

LAB. MANUAL 6



**MANUAL OF METHODS
OF
ANALYSIS OF FOODS**

***MEAT AND MEAT PRODUCTS
&
FISH AND FISH PRODUCTS***



**FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA
MINISTRY OF HEALTH AND FAMILY WELFARE
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**MANUAL FOR ANALYSIS OF MEAT AND MEAT PRODUCTS &
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 in Section 2.5 of Food Safety and Standards (Food Product Standards and Food Additives) Regulations, 2011. 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 homogenizer / blender to a uniform mass as appropriate. Transfer to a wide mouth glass or other suitable container with a 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 vacuum 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))

Alternatively following official method can be used:

- 1) Meat and Meat Products- Method of Sampling- IS/ISO 3100-1: 1991

1.2 Determination of Nitrite**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- 1 ppm - 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 volume 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 NaNO_2 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:-

- a) 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.

b) 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.A.1 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.A.2 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

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

(ii) Dissolve 0.25gm 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

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

1.2.A.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.A.4 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.A.5 Calculation

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

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 $\mu\text{g} / \text{ml}$ 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)

1.2.B Determination of Nitrite in Processed meat and meat products / fish and fish products like Ready to eat / ready to cooked products (Ion exchange chromatography method)

1.2.B.1 Principle

Sample is extracted and purified using relevant method after protein precipitated and fat skimmed before separated by anion exchange column with KOH solution as an eluate and detected with a conductivity detector. It is then determined with an external standard method by taking retention time as for quantitative analysis.

1.2.B.2 Reagents and materials

1. Ultrapure water: with its conductivity of $18.2\text{M}\Omega.\text{cm}$.
2. CH_3COOH : analytically pure
3. KOH: analytically pure
4. CH_3COOH solution (3%): 3ml CH_3COOH (3.2) into 100ml volumetric flask, diluted to a mark with water and fully homogenized.
5. Nitrite ion (NO_2^-) stock solution (100mg/L, aqueous solution).
6. Nitrate ion (NO_3^-) stock solution (1000mg/L, aqueous solution).
7. Mixed standard solution of nitrate (counted on NO_3^- ion, the same herein below) and nitrite (counted on NO_2^- ion, the same herein below): accurately pipette 1.0mL of nitrite ion (NO_2^-) stock solution and nitrate ion (NO_3^-) stock solution to 100mL volumetric flask, diluted to a mark with water, which 1mL of this solution contains $1.0\mu\text{g}$ of nitrite ion and $10.0\mu\text{g}$ of nitrate ion.

1.2.B.3 Instruments and equipments

1. Ion chromatograph: including a conductivity detector, suppressor, high capacity anion exchange column, measuring ring in 25 μ l.
2. Food disintegrator.
3. Supersonic cleaner.
4. Analytical balance: readability 0.1mg and 1mg.
5. Centrifuge: rotational speed no less than 10000rpm with 5ml or 10ml centrifugal tubes.
6. 0.22 μ m syringe filters with hydrophilic filterable membrane.
7. Decontaminating column: including C₁₈ column, Ag column and Na column or its equivalent.
8. Syringe: 1.0ml and 2.5ml. All glassware should be soaked in 2mol/L of NaOH solution and water for 4h, respectively, followed by rinsing with water for 3-5 times before ready for use later.

1.2.B.4 Analytical Procedures

1.2.B.4.1 Sample pre-treatment

(a) Fresh vegetable and fruit: the whole piece of vegetable and fruit is washed with deionized water, the edible portion of these vegetable and fruit is then disintegrated to uniformity after air dried. The adequate amount of disintegrated sample is then taken by quartering, and prepared into slurry with a stamp mill for use later. Water addition should be recorded if water is required to add.

(b) Meat, egg, aquatic products and their processed products: an adequate amount or full of materials is taken with quartering, and then prepared into slurry with a stamp mill for use later.

(c) Solid dairy products such as milk powder, soybean milk powder, and infant formula

powder (excluding cheese): sample is put in a container with lid in capacity of two folds of sample; the sample is finally homogenized by repeatedly shaking and reversing the container.

(d) Fermented milk, milk, condensed milk and other liquid dairy products: the sample is or repeatedly shaking and reversing the container.

(e) Cheese: an adequate amount of sample is uniformly ground to a muddy form. In order to avoid the loss of water content the excessive heat should be avoidable during the process of grinding.

1.2.B.4.2 Extraction

(a) Fruit, vegetable, fish, meat, egg and their processed products: 5g (accurately weighed to 0.001g) of sample in a homogeneous slurry form are taken and washed into an 100mL volumetric flask with 80mL water, extracted for 30min with an ultrasonic generator, shaken once every 5min to make sure that the solid phase is fully distributed. Leave it on a water bath at 75°C for 5min before making volume with water. A portion of solution after filtered is then subjected to centrifuge in 10000rpm for 15min; the supernatant is ready for use later.

(b) Salted fish, salted meat, and other processed products: 2g (accurately weighed to 0.001g) of sample in a homogeneous slurry form are taken and washed into an 100mL volumetric flask with 80mL water, extracted for 30min with an ultrasonic generator, shaken once every 5min to make sure that the solid phase is fully distributed. Leave it on a water bath at 75°C for 5min before making volume with water. A portion of solution after filtered is then subjected to centrifuge in 10000rpm for 15min; the supernatant is ready for use later.

(c) Milk: 10g (accurately weighed to 0.001g) of sample are put into an 100mL volumetric

flask with 80mL water added, shaken uniformly, ultrasonic treated for 30min, 2mL of 3% of glacial acetic acid is then added before leaving it at 4°C for 20min and resting it to ambient temperature, making volume with water. The supernatant after filtered is ready for use later.

(d) Milk powder: 2.5g (accurately weighed to 0.001g) of sample are put into an 100mL volumetric flask with 80mL water added, shaken uniformly, ultrasonic treated for 30min, 2mL of 3% of glacial acetic acid is then added before leaving it for 20min at 4°C and resting it at ambient temperature, making volume with water. The supernatant after filtered is ready for use later.

(e) 15mL of supernatant are taken to run through a 0.22µm syringe filters with hydrophilic filterable membrane and C18 column, the front segment in 3mL is discarded (if Cl⁻ ion is over 100mg/L, the supernatant should be successively run through syringe filters, C18 column, Ag column and Na column, the front segment in 7mL shall be discarded), the eluate collected is then determined. The solid phase extraction column should be activated before applied. The activation is carried out as follows: if OnGuard II RP column (1.0mL), OnGuard II Ag column (1.0mL) and OnGuard II Na column (1.0mL) are engaged in the application: OnGuard II RP column is run through with 10mL of methanol, 15mL of water before use, and then activated by resting for 30min. OnGuard II Ag column (1.0mL) and OnGuard II Na column (1.0mL) are run through with water before activated with resting for 30min.

1.2.B.4 .3 Chromatographic conditions for reference

(a) Chromatographic conditions for reference Chromatographic column: selectivity of hydroxide, high capacity anionic exchange column compatible to gradient elution, such as Dionex IonPac AS11-HC 4mm×250mm (with a protection column in IonPac AG11-HC type 4mm×50mm)1), or equivalent ion chromatographic column.

(b) Elution solution

- i) General samples: KOH solution with its concentration of 6 mmol/l-70mmol/l, elution gradient is 6mmol/l for 30min, 70mmol/l for 5min and 6mmol/l for 5min. Flow rate is 1.0ml/min.

- ii) Powder infant formula foods: KOH solution with its concentration of 5 mmol/l-50mmol/l, elution gradient is 5mmol/l for 33min, 50mmol/l for 5min and 5mmol/l for 5min. Flow rate is 1.3ml/min.

(c) Inhibitor

Anion inhibitor with regenerated membrane in automatic and continuous mode, or its equivalent.

(d) Detector

Conductivity detector with its temperature of detector cell at 35°C.

(e) Sample volume

25µL (enabled to be modified according to the content of ion to be measured).

1.2.B.4.4 Determination

Standard curve

The mixed standard solution of nitrite and nitrate pipetted is diluted with water to prepare a series of standard solutions with nitrite ion concentration of 0.00mg/L, 0.02mg/L, 0.04mg/L, 0.06mg/L, 0.08mg/L, 0.10mg/L, 0.15mg/L, 0.20mg/L, and with nitrate ion concentration of 0.0mg/L, 0.2mg/L, 0.4mg/L, 0.6mg/L, 0.8mg/L, 1.0mg/L, 1.5mg/L, 2.0mg/L. The chromatographic diagram of standard solution above with each concentration is obtained by successive injection of samples one by one from the lowest concentration. The calibration curve is plotted using concentration (mg/L) of nitrite and nitrate ions as abscissa and peak height (µS) and peak area as ordinate to calculate

the linear regression equation

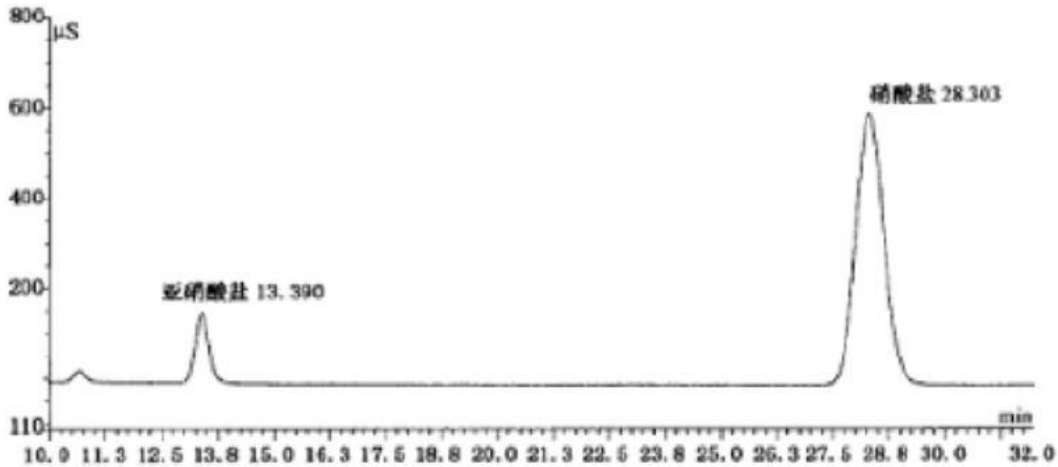


Fig.1 Chromatographic diagram of mixed standard solution of nitrite and nitrate

Determination of samples

50ul blank solution and 50ul sample solution are injected into ion chromatograph one by one at the same working condition, respectively, chromatographic diagrams are then recorded. The peak height (μS) and peak area are individually measured using retention time for qualitative analysis.

1.2.B.4.5 Formulation of analytical results

The contents of nitrite (counted on NO²⁻ ion) and nitrate (counted on NO₃⁻ ion) in samples are calculated in accordance with formula (1):

$$X = \frac{(e - e_0) \times V \times f \times 1000}{m \times 1000} \dots\dots\dots (1)$$

Where,

X – the content of nitrite or nitrate in samples, mg/kg;

C— the content of nitrite or nitrate in samples for measurement, mg/L;

C₀— the content of nitrite or nitrate in blank solution, mg/L;

V— the volume of sample solution, mL;

f— dilution factor of sample solution;

m— sample taken, g.

Note: The content of NO²⁻ in the sample multiplies by 1.5, to represent the nitrite content (calculation per sodium nitrite). The content of NO³⁻ in the sample multiplies by 1.37, to represent the nitrate content (calculation per sodium nitrate).

The result is represented by the mean arithmetical value from two independent determination results under the same condition, and keeps two digits.

1.2.B.4.6 Precision

The absolute difference of two independently measured results under the same condition will not be over 10% of arithmetic mean.

(Reference: Chinese standard method for determination of Nitrate and Nitrite in Foods; GB5009.33-2010)

1.3 Determination of Ascorbic acid

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_3COOH + 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 =

$$\frac{\text{Sample titre} - \text{blank} \times \text{mg ascorbic acid/ml} \times \text{Vol made} \times 100}{\text{Aliquot taken} \times \text{wt of sample}}$$

(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)

Alternatively following method can be used:

1) Methods of test for meat and meat products – IS 5838 : 1970

1.3.B Determination of Ascorbic acid using HPLC with UV Detection

(Applicable to the determination of vitamin C in infant, pediatric, and adult powdered, liquid, and ready-to-drink nutritional products by high-performance liquid chromatography. It allows for the separation and quantitation of L-ascorbic acid.)

See Tables 2012.21A and B for results of the study supporting acceptance of the method.

1.3.B.1 Principle

Vitamin C is extracted from products with a combination of ethylenediamine tetraacetic acid (EDTA), tris (2-carboxy-ethyl) phosphine (TCEP) hydrochloride, and metaphosphoric acid. These reagents precipitate proteins and stabilize vitamin C. There are two chromatographic options available for use with this method. Option 1.—A portion of prepared sample is injected onto a 4.6 × 75 mm, 4 μm, 80 Å, reversed-phase phenyl column that is compatible with 100% aqueous mobile phases where vitamin C is separated from other early- and late-eluting compounds present in the sample with a sodium acetate/EDTA mobile phase containing the ion pairing agent, dodecyltrimethylammonium bromide. The fraction of eluant from the 4.6 × 75 mm phenyl column containing vitamin C is collected on a 4.6 × 150 mm, 4 μm, 80 Å, reversed- phase phenyl column where vitamin C is further separated from compounds present in the sample. After vitamin C elutes from the

4.6 × 150 mm column, it is detected and quantitated by UV at 254 nm.

Option 2. — A portion of prepared sample is injected onto a 3 × 100 mm, 2.5 μm, 100 Å, reversed-phase phenyl column where vitamin C is separated from other compounds present in the sample with a sodium acetate/EDTA mobile phase containing the ion pairing agent, dodecyltrimethylammonium bromide. After vitamin C elutes from the column, it then passes through the UV detector cell. Option 1 is ideal for laboratories with high sample throughput analyzing multiple samples daily every week. With option 1, thousands of samples can be analyzed before the columns need to be cleaned. Option 2 is ideal for laboratories with low sample throughput analyzing a few samples every week. With option 2, the column will typically need to be cleaned after approximately 200 injections. Also with option 2 it may be necessary to clean and store the column in 50% acetonitrile if the system is not used daily or weekly.

1.3.B.2 Apparatus

(a) HPLC instrument.—Autosampler, vacuum degasser, pump, UV detector, and column switching valve, or equivalent.

(b) HPLC autosampler vials and caps.

(c) HPLC columns.—Option 1.—Phenomenex Synergi Polar-RP, 4 μm, 80 Å, 4.6 × 75 mm [Part No. 00C-4336-E0 (1)] and Synergi Polar-RP, 4 μm, 80 Å, 4.6 × 150 mm [Part No. 00F-4336-E0 (1)] each with an appropriate guard column, or equivalent.

Table 2012.21A. Accuracy and within-day precision data

Product	Within-day precision data ^a		Spike recovery data ^a		
	RSD, %	<i>n</i> , Days	Accuracy, %	<i>n</i> , Days	RSD, %
Milk-based infant formula, concentrated liquid	1.96	3	98.40	3	2.49
Lactose-free milk-based infant formula, concentrated liquid	1.94	3	99.30	3	1.59
Milk-based starch-containing infant formula, ready-to-feed liquid	1.46	5	98.60	4	0.77
Milk-based specialty infant formula, ready-to-feed liquid	0.97	6	99.10	4	1.64
Soy-based infant formula, ready-to-feed liquid	1.23	5	98.00	4	2.14
Soy-based carbohydrate-free infant formula, concentrated liquid	0.786	5	98.60	4	1.65
Hydrolyzed protein milk-based pediatric, ready-to-feed liquid	1.56	4	100.3	3	4.72
Milk-based pediatric, ready-to-feed liquid	2.96	3	99.3	3	4.10
High protein, milk-based adult, ready-to-feed liquid	2.40	3	98.1	3	2.68
Moderate protein, moderate fat casein and soy adult, ready-to-feed liquid	2.99	5	97.2	5	1.73
Moderate protein, moderate fat soy and casein adult, ready-to-feed liquid	0.784	5	100.5	5	2.24
High protein, high fat milk-based adult, ready-to-feed liquid	1.54	4	98.2	3	2.19
Moderate protein, low fat, hydrolyzed soy adult, ready-to-feed liquid	2.20	3	99.4	3	3.59
Moderate protein, high fat milk-based adult, ready-to-feed liquid	0.673	3	100.9	3	0.88
Moderate protein, low fat, soy and milk adult, ready-to-feed liquid	1.02	3	100.5	3	1.59
High protein, moderate fat hydrolyzed milk adult, ready-to-feed liquid	3.17	3	97.0	3	2.64
Moderate protein, high fat milk-based adult, ready-to-feed liquid	0.740	3	100.7	3	1.39
High protein, high fat milk-based adult, ready-to-feed liquid	1.79	4	100.6	3	3.69

^a Samples were prepared and analyzed in triplicate on each day.

Table 2012.21B. Intermediate precision data^a

Product	Powder, mg/kg	<i>n</i> , Days	RSD, %
SRM 1849	1002	10	1.32
Whey protein-based infant formula powder	1370	7	2.41
Hydrolyzed milk protein, free amino acid-based infant formula powder	1780	8	1.53
Milk-based pediatric formula powder	1500	9	2.94
Elemental infant formula powder	3280	7	3.35
Milk-based specialty infant formula powder	720	10	2.67

Option 2.—Synergi Polar-RP, 2.5 μm , 100 \AA , 3 \times 100 mm (Part No. 00D-4371-Y0) with an appropriate guard column, or equivalent.

(d) Analytical and top-loading balances.—Capable of weighing 0.0001 g and up to 1000 g.

(e) Filtering apparatus.—Vacuum filter apparatus for membrane filters, including 2 L flask (Millipore, or equivalent).

(f) Filter membrane.—Nylon, 0.45 μm .

(g) Filter paper.—Whatman 2 V, or equivalent.

- (h) Vacuum flasks.—2000 mL.
- (i) Homogenizer or blender.
- (j) Light shields.—Yellow or clear shields with a cutoff of 385 nm.
- (k) pH meter.
- (l) Pipets.—Volumetric, Class A, assorted sizes.
- (m) Pipettors.—Mechanical, 1–5 mL, 100–1000 µL, and 10–100 µL with tips, repipet heads, or equivalent.
- (n) Syringe.—Disposable, 1 mL.
- (o) Syringe filters.—Nylon, 13 mm, 0.45 µm (Whatman; Part No. 6768-1304), or equivalent.
- (p) Ultrasonic bath.
- (q) Vials.—Glass with screw cap, to hold approximately 5.5 ml vitamin C stock standard.

1.3.B.3 Reagents

Storage of chemicals or reagents at any temperature between 2 and 30°C in an air-tight, inert container is appropriate unless otherwise stated. Also, unless otherwise stated, the reevaluation date for all opened reagents is 2 years from the date received. Regardless of expiration or reevaluation dates, discontinue use of any chemicals or solutions whenever indications of contamination, chemical degradation, or changes in concentration are evident.

- (a) Acetonitrile.—HPLC grade.
- (b) Control sample.—A representative sample analyzed with each batch of samples to monitor method performance.
- (c) Dodecyltrimethylammonium bromide.—Reagent grade (Sigma; Part No. 8638), or equivalent.
- (d) Drierite (desiccant).—Anhydrous calcium sulfate, 8 mesh, or equivalent.
- (e) EDTA, disodium salt.—ACS, or equivalent.
- (f) Laboratory water.—Distilled or deionized water.
- (g) Meta-phosphoric acid.—ACS, or equivalent.

- (h) Phosphoric acid.—85% ACS, or equivalent.
- (i) Sodium acetate.—Anhydrous; ACS, or equivalent.
- (j) TCEP hydrochloride.—Sigma (Part No. C4706), or equivalent.
- (k) Takadiastase.—Accurate Chemical Co., or equivalent.

1.3.B.4 Standards

- (a) Ascorbic acid.—USP reference standard for instrument calibration.
- (b) NIST SRM 1849.—NIST infant/adult nutritional formula with certified values for evaluation of method performance.

1.3.B.5 Solutions Preparation

Solutions can be stored at 2–30°C in tight inert containers unless otherwise noted. Preparation should be performed under shielded fluorescent lighting with a minimum UV cutoff of 385 nm.

(a) 6% Metaphosphoric acid.—Weigh 30.0 g ($\pm 10\%$) metaphosphoric acid into a 500 mL volumetric flask. Dissolve and dilute to volume with laboratory water. Mix well. Store refrigerated. Expiration: 1 week.

(b) 3% Metaphosphoric acid.—Weigh 15.0 g ($\pm 10\%$) metaphosphoric acid into a 500 mL volumetric flask. Dissolve and dilute to volume with laboratory water. Mix well. Store refrigerated. Expiration: 1 week.

(c) 0.2% EDTA.—Weigh 2.0 g ($\pm 10\%$) EDTA into a 1000 mL volumetric flask. Dissolve and dilute to volume with laboratory water. Mix well. Store at room temperature. Expiration: 6 months.

(d) 1% TCEP.—Weigh 0.1 g ($\pm 10\%$) TCEP hydrochloride into a 10 mL volumetric flask. Dissolve and dilute to volume with laboratory water. Mix well. Expiration: 2 months.

(e) Ascorbic acid stock standard (2000 mg/L).—Weigh 0.2000 g ($\pm 2\%$) ascorbic acid into a 100 mL volumetric flask. Dissolve and dilute to volume with 3% metaphosphoric acid. Mix well. Store approximately 5.5 mL aliquots frozen in individual vials. Expiration: 2 years.

(f) Ascorbic acid intermediate standard (100 mg/L).—Quantitatively transfer 5.0 mL

ascorbic acid stock standard into a 100 mL volumetric flask with a volumetric pipet. Dilute to volume with 3% metaphosphoric acid. Mix well. Expiration: 1 day. Discard after use.

(g) Ascorbic acid routine working standards (15, 7, and 2 mg/L).—Quantitatively transfer 15.0, 7.0, and 2.0 mL ascorbic acid intermediate standard into separate 100 mL volumetric flasks with volumetric pipets. Add 5 mL ($\pm 10\%$) 0.2% EDTA, 100 μL ($\pm 10\%$) 1% TCEP and 5 mL ($\pm 10\%$) 6% metaphosphoric acid and dilute to volume with laboratory water. Mix well. Filter the working standards through 0.45 μm syringe filters. Discard the first milliliter and collect the second milliliter in HPLC autosampler vials. Store refrigerated. Expiration: 14 days.

(h) Ascorbic acid working standards for low sample concentrations (1, 0.5, 0.25, and 0.125 mg/L).—To make the 1.0 and 0.5 mg/mL working standards, quantitatively transfer 1 and 0.5 mL ascorbic acid intermediate standard into separate 100 mL volumetric flasks with volumetric pipets. Add 5 mL ($\pm 10\%$) 0.2% EDTA, 100 μL ($\pm 10\%$) 1% TCEP, and 5 mL ($\pm 10\%$) 6% metaphosphoric acid and dilute to volume with water. Mix well. To make the 0.25 and 0.125 mg/mL working standards quantitatively transfer 5 mL of the 0.5 mg/mL and 5 mL of the 0.25 mg/mL working standard into a 10 mL volumetric flask. Add 0.5 mL ($\pm 10\%$) 0.2% EDTA, 10 μL ($\pm 10\%$) 1% TCEP, and 0.5 mL ($\pm 10\%$) 6% metaphosphoric acid and dilute to volume with laboratory water. Mix well. Filter the working standards through 0.45 μm syringe filters. Discard the first milliliter and collect the second milliliter in HPLC autosampler vials. Expiration: 1 day.

(i) 0.25 M sodium acetate buffer.—Weigh 41.0 g ($\pm 10\%$) sodium acetate anhydrous into a weighing dish. Quantitatively transfer to a 2000 mL beaker containing approximately 1800 mL laboratory water and dissolve. Adjust pH to 3.0 (± 0.1) with concentrated phosphoric acid. Quantitatively transfer solution to a 2000 mL volumetric flask and dilute to volume with laboratory water. Expiration: 1 month.

(j) Mobile phase.—Quantitatively transfer 200 mL ($\pm 10\%$) 0.25 M sodium acetate buffer and 100 mL ($\pm 10\%$) 0.2% EDTA to a beaker containing approximately 1650 mL laboratory water. Weigh 1.0 g ($\pm 10\%$) dodecyltrimethylammonium bromide and quantitatively

transfer to the beaker. After all of the dodecyltrimethylammonium bromide has dissolved, adjust the pH of the solution to 3.15 (± 0.05) with phosphoric acid. Quantitatively transfer the solution to a 2000 mL volumetric flask and dilute to volume with laboratory water. Filter solution through a 0.45 μm filter and degas. Expiration: 1 month.

(k) Precipitant.—Dilute 1 part 6% metaphosphoric acid with 1 part 0.2% EDTA. Mix well. Expiration: 16 h.

(l) 6% Takadiastase solution.—Weigh 0.6 g ($\pm 10\%$) into a 50 mL beaker. Add approximately 10 mL laboratory water. Stir until dissolved. Expiration: 8 h.

1.3.B.6 Procedure

Sample preparation.—Liquid samples must be freshly opened and sample containers tightly closed between samplings. Sample preparations must be completed within 20 min after the container is opened. Sample preparations are good for up to 18 h. After 18 h sample preparations cannot be injected and must be prepared again.

All samples, liquids, semisolids, and powders should be as uniform and representative of the product as possible. This should be accomplished by thoroughly mixing or stirring the product prior to sampling. Mixing of liquid products should be performed to minimize the production of foam. Powdered products may require a preliminary reconstitution. Sampling of liquid products should be performed immediately after a final, gentle mixing or stirring to prevent inaccurate sampling due to stratification induced by foaming or creaming.

Powdered products, which are not homogeneous at the subgram level, should first be reconstituted. Some products may require homogenization in order to assure accurate sampling. For powder products which are not homogeneous at the subgram level, reconstitute the samples by dissolving appropriate amounts of the powders in water or precipitant. If necessary, homogenize or blend the reconstitutions to breakup any large clumps. Appropriate sample sizes can be calculated from the following equation: $\text{Sample size} = 400/E$

where sample size is the theoretical sample size, in grams; E is the expected ascorbic acid concentration in mg/L or mg/kg of the liquid or reconstituted sample; and 400 is the desired amount, in micrograms (μg), of ascorbic acid in the sample preparation. The net conversion factor for μg to mg and kg to grams is unity.

Weight and volume sample sizes are interchangeable where feasible provided appropriate corrections are made to the calculations using the product density. All sample weights should be recorded to at least three significant figures.

(a) Immediately after preparing sample reconstitutions or after opening samples, weigh the sample into a 100 mL volumetric flask and record the weight to the nearest 0.0001 g. If the sample contains a significant amount of starch, add 0.5 mL 6% takadiastase and allow it to react for 1 min before continuing.

(b) Prepare a control sample with each sample set.

(c) Immediately after weighing the sample into a 100 mL volumetric flask, add 5 mL ($\pm 10\%$) 0.2% EDTA and 100 μL ($\pm 10\%$) 1% TCEP and swirl the flask for a few seconds to thoroughly mix. Immediately after mixing, add 5 mL ($\pm 10\%$) 6% metaphosphoric acid and dilute to volume with laboratory water. For directly weighed powder samples, make sure that the powder is completely dissolved before adding metaphosphoric acid. Mix well.

(d) Samples can either be filtered using 2 V filter paper or the 100 mL flask can set undisturbed for a few minutes. An aliquot of the prepared sample can then be filtered through a 0.45 μm nylon syringe filter. Discard the first milliliter through the filter and collect next milliliter in an autosampler vial.

1.3.B.7 Instrument Operating Conditions

(a) Instrument conditions.—Option 1.—Note: Option 1 is ideal for laboratories with high sample throughput analyzing multiple samples daily. With option 1 thousands of samples can be analyzed before the columns need to be cleaned.

(1) Mobile phase flow rates (both pumps).—1.0 mL/min.

(2) Columns.—Synergi Polar-RP, 4.6 \times 75 mm, 4 μm , 80 Å and Synergi Polar-RP, 4.6 \times 150 mm, 4 μm , 80 Å.

- (3) Injection volume.—20 μ L.
- (4) Detector wavelength.—254 nm.
- (5) Run time.—11 min.
- (6) System configuration.—See Figure **2012.21A**.
 - (a) Configuration 1.—0–2 min.
 - (b) Configuration 2.—2–3 min.
 - (c) Configuration 1.—3–11 min.

(b) Instrument conditions— Option 2.— Option 2 is ideal for laboratories with low sample throughput analyzing a few samples every week. With option 2 the column will typically need to be cleaned after approximately 200 injections. Also with option 2 it may be necessary to clean and store the column in 50% acetonitrile if the system is not used daily or weekly.

- (1) Mobile phase flow rate.—0.4 mL/min.
- (2) Column.—Synergi Polar-RP, 3 \times 100 mm, 2.5 μ m, 100 \AA .
- (3) Injection volume.—20 μ L.
- (4) HPLC detector wavelength.—254 nm.
- (5) Run time.—15 min.

(c) Instrument start-up.— Equilibrate the instrument by pumping mobile phase through the columns for at least 0.5 h before injecting standards and samples onto the column. When new columns are installed, mobile phase must pass through the columns for at least 3 h to equilibrate the columns with ion pairing agent.

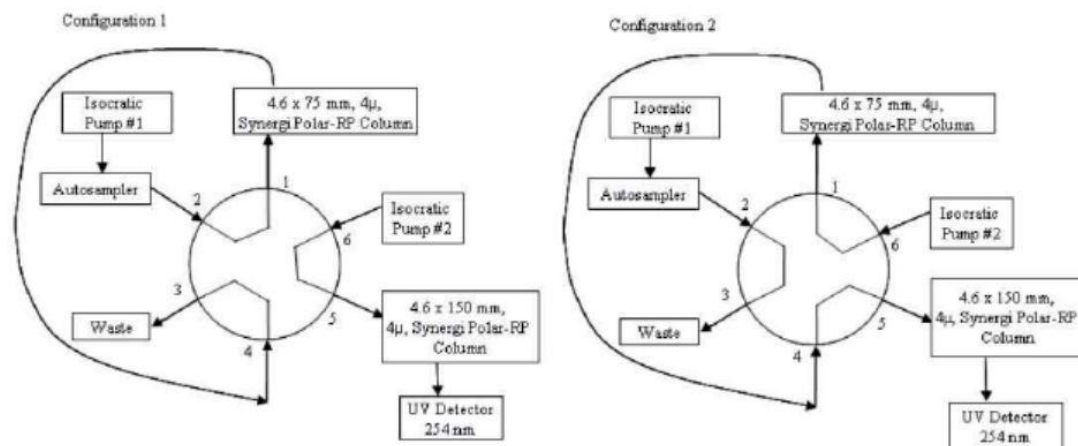


Figure 2012.21A. System configuration.

(d) HPLC analysis of standards and samples.—Inject the most concentrated standard 3–4 times and note the peak areas. Confirm that the precision of the peak areas is $\leq 2\%$ RSD and the peak areas are not steadily increasing or decreasing by more than 4% from the first injection to the third or fourth injection. If the RSD $> 2\%$, locate the source of the imprecision and correct it before beginning the sample analysis. Once the system has been calibrated, inject the control and sample preparations. The control sample preparation should be the first injection after the system has been calibrated. For batch analyses, all working standards should be injected every 8 h which will allow analysis of the control sample and up to 44 samples before another set of working standards must be analyzed.

(e) HPLC column and system maintenance.—Option 1.—If the vitamin C peak responses drop and curves have acceptable linearity, clean off the columns using 50% water–acetonitrile solution at 0.6–0.8 mL/min for 1 h. After cleaning the columns, reequilibrate the columns with mobile phase for at least 3 h.

Option 2.— After approximately 200 sample injections, the vitamin C retention time will decrease and the vitamin C peak will begin to merge with other peaks present in the chromatograms. When this happens, clean off the columns using 50% water–acetonitrile solution at 0.6–0.8 mL/min for 1 h. After cleaning the columns, reequilibrate the columns with mobile phase for at least 3 h.

1.3.B.8 Calculations and Results

The ascorbic acid concentrations of samples analyzed with the HPLC system are determined by comparison of the ascorbic acid peak areas from samples of known weight with the peak areas of standards of known concentration.

(a) Calculation of the standard concentrations.

(1) Concentration of stock standard.

(2) Concentration of intermediate standard.

$$C_i = \frac{C_s \times 5.0}{100} = C_s \times 0.05 = W \times P \times 500$$

where C_i is the concentration of the intermediate standard solution in mg/L; 5.0 is the volume of the stock standard solution aliquot in mL; 100 is the dilution volume in mL.

(3) Concentration of working standards.

$$C_w = (C_i \times A)/100 = W \times P \times A \times 5$$

where C_w is the concentration of the working standard in mg/L; A is the volume of the intermediate standard in mL; 100 is the dilution volume in mL.

(b) Measurement of peak areas.—Peak areas are measured with a data system. Before calculating concentrations, compare the ascorbic acid standard peaks with the ascorbic acid sample peaks to make sure that there are not any interfering compounds and that ascorbic acid is separated from all other components in the sample. The concentration of ascorbic acid cannot be calculated if there are interferences or if there is poor separation. Also check to see that the ascorbic acid peak areas of the samples fall within the range of the standards. Peak areas of the same standards injected before and after a set of samples should not change by more than 6%. If they do, the system was not equilibrated or the columns need to be cleaned or replaced and the data are not acceptable.

(c) Preparation of the standard curve.—Average the standard peak responses from the

standards injected before and after a set of samples. Prepare a standard curve by performing a linear least squares routine (regression) on the concentration of the working standards versus their corresponding averaged peak areas. The linear correlation coefficient (r) of the curve should be >0.999 .

(d) Calculation of ascorbic acid concentration in a sample.—

$$C_{(AA)} = \frac{C_{(1)} \times 100}{S} \times R \times D$$

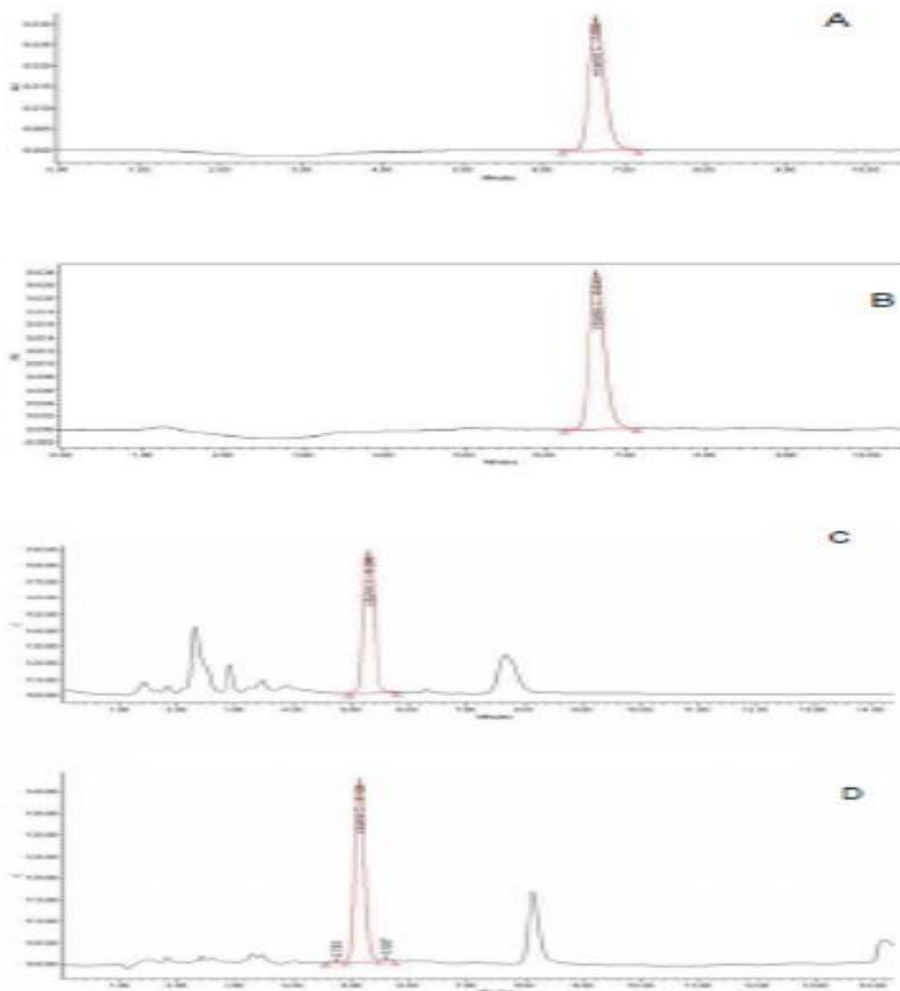


Figure 2012.21B. HPLC analysis of (A) vitamin C standard, option 1; (B) infant nutritional product, option 1; (C) infant nutritional product, option 2; and (D) adult nutritional product, option 2.

where C(AA) is the concentration of ascorbic acid in the original sample in mg/kg or mg/L; 100 is the dilution volume of the sample in mL; S is the sample size in grams or mL; C(I) is the concentration of ascorbic acid in the injected sample determined from the standard curve in mg/L; R is the reconstitution rate (total weight of sample reconstitution in grams divided by the weight of powder or solid used in grams), if no sample reconstitution is required R = 1; and D is the density of the product in g/mL (if results for weighed samples need to be converted from mg/kg to mg/L).

(e) Calculation of ascorbic acid concentrations in control samples.—Calculate the concentration of ascorbic acid in the control sample. The control result must be within ± 3 standard deviations of the control mean. If the control result is outside 3 standard deviations of the control mean, the samples must be prepared and analyzed again.

1.3.B.9 Method Validation

See Table **2012.21A** for accuracy and within-day precision data and Table **2012.21B** for intermediate precision data. See Figure **2012.21B** for sample chromatograms. References: J. AOAC Int. (future issue); AOAC SMPR 2012.012 J. AOAC Int. (future issue)

1.4 Determination of Total Phosphorous content

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 $260 \pm 20^\circ \text{C}$
- (8) Conical suction flask
- (9) Desiccator

1.4.4 Procedure

Make the sample homogeneous by passing it at least twice through the meat mincer and mixing. Keep the homogenized 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 ± 2 °C, cooled in a dessicator 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 ± 2 °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

$$\text{Phosphorous (\%)} \text{ as } P_2O_5 = 0.03207 \times \frac{m_1}{m_0} \quad \text{or} \quad = 3.207 \times \frac{m_1}{m_0}$$

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

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 ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), 300 mg of tetra sodium diphosphate decahydrate ($\text{Na}_4\text{P}_2\text{O}_7 \cdot 10\text{H}_2\text{O}$), 200

mg of penta sodium triphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$) and 200 mg of sodium hexametaphosphate ($(\text{NaPO}_3)_6$) [$\lambda > 10$]

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 trichloroacetic 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 $[(\text{NH}_4)_6\text{MoO}_7 \cdot 4\text{H}_2\text{O}]$ 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 μ l 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 μ l of serum or 6 μ l 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 μ l at a time. Use a warm air stream from hair dryer for drying. In the same way apply 3 μ l 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 60°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 100°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 /1). 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 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

$A_2 B$ = Absorbance ($T = 0$) of the blank solution

1.6.3 Calculation

$$A = (A_2 - A_1) - (A_{2B} - A_{1B})$$

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

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)

Alternatively following method can be used-

- 1) Methods of test for meat and meat products: Part 11 – IS 5960 : Part 11 :1998
Determination of glucone- delta-lactone content

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 NH_4OH 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 Imethod).

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

- a) Kjeldahl catalyst:- 15gm Pot. Sulphate + 0.5gm Copper sulphate
- b) Sulphuric Acid - Concentrated
- c) NaOH solution- 50% (1+1). Let stand until clear
- d) Standard NaOH solution-0.1 N=0.1 M (4.00gm/litre)
- e) Standard acid solution- Prepare either HCl or H_2SO_4 solution HCl sol-0.1
- f) N= 0.1 M (3.646gm/litre)
- g) H_2SO_4 sol - 0.1N=0.05 M (4.9gm/litre)
- h) 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, 1 ml of 0.1 HCl or H₂SO₄ is equivalent to 0.0014 of N.

Total protein is equal to $N \times 6.25$.

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

(Alternative II)).

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

Combustion Method (DUMAS Method) for Determination of Nitrogen Content validated ISO method (EN ISO 16634 series (2008)).

3.0 Tests for determination of Physico-Chemical quality of meat and meat products:

3.1 Determination of pH

The pH is a measure of the acidity or alkalinity in solutions or water containing substances. pH values lower than 7 are considered acidic, while pH values higher than 7 are

considered alkaline. A pH of 7 indicates neutrality. pH values are related to the concentration of hydrogen ions (H^+) in the substance.

Typical pH values for meat and meat products are:

Product	pH value (range)
Meat mixes in jelly + vinegar added	4.5 to 5.2
Raw fermented sausage	4.8 to 6.0
Beef	5.4 to 6.0
Pork	5.5 to 6.2
Canned meats	5.8 to 6.2
Curing brines	6.2 to 6.4
Blood sausages	6.5 to 6.8
Muscle tissues, immediately after slaughter	7.0 to 7.2
Blood	7.3 to 7.6

pH measurement is useful for:

- Evaluation of meat quality for further processing, in particular the water binding capacity
- Control of ripening of raw fermented products, which is connected with drop in pH
- Control of acidity of ingredients such as brines, marinades etc.

The pH can be measured by following methods –

1. Digital pH meter
2. Chemical indicator method (Nitrazine yellow)

a. Digital pH meter

Portable instruments are battery driven and have glass electrodes. The pH-value in meat and meat products can be measured by direct contact between the sensitive diaphragm of the electrode and the meat tissue. Through the diaphragm differences in

electrical load between the meat and electrolyte solution (e.g. Potassium chloride KCl) inside the glass electrode are measured and directly indicated as the pH-reading. In raw fresh meat, it is recommended to spray small amounts of distilled water onto the tissue at the point of measurement (prior to inserting the electrode), because the operation requires some fluidity in the sample and the glass electrode should be thoroughly wet. The amount of water necessary will not appreciably alter the pH. For accurate pH readings the pH-meter should be calibrated before use and adjusted to the temperature of the tissues to be measured. The electrode must be rinsed with distilled water after each measurement.

Requirement- Digital pH meter, distilled water, beaker, electrolyte solution.

Procedure:

1. Blend 15 gm meat with 30 ml distilled water at 27-30°C.
2. Note the pH with a glass electrode pH meter.

Reference method: Chicken broth flavor and pH by Pippen et al. (1965), Poultry Sci. 44: 816-823

b. Nitrazine- Yellow Test

This test determines the acidity of meat.

Requirements: Nitrazine- Yellow indicator, glass rod, petri plate

Procedure:

- i. Take a piece of meat free of blood, fat, and connective tissue in a petri dish.
- ii. Add nitrazine yellow indicator (1:10000) sufficient to cover the meat piece
- iii. Mix with stirring rod
- iv. Note the colour change with standard chart provided.

Interpretation:

pH	Colour	Inference
6.0	Yellow	Good keeping quality
6.4	Olive Green	Not having same good keeping quality
6.8	Bluish violet	suspect on signs of incipient spoilage

3.2 Determination of Extract Release Volume (ERV):

Homogenize 20 gm meat with 100 ml distilled water for 2 minutes. Pour the homogenate directly into the funnel lined with Whatman filter paper No.1, folded thrice so as to make eight sections. Allow the homogenate to seep between the folds and the collect the extract in 100 ml graduated cylinder for 15 min.

Reference method:

1. Release of aqueous extracts by beef homogenates and factors by Jay (1964), Food Technol. 18 : 129-132.
2. Beef microbial quality determined by extract release volume (ERV) by Jay (1964), Food Technol. 18 : 132-137.

Alternatively BIS standard method IS 5960: part 10 : 2011 (methods of test for meat and meat products: Part 10 Measurement of pH – Reference) may be used

3.2 Determination of Extract Release volume (ERV)

The technique was first described in 1964, has been shown to be a value in determining incipient spoilage in meat as well as in predicting refrigerator shelf life.

Principle: The technique is based on the volume of aqueous extract released by homogenate of meat when allow to pass through the filter paper for a given period of time, by this meat of good organoleptic and microbial quality release large volume of extract, whereas meat of poor quality releases smaller volume or none.

Requirements: Beaker, distilled water, Whatman No. 1 filter paper, pestle and mortar, graduated cylinder.

Procedure

- a) Take 25 g meat sample in 100 ml distilled water
- b) Blend it with in pestle and mortar
- c) Filter through Whatman No. 1 filter paper, folded thrice so as to make eight sections.
- d) Allow the homogenate to seep between the folds
- e) Collect the extract in 100 ml graduated cylinder for 15 min.
- f) Record extract release volume and interpret results

Interpretation:

<u>ERV (ml)</u>	<u>Meat quality</u>
> 25 ml	Good quality
> 20 ml	Incipient spoilage
< 20 ml	Spoiled meat

Reference method

Jay (1964), Release of aqueous extract by beef homogenates and factors. Food Technol. 18:129-132.

Jay (1964), Beef microbial quality determined by extract release volume (ERV) (1964), Food Technol. 18: 132-137.

3.3 Determination of Meat Swelling Capacity (MSC):

Blend 25 gm of meat with 100 ml distilled water for 2 minutes. Centrifuge 35ml of homogenate at 2000rpm for 15 minutes. Measure the volume of supernatant (S). % Meat Swelling = $(35-S)/7 \times 100$.

Reference method:

1. Determination of meat swelling capacity as a method for investigating the water binding capacity of muscle proteins with low water holding forces. II Application of the swelling methodology by Wierbicky et al (1963) Fleischwirtschaft 15: 404.

3.3 Determination of meat swelling Capacity**3.3.1 Principle**

This test determines the freshness of meat Swelling capacity of meat increases during spoilage due to protein degradation and penetration of more amounts of water in protein matrix. A method of measuring the water binding capacity of muscle proteins with low water holding forces known as meat swelling (SW).

3.3.2 Requirements: distilled water, centrifuge, blender, graduated cylinder

3.3.3 Procedure

- Take 25 g of meat in 100 ml of distilled water
- Blend it for 2 min
- Centrifuge 35 ml of homogenate at 2000 rpm for 15 min
- Measure the volume of supernatant (S)
- Record the volume and denote it as "S".

Percent meat swelling can be determined as

$$\% \text{ swelling} = \frac{(35 - S - 7)}{7} \times 100$$

3.3.4 Reference method

- a) Wierbicky et al. (1963) Determination of meat swelling capacity as a method for

investigating the water binding capacity of muscle proteins with low water holding forces. II Application of the swelling methodology *Fleischwirtschaft* 15: 404.

3.4 Determination of Total Volatile Basic Nitrogen (TVBN)

3.4.1 Determination of TVBN by Micro diffusion technique

The volatile bases in most species of fish consist of ammonia together with appreciable quantities of amines. In meat trimethylamine is only present in significant quantities and total volatile nitrogen consist almost entirely as ammonia. As ammonia production due to deamination of protein increases during spoilage, its determination represents a simple method of following the course of determination of the quality lean meat.

3.4.2 Principle – Meat extract is treated with relatively weak alkali and the volatile base is distilled or diffused over into standard acid or boric acid.

3.4.3 Reagent – Boric acid reagent: Dissolve 5 g of boric acid in 100 ml of 95% Alcohol and add 350 ml of water. After the acid had dissolved add 5ml of indicator (0.066% methyl red and 0.33% bromocresol green in alcohol). Add alkali (40% sodium hydroxide) until a faint Reddish colour is produced. Make the volume up to 500 ml with alcohol.

i) Preparation of trichloroacetic acid (TCA) extract : Take 10 g of meat sample blend with 90 ml of distilled water for 2 min. To 5 ml of this homogenate add equal volume of 10% TCA (w/v in distilled water), allowed to stand for 15 min. Filtered through Whatman's filter paper no.1. The clear TCA extract thus obtained is used to determine TVBN value following the technique of Conway (1947) and Pearson (1968 b).

ii) Preparation of TVBN reagent: Take 92 ml of 2% boric acid, 4 ml of 0.1% of alcoholic solution of bromocresol green and 4 ml of 0.1% of alcoholic methyl red are mixed to make 100 ml TVBN reagent.

3.4.5 Procedure

- a) Add One ml of TVBN reagent in to the inner well of the Conway unit.
- b) In outer well one ml of TCA extract is deposit followed by the addition of one ml saturated potassium carbonate (K_2CO_3).
- c) Close the Conway unit immediately with an airtight ground glass plate
- d) Rotate clockwise and antilock wise and incubate at room temperature for 3 hours.
- e) The TVBN reagent in the inner well is back-titrated with 0.02 N sulphuric acid (H_2SO_4) till the blue color changes to pink.

3.4.6 Calculations

$$14 \times a \times b = 'N' \text{ mg/ml of extract.}$$

$$C = 100 \times N$$

Where, 14 = Molecular weight of Nitrogen

a = Normality of H_2SO_4

b = volume of H_2SO_4 (Titration value)

c = mg% of TVBN value

TVBN values are expressed as mg%.

Reference method:

1. Micro-diffusion analysis and volumetric error by Conway (1947), D.Van Nostrand Co. Inc., New York
2. Application of chemical methods for the assessment of beef quality II Methods related to protein breakdown by Pearson (1968), J. Sci. Fd. Agric. 19: 366-369.
3. Alternatively BIS reference method IS 5960 : Part 3 : 1970 may be used.

3.5 Determination of Picric Acid Turbidity (PAT):

Blend 5gm meat with 20ml of 70% ethanol for 30 seconds in which add 5 ml of saturated aqueous picric acid solution and re-blend the slurry for about 20 seconds. Filter the slurry through Whatman filter paper No.41. Measure the optical density of the filtrate at 540 nm wavelength with visible spectrophotometer as a turbidity of the solution.

Reference method:

- a) Kurtzman and Synder (1960), The picric acid turbidity: A possible practical freshness test for ice shrimps. Food Technol. 14(7) : 337.

3.5 Determination of Picric Acid Turbidity

Requirement- 70% ethanol, saturated aqueous picric acid, Whatman filter paper no. 41., spectrophotometer.

Protocol-

- a) Blend 5 gm of meat with 20 ml of 70 % ethanol for 30 sec
- b) Add 5ml of saturated aqueous picric acid solution and reblend the slurry for about 20 sec.
- c) Filter the slurry through Whatman filter paper no. 41.
- d) Measure the optical density of the filtrate at 540 nm wavelength with visible spectrophotometer as a turbidity of the solution.

Reference:

- a) The picric acid turbidity: A possible practical freshness test for ice shrimps by Kurtzman and Synder (1960), Food Technol. 14(7) : 337.

3.6 Determination of Dye Reduction Capacity

a. Resazurin dye reduction test (RDRT):

Reference method:

1. Resazurin dye reduction tests for shelf life estimation of poultry meats by Wells (1959), Food Technol. 13: 584-586.

b. Methylene blue reduction test (MBRT):

Reference method:

1. Rapid procedure for approximation of bacterial counts in shrimps and oysters by Novak *et al* (1955), Food Technol. 10 : 66 – 67.

Dye reduction test:

Principle- This test estimates bacterial population in meat sample indirectly.

Requirements – resazurin dye / tablet, filter paper strips, polythene bag, nutrient broth, swab, distilled water

Procedure- A.

- a) Four Resazurin tablet dissolved in 100 ml of water sample.
- b) Filter paper strips are dipped in above solution and dried in dark and cool room.
- c) For testing , the strip is moistened and a drop of meat juice to be tested is placed on it for 1 minute
- d) The strip is then placed in polythene bag and kept in dark room(22-23⁰ C)
- e) Time taken for the blue colour to the paper to change to red is noted

Procedure- B.

- a) Soak the swab in nutrient broth
- b) Sample 1 cm² area of meat
- c) Break the swab in 10 ml nutrient broth yeast extract medium
- d) Collect the washings in a sterile glass beaker containing 0.3 ml freshly prepared 0.05%

aqueous Resazurin dye

- e) Incubate the beaker at 30°C in a dark room
- f) Note the time taken for the change in colour from violet/ blue to colorless

Interpretation -

Reduction time	Meat quality
10 min	Meat not acceptable
10-30 min	Doubtful
30-60 min	Good quality
> 60 min	Very good quality

Reference:

Textbook of “Methods in Meat Science”

4.0 Official analytical methods for microbiological testing of meat and fish products

4.1 Determination of Microbial load in meat and meat products

Samples of meat and meat products can be collected following specified methods of Indian standards viz. IS/ISO 3100-1:1991 (Meat and Meat Products- method of sampling) and IS 15478 : Part 2 : 2004 (Meat and Meat Products- Sampling and preparation of test samples- Part 2 : Preparation of test samples for microbiological test samples.

4.1.1 Enumeration Total Viable Count (TPC) / Aerobic Plate Count (APC)

For evaluating total viable counts of the microorganisms, standard pour plate technique is used. Take 0.1ml of 10^{-4} , 10^{-5} and 10^{-6} dilutions (in duplicate) of inoculum in the Petri plates

to which pour molten agar having temperature around 45-50°C and mix thoroughly by rotating plate clockwise and anticlockwise for five times. Allow the plates to solidify and then keep the plates for incubation at 37°C for 24-48 hrs.

4.1.2 Enumeration *Staphylococcus aureus*

4.1.3 Enumeration *E coli* : IS 15463:2004/ISO 6391:1997 Meat and Meat products – Enumeration of *Escherichia coli* – Colony count technique at 44 °C using membranes
Alternatively, official Indian method IS 15463: 2004 (Meat and Meat products- enumeration of *e. coli*- colony- count technique at 44 °C using membranes)

4.1.4 Enumeration *Salmonella*

4.1.5 Enumeration *Faecal Coliform*

4.1.6 Enumeration *Clostridium* species

4.1.7 Enumeration *Yeast and molds*: BIS standard procedure IS:14920:2001/ISO 13681:1995 Meat and Meat products- Enumeration of yeasts and mould - Colony count technique.

4.1.8 Enumeration *Pseudomonas* species: IS 14843:2000/ISO 13720:1995 Meat and Meat products – Enumeration of *Pseudomonas species*.

Listeria monocytogens

Indian official method IS 14988: Part 2 :2002 (microbiology of food and animal feeding stuffs- horizontal method for detection and enumeration of *Listeria monocytogenes*- part 2 : enumeration method) may be used

4.2 Determination of Microbial Toxins in Meat and Meat Products

4.2.1 Determination of *Shiga* toxin:

Reference method:

1. USDA (Laboratory Guide)

4.2.2 Determination of *Clostridium botulinum* toxins

Reference method:

1. *Clostridium Botulinum* toxins -USDA/FSIS Microbiology Laboratory Guidebook, 3rd Edition.

Standard reference books for guidance:

- i. USDA (Laboratory Guide)
- ii. Manual for the Identification of Medical Bacteria by S.T. Cowan and K.J. Steel, Cambridge University Press, Cambridge 1970)
- iii. Medical laboratory manual for tropical countries, Vol. II Microbiology, by Monica Cheesbrough, Cambridge University Press, Cambridge (1985)
- iv. Bergay's Manual of systematic Bacteriology, Vol. 1 & 2 by Sneath *et al*, Williams and Wilkins Co., Baltimore. M.S. 2102, U.S.A.(1986)

NOTE: For any other methods on microbiological analysis of meat and fish products the manual on microbiological analysis of foods may be referred.

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 40°C.

(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

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

Where

V₁ = 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 µl of extract and 0.5, 2, 5, and 10 µl of histamine solution (eqvt to 0.1 , 0.4 , 1 and 2 µg 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 (µg / µl) 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.A Determination of Histamine - Alternate method

1.3.A.1 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 precipitates 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 µg histamine. Dilute 5 ml of this dilute standard solution and 5 ml of methanol to 100 ml with water (1 ml = 5 µg 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.A.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.A.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 H_2SO_4 (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 for 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 $Na_2B_4O_7 \cdot 10 H_2O$ 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 μg

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 µg / 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:

$$\text{Histamine, mg} = \frac{\text{DA sample} \times 25}{\text{DA standard}}$$

(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

$$\text{Moisture} = \frac{M1 \times 100}{M2}$$

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

$$\text{(on dry basis) } m / m = \frac{5.85 \times (V_1N_1 - V_2N_2)}{M}$$

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 weighing is not more than 1 mg. Note the lowest weight.

2.4.3 Calculation

Ash insoluble in dil HCl

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

Where,

M₂ = Lowest mass of dish with acid insoluble ash

M = Mass of empty dish

M₁ = 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

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

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

3.3 Modified Starches in the packing medium

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

Follow method given in Meat and Meat Products Clause 1.2

3.5 Determination of polyphosphate

Follow method given in Meat and Meat Products Clause 1.4

3.6 Determination of Sulphur Dioxide

Follow method given in Processed Fruits and Vegetables clause 16.7

MEAT SPECIATION FOR THE DETECTION OF MEAT ADULTERATION

1.0 Introduction

There has been unprecedented growth in the food industry consequent to the growing demand for quality food by an ever increasing population. Nowadays, consumers have become health conscious and thus demand quality food. The adulterated food however, often enters the supply chain and jeopardizes the sentiments as well as health of the people. Substantial proportion of population has got religious considerations towards the consumption of meat of a particular animal species and any deviation may result into detraction from its consumption.

The meat adulteration has got social, religious, economic and public health concerns. The legal enforcement also restricts consumption of meat of wild animal species. In view of all these catastrophes that often challenge the analyst, there is an urgent need to have reliable techniques which may aid in the authentic identification of meat species. Although, numerous analytical methodologies viz. anatomical, histological, microscopic, organoleptic, chemical, electrophoretic, chromatographic and immunological techniques have been employed but none of these are considered to be complete for the purpose of species identification.

The traditional methods such as anatomical, chemical, electrophoretic, chromatographic and immunologic techniques have one or the other limitations. These procedures are either cumbersome or lack repeatability and reproducibility. For instance, immunoassays fail to differentiate the closely related species as the protein markers may possess similar isotypes in different species of animals and different components of the same animal species could behave differently making the judgment difficult. Further, techniques based on the variation in the protein profile between species could not be used for the processed, stored and comminuted meat and meat product samples.

Recently, DNA based molecular techniques have become popular and could provide satisfactory solution to the problem of identification of meats of different animal species. Targeting DNA for the animal speciation offers many advantages over the protein-based techniques. The DNA molecule is more stable and the composition of DNA is same in any cell of the individual. Hence, DNA based techniques namely Polymerase Chain Reaction (PCR) and its variants; Restriction Fragment Length Polymorphism (RFLP); Random Amplified Polymorphic DNA (RAPD) finger printing; DNA Hybridization, PCR-Sequencing; Arbitrarily Primed-PCR (AP-PCR) and PCR-Single Strand Conformation Polymorphisms (SSCP) have been employed for the identification of animal species in the recent past. Further, with the advent of Real Time PCR, there has been a major breakthrough in the quantitative analysis of meats of different animal species. The PCR-based techniques provide high level of specificity, sensitivity, accuracy and precision than the techniques hitherto being used for the purpose of animal species identification. Following methods can be used in the laboratories for speciation of meat after standardization in the laboratories.

2.0 Meat speciation using DNA-based molecular techniques

The reasons why DNA is the molecule of choice for the purpose of species identification include,

- (i) Its stability allows the species identification in heated and processed products;
- (ii) DNA structure is conserved in all the tissues of an individual;
- (iii) Higher discrimination of closely related species is possible as vast information is present in the nucleotide algorithm consisting of A, T, G and C.

The DNA based animal speciation techniques are the most preferred of all the techniques, since the DNA carries an organisms' total genetic information and is stably functioning as long as the animal is alive. The DNA isolated from any cell/tissue of an individual is identical irrespective of the organs or tissues. Further, the information available in the DNA is enormous as compared to the proteins due to the degeneracy of the

genetic code. Hence, different DNA-based techniques used for animal species identification include,

- a) DNA hybridization
- b) PCR (Polymerase Chain Reaction) and its variants
- c) PCR-RFLP (Polymerase Chain Reaction- Restriction Fragment Length Polymorphism)
- d) RAPD-PCR (Randomly Amplified Polymorphic DNA- Polymerase Chain Reaction)
- e) PCR-SSCP (Polymerase Chain Reaction- Single Strand Conformational Polymorphism)
- f) PCR-sequencing of which most widely methods for meat species identification are,
- g) PCR (Species-specific & Multiplex)
- h) RFLP fingerprinting
- i) FINS (Forensically Informative Nucleotide Sequencing)

All these techniques require extraction of DNA from tissues.

3.0 Isolation of DNA from tissue/ meat samples

3.1 DNA extraction

Any tissue like muscle, organ, skin, blood, excretion and secretion can act as a source of DNA.

3.1.1 Principle

Tissue sample is trichurated and homogenized to get cells. The cells are first ruptured to release cellular contents including DNA. Interfering substances such as proteins and RNA are lysed and then DNA is separated from by precipitation. Extracted DNA is checked for quality, purity and concentration.

3.1.2 Reagents

- a) Tris saturated Phenol (equilibrated with 0.1 M Tris-Cl, pH 8.0)
- b) Chloroform
- c) DNA lysis buffer (pH 8)
- d) Ribonuclease-A
- e) Proteinase-K solution (20 mg/ml)
- f) Isoamylalcohol
- g) 10 M ammonium acetate
- h) Absolute ethanol
- i) 70% alcohol
- j) 1X TE (Tris-EDTA) buffer

3.1.3 Apparatus

- a) Scissors
- b) Forceps
- c) Refrigerated Centrifuge
- d) Dry bath
- e) Water bath

3.1.4 Procedure

Phenol: Chloroform method can be used for DNA extraction as per the protocol of Sambrook and Russel (2001). The tissue samples (0.2-1 g) are cut into very small (<1 mm) pieces or pulverized in liquid nitrogen and 10 volumes (w/v) of DNA lysis buffer (pH 8) containing Ribonuclease-A @ 100 µg/ml (20 µg/ml) is added and incubated at 37°C for 1 h. Then, proteinase-K solution (20 mg/ml) was added @ 200 µg/ml and again incubated at 50°C for not less than 3 h or overnight. During incubation, regular swirling of the tubes is undertaken gently from time to time. Equal vol. of Tris-saturated phenol (equilibrated with 0.1 M Tris-Cl, pH 8.0) is added and the contents of the tubes are subjected to mixing end to

end for 10 min. and the contents are then centrifuged at 6,500 RPM for 15 min. The upper aqueous phase is collected into a fresh tube and equal volume of phenol: chloroform: isoamylalcohol (25:24:1) mixture is added and centrifuged. The upper layer is collected and again equal volume of phenol: chloroform: isoamylalcohol (25:24:1) is added and centrifuged. Finally, the upper aqueous phase is collected in a fresh tube and equal volume of chloroform was added and then centrifuged. The upper phase is again collected in to a fresh tube containing 0.2 volumes of 10 M ammonium acetate and 2 volumes of absolute ethanol and is mixed well for precipitation of DNA. The mixture containing visible DNA threads were centrifuged at (10,000 RPM for 10 min). The DNA pellet is washed twice with 70% alcohol by centrifugation (10,000 RPM for 5 min each), dried over a dry bath at 60°C and then dissolved in 1X TE (Tris-EDTA) buffer (50- 100 µl) or nuclease free water either. These DNA samples are used for PCR or stored at -20°C until further use.

3.1.5 Reference

- a) Sambrook, J. and Russell, D. W. 2001. *Molecular cloning: A laboratory manual*. 3rd edition. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.

3.2 Checking the quality, purity and concentration of DNA

For further molecular analysis, isolated DNA has to undergo quality checks.

3.2.A Determination of quality of DNA

A good quality DNA is a pre-requisite for its further molecular analysis.

3.2.A.1 Principle

In order to ensure absence from contaminants such as protein and RNA, the isolated DNA is checked for quality. The DNA must be intact and free from shearing.

3.2.A.2 Reagents

- a) Agarose gel (0.6-0.8%, w/v)
- b) TE buffer
- c) 6X gel loading dye (Xylene Cyanol and Bromophenol Blue)
- d) 1 kb molecular weight marker
- e) Ethidium bromide

3.2.A.3 Apparatus

- a) Electrophoresis unit
- b) UV trans-illuminator
- c) Gel documentation

3.2.A.4 Procedure

The quality of isolated DNA is checked by agarose gel (0.6-0.8%, w/v) electrophoresis. About 5 µl of dissolved DNA (in TE buffer or NFW) is mixed with 1 µl of 6X gel loading dye (Xylene Cyanol and Bromophenol Blue) and loaded on to a well along with a 1 kb molecular weight marker. Electrophoresis is undertaken @ 5-8 V/cm and ethidium bromide (added @ 0.5 µg/ml in the agarose) stained DNA bands are visualized under UV trans-illuminator and documented over a gel documentation system to assess the quality of isolated DNA.

3.2.B. Determination of purity of DNA

3.2.B.1 Principle

Based on wavelength maximum of DNA and protein, purity is determined.

3.2.B.2 Reagents

- a) nuclease free water

3.2.B.3 Apparatus

- a) UV/ VIS spectrophotometer

3.2.B.4 Procedure

The purity of the isolated DNA is checked by using UV/ VIS spectrophotometer. The DNA samples with OD₂₆₀:OD₂₈₀ ratios between 1.7 and 1.9 are used for PCR amplification.

3.2.B.5 Calculation

The concentration of the DNA can be estimated by using following formula-

$$\text{DNA concentration } (\mu\text{g/ml}) = \frac{\text{OD}_{260} \times \text{Dilution Factor} \times 50}{1000}$$

(1 OD value at 260 nm is equivalent to ~50 µg of dsDNA / ml)

4.0 Comprehensive DNA-based methods of meat speciation

Of the several DNA based molecular tools, PCR, RFLP and FINS are most widely used for meat speciation.

4.1 Method 1: Polymerase chain reaction (PCR)

PCR provides a wide range of options to the analysts by virtue of its versatility and hence it is considered as state-of-the-art technique in animal species identification. It is a rapid means of making multiple copies of specific piece of DNA sequence *in vitro* and possesses high level of selectivity as well as sensitivity and hence it is an ideal diagnostic analytical tool.

There are several types of PCR, but most widely used in meat speciation are species-specific PCR and multiplex PCR.

4.1.1 Species-Specific PCR

Species specific DNA in femto grams (fg) and pico grams (pg) could be detected in both processed and unprocessed samples using targeted amplification of rRNA genes (12S, 16S, 18S), actin-multigene families (which are highly conserved in all the eukaryotes), satellite DNA, cytochrome-b gene, cytochrome oxidase II, growth hormone gene, melanocortnin gene, mt D-loop, myofibrillar components and Satellite I DNA using PCR. Some highly conserved regions on the mitochondrial genes such as cytochrome b have been most widely used compared to others for the species identification where pair of specific primers amplifies a specific region in PCR. Although both genomic and mt targets are used, mt sequences are preferred since it possess high rate of mutation allowing species identification and also help in the differentiation of most closely related species.

4.1.1.1 Principle

It is a process of *in vitro* amplification of target DNA, a polymerase enzyme synthesises new DNA which is specific to the target using specific primers and other ingredients required for DNA synthesis. PCR produces millions of copies of target DNA in a span of 2-3 hours thereby producing DNA that could be documented thereby allowing meat speciation.

4.1.1.2 Reagents

- a) PCR reaction mixture i.e. 2.5 μ l of 10X assay buffer [160 mM $(\text{NH}_4)_2\text{SO}_4$, 670 mM Tris-HCl, pH 8.8, 0.1% tween-20, 25 mM MgCl_2]
- b) dNTP mix [Sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water i.e., 40 mM total pH 7.5]
- c) Forward and reverse primers (Species-specific primers as shown in table) 20 Pico moles each
- d) 1U Taq DNA polymerase
- e) 50 ng of purified DNA
- f) Nuclease Free Water
- g) Agarose gel (2%)
- h) 1X TBE buffer
- i) 6X gel loading dye (1 μ l)
- j) 100 bp DNA ladder

4.1.1.3 Apparatus

- a) Thermal cycler
- b) Pipettes 1 ml, 01 ml
- c) Laminar air flow cabinet
- d) Electrophoresis unit
- e) Gel documentation system

4.1.1.4 Procedure

The PCR is set up in a 25 μ l volume reaction mixture consisting of 2.5 μ l of 10X assay buffer [160 mM $(\text{NH}_4)_2\text{SO}_4$, 670 mM Tris-HCl, pH 8.8, 0.1% tween-20, 25 mM MgCl_2], 0.5 μ l (200 μ M each) of dNTP mix [Sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water i.e., 40 mM total pH 7.5], 0.5 μ l (20 Pico moles) each of forward and reverse primers

(Species-specific primers as shown in Table1), 1U Taq DNA polymerase, 50 ng of purified DNA and NFW to make the volume. The tubes are flash spun and the PCR was performed in a Thermal cycler.

Table 1: Some species-specific primers found suitable for meat speciation*

Species	Target	Primer	Direction	Sequence	Primer size	Tm	Spp. Specific amplicon
Cattle	mt loop	D- VPH-CF	Forward	5'-TAT CAA AAA TCC CAA TAA CTC AAC ACA-3'	27 bp	52.0	381 bp
		VPH-CR	Reverse	5'-GGG CCC GGA GCG AGA AG-3'	17 bp	61.6	
Buffalo	mt loop	D- VPH-BF	Forward	5'-TAG AAA TAA CTG CAA CCA TCA ACA C-3'	25 bp	53.0	534 bp
		VPH-BR	Reverse	5'-GTC CAA GCA TCC CCC AAA AT-3'	20 bp	52.0	
Sheep	mt loop	D- VPH-SF	Forward	5'-CCA CCC ACG GAC ACG AG-3'	17 bp	58.5	329 bp & 404 bp
		VPH-SR	Reverse	5'-AGT TCA ATG CCC TAT ATG CTT CAG-3'	24 bp	55.0	
Goat	mt loop	D- VPH-GF	Forward	5'-TCC CAC TCC ACA AGC TTA CAG ACA-3'	24 bp	59.9	436 bp
		VPH-GR	Reverse	5'-GCT TAT ATG CAT GGG GGA GAT-3'	21 bp	54.5	
Pig	mt loop	D- VPH-PF	Forward	5'-AAT TTT TGG GGA TGC TTA GAC T-3'	22 bp	52.5	712 bp
		VPH-PR	Reverse	5'-TAT TTT GGG AGG TTA TTG TGT TGT A-3'	25 bp	52.6	

Chicken	5-amino levulina te sythase gene	VPH- ChF VPH- ChR	Forwar d Revers e	5'-CCG GCA GCA GAG CAG ACT AAC AAC-3' 5'-AGG GGA TAC GCC GAC TGC TGA G-3'	24 bp 22 bp	62.0 63.1	288 bp
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[* These primers are designed and validated under a DBT sponsored project at Deptt. Veterinary Public Health, College of Veterinary & Animal Sciences, G.B. Pant Universty of Agriculture & Technology, Pantanagar].

4.1.1.5 PCR Program:

The cycling conditions include an initial denaturation (95°C for 5 min) followed by 30 cycles of denaturation (95°C, 30s), primer annealing (55°C, 30s) and extension (72°C, 30s). After the final extension (95°C, 5 min), the PCR products are held at 4°C until electrophoresis.

4.1.1.6 Electrophoresis

Agarose gel (2%) was prepared in 1X TBE buffer and the PCR products (5 µl) stained with 6X gel loading dye (1 µl) are electrophoresed at 40-60 V for 1-3 hrs. The amplified products are visualized and confirmed over a gel documentation system. The relative molecular weight of the amplicons is calculated against a 100 bp ladder (Fermentas).

4.1.1.7 Refernces

- a) Nagappa S. Karabasanavar, S. P. Singh, Umapathi V, Girish Patil, Sunil N. Shebannavar and Deepak Kumar. 2011. Authentication of carabeef (water buffalo, *Bubalus bublis*)

using highly specific polymerase chain reaction. *European Food Research and Technology*. 233:985-989.

- b) Nagappa S. Karabasanavar, S. P. Singh, Umapathi V, Deepak Kumar, Girish Patil and Sunil N. Shebannavar. 2011. A highly specific PCR assay for identification of raw and heat treated mutton (*Ovis aries*). *Small Ruminant Research*. 100: 153-158.
- c) Nagappa S. Karabasanavar, S.P. Singh, Umapathi V., Deepak Kumar and Sunil N. Shebannavar. 2011. Identification of goat meat using highly species-specific polymerase chain reaction. *Journal of Food Quality*, 34 (2): 142–149.
- d) Nagappa S. Karabasanavar. S.P. Singh, Umapathi V., Deepak Kumar and Sunil N. Shebannavar. 2011. Identification of goat meat using highly species-specific polymerase chain reaction. *Journal of Food Quality*, 34 (2): 142–149.
- e) Nagappa S. Karabasanavar. 2008. Molecular identification of animal species using Polymerase Chain Reaction based techniques. Ph.D Thesis Submitted to the Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India.

4.1.2 Multiplex PCR

Multiplex PCR is used to detect several meat species simultaneously.

4.1.2.1 Principle

Multiplex PCR offers a short cut to many individual PCRs as many targets are simultaneously amplified. Keeping a primer common, species specific forward/reverse primers could be used for the simultaneous detection of many species. Multiplex PCR saves time, labor and it is a good tool for the mass screening of samples that might be confirmed later by species-specific PCR.

4.1.2.2 Reagents

A) PCR reaction mixture

- a) 10X buffer (5 μ l)
- b) dNTP mix (2.5 μ l of 10 mM dNTP solution)
- c) MgCl₂ (3mM, 6 μ l of 25 mM MgCl₂ solution)
- d) Common forward primer (20 pmoles)
- e) Species-specific reverse primers (20 pmoles each)
- f) Taq DNA polymerase (3U)
- g) Purified DNA (50 ng each)
- h) Autoclaved milli Q water

4.1.2.3 Apparatus

- a) Thermocycler
- b) Pipettes: 0.1 & 1 ml

4.1.2.4 Procedure

The multiplex PCR technique as described by Matsunaga *et al.* (1999) is widely followed for meat spectation; PCR is set up in 50 μ l reaction volume. The reaction mixture is optimized by using 10X buffer (5 μ l), dNTP mix (2.5 μ l of 10 mM dNTP solution), MgCl₂ (3mM, 6 μ l of 25 mM Mgcl₂ solution), common forward primer (20 pmoles) and species-specific reverse primers (20 pmoles each), Taq DNA polymerase (3U), purified DNA (50 ng each) and autoclaved milli Q water to make up the reaction volume. The multiplex reaction mixture is optimized by using species-specific reverse primers at different ratios viz. goat:chicken:beef:sheep:pig:horse: :0.3:2.5:0.5:2.5: 0.5:1.8. The PCR tubes are splash spun in a centrifuge to get the reactants at the bottom. The reactions are performed in a PCR System.

4.1.2.5 PCR Program

The cycling conditions are optimized with 1 cycle of initial denaturation (at 94°C for 5 minutes), 33 cycles each of denaturation (94° for 45 seconds), annealing (61°C for 45

seconds), extension (72°C for 45 seconds) and a final extension (at 72°C for 10 minutes). After completing the reaction, tubes with PCR products are held at 4°C until further analysis.

4.1.2.6 Analysis of PCR products by gel electrophoresis

4.1.2.6.1 Principle

After PCR amplification, the products require to be resolved for detection of species.

4.1.2.6.2 Reagents

- a) 3% (w/v) agarose gel
- b) 5X TBE buffer (54 gram Tris base, 27.5 gram boric acid and 20ml of 0.5M EDTA (pH 8.0)
- c) Ethidium bromide (1:10 diluted 20 mg/ml)
- d) 1.0X TBE buffer
- e) 6X Orange loading dye
- f) 100 bp DNA ladder

4.1.2.6.3 Apparatus

- a) Horizontal submarine gel electrophoresis unit
- b) UV trans-illuminator
- c) Gel documentation system.

4.1.2.6.4 Procedure

The amplified PCR products are analyzed using horizontal submarine gel electrophoresis. The casting tray is prepared and the acrylic gel comb was placed over the tray in such a way that a gap of 0.5 mm space is left between the tips of the comb teeth and

the floor of the casting tray. A 3% (w/v) agarose suspension [1.2 gram agarose + 4 ml 5X TBE buffer (54 gram Tris base, 27.5 gram boric acid and 20ml of 0.5M EDTA (pH 8.0) and volume was made upto 1 litre) + 36 ml distilled water] in 0.5X TBE buffer is prepared and heated on an electric heater until the suspension become transparent. The solution is then cooled to 60°C and 10 µl of ethidium bromide (1:10 diluted 20 mg/ml) is added and mixed thoroughly by gentle agitation.

The agarose solution is then poured into a leveled casting tray in such a way that a 4 mm thickness gel was formed. Subsequently, the agarose is allowed to set and the comb is gently removed. The casted gel along with the running tray is submerged in the electrophoresis tank containing 1.0X TBE buffer.

A volume of 6 µl of PCR product is mixed with 2 µl of 6X Orange loading dye and then loaded into the well. A separate well charged with 100 bp DNA ladder is also allowed to run along with the PCR products. Electrophoresis is performed at 7 volts/cm (60-70 volts) for 2 hours and the gel is observed under UV trans-illuminator and analyzed through gel documentation system.

4.1.2.2. Primers

The primers for mitochondrial cytochrome b gene used in multiplex PCR are custom synthesized. The common forward primer as well as species-specific reverse primers as reported by Matsunaga et al. (1999) are used in multiplex PCR technique. The details of the primers used are given below.

Common forward primer*

SI.No.	Sequence	Length
1.	5'-GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA-3'	38

Species-specific reverse primers*

SI.No.	Species	Sequence	Length
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1.	Goat	5'-CTCGACAAATGTGAGTTACAGAGGGA-3'	26
2.	Chicken	5'-AAGATACAGATGAAGAAGAATGAGGCG-3'	27
3.	Cattle	5'-CTAGAAAAGTGTAAGACCCGTAATATAAG-3'	29
4.	Sheep	5'-CTATGAATGCTGTGGCTATTGTCGCA-3'	26
5.	Pig	5'-GCTGATAGTAGATTTGTGATGACCGTA-3'	27
6.	Horse	5'-CTCAGATTCACTCGACGAGGGTAGTA-3'	26

*Matsunaga *et al.*, 1999

4.1.3 Interpretation

The size of the amplicons obtained are 157, 227, 274, 331, 398 and 439 bp in goat, chicken, cattle, sheep, pig and horse, respectively

4.1.4 References

- a) Sambrook, J. and Russell, D. W. 2001. *Molecular cloning: A laboratory manual*. 3rd edition. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York.
- b) Matsunaga, T., Chikuni, K., Tanabe, R., Muroya, S., Shibata, K., Yamada, J. and Shinmura, Y. 1999a. A quick and simple method for the identification of meat species and meat products by PCR assay. *Meat Science*. 51: 143-148.

4.2. Method 2: Restriction Fragment Length Polymorphism (RFLP)

4.2.1 Principle

In PCR-RFLP, the conserved target gene is amplified and the resultant PCR product is digested with specific restriction endonuclease enzyme (s) to get a restriction pattern (fingerprint). These restriction enzymes splice the DNA at specific sites to yield species-specific pattern. PCR-RFLP is a rapid and versatile tool but often requires technical

competence in interpretation of results. The mutated nucleotides are recognized by the restriction enzymes leading to a specific recognition of the sequence. The mitochondria accumulate 10 times more mutations per unit time compared to the nuclear sequences. Hence, mt DNA has been the most targeted sequence. Also, PCR-RFLP has been widely used for species identification especially targeting the mt *cyt-b* gene.

Different restriction enzymes (*AfiIII*, *AluI*, *ApaI*, *AvaiI*, *BamHI*, *DraI* *EcoRI*, *Hinfl*, *NcoI*, *NspI*, *PstI*, *RsaI*, *SfuI*, *StyI* and *TaqI*) could be used depending on the enzyme recognition and splicing sites. The PCR-RFLP has been one of the most commonly used techniques for the differentiation of meat species.

4.2.3 Reagents

- a) 10X reaction buffer
- b) 200 μ M of each dNTP
- c) 2.5mM MgCl₂
- d) 20 pmoles each of forward and reverse primers
- e) 2U of Taq DNA polymerase
- f) 50ng of purified DNA
- g) Autoclaved milli Q water

4.2.4 Apparatus

- a) 500 μ l microtubes
- b) Thermocycler
- c) Pipettes, 0.1 ml, 1 ml

4.2.5 Procedure

The method as described by Partis *et al.* (2000) is followed for PCR-RFLP. PCR amplification reaction is set in a volume of 50 μ l with 5 μ l of 10X reaction buffer, 200 μ M of

each dNTP, 2.5mM MgCl₂, 20 pmoles each of forward and reverse primers, 2U of Taq DNA polymerase, 50ng of purified DNA and autoclaved milli Q water to make up the reaction volume. The reaction is performed within 500 µl microtubes and PCR amplification is carried out using a PCR System 9700. The reaction conditions used for PCR amplification consisted of 1 cycle of initial denaturation (at 94°C for 5 minutes), 35 cycles each of denaturation (94°C for 45 seconds), annealing (60°C for 45 seconds), extension (72°C for 45 seconds) and a final extension at 72°C for 10 minutes. After completing the reaction, tubes with PCR products are held at 4°C until further analysis.

4.2.5 Universal primers used for PCR-RFLP*

Sl. No.	Sequence	Length
1.	5'-CCATCCAACATCTCAGCATGATGAAA-3'	26
2.	5'-GCCCCTCAGAATGATATTTGTCCTCA-3'	26

* Carr and Marshall, 1991

4.2.6 Purification of PCR amplified product for RFLP

The PCR amplified products are subjected to purification using commercially available purification kit as per the protocol of the kit.

4.2.7 Restriction Fragment Length Polymorphism Analysis

The PCR-RFLP is performed as per the protocol described by Partis *et al.* (2000). PCR products obtained from cytochrome b gene are subjected to digestion with suitable restriction enzymes (*Alu* I, *Hinf* I, *Taq* I and *Rsa* I).

4.2.7.1 Reagents

- a) Enzyme buffer mix
 - a. 2 µl of 10X buffer and 1 µl of restriction enzyme

- i. *Alu* I
 - ii. *Hinf* I
 - iii. *Rsa* I
- b) Reaction mix
 - a. 10 μ l of purified PCR product and 3 μ l of enzyme buffer mix and the volume is made upto 30 μ l using autoclaved Milli Q water
- c) 3 μ l of loading dye
- d) 100 bp ladder
- e) 2.5% agarose gel

4.2.7.2 Apparatus

- a) Water bath (37°C)
- b) Electrophoresis unit
- c) UV trans-illuminator
- d) Gel documentation system

4.2.7.3 Procedure

Enzyme buffer mix is prepared by mixing 2 μ l of recommended 10X buffer and 1 μ l of restriction enzyme. The reaction mix is prepared by mixing 10 μ l of purified PCR product with 3 μ l of enzyme buffer mix and the volume is made upto 30 μ l using autoclaved Milli Q water. The reaction mixture is subsequently incubated at 37°C for *Alu* I, *Hinf* I and *Rsa* I and 65°C for *Taq* I for 12 hours. Thereafter, the enzyme activity is arrested by adding 3 μ l of loading dye to the above reaction mixture. The digested product is finally subjected to electrophoresis along with 100 bp ladder in 2.5% agarose gel at 7 volts/cm for 2 hours. The bands are then visualized under UV trans-illuminator and analyzed under gel documentation system for bands of desired molecular weight.

4.2.7.4 Interpretation

Based on the following table, meat species are identified.

Table 2: Restriction enzymes and their digested fragments (in bp) for PCR-RFLP

RE	Cattle	Buffalo	Sheep	Goat	Pig	Chicken	Horse
<i>Alu I</i>	170,189	189,170	*	*	114,245	*	303
<i>Hinf I</i>	117,198	*	296	161,198	*	161,188	234
<i>Rsa I</i>	*	325	*	*	*	148,211	*
<i>Taq I</i>	355	161,198	*	141,218	141,218	*	*

* indicates uncut products

4.2.8 References

- a) Partis, L., Croan, D., Guo, Z., Clark, R., Coldham, T. and Murby, J. 2000. Evaluation of a DNA fingerprinting method for determining the species origin of meats. *Meat Science*. 54: 369-376.
- b) Carr, S. M. and Marshall, H. D. 1991. Detection of intra-specific DNA sequence variation in the mitochondrial cytochrome b gene of Atlantic cod (*Gadus morhua*) by the polymerase chain reaction. *Can. Bull. Fish. Aquat. Sci./ Bull. Can. Sci. Halieut. Aquat.* 48 (1): 48-52.

4.3 Method 3: Forensically Informative Nucleotide Sequencing (FINS)

For meat species identification, a forensically informative fragment of a particular gene is made use of to solve the legal problems. The conserved region of a gene specific to a species is amplified and the PCR product is analyzed. A species-specific PCR would be rather appreciable better if the samples are sub-optimal in the forensic analysis over the RFLP.

4.3.1 PCR amplification of a conserved sequence

For the identification and confirmation of the unknown species, the DNA is isolated as described previously and PCR is performed using universal primers as reported by Kocher *et al.*, 1989 against the flanking sequence of mt 12S rRNA gene.

4.3.1.1 Principle

Conserved sequences in sufficient length carry information in the algorithm of ATGC that could establish origin of species thereby aiding in meat speciation.

4.3.1.2 Reagents

a) PCR reaction mix

- a. 10X Assay buffer [160 mM $(\text{NH}_4)_2\text{SO}_4$, 670 mM Tris-HCl, pH 8.8, 0.1% tween-20, 25 mM MgCl_2]
- b. 1 μl (200 μM each) of dNTP mix [sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water i.e., 40 mM total pH 7.5],
- c. 1 μl or 20 Pico moles each of forward (5'-CAA ACT GGG ATT AGA TAC CCC ACT AT-3' 26 mer) and reverse (5'- GAG GGT GAC GGG CGG TGT GT-3' 20 mer) primers
- d. 1.66 U Taq DNA polymerase
- e. 50 ng of purified DNA
- f. Autoclaved nuclease free water
- g. Agarose gel (2% in 1x TBE and 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide)

4.3.1.3 Apparatus

- a. Thermal cycler.
- b. Electrophoresis unit

- c. UV illumination
- d. Gel documentation system

4.3.1.4 Procedure

PCR is performed in a 50 µl reaction volume; consisting of 5 µl of 10X Assay buffer [160 mM (NH₄)₂SO₄, 670 mM Tris-HCl, pH 8.8, 0.1% tween-20, 25 mM MgCl₂]; 1 µl (200µM each) of dNTP mix [sodium salts of dATP, dCTP, dGTP and dTTP 10 mM each in water i.e., 40 mM total pH 7.5], 1µl or 20 Pico moles each of forward (5'-CAA ACT GGG ATT AGA TAC CCC ACT AT-3' 26 mer) and reverse (5'- GAG GGT GAC GGG CGG TGT GT-3' 20 mer) primers; 1.66 U Taq DNA polymerase, 50 ng of purified DNA and autoclaved NFW to make the final volume. The tubes flash spun and the PCR was performed using a Thermal cycler.

4.3.1.5 PCR program

The cycling conditions involve an initial denaturation (94°C for 5 min) followed by 30 cycles of denaturation (94°C, 45s), primer annealing (60°C, 45s,) and extension (72°C, 1 min); after the final extension (72°C, 5 min), the PCR products are held at 4°C. Agarose gel (2% in 1x TBE and 0.5 µg/ml ethidium bromide) electrophoresis (50 V, 2 hrs) is performed and the amplified products are visualized under UV illumination and the gel is documented.

4.3.2 Sequencing of PCR products from mt 12S rRNA gene

The PCR products are sequenced using automatic DNA sequencer at a DNA sequencing facility. The sequences obtained are aligned using '*Megalign*' and compared by '*Clustal*' method (DNASTar, Inc. 1996).

Any other equivalent software could also be used for this purpose.

4.3.3 BLAST analysis of mt 12S rRNA gene sequence

- a. Log in to the NCBI site (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

- b. Nucleotide blast (*BlastN*) is selected.
- c. The unknown sequence (Accession number, gi, or FASTA) is entered.
- d. Nucleotide collection and highly similar sequences (*Megablast*) option are selected.
- e. The sequence is subjected to Blast.
- f. The data retrieval is in two modes, i.e., distance tree and alignments.

(i) Distance tree of results: Searched by sequence ID, taxonomic name, sequence title or the Blast name. Further, a focused sub-tree as well as the alignment report of the desired species (or accessions) was browsed.

(ii) In '*Alignments*' option, the sequence show significant alignments along with their accession numbers, title, score, E (bits) value, followed by the detailed information about the length of sequence, percent identity, gaps, etc.

Based on the distance tree and alignments with the query sequence, the nearest species with the maximum score was found and the animal species is identified.

4.3.4 References

- a) Kocher, T. D., Thomas, W. K., Meyer, A., Edwards, S. V., Paabo, S., Villablanca, F. X. and Wilson, A. C. 1989. Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proc. Natl. Acad. Sci. U.S.A.* 86:6196-6200.
- b) Nagappa Karabasanavar, Suresh P. Singh, Sunil N. Shebannavar, Kotresh M. Angadi, Girish S. Patil and Umapathi. V. Pillai. 2010. Identification of avian species using polymerase chain reaction and sequence analysis of mitochondrial 12s rRNA gene. *Veterinarski Arhiv.* 80 (5): 653-661.
- c) Website: <http://www.ncbi.nlm.nih.gov/blast>

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*The methods mentioned in the manual needs to be verified/ validated before they are put in use by the laboratory.



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