIS OF MEAT AND MEAT PRODUCTS **AND FISH AND FISH PRODUCTS**

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MANUAL FOR ANALYSIS OF MEAT AND MEAT PRODUCTS **AND FISH AND FISH PRODUCTS**

Meat, fish and their products are important components of diet of a large majority of people. Their nutritive value and palatability are widely appreciated. Standards for meat and meat products and fish and fish products are laid down in item A 34 and A 35 respectively of Appendix B to P.F.A Rules (1955). These standards contain microbiological requirements in addition to chemical requirements. A separate manual has been prepared for microbiological examination of these products

1.0 Meat and Meat Products

1.1 Preparation of sample: -

To prevent loss of moisture during preparation and subsequent handling, do not use small test samples. Keep ground material in glass or similar containers with air and water tight covers

(a) Fresh and frozen meat, cured meats, smoked meats etc

Separate as completely as possible from any bone, pass rapidly three times through food chopper with plate opening equal to 1/8th inch (3 mm), mixing thoroughly after

each grinding and begin all determinations promptly. If any delay occurs chill sample to inhibit decomposition. In case of cured meats, mix thoroughly with a spatula or pass through a food chopper or mix in a homogeniser / blender to a uniform mass as appropriate. Transfer to a wide mouth glass or other suitable container with an airtight stopper. Carry out analysis as soon as possible

(b) Canned meats

Pass entire contents of the can through the food chopper or blender to obtain a uniform mass.

Dry portions of (a) and (b) not needed for immediate analysis either in vacuo at less than 60 ° C or by evaporating on steam bath 2 -3 times with alcohol. Extract fat from dried product with petroleum ether (b. p. less than 60 ° C) and let petroleum ether evaporate spontaneously, finally expelling last traces by heating short time on steam bath. Do not heat test sample or separated fat longer than necessary because of tendency to decompose.

(Ref:- A.O.A.C 17th edition,2000, 983.18 Meat and Meat Products, Preparation of test sample (a)and (b))

1.2 Determination of Nitrite (rule 55)

1.2.1 Reagents:-

(a) NED reagent - Dissolve 0.2 gm N- (1 Naphthyl) ethylenediamine dihydrochloride in 150 ml, 15% (v/v) acetic acid. Filter if necessary and store in a glass stoppered brown glass bottle.

(b) Sulphanilamide reagent- Dissolve 0.5gm sulphanilamide in 150 ml 15% acetic acid (v/v). Filter, if necessary and store in a glass stoppered brown bottle.

(c) Nitrite standard solution-

(i) Stock solution - 1000 ppm. NaNO_2 - Dissolve 1.000 gm pure NaNO_2 in water and make upto 1 litre.

(ii) Intermediate solution - 100 ppm. - Dilute 100ml of stock solution to 1 litre with water.

(iii) Working solution- 1ppm - Dilute 10ml of intermediate sol to 1 litre with water.

(d) Filter paper - Test for nitrite contamination by analyzing 3-4 sheets at random. Filter approx 40ml water through each sheet. Add 4ml of Sulphanilamide reagent, mix, let stand 5 minutes, add 4ml of NED reagent, mix and wait for 15 minutes. If any sheets are positive do not use them.

1.2.2 Procedure:-

Weigh 5 gms prepared sample in a 50 ml beaker. Add about 40ml of water heated to 80°C. Mix thoroughly with glass rod taking care to break all lumps and transfer to 500ml volumetric flask. Thoroughly wash beaker and glass rod with successive portions of hot water adding all washings to flask. Add enough hot water to bring vol to about 300ml. Transfer flask to steam bath and let stand 2 hours shaking occasionally. Cool to room temperature, dilute to volume with water and remix. Filter. If turbidity remains after filtration, centrifuging will usually clear the solution. Add 2.5ml of sulphanilamide sol to aliquot containing 5-50 ug NaNO₂ in 50 ml vol flask and mix. After 5 minutes add 2.5 ml NED reagent, mix dilute to vol, mix and let colour develop 15 minutes. Transfer portion of solution to photometer cell and determine absorbance at 540 nm against blank of 45ml water and 2.5ml of sulphanilamide reagent and 2.5ml of NED reagent.

Determine Nitrite present by comparison with standard curve prepared as follows:-

Add 10, 20, 30, 40 ml of nitrite working solution to 50ml vol flasks. Add 2.5 ml of sulphanilamide reagent and after 5 minutes add 2.5ml of NED reagent and proceed as above.

Standard curve is straight line upto 1 ppm Na NO₂ in final solution

(Ref :- A.O.A.C Official method 17th edition 2000, 973.31 Nitrites in cured meats - Colorimetric method, Adopted as Codex Reference method (Type II))

1.2 A Alternate method for determination of Nitrite

1.2.1. A Principle

Extraction of a test portion in hot water, precipitation of the proteins and filtration. In the presence of nitrite development of a red colour by the addition of sulphanilamide and N - naphthylethylenediamine dihydrochloride to the filtrate and photometric measurement at 538 nm

1.2.2 A Reagents

(a) Solutions for precipitation of proteins

- (1) Dissolve 106 gm of Potassium ferrocyanide trihydrate in water and dilute to 1000 ml
- (2) Dissolve 220 gm of Zinc acetate dihydrate and 30 ml glacial acetic acid in water and dilute to 1000 ml
- (3) Dissolve 50 gm of disodium tetraborate decahydrate in 1000 ml of tepid water and cool to room temperature

(b) Standard Sodium nitrite solution - Dissolve 1.000 gm pure sodium nitrite in water and dilute to 100 ml in a volumetric flask. Pipette 5 ml of the solution into a 1000 ml volumetric flask and make upto volume.

Prepare a series of standard solutions by pipetting 5 ml, 10 and 20 ml of the solution into 100 ml volumetric flasks and diluting to mark with water. These standard solutions contain 2.5 μg , 5.0 μg , and 10 μg sodium nitrite respectively. The standard solutions and the 0.05 gm /1 solution from which they are prepared shall be made on the day of the use.

(c) Solution for colour development

(1) Dissolve by heating on a water bath, 2 gm of sulphanilamide in 800 ml water. Cool, filter if necessary and add 100 ml of conc HCl while stirring. Dilute to 1000 ml with water.

(2) Dissolve 0.25 gm of N – naphthyl ethylenediamine dihydrochloride in water. Dilute to 250 ml with water. Store in a stoppered brown bottle in a refrigerator for not more than one week

(3) Dilute 445 ml of Concentrated HCl (sp.gr 1.19) to 1000 ml with water.

1.2.3 Apparatus

- (1) Meat mincer - fitted with a perforated plate with holes not greater than 4 mm in diameter.
- (2) Analytical Balance
- (3) Volumetric flasks - 100 ml, 250 and 1000 ml
- (4) Pipette 10 ml
- (5) Conical flask
- (6) Boiling water bath
- (7) Fluted filter paper
- (8) Photoelectric colorimeter or spectrophotometer.

1.2.4. A Procedure

Weigh to the nearest 0.001 gm, about 10 gm of the test sample, transfer quantitatively to a 300 ml conical flask and add successively 5 ml of saturated borax solution and 100 ml water at a temperature not below 70 °C Heat the flask for 15 minutes on the boiling water bath and shake repeatedly. Allow the flask and its contents to cool to room temperature and add successively 2 ml of Pot ferrocyanide followed by 2 ml of zinc acetate. Mix thoroughly after each addition. Transfer the contents to a 200 ml volumetric flask. Dilute to mark with water and mix. Allow the flask to stand for 30 minutes at room temperature. Carefully decant the supernatant liquid and filter it through fluted filter paper to obtain clear solution.

Colour Development - Pipette an aliquot of the filtrate (v ml) not more than 25 ml into a 100 ml volumetric flask and add water to make upto 60 ml. Add 10 ml of sulphanilamide solution followed by 6 ml of cone HC1 and leave the solution in the dark for 5 minutes. Add 2 ml of N -Naphthylethylenediamine solution and leave for 5- 10 minutes in the dark. Dilute to mark with water. Measure the absorbance of the solution in a 1 cm cell using a photoelectric colorimeter or spectrophotometer at a wave length of about 538 nm

Prepare a calibration curve by taking 10ml water in 4 separate volumetric flasks, adding 10 ml each of the standard sodium nitrite solution containing 2.5, 5.0 and 10 µg of nitrite / ml, developing the colour and measuring as above

1.2.5 A Calculation

Nitrite content expressed as $\text{NaNO}_2 = \frac{c \times 2000}{M \times V}$

Where

V = volume in ml of aliquot portion of filtrate taken for test
M = mass in gm of sample taken
c = concentration of sodium nitrite in ug / ml read from the calibration curve that corresponds with the absorbance of the solution prepared from the sample

(Ref:- I.S 5960 (Part VII): 1996 / I.S.O 2918 : 1975 Meat and Meat Products Methods of Test - Determination of Nitrite content)

1.3 Determination of Ascorbic acid (Rule 72):

1.3.1 Principle:-

Ascorbic acid reduces oxidation-reduction indicator dye 2, 6 dichlorophenol indophenol to colorless solution. At end point excess unreacted dye is rose pink in acid solution. Vitamin is extracted and titration performed in presence of metaphosphoric acid-acetic acid solution to maintain proper acidity and avoid auto oxidation of ascorbic acid at high pH

1.3.2 Reagents:-

(a) Extracting solution -

Metaphosphoric acid-acetic acid solution-Dissolve with shaking 15gm HPO_3 pellets or freshly pulverized sticks in 40ml acetic acid and 200ml water. Dilute to 500ml. Filter

rapidly through fluted filter paper into a glass stoppered bottle. Store in a refrigerator to prevent change to H_3PO_4 . Solution remains satisfactory for 7-10 days.

(b) Ascorbic acid standard solution (1 mg/ ml) - Accurately weigh 50mg USP Ascorbic acid reference standard that has been stored in a dessicator away from sunlight. Transfer to 50ml vol flask. Dilute to vol with metaphosphoric- acetic acid extracting solution before use.

(c) Indophenol standard solution:-Dissolve 50mg 2,6 dichlorophenol indophenol sodium salt in 50ml of water to which have been added 42 mg of $NaHCO_3$. Shake vigorously and when dye dissolves dilute to 200ml with water. Filter through fluted filter in an amber colored glass bottle. Keep stoppered and store in a refrigerator.

(Note:-Decomposition products that make end point indistinct occur in some batches of dry indophenol and also develop with time in stock solution. Add 5ml of extracting solution with excess ascorbic acid to 15ml of dye solution. If reduced solution is not practically colorless discard and prepare new stock solution.)

Standardisation of Indophenol solution - Transfer 3 aliquots of 2.0 ml Ascorbic acid standard sol to 3 conical flasks containing 5ml of metaphosphoric- acetic acid extracting solution. Titrate rapidly with indophenol dye from 50ml burette until a light distinct rose pink remains for 5 seconds. Each titration should require about 15ml indophenol solution and differ from each other by 0.1 ml. Similarly titrate 3 blanks composed of 7ml of $HPO_3 - CH_3 COOH +$ water equal to the vol of indolphenol sol used in earlier titration. Titrate with indolphenol. Titre for blank should be approx 0.1 ml. Subtract blank from earlier titration and calculate concentration of indophenol

solution as mg ascorbic acid equivalent to 1 ml of solution.

Standardise indophenol solution daily with freshly prepared ascorbic acid standard solution.

1.3.3 Procedure

Take about 25-50 gm of prepared sample (containing 100 to 200mg ascorbic acid). Dilute with 100ml of metaphosphoric-acetic acid solution and mix thoroughly in a laboratory homogeniser. Centrifuge and decant the supernatant liquid through an acid washed filter paper (Whatman 541). Take 3 sample aliquots containing about 2.0mg of ascorbic acid, add 5ml of metaphosphoric-acetic acid solution. Make a blank using 7ml of extracting solution Titrate with indophenol solution Titrate blank also by diluting it with water to the extent of the indophenol sol used in the sample.

1.3.4 Calculation

Ascorbic acid mg/100 gm =

Sample titre - blank x mg ascorbic acid/ml x Vol made x 100

Aliquot taken x wt of sample

(Reference:- A.O.A.C 17th edition,2000, Official method 967.21 Ascorbic acid in vitamin preparation and juices)

1. 3. A - Alternate method for Ascorbic acid

Extract 50 gm prepared sample with 100 ml meta phosphoric acid - acetic acid mixture prepared by dissolving 30 gm metaphosphoric acid in 1000 ml water containing 80 ml glacial acetic acid. Mix thoroughly in a laboratory homogenizer. Centrifuge and decant the supernatant through an acid washed filter paper(Whatman 541). Titrate 2 ml of the extract with a solution of 2 , 6 - dichloro - N - p -hydroxy phenyl- p - benzoquinone monoamine (30 gm in 200 ml water) until a permanent pink colour persists for at least 1 minute.

0.1 ml = 7 mg of total ascorbate

(Ref:- Pearsons Composition and Analysis of Foods 9th edn1991 , page 500)

1.4 Determination of Total Phosphorous content (rule 72) :-

1.4.1 Principle

Mineralization of a test portion (wet digestion) with sulphuric and nitric acid, precipitation of phosphorous as quinoline phosphomolybdate and drying and weighing of the precipitate. Alternatively the sample can be ashed and ash taken up in 15 ml cone nitric acid in a conical flask adding water to make upto 75 ml, heating on a boiling water bath for 30 minutes , cooling and making upto a known volume.

1.4.2 Reagents

- (1) Cone. Sulphuric acid - 1.84 gm / ml
- (2) Cone. Nitric acid - 1.40 gm / ml
- (3) Precipitating reagent - Dissolve 70 gm of Sod. Molybdate dehydrate in 150 ml water. Dissolve 60 gm of Citric acid monohydrate in 150 ml water and add 85 ml of cone nitric acid. Mix the two solutions and stir slowly. To another 100 ml water add 25 ml nitric acid and 5 ml of distilled quinoline. Gradually add this solution to the first solution while stirring. Leave for 24 hrs at room temperature. Store the reagent in a stoppered plastic bottle in the dark

1.4.3 Apparatus

- (1) Mechanical meat mincer - fitted with a plate with holes of dia not exceeding 4 mm.
- (2) Analytical balance
- (3) Kjeldahl flask
- (4) Heating device on which the flask can be heated in an inclined position in such a way that the source of heat only touches the wall of the flask which is below the level of the liquid.
- (5) Suction device to remove the acid fumes formed during the digestion.
- (6) Fritted glass filter - pore diameter 5-15 mm.
- (7) Drying oven capable of being adjusted to 260 — 20 ° C
- (8) Conical suction flask
- (9) Dessicator

1.4.4 Procedure

Make the sample homogeneous by passing it at least twice through the meat mincer and mixing. Keep the homogenised sample in a completely filled airtight closed container and store it in such a way that deterioration and change in composition is prevented. Analyse the sample as soon as possible, but in any case within 24 hrs. If the sample is not immediately analysed after passage through the mincer, liquid separation may occur. Therefore homogenize the sample thoroughly immediately before testing.

Weigh to the nearest 0.001 gm about 3 gm of sample into the flask, add 20 ml nitric acid and some glass beads. Place the flask in an inclined position on the heating device and heat for 5 minutes. Cool and then add 5 ml of sulphuric acid. Heat the flask gently until the foaming has ceased, then heat more strongly. Add more nitric acid and continue heating. Repeat the operation until evolution of brown fumes has ceased. Finally when the liquid has become colourless heat until white fumes appear. Cool add 15 ml water and boil gently. Transfer the liquid to a 250 ml beaker or conical flask rinsing the flask with water. Add 10 ml nitric acid. The total volume should then be 50ml.

1.4.4.1 Determination

Add 50 ml of the precipitating reagent to the liquid in the beaker or conical flask. Cover with watch glass and boil for 1 minute on a hot plate. Allow to cool to room temperature, during cooling swirl the contents three or four times. Filter under the suction through the fritted glass filter which has been previously heated for 30 minutes at a temperature of 260 ± 20 ° C, cooled in a desiccator and weighed to the

nearest 1 mg. Wash the ppt on the filter five times with 25 ml water using the same water to wash away any remaining ppt from the conical flask onto the filter. Dry in the oven at $260 \pm 20^{\circ} \text{C}$ for 1 hr. Cool in a dessicator and weigh to the nearest 1 mg. Carry out a blank test using same procedure and same quantity of reagents but omitting the test portion

Calculation

$$\text{Phosphorous (\%)} \text{ as } \text{P}_2\text{O}_5 = 0.03207 \times m, \times \frac{100}{m_0} \text{ or } = 3.207 \times \frac{m_1}{m_0}$$

Where

m_0 = mass in gm of the test portion

m_1 = mass in gm of the quinoline phosphomolybdate precipitate.

Report the result to the nearest 0.01 gm of phosphorous pentoxide / 100 gm

(Ref:- I.S 5960 (Part 9) : 1988 / I.S.O 2294 : 1974 Meat and Meat Products-Methods of Test - Determination of Total Phosphorous Content)

1.5 Test for presence of Poly phosphates:-

1.5.1 Principle

Extraction of meat or meat product with trichloro acetic acid, cleaning of the serum

obtained with ethanol / diethyl ether mixture, separation of the phosphates by thin layer chromatography and detection of polyphosphates by spraying with reagents for colour development.

1.5.2 Reagents

- (1) Trichloro acetic acid
- (2) diethyl ether
- (3) Ethanol 95% (v/v)
- (4) Cellulose powder for TLC
- (5) Soluble starch

1.5.3 Reference mixture –

Dissolve in 100 ml water 200 mg of Sodium dihydrogen phosphate monohydrate ($\text{Na H}_2 \text{PO}_4, \text{H}_2\text{O}$), 300 mg of tetra sodium diphosphate decahydrate ($\text{Na}_4 \text{P}_2\text{O}_7, 10 \text{H}_2\text{O}$), 200 mg of penta sodium triphosphate ($\text{Na}_5 \text{P}_3 \text{O}_{10}$) and 200 mg of sodium hexametaphosphate (NaPO_3)₂ [$1x >10$]

The reference mixture is stable at 4 ° C for at least 4 weeks.

1.5.4 Developing Solvent

Mix 140 ml isopropyl alcohol, 40 ml of a 135 gm / litre solution of trichloro acetic acid and 0.6 ml of ammonium hydroxide 0.9 gm /ml, about 25 % (m/m) solution.

1.5.5 Spray Reagent I

Mix equal volume of a 75 gm / litre solution of ammonium molybdate tetrahydrate

$[(\text{NH}_4)_6\text{Mo}_2]_{24}\cdot 4\text{H}_2\text{O}$] and conc nitric acid (1.4gm / ml) and dissolve 10 gm tartaric acid in 100 ml of this mixture

1.5.6 Spray reagent II

Dissolve 0.5 gm of 1 amino 2 naphthol- 4 sulphonic acid in a mixture of 195 ml of a 150 gm /l solution of sodium disulphite (Sod metabisulphite) and 5 ml of a 200 gm /l solution of sodium sulphite ($\text{Na}_2\text{S}_2\text{O}_3$). Dissolve 40 gm of sod acetate trihydrate in the mixture. Store the reagent in a tightly closed brown bottle in refrigerator, Discard the solution after 1 week.

1.5.6 Apparatus

- (1) Glass plates - 10 cm x 20 cm
- (2) Spreading device for preparing layers of 0.25 mm thickness
- (3) Laboratory mixer
- (4) Dessicator
- (5) Mechanical meat mincer fitted with a plate with holes of diameter not more than 4 mm.
- (6) Fluted filter paper, 15 cm dia
- (7) Micropipette - 1 μl or micrometer syringe
- (8) Paper lined glass tank.
- (9) Hair dryer
- (10) Sprayer
- (11) Oven capable of being maintained at 60 ° C

1.5.7 Preparation of TLC plates

Dissolve 0.3 gm starch in 90 ml boiling water, add 15 gm of cellulose powder and homogenize in the laboratory mixer for 1 minute. Apply the slurry onto

glass plates with the spreading device adjusted to obtain a layer of 0.25 mm. Air dry the plates at room temperature and heat them finally for 10 minutes at 100 °C. Store the plates in a dessicator. Alternatively ready to use plates may be used.

1.5.8 Preparation of serum

Macerate 50 gm of test sample with 15 ml water at 40 - 60 °C in a beaker with a spatula or flattened stirring rod until a homogeneous mass is obtained taking no more than 5 minutes. Add 10 gm of trichloroacetic acid and mix again.

Immediately place in a refrigerator for 1 hr and then collect the separated serum by decanting through the fluted filter paper. If the filtrate is turbid shake once with an equal volume of diethyl ether. Remove the ether layer with a small pipette and add an equal volume of ethanol to the aqueous phase.

Shake for 1 minute. Allow the mixture to stand for few minutes and filter through a fluted filter paper.

1.5.9 Chromatographic separation

Pour developing solvent in the developing tank to a depth of 5 - 10 mm and close the tank with its lid. Allow to stand for at least 30 minutes at ambient temperature. Apply 3 µl of serum or 6 µl if the clearing procedure was carried out to the cellulose layer on a pencil line drawn at about 2 cm from the bottom. Keep the spots small by applying 1 µl at a time. Use a warm air stream from hair dryer for drying. In the same way apply 3 µl of reference mixture to the plate at a distance of 1 - 1.5 cm from the sample spot. Remove the lid from the tank and

quickly place the cellulose plate in the tank. Develop the plate until the solvent front has ascended to approx 10 cm from the pencil line. Remove the plate, dry for 10 minutes in oven at 60⁰ C or for 30 minutes at room temperature. Spray the plates lightly but uniformly with spray reagent No 1. Yellow spots appear immediately. Dry the plate in a stream of warm air from a hair dryer and heat in oven for 1 hr at 100⁰ C to remove last traces of nitric acid. Check that the plate is free from pungent smell of nitric acid. Allow the plate to cool and spray lightly with spray reagent no 2. Blue spots appear immediately. Spraying with reagent 2 is not an absolute necessity but the intense blue spots produced improve the detection considerably.

1.5.10 Interpretation

Compare the migration distance of the phosphate spots from the sample and the reference mixture. An orthophosphate spot is always present. If the sample contains condensed phosphates, a diphosphate spot and / or spots of more highly polymerized phosphates are visible.

(Ref:- I.S 5960 (Part 13) : 1988 / I.S.O 5553 : 1980 Meat and Meat Products - Methods of Test - Detection of Polyphosphates)

1.6 Determination of Glucono- delta- lactone (rule 72) :-

This is an enzyme ultraviolet procedure recommended by I.S.O and B.S.I (I.

S.O 4133 and B.S 4401, part 13). A test combination kit is available.

1.6.1 Reagents

- (1) Perchloric acid - 0.4 M - Dilute 17.3 ml Perchloric acid (70 % m /m) to 500 ml with water
- (2) Potassium hydroxide - 2 M - Dissolve 56.1 gm Pot. Hydroxide in water - Dilute to 500 ml
- (3) Buffer Solution - pH 8.0 - Dissolve 2.64 gm glycylglycine and 0.284 gm magnesium chloride hexahydrate in 150 ml water. Adjust to pH 8 with potassium hydroxide. Dilute to 200 ml with water
- (4) Nicotinamide adenine dinucleotide phosphate (NADP) - Dissolve 50 mg of NADP disodium salt in 5 ml water.
- (5) Adenosine -5- triphosphate(ATP) - Dissolve 250 mg ATP disodium salt and 250 mg sodium hydrogen carbonate in 5 ml water.
- (6) 6 - Phosphogluconate dehydrogenase (6 PGDH)- Commercial suspension containing 2 mg 6- PGDH / ml from yeast
- (7) Gluconate kinase (GK)- Suspension containing 1 mg / ml from E.Coli.

1.6.2 Procedure

Weigh 50 gm of prepared sample into a homogenizer. Add 100 ml of cold (0 °C) 0.4 M perchloric acid and homogenize. Transfer the slurry to a 100 ml centrifuge tube. Centrifuge at 3000 rpm for 10 minutes. Move the fat layer. Decant the supernatant through a fluted filter paper into a 200 ml conical flask and discard the first 10 ml.

Transfer 50 ml of the filtrate to a 100 ml beaker. Adjust to pH 10 with Pot. Hydroxide and make up to 100 ml in a volumetric flask with water. Cool in ice for 20 minutes. Filter through a fluted filter paper. Discard the first 10 ml. Pipette 25 ml of the filtrate (V ml) into a 250 ml volumetric flask. Dilute to mark with water (maximum concentration of D (+) gluconate is 400 mg /l). this is the prepared extract. Pipette into each of 2 photometric cells - 2.5 ml of pH 8 buffer, 0.1 ml NADP, 0.1 ml ATP.

Into one of the cells pipette 0.2 ml extract, into the other 0.20 ml water. Pipette 0.05 ml of 6 - PGDH suspension on to a plastic spatula, mix with the contents of one of the cells. Repeat the operation with the second cell. Read the absorbance of each cell against air at 365 nm after 5 minutes. Retain the cells for reaction

A_1 = absorbance of test solution
 A_{1B} = absorbance of blank

Pipette 0.01 ml of GK suspension on to the plastic spatula. Mix with the contents of one of the cells. Repeat the operation with the other cell. Read the absorbance of each cell at 365 nm after 10 minutes and again after 2 minutes until a constant rate of absorbance is obtained. Plot the absorbance against time and extrapolate the linear part of the curve back to zero time.

A_2 = Absorbance (T = 0) of the test solution

A_{2B} = Absorbance (T = 0) of the blank solution

1.6.3 Calculation

$$? A = (A2 - A1) - (A2B - A1B)$$

$$\text{Glucona - delta lactone \% by mass} = \frac{15058 \times ? A}{V \times m} \frac{(100 + M \times m)}{(100)}$$

Where V = volume in ml of filtrate to make prepared extract M = moisture content of prepared sample percent m/m m = mass in gm of test sample.

(Ref:- Pearsons Composition and Analysis of Foods 9th edn1991 , page 502)

2.0 Additional tests as per label declaration if necessary

2.1 Total Fat :-

Weigh accurately 3-4gms of well mixed sample in a 100ml beaker. Add a few drops of NH₄ OH and warm on a steam bath. Add 10ml of cone HCl and boil for approx 30 minutes. Cool, filter through a wetted filter paper. Wash filter paper with hot water. Dry the filter paper containing the residue of the sample, roll and insert in an extraction thimble and extract fat in a soxhlet apparatus using ethyl ether or petroleum ether, transfer to another flask. Remove solvent. Keep flask in an air oven maintained

at 100°C for 30 minutes to remove residual solvent if any. Transfer flask to a desiccator to allow it to cool. Weigh the residue and calculate total fat.

(Ref:- ISO 1443- 1973 Codex approved method - Extraction / gravimetry Type I method)

PI also see I.S: 5960 (Part 3) 1970 Methods of test for meat and meat products - Determination of total fat content

2.2 Total Protein (Kjeldahl method) :-2.2.1

Reagents :-

Kjeldahl catalyst:- 15gm Pot. Sulphate + 0.5gm Copper sulphate

Sulphuric Acid - Concentrated

NaOH solution- 50% (1+1). Let stand until clear

Standard NaOH solution-0.1 N=0.1 M (4.00gm/litre)

Standard acid solution- Prepare either HCl or H₂SO₄ solution HCl sol-0.1

N= 0.1 M (3.646gm/litre)

H₂SO₄ sol - 0.1N=0.05 M (4.9gm/litre)

Methyl Red Indicator - 0.5gm in 100ml ethanol

2.2.2 Procedure :-

Weigh 1-1.5 gm of prepared sample and transfer to a kjeldahl digestion flask. Add

15gm of Pot sulphate, 0.5gm of copper sulphate and 25-40ml of Sulphuric acid. Heat the flask gently in an inclined position until frothing ceases then boil briskly for 2 hours. Allow to cool. Add approx 200ml of water and 25ml of Sod thiosulphate solution (80gm/l) and mix. Add a piece of granulated Zinc or anti bump granules and carefully pour down the side of the flask sufficient Sodium Hydroxide sol (1+1) to make the contents strongly alkaline (about 110ml). Before mixing the acid and alkaline layers connect the flask to a distillation apparatus incorporating an efficient splash head and condenser. To the condenser fit a delivery tube which dips just below the surface of a pipetted vol of the digestion flask and boil until about 150ml of the distillate has been collected. Add 5 drops of methyl red indicator and titrate with 0.1N NaOH. Carry out a blank, 1ml of 0.1 HCl or H₂SO₄ is equivalent to 0.0014 of N.

Total protein is equal to N X 6.25.

(Ref:- A.O.A.C 17th edition,2000, Official Method 928.08 Nitrogen in Meat
(Alternative II))

PI also see I.S-5960 (Part 1) 1996 / I.S.O 937-1978 Meat and Meat
Products - determination of Nitrogen Content

FISH AND FISH PRODUCTS

1.0 FROZEN FISH

Fish stored in ice spoils as a result of bacterial and enzyme action which results in the formation of volatile bases, in particular trimethylamine (TMA), dimethylamine (DMA) and ammonia.

Trimethylamine is the reduction product of Trimethylamine oxide during spoilage while ammonia is formed mainly as the end product of protein breakdown. The amounts of trimethylamine(TMA) and total volatile nitrogen (TVN) present in fish are commonly used as indices of spoilage.

1.1 Preparation of Sample

1.1.1 Frozen Fish

Place frozen fish in a plastic bag and thaw by immersion in cold water at a temperature not exceeding 4 °C. Judge the completion of thawing by gently squeezing the bag until no core or ice crystals can be felt.

Transfer to a blender or homogenizer to mince the sample. Ensure that the product remains at 4 ° C by pre cooling the blender. Analyse the sample as quickly as possible. When storage is necessary keep at a temperature not exceeding 4⁰C

(Ref:- Pearsons Composition and Analysis of Foods 9th edn,1991, page509)

1.2 Determination of Total Volatile Bases

1.2.1 Principle

The method is based on a semi micro distillation procedure. Extracts or solutions are made alkaline with sodium hydroxide and bases are steam distilled into standard acid and back titrated with standard alkali.

1.2.2 Apparatus

- (1) Blender
- (2) Semi micro distillation apparatus
- (3) Burette, Pipette, Conical flask

1.2.3 Reagents

- (1)Trichloro acetic acid - 5 %
- (2) Sodium Hydroxide - 2 N
- (3)Hydrochloric acid - 0.01N
- (4)Rosolic acid indicator - 1 % in 10 % ethanol (v /v)
- (5)Sodium Hydroxide - 0.01N

1.2.4 Procedure

Weigh 100 ± 0.5 gm prepared sample into a homogenizer with 300 ml of Trichloroacetic acid. Run the homogenizer to obtain a uniform slurry. Filter or centrifuge to obtain a clear extract. Pipette 5 ml of the extract into a semi micro distillation apparatus. Add 5 ml of 2 N NaOH. Steam distill. Collect distillate in 15 ml of 0.01 N standard hydrochloric acid. Add indicator (Rosolic acid). Titrate the liberated acid to a pale pink end point with 0.01 N sodium hydroxide. Do a blank determination.

1.2.5 Calculation

$$\text{TVBN (mg / 100 gm)} = (N) 14 \frac{(300 + W) \times V_1}{500}$$

Where

V_1 = Volume of standard acid consumed

W = water content of sample (gm / 100 gm)

(Ref:- Pearson's Composition and Analysis of Foods 9th edn,1991, page510)

1.3 Determination of Histamine

The presence of Histamine is also an indicator of decomposition and has been linked to scomboid poisoning. The natural level in fresh fish is less than 5 mg per 100 gm and the higher values in decomposing fish are due to decarboxylation of

histidine. Histamine is produced during the storage of pelagic species e.g tuna, mackerels, carangidaie species at elevated temperatures.

1.3.1 Principle

Bacterial enzyme decarboxylase free histidine in the muscle to histamine
The concentration of histamine is an indicator of bacterial spoilage. Free histamine is extracted from fish with methanol. The extract is chromatographed on silica gel plates. Histamine is visualized with ninhydrin

1.3.2 Apparatus

- (1) Chromatographic tank
- (2) Silica gel thin layer (TLC) plates or ready coated plates

1.3.3 Reagents

- (1) Histamine standard (0.2 mg / ml) - Dissolve 16.4 mg histamine dihydrochloride in 50 ml methanol
- (2) Solvent system - Methanol: Cone ammonia (95 : 5)
- (3) Ninhydrin spray reagent - Dissolve 0.3 gm ninhydrin in 100 ml n - butanol and add 3 ml glacial acetic acid

1.3.4 Procedure

Homogenise 10 gm fish with 50 ml methanol and transfer with methanol rinsings to a 100 ml volumetric flask. Immerse stoppered flask in a water bath at 60 ° C for 15 minutes. Cool, make upto 100 ml with methanol and centrifuge a portion to

produce clear extract for TLC

Spot extract and histamine solution on TLC plate. A useful spotting regime is 1, 5, 10 ul of extract and 0.5, 2, 5, and 10 ul of histamine solution (eqvt to 0.1 , 0.4 , 1 and 2 ug standard.). Develop plates in the solvent mixture. Thoroughly dry the plate with a hair dryer (residual ammonia will react with spray reagent) and spray with ninhydrin reagent. Dry and gently warm plate with a hair dryer to accelerate colour development. Estimate histamine level in the extract (ug / ul) by comparison of spot size and intensities with those of standards. Rerun plate with different quantities of sample extract and standard if necessary.

1.3.5 Calculation

Histamine in fish (mg / 100 gm) = Histamine in extract (ug / ul x 1000)

(Ref:- Lieber, E.R and Taylor, S.L 1978, TLC screening methods for histamine in tuna fish - J . Chromatgr 153 , 143 - 52)

1.3.0 A Determination of Histamine - Alternate method

1.3.0 A.I Reagents

- (a) Benzene - n - butanol mixture -3 + 2 (v/v)
- (b) Cotton acid succinate - Dissolve 5 gm anhydrous sodium acetate

fused just before use, and 40 gm succinic anhydride in 300 ml acetic acid in 500 Erlenmeyer flask. Immerse 10 gm absorbent cotton, cut into strips in solution, attach drying tube containing drying agent and heat 48 hrs at 100 ° C.(flask may be immersed to neck in active steam bath). Filter, wash well with water, HCl (1+9), water and finally with alcohol. Dry in vacuum at 100 ° C

- (c) Diazonium reagent - Dissolve 0.1 gm p - nitroaniline recrystallised from hot water and dilute to 100 ml with 0.1 N HCL. Store in refrigerator. Dissolve 4 gm of NaNO₂ in water and dilute to 100 ml. Store in refrigerator. Just before use place 10 ml p - nitroaniline solution in ice bath for 25 minutes, add 1 ml of NaNO₂ solution, mix and let stand in bath 5 minutes before use.
- (d) Coupling buffer - Dissolve 7.15 gm sodium metaborate and 5.7 gm sod.carbonate in water and dilute to 100 ml. Store in polyethylene bottle
- (e) Barbital buffer - Dissolve 10 gm of sodium barbital in 1 litre water and adjust to pH 7.7 with acetic acid (1+ 15) (about 25 - 30 ml), using pH meter. Store in refrigerator to prevent mould growth. Dissolve any ppt by warming before use (50 - 250 ml bottle of the buffer may be kept at room temperature and replenished from main supply when mould growth is apparent)
- (f) Histamine standard solution - Dry Histamine dihydrochloride 2 hrs over H₂SO₄. Dissolve 0.1656 gm dried histamine 2 HCl in water and dilute to 100 ml (1 ml = 1 mg histamine). Dilute 10 ml of this stock solution to 100 ml with water (1 ml = 100 ug histamine. Dilute 5 ml of this dilute standard solution and 5 ml of

methanol to 100 ml with water (1 ml = 5 ug histamine. Store in cold. Prepare fresh standards weekly.

(g) 4 - methyl - 2 pentanone (methyl isobutyl ketone). To recover used ketone wash once with saturated sodium bicarbonate solution and 3 times with water, distill retaining fraction boiling at 115-118 ° C and check A at 475 nm.

(h) Benzaldehyde - chlorine free

(i) Dilute sulphuric acid - 0.01 M accurately standardized.

1.3.0A 2 Preparation of CAS column

Prepare column by firmly lacing small plug of cotton acid succinate (CAS ca 50 mg) in column by cutting off or blowing out bottom of 15 ml centrifuge tube. Wash plug with 15 ml portions of water and two 3 ml portions of alcohol. Let solvents drip through CAS syringing out column by blowing out last portion of each solvent, using 10 ml syringe with needle inserted through rubber stopper. CAS plugs may be used for months by washing shortly after use with water and alcohol as above and protecting from dust with inverted beaker

1.3.0A 3 Determination

Transfer 10 gm prepared sample to semi micro container of high speed blender, add about 50 ml methanol and blend about 2 minutes. Transfer to 100 ml glass stoppered volumetric flask. Rinsing lid and blender jar with methanol and adding rinsings to flask, Heat in water bath to 60 ° C and let

stand 15 minutes at this temperature. Cool to 25 °C, dilute to volume with methanol and filter through folded filter paper. Alcohol filtrate may be stored in refrigerator for several weeks. (Light powdery ppt separating on storage may be ignored)

Dilute 5 ml of filtrate to 100 ml with water (disregard turbidity). Pipette 5 ml aliquot into 16 x 150 mm glass stoppered test tube and add 1 drop benzaldehyde and 0.2 ml 20 % (w/v) NaOH (pH after adding alkali should be about 12.4 - 12.5). Shake vigorously about 25 times. Let stand 5 minutes and add 5 ml benzene - n - butanol mixture. Shake vigorously about 25 times and let stand 5 minutes to separate. If emulsion forms centrifuge.

Transfer upper layer with fine tipped tube equipped with rubber bulb to previously prepared CAS column, avoiding transfer of any aqueous phase. Re extract aqueous solution with 5 ml of benzene butanol mixture as before, shaking, letting stand 5 minutes and transferring upper layer to column. Rinse lip and sides of column with fine stream of alcohol from wash bottle syringing out CAS. Wash column with two 3 ml portions water and syringe out. Discard solvents and washes.

Elute histamine from CAS into 25 ml glass stoppered Erlenmeyer by washing down sides of tube with 2 ml 0.01M H₂SO₄ (volume and concentration of acid are critical) followed by 3 ml water. Syringe out after dripping ceases.

Cool eluate in ice bath, weighting flask with clamp to prevent tipping and let

stand 5-10 minutes. Add 0.5 ml of cooled diazonium reagent and let stand 5 minutes in ice bath. Add 0.5 ml coupling buffer (volume is critical, oswald pipette is convenient) with continuous shaking or swirling to avoid localized alkalinity (pH after addition of coupling buffer 5-6). Let stand 5 minutes in ice bath, saturated solution with about 0.25 gm powdered $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$ added in one portion. Shake solution immediately and continuously about 30 seconds to ensure rapid and complete saturation (final pH about 8.6). Let stand 15 minutes in ice bath.

Pipette in 5 ml methyl isobutyl ketone and shake vigorously 25 times. Immediately transfer both layers to 16 x 150 mm test tube and let stand 10 minutes at room temperature to separate and warm up. Transfer upper layer with fine tip dropper to second 18 x 150 mm glass stoppered test tube containing 5 ml barbital buffer. Avoid transferring aqueous and solid phases if present (transfer need not be quantitative). Shake vigorously about 25 times (pH of barbital buffer after washing about 8.3- 8.4). Let stand 10 minutes to separate.

Transfer upper layer with fine tip dropper to 1 cm cell and determine A at 475 nm against methyl isobutyl ketone. Repeat determination on samples yielding A values > 25 ug standard by diluting 1 ml methanol filtrate to 100 ml with water. Alternatively, aqueous solutions may be diluted 1+ 4 or more with water.

Conduct standard and blank determinations as follows. Pipette 5 ml of 5 ug / ml histamine standard solution into 16 x 150 mm glass stoppered test tube.

Pipette 5 ml of 5% methanol into a similar tube for blank. Add 1 drop benzaldehyde and 0.2 ml of 20 % NaOH. Shake vigorously 25 times. Let stand 2 minutes and add 5 ml benzene - n - butanol mixture. Follow procedure mentioned above beginning " transfer upper layer with fine tip tube equipped with rubber bulb to previously prepared CAS column avoiding transfer of any aqueous phase"

Subtract blank A from A of standard (A) and sample (A) and calculate histamine as under

$$\text{Histamine , mg} = \frac{? A \times 25}{? A}$$

(Ref: - A.O.A.C 17th edn ,2000 Official method 957.07 Histamine in sea food - chemical)

(A Fluorimetric method - A.O.A.C Official method 977. 13 is also available as another alternative)

2.0 Dried Fish

2.1 Sampling

Cut large pieces into small size and mix. Grind the pieces to obtain a homogeneous mass. Transfer to an airtight container to prevent loss of moisture

(Ref:- I.S 14950 : 2001 Fish Dry and dry salted)

2.2 Determination of moisture

Weigh accurately about 5 gm of the prepared sample in a moisture dish with slip on cover. Dry in an air oven at $100 \pm 1^\circ \text{C}$ for 5 hours. Place lid on the dish and cool in a dessicator. Quickly weigh the dish. Return the dish with the cover to the oven and dry for another 1/2 hour. Cool in the dessicator and weigh again. Repeat until successive weights do not differ by more than 1 mg.

Calculate moisture as under

$$\text{Moisture} = \frac{M_1 \times 100}{M_2}$$

Where

M_1 = Loss in gm in the mass of sample

M_2 = Mass in gm of the sample taken for test

(Ref:- I.S 14950 : 2001 Fish Dry and dry salted)

2.3 Determination of Sodium Chloride

2.3.1 Reagents

- (1) Standard Silver Nitrate - 0.1 N
- (2) Dilute Nitric acid - 1 + 4
- (3) Ferric alum indicator - Prepare a saturated solution of Ferric ammonium sulphate
- (4) Standard Potassium thiocyanate solution - 0.1 N

2.3.2 Procedure

Take 1-2 gm of the dried material (obtained after determination of moisture) in a 250 ml beaker and add 50 ml of distilled water free from chloride and heat on a water bath till all the Sod.Chloride goes into solution. Filter in a 250 ml conical flask and wash with distilled water till the washings are free from chloride. Add 20 ml of dilute nitric acid and a known volume of standard silver nitrate sufficient to precipitate all the chloride. Add 1 ml of ferric alum indicator and titrate with standard Potassium thiocyanate solution until a permanent light brown colour appears

2.3.3 Calculation

Sodium Chloride

$$\text{(on dry basis) } m / m = 5.85 \times \frac{(V_1 N_1 - V_2 N_2)}{M}$$

Where

V_1 = Vol of standard solution of silver nitrate

N_1 = Normality of standard silver nitrate solution V_2 = Vol of standard Pot. Thiocyanate solution

N_2 = Normality of standard Pot. Thiocyanate sol

M = Mass of dried material taken for test

(Ref:- I.S 14950 : 2001 Fish Dry and dry salted)

2.4 Determination of Ash insoluble in dil HCl

2.4.1 Reagent

Dilute Hydrochloric acid - 1+1

2.4.2 Procedure

Weigh accurately 2 gm of the dried material (obtained after determination of moisture) in a silica or platinum dish. Ignite on a burner till all organic matter is charred. Transfer to a muffle furnace maintained at 550 °C and keep for few hours till grey ash is obtained. Cool in a desiccator. Weigh to determine total ash, if desired. Add 25 - 30 ml of dilute HCl to the dish and boil it for 10 minutes. Cool and filter it through Whatman filter paper No 42 or its equivalent. Wash the residue with water until the washings are free from chloride as tested with silver nitrate. Return the filter paper and residue to the dish. Dry in an air oven for 2 hours and ignite in the muffle furnace for 1 hour. Cool and weigh. Return the dish to the furnace again for 30 minutes, cool and weigh again. Repeat the process till the difference between two successive weighings is not more than 1 mg. Note the lowest weight.

2.4.3 Calculation

Ash insoluble in dil HCl

$$\text{(on dry basis) } m / m = 100 \times \frac{(M_2 - M)}{M_1 - M} \text{ Where}$$

M_2 = Lowest mass of dish with acid insoluble ash

M = Mass of empty dish

M_1 = Mass of dish with the dried material taken for test.

(Ref:- I.S 14950 : 2001 Fish Dry and dry salted)

3.0 Canned Fish

3.1 Preparation of sample

Place entire contents of can (meat and liquid) in a blender and blend until homogeneous or grind three times through meat chopper.

(Ref: - A,O,A,C 17th edn,2000, Official method 937.07 Fish and Marine Products - Treatment and Preparation of Sample)

3.2 Determination of Acidity of Brine

3.2.1 Reagents

- (1) Standard Sodium Hydroxide solution - 0.1 N
- (2) Phenolphthalein indicator solution - Dissolve 1 gm phenolphthalein in 100 ml of 95% alcohol

3.2.2 Procedure

Take 25- 40 ml of the brine solution,(previously filtered to remove suspended matter if any) in a 200 ml flask., add 25- 50 ml water if desired and titrate against standard Sodium hydroxide solution using phenolphthalein as indicator till a faint pink colour persists for 15 seconds

3.2.3 Calculation

$$\text{Acidity as citric acid (m/m)} = \frac{0.0070 \times \text{ml } 0.1 \text{ N NaOH} \times 100}{\text{Volume of brine taken}}$$

(Ref:- I.S.I Handbook of Food Analysis (Part XII) - 1984, page 50) **3.3**

3.30 Modified Starches in the packing medium (Appendix C)

3.3.1 Detection

Add a few drop of iodine solution to a small portion of the packing medium in a test tube. Boil for a minute. The presence of dark blue to red colour indicates presence of starch

(Ref:- Specifications for identity and purity of certain food additives - F.A.O Food and Nutrition Paper 49 , page 29)

3.3.2 Quantitative Determination

Take 50 ml of the packing medium. Precipitate starch by adding 95 % alcohol. Centrifuge, throw away the supernatant liquid. Wash residue with water in a boiling flask. Add 10 ml of dilute HCl (1+1) and about 50 - 70 ml of water, mix and reflux for 2 hours. Cool. Make the solution alkaline by adding cone Sodium hydroxide and sodium carbonate at the end. Make up to 100 ml Determine reducing sugars using Lane and Eynon method and convert total sugars to starch by multiplying with 0.9

(Ref:- A.O.A.C 17th edn 2000, Modified Official Method 925.5. for Starch in Confectionery)

3.4 Determination of Ascorbic acid (Appendix C)

Follow method given in Meat and Meat Products Clause 1.2

3.5 Determination of polyphosphate (Appendix C)

Follow method given in Meat and Meat Products Clause 1.4

3.6 Determination of Sulphur Dioxide (Appendix C)

Follow method given in Processed Fruits and Vegetables clause 16.7