Standard Laboratory Protocol for
Conducting Pork Safety Tests

Prepared under the
Assam Agribusiness & Rural Transformation Project (APART)
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For
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GOVERNMENT OF ASSAM
Assam Rural Infrastructure and Agricultural Services Society

ANIMAL HUSBANDRY & VETERINARY DEPARTMENT
Government of Assam

ILRI
INTERNATIONAL LIVESTOCK RESEARCH INSTITUTE

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Resident Consultant, ILRI-APART
International Livestock Research Institute (ILRI)

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Abbreviation

AHVD	:	Animal Husbandry and Veterinary Department
AIB	:	Absorbance of Blank
APART	:	Assam Agribusiness and Rural Transformation Project
APC	:	Aerobic Plate Count
APC	:	Agriculture Production Commissioner
BGLB	:	Brilliant- Green Lactose Bile Broth
BHI	:	Brain Heart Infusion
DDL	:	Disease Diagnostic Laboratory
ERV	:	Extract Release Volume
FSSAI	:	Food Safety and Standards Authority of India
GK	:	Gluconate Kinase
HEA	:	Hektoen Enteric Agar
LIA	:	Lysine Iron Agar
LST	:	Lauryl SulphateTryptose Broth
MPN	:	Most Probable Number
MSC	:	Meat Swelling Capacity
NADP	:	Nicotinamide Adenine Dinucleotide
PAT	:	Picric Acid Turbidity
PCA	:	Plate Count Agar
PGDH	:	Phosphogluconate Dehydrogenase
RDRT	:	Resazurin Dye Reduction Test
TCA	:	Trichloroacetic Acid
TVBN	:	Total Volatile Based Nitrogen
VRBA	:	Violet Red Bile Agar
XLD	:	Xylose Lysine Deoxycholate

1. Background

To create a demand-driven production system, quality assurance of livestock products is important and therefore under the World Bank sponsored Assam Agribusiness and Rural Transformation Project (APART), the Animal Husbandry and Veterinary Department (AHVD) has decided to strengthen seven District Disease Diagnostic Laboratories (DDL) by restructuring them additionally as food safety laboratories apart from their normal role of disease diagnosis.

The food safety laboratories under the Department of Health Services carry out all sorts of food safety tests for regulatory purpose under the aegis of Food Safety and Standards Authority of India (FSSAI), while AHVD intends to conduct the safety tests for pork on a surveillance purpose. By doing so, AHVD will support the pig producers, pig butchers and pork retailers by checking quality of their produce and helping them for improvement of pork quality and safety on a risk mitigating approach. These laboratories once upgraded to food safety laboratories along with their disease diagnosis facilities may also help the existing Food Safety Laboratories under FSSAI, if required, for performing some specialized tests. Besides, AHVD has the plan to designate some of the Veterinary Officers as Food Safety Officers as per the guidelines of FSSAI. The proposal is under consideration and if it is realized, the Department may need a full-fledged Central Pork Safety Laboratory in the state. The state laboratory may act as an FSSAI accredited independent laboratory of Level- 1 or Level-2 as per FSSA regulation 2011, while the district laboratories may keep functioning towards meeting the development/surveillance purpose of the department.

As per FSSAI, about 15 tests are required to be conducted on meat safety (Table 1). Some of these tests are very important to conduct in Assam's context, while some tests are less important as per the opinion of local meat experts in Assam. Further, there are few additional tests which are more essential in the context of Assam as opined by the meat experts. Therefore, the tests are presented in a tabular form (Table 2) to present the same in separate categories; such as tests recommended by FSSAI and the tests recommended by ILRI in consultation with local meat experts for the state laboratories as well as district laboratories. Since the department is yet to have state meat laboratory in place, the focus is now more on the tests recommended for the district laboratories for surveillance purposes. To conduct these tests by the departmental officials, they need a standard test protocol to follow. FSSAI has ready standard test protocol for all the tests recommended by them (for all sorts of meat and not specific to pork). Since test protocol is purely a scientific approach of conducting test and needs authenticity, the already established test protocol mainly developed by FSSAI needs to be followed. Thus, the present protocol is only the compilation of the test protocol on meat safety produced by FSSAI. ILRI has, however, made efforts to present the protocol in a simpler and easily understandable form.

Table 1: List of tests to be conducted as per FSSAI for meat safety, 2016

Name of the test	Requirements as per FSSAI
Determination of Nitrite	√
Determination of Ascorbic Acid	√
Determination of total Phosphorous	√
Determination of Glucono-Delta-Lactone	√
Total Fat	√
Total Protein	√
Determination of pH	√
Determination of Extract Release Volume (ERV)	√
Determination of Meat Swelling Capacity (MSC)	√

Name of the test	Requirements as per FSSAI
Determination of Total Volatile Basic Nitrogen (TVBN)	√
Determination of Picric Acid Turbidity (PAT)	√
Determination of Dye Reduction Capacity	√
Determination of Microbial Load	√
Determination of Microbial Toxins	√
Meat speciation using DNA based molecular technique	√

Among the tests, there are again certain easy and quick tests that can be conducted in district laboratories with limited infrastructure, equipment, manpower, skill and resources. The tests requiring more sophisticated equipment, skilled manpower and resources are recommended for the state laboratory only (Table 2).

Table 2: Recommended tests for the state and district laboratories with particular reference to pork safety

	Name of the test	Degree of difficulties to conduct *	Recommended by FSSAI	Recommended tests	
				For state laboratory	For district laboratory
3.1 Physical tests					
3.1.1	Visual observation of the muscle for extraneous matter, colour and smell, measly pork, etc.	+		√	√
3.1.2	Colour and smell	+		√	√
3.1.3	Presence of *Cysticercus cellulosae (Larval form of Taenia solium)*	+		√	√
3.2 Compositional tests					
3.2.1	Determination of Nitrite	++	√	√	√
3.2.2	Determination of Ascorbic acid	++	√	√	
3.2.3	Determination of Total Phosphorous	+++	√	√	
3.2.4	Determination of Glucono-Delta-Lactone	++++	√	√	
3.2.5	Estimation of Total Fat	++	√	√	√
3.2.6	Estimation of Total Protein	++	√	√	
3.2.7	Determination of pH	+	√	√	√
3.2.8	Determination of Protein Degradation (Tyrosine value)	++++		√	√
3.2.9	Determination of Extract Release Volume (ERV)	+++	√	√	√
3.2.10	Determination of Meat Swelling Capacity (MSC)	+	√	√	
3.2.11	Determination of Total Volatile Basic Nitrogen (TVBN)	++	√	√	
3.2.12	Determination of Picric Acid Turbidity (PAT)	+	√	√	
3.2.13	Determination of Dye Reduction Capacity	++	√	√	
3.2.14	Determination of Fat Oxidation	+++		√	√

	Name of the test	Degree of difficulties to conduct *	Recommended by FSSAI	Recommended tests	
				For state laboratory	For district laboratory
3.2.15	Determination of Water Holding Capacity	++		√	√
3.3 Microbiological tests					
3.3.1	Determination of Total Microbial Load	+++	√	√	√
3.3.2	Coliform Count	++++		√	√
3.3.3	Test for *Salmonella*	++++		√	
3.3.4	Test for *Staphylococcus aureus*	++++		√	
3.3.5	Test for *Listeria monocytogenes*	++++		√	
3.4 Other tests					
3.4.1	Determination of Microbial Toxins	++	√	√	
3.4.2	Determination of Antimicrobial Residues by using CHARM Rosa platform	++		√	
3.4.3	Detection of Heavy Metals	++++		√	
3.4.4	Determination of Insecticide & pesticide residues by using CHARM Rosa platform	++		√	
3.4.5	Formalin test	++		√	√
3.4.6	Meat speciation using DNA based Molecular Technique	++++	√	√	

*More '+' means increased in level of difficulties in conducting the test

In addition to the test protocol, a separate protocol for bio-safety has been prepared. The same may be followed by the departmental officials in carrying out the lab tests.

2. Method of sample collection, labeling, storing, and dispatching

Sample collection

Appropriate sample collection is one of the prerequisites for accurate test results.

2.1 General requirement for sample collection:

- Make arrangement of all necessary materials before going to sample collection.
- Take sterile container and zip lock bags for tissue samples.
- Take a clean, dry, leak-proof, wide-mouthed and sterile container of a size suitable for the pork sample
- Take a couple of stainless steel knives.
- Take a small weighing balance, if possible.
- Take a cool box/ thermos flask to carry the sample.
- Take required ice/gel packs in cool box to keep the sample cool during transportation.
- Take personal protective clothing like apron, gloves, mask etc.
- Take sticker tags, marker, note pad, gloves, mask, sanitizers and biohazard bag.
- Take a disposal bag for carrying disposable materials like leftover meat, gloves, mask etc.
- Take a spirit lamp/alcohol for sterilization of the instruments on the spot

2.2 Information to be submitted along with the sample:

- Type of the sample (e.g. meat/offal/eye/tongue/others)
- Species: pig
- Type of animal: fattener/ boar/sow/piglet
- Sample no.
- Weight/ volume of the sample
- Place of collection
- Date and time of collection
- Name of the collector
- Purpose of collecting the samples
- Name, address and signature or thumb impression of the person from whom the sample has been taken

2.3 General considerations in sample collection, handling and storage:

- The samples should never be touched with bare hands. Gloves and mask should always be used in the process of collection.
- Outer and inner surface of the container should be cleaned thoroughly.
- Knife/instruments used for cutting, removing and manipulating samples should be sterilized with hot water before and after use.
- Sample should not be allowed to expose to dirty materials/environment after collection and should not be mixed with other biological samples.
- Temperature and pH shall be recorded at the collection stage and after transporting to the laboratory.
- Disinfect the surface of the work area before opening the samples for weighing, packaging, etc. at the laboratory

- Sample should be weighed directly in the sterile container;
- Gloves, mask and other materials in contact with the sample must be incinerated or disposed properly.
- The stopper/cover of the container shall be securely fastened to prevent leakage of the contents in transit.
- The container shall be completely wrapped in fairly strong thick paper.
- The ends of the paper shall be neatly folded in and affixed by means of gum or other adhesive.
- Always collect the samples as fast as possible after slaughter to prevent post slaughter changes.

2.4 Sample collection, labelling and storing

- All meat samples should be collected in the sterile container or polythene bags.
- Pork sample should be taken by cutting the meat in the form of a 100 g meat block with a sterile knife and then placed in sterile container or bag. However, in order to avoid any unpredictable loss of the sample due to handling or else, it is advisable to take a block of 250 g of pork sample.
- Separate sterile container or plastic bag should be used for each sample.
- Collected sample should be distinguish from other samples by writing the details of the sample on the body of the container by a good quality marker and on an sticker tag and pasting the same on the container immediately after collection.
- The detail information of the sample should be written in a piece of paper and should put the same in a polythene zip bag and stick it at the body of the container by a transparent adhesive tape.
- Alternatively (mainly for sample that is collected for regulatory purpose as advised by Food safety Officer), a paper slip of the size that goes round completely from the bottom to top of the container, bearing the signature of the Designated Officer and number of the sample, shall be pasted on the wrapper, the signature or thumb impression of the person from whom the sample has been taken, shall be affixed in such a manner that the paper slip and the wrapper both carry a part of this signature or the thumb impression. The outer covering of the packet shall also be marked with the same number of the sample
- The labeled container should immediately be transferred to the cool box/ thermos flask filled with ice packs.
- The collected container shall be properly secured and sealed so that no tempering is possible after collection. To ensure this, signature of the pork seller/pig slaughter and a witness should be taken on the sealed pack.
- AHVD officers will generally collect and test pork samples for surveillance purpose but if the sample is collected as per the request of Health Services Department for regulatory purposes, some additional process may need to be followed as suggested by them.
- All samples should be transported to the laboratory by maintaining the cold chain in a cool box/ thermos flask with gel/ice packs.
- After arriving the sample at the laboratory, a separate code should be assigned to each sample at the reception desk. The sample should be processed in the laboratory and results should be mentioned against that code only.
- No personal details of the owner of the sample should be supplied to the laboratory technicians who conduct the tests to avoid any potential pre-judgment by the lab technicians.
- The sample should be stored at 4°C and processing should be immediately.
- In case of delay, samples should be refrigerated at 4°C and processing should be done within 96

hours of sampling. If sample needs to be preserved for more than 96 hours, it should be stored at -200C

2.5 Packaging and dispatching of samples to other laboratories

- Basic triple packaging system, i.e. a leak-proof primary receptacle, a leak-proof secondary container and an outer box should be used for transport of all infectious substances as recommended by 'United Nations Committee of Experts as per Transport of Dangerous Goods (UNCETDG).
- For dispatching the samples to other laboratories, sample with their details should be put in a thermo-cool box. Adequate quantity of cool ice pack/jel pack should be put in the box to keep the sample cool during the time of transportation.
- The outside of the thermo-cool box should be wrapped up with white paper and address of 'From' and 'To' should be clearly written on it preferably in all capital letters.
- A certificate should also be enclosed with the box stating the nature of the materials and purpose of sending.
- Packages should be marked clearly to provide information about the contents of package and, nature of the hazard, if any.
- Before transporting biological materials, its nature must be determined whether material should be classified as dangerous (hazardous or infectious) goods or not.
- The infectious substances can be classified as Category A or Category B as per criteria provided in WHO guidance document39
- A category A [UN 2814 (affecting humans) or UN 2900 (affecting animals)] infectious substance affects humans or animals". While Category B (UN 3373) infectious material contain pathogens that do not affect humans or animals.
- Sample should be sent by the mode of transportation that can deliver the sample at the quickest possible time in the destination. If the transportation time is more, the ice/jel pack may come to normal temperature and the sample may get spoiled.
- The thermos-cool box should also be marked with 'handle with care' an 'arrow mark' showing upside of the box, in order to guide the handlers during transportation.

3. Test protocol for pork safety tests

Some important terminologies as defined by FSSAI [Food Safety and Standards Authority of India (food products standards and food additives) regulations, 2011: Version-IX (29.03.2019)): 2.5. Meat and meat products] are as follows:

Pork: means the edible portion of domestic pigs;

Fresh pork: means pork that has not been treated in any manner to ensure its preservation;

Chilled pork: means fresh pork subjected to chilling in such a manner that the product is maintained at a temperature between 0°C to 4° C;

Frozen pork: means chilled pork subjected to freezing in appropriate equipment in such a manner that the product is maintained at a temperature of -18° C or lower;

Pork edible offal: means edible by-products derived from the slaughtered pig which includes brain, liver, gut, paunches, tripe, lungs, and other edible parts.

Pork should be stored at 4º C for short-term storage and at -18ºC or below for long-term storage.

The chilled pork shall be consumed within two to four days under normal chilling conditions of storage and frozen pork shall be consumed within four to six months.

Every test protocol has been explained in the following lines

1. Reagents required
2. Test procedure to be followed
3. Interpretation of the results

3.1 Physical Test

Physical observation of the pork samples is required to assess:

- Presence of extraneous/foreign matters: Visual observation of foreign matter such as hair, straw, grass, dirt, sand, soil, etc. or other similar particles that may reduce consumers' preference. This may indicate the non-compliance of good sanitary and hygiene practices in slaughtering and displaying pork.
- Colour and smell: Examine the colour and smell of pork samples to recognise if the pork sample has any abnormalities in terms of colour, consistency or smell. Some pork may be giving off the peculiar smell of meat of an uncastrated boar which is termed as "Boar Taint".Examine the liver and lungs for any discoloration.
- Presence of Cysticercus cellulosae (Larval form of Taenia solium): Observation of cotton seed like white follicle (measly pork) in muscle, mucus layer of eye, tongue etc. by making multiple incisions. Also observe for presence of tubercles in lungs or other organs which is indicative of tuberculosis.

Appliances required

- Hand gloves
- Butchers' knife
- Apron
- Forceps
- Magnifying glass

Procedure:

3.1.1 Visual observation of the muscle

- Examine the pork sample for presence of extraneous/foreign matters -such as- hair, straw, grass, dirt, sand, soil, etc. or other similar particles which are readily recognized by naked eyes. Discard the pork if found with presence of any of the above mentioned impurities;
- Examine the pork sample under a magnifying glass if required.
- Sometimes, Pale Soft Exudative (PSE) pork may be observed. This is due to stress condition prior to slaughter and this kind of pork is known as "Watery pork". Though not pathological, it may cause consumers' dissatisfaction and lowering of price.
- There may be spoilage of pork due to fat oxidation i.e., lipolysis. Here,discoloration and liquefaction fat is observed. This is also not pathological but may cause consumers' dissatisfaction and lowering of price.

3.1.2 Colour and smell

- Examine the colour of the pork sample with naked eyes for any abnormal colour. Pork having any off colour or colour not fit to be described as the normal colour of pork should be discarded. Examine the liver and lungs for any discoloration. This discoloration is not pathological and pork should not be discarded.
- Smell the odour of the pork sample to get a sense of any abnormality. Pork having any off smell/ odour not fit to be described as the normal smell/odour of pork should be discarded.

3.1.3 Presence of *Cysticercus cellulosae (Larval form of Taenia solium)*-

- Make multiple incisions on the pork sample and examine it for presence of Cysticercus cellulosae (Larval form of Taenia solium))-which can be seen as Cotton seed like white follicle (measly pork) in

muscle, mucus layer of eye, tongue etc. Pork having this kind of appearance should not be sold or consumed.

- Also observe for presence of tubercles in lungs or other organs which is indicative of tuberculosis. Pork having this kind of appearance should not be sold or consumed.

Closely observe the muscle and record for any abnormalities in case of all the above four physical tests.

3.2 Compositional Test

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 the 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))

3.2.1 Determination of Nitrite

Nitrite and nitrates are used in pork to form cured meat colour, to enhance flavour and to provide antimicrobial and antioxidant effect in pork **and** pork **products**. It is a well-known fact that nitrite reacts with secondary amines and other nitrogenous compounds, causing the formation of carcinogenic, immunosuppressive and mutating N-nitrosamines. This test is aimed to assess the technologically acceptable quantity of nitrate and nitrite , (must not exceed 15 mg% of NaNO2)

Reagents Required:

(a) NED reagent-

- Dissolve 0.2 g N- (1 Napthyl) 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 0.5 g sulphanilamide in 150 ml 15% acetic acid (v/v).
- Filter, if necessary and store in a glass stoppered brown bottle.

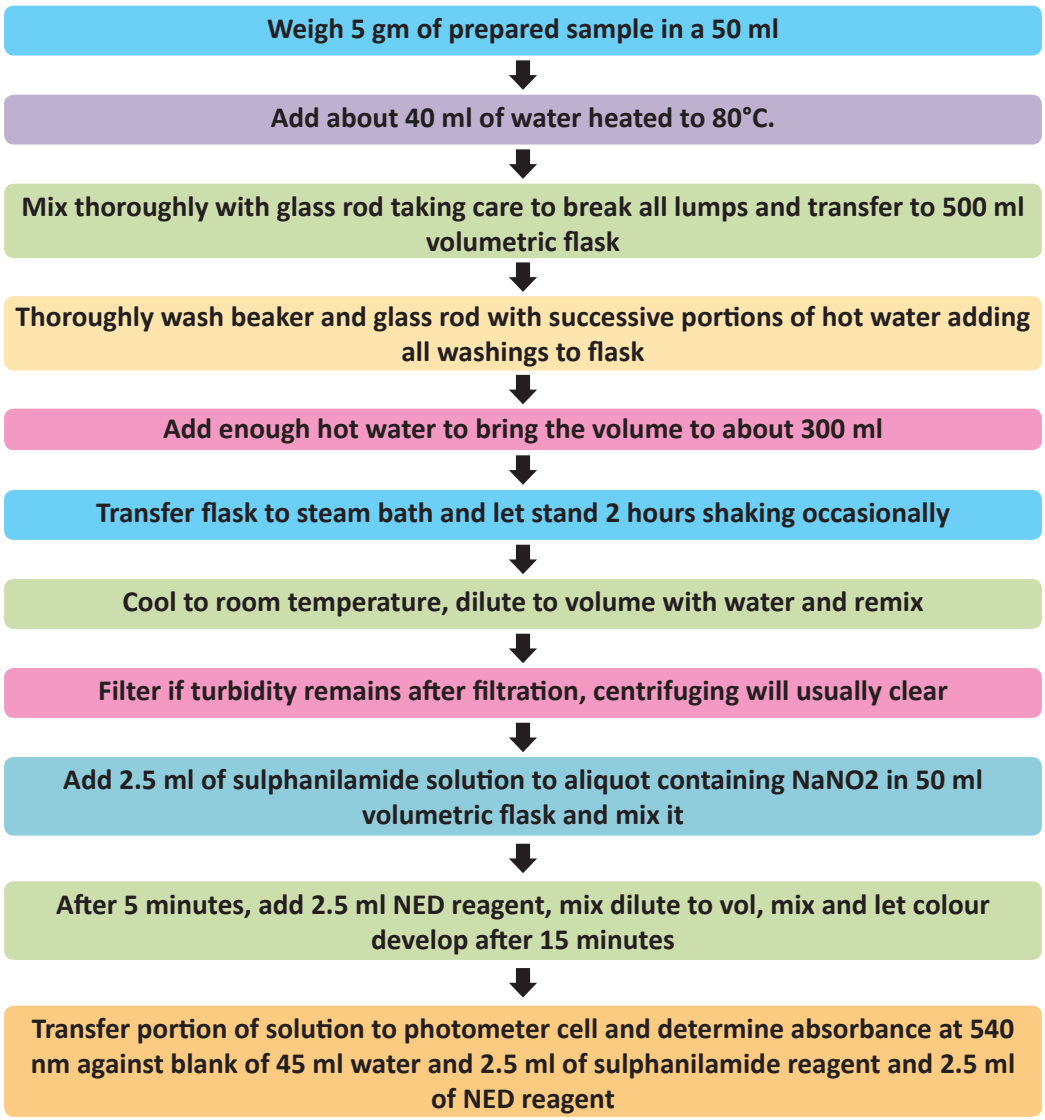
(c) Nitrite standard solution-

- Stock solution - 1000 ppm $NaNO_2$
- Dissolve l.0 g pure NaNO2 in water and make up to 1 litre.
- Intermediate solution 100 ppm
- Dilute 100 ml of stock solution to 1 litre with water.
- Working solution- l ppm

(d) Filter paper-

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

Procedure:



Determine presence of Nitrite by comparison with standard curve prepared as follows:

- Add 10, 20, 30, 40 ml of nitrite working solution to 50 ml volumetric flasks.
- Add 2.5 ml of sulphanilamide reagent and after 5 minutes add 2.5 ml of NED reagent and proceed as above.
- Standard curve is straight line upto 1 ppm $NaNO_2$ in final solution.

(Reference- AOAC Official method 17th edition 2000, 973.31 Nitrites in cured meats

Colorimetric method, Adopted as Codex Reference method (Type II).

3.2.2 Determination of Ascorbic Acid

Ascorbic acid is a commonly-used food additive, antioxidant, and stabilizer permitted in pre-packed fresh meat preparations. Considering that the addition of this food additive is generally considered safe, these low concentrations may be useful for the determination of an allowable limit for the ascorbic acid in fresh meat preparations.

Reagents Required:

a) Extracting solution - Metaphosphoric acid-acetic acid solution-

- Dissolve with shaking 15 g HPO_3 pellets or freshly pulverized sticks in 40 ml acetic acid and 200 ml water.
- Dilute to 500 ml. Filter rapidly through fluted filter paper into a glass stoppered bottle. Store in a refrigerator. Solution remains satisfactory for 7-10 days.

b) Ascorbic acid standard solution (1 mg/ml) –

- Accurately weigh 50 mg USP Ascorbic acid reference standard that has been stored in a desicator away from sunlight.
- Transfer to 50 ml volumetric flask. Dilute to volume with metaphosphoric- acetic acid extracting solution before use.

c) Indophenol standard solution:-

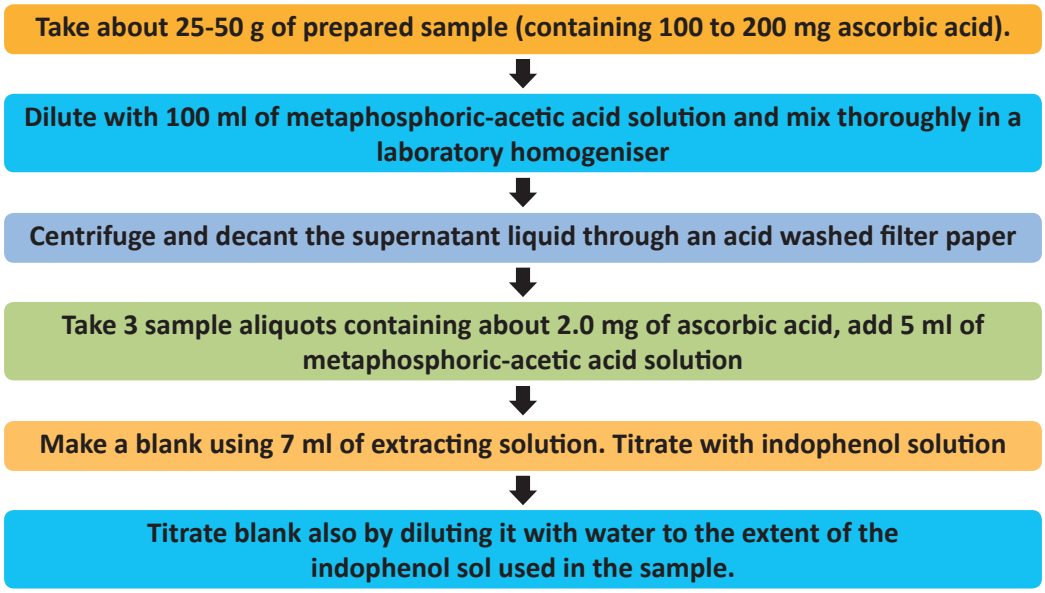
- Dissolve 50 mg 2, 6 dichlorophenol indophenols sodium salt in 50 ml of water to which have been added 42 mg of $NaHCO_3$.
- Shake vigorously and when dye dissolves dilute to 200 ml with water.
- Filter through fluted filter in an amber colored glass bottle. Keep stoppered and store in a refrigerator.

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

d) Standardization of Indophenol solution –

- Transfer 3 aliquots of 2.0 ml Ascorbic acid standard sol. to 3 conical flasks containing 5 ml of metaphosphoric- acetic acid extracting solution.
- Titrate rapidly with indophenol dye from 50 ml burette until a light distinct rose pink remains for 5 seconds.
- Each titration should require about 15 ml indophenol solution and differ from each other by 0.1 ml.
- Similarly, titrate 3 blanks composed of 7 ml of metaphosphoric- acetic acid 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.
- Standardize indophenol solution daily with freshly prepared ascorbic acid standard solution.

Procedure:



Calculation :

Ascorbic acid mg per 100 gm

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

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

3.2.3 Determination of Total Phosphorous

Phosphate and polyphosphate additives are added to meat because they serve as an emulsifier of fat, water and protein, and stimulate water binding. Phosphates affect the texture of the product, prevent discoloration, improve emulation of fat and improve flavor, prevent oxidation of unsaturated fatty acids and affect the pH value. This test is done to assess whether the meat sample is containing the maximum admissible amount of phosphorus (expressed as P_2O_5), that can be added to meat products and which is 5 g/kg. **Reagents required**:

a) Conc. Sulphuric acid - 1.84 g/ml

b) Conc. Nitric acid - 1.40 g/ml

c) Precipitating reagent –

- Dissolve 70 g of Sod. Molybdate dehydrate in 150 ml water.
- Dissolve 60 g 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.

Apparatus

- Mechanical meat mincer - fitted with a plate with holes of dia not exceeding 4 mm.
- Analytical balance
- Kjeldahl flask
- 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.
- Suction device to remove the acid fumes formed during the digestion.
- Fritted glass filter - pore diameter 5-15 mm.
- Drying oven capable of being adjusted to 260 — 20 ° C
- Conical suction flask
- Desiccator

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

↓

Analyze the sample as soon as possible, within 24 hrs

↓

If the sample is not immediately analyzed after passage through the mincer, separation of liquid may occur. Therefore, homogenize the sample thoroughly and immediately before testing

↓

Weigh to the nearest 0.001 g 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 solution

↓

Finally, when the liquid has become colorless heat until white fumes appear

↓

Cool and 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.

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 precipitate on the filter five times with 25 ml water using the same water to wash away any remaining precipitate 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:

Phosphorous (%)as P_2 0_5=0.03207×m_1×$\frac{100}{m_0}$ or 3.207×$\frac{m_1}{m_0}$

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

3.2.4 Determination of Glucono-Delta-Lactone (Rule 72)

GDL (**glucono-delta-lactone)** is an acidifier, preservative and leavening agent .

It is a widely used acidulant (a food additive used to increase acidity or to give a tart test) in meat products, especially for dry cured sausages. It is used when a slow release of acid is required. In the presence of water it reverts to gluconic acid. The lowering of the pH inhibits growth of bacteria and accelerates the drying .

High levels of GDL promote the growth of peroxide-forming Lactobacillus spp., which can lead to rancidity and poor curing color. The acidic flavor from gluconic acid, from GDL, is less favorable compared with lactic acid .

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.

Reagents Required:

- Perchloric acid - 0.4 M - Dilute 17.3 ml Perchloric acid (70 % m /m) to 500 ml with water.
- Potassium hydroxide - 2 M - Dissolve 56.l g Pot. Hydroxide in water- Dilute to 500 ml.
- Buffer Solution - pH 8.0 - Dissolve 2.64 g glycylglycine and 0.284 g magnesium chloride hexahydrate in 150 ml water. Adjust to pH 8 with potassium hydroxide. Dilute to 200 ml with water.
- Nicotinamide Adenine Dinucleotide Phosphate (NADP) - Dissolve 50 mg of NADP disodium salt in 5 ml water.
- Adenosine -5- triphosphate (ATP) - Dissolve250 mg ATP disodium salt and 250 mg sodium hydrogen carbonate in 5 ml water.
- 6 - Phosphogluconate dehydrogenase (6 PGDH)- Commercial suspension containing 2mg 6- PGDH / ml from yeast.
- Gluconate kinase (GK)- Suspension containing mg/ml from *E. coli*.

Procedure:

Weigh 50 g of prepared sample into a homogenizer

↓

Add 100 ml of cold (0°C) 0.4M 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 flter paper. Discard the first 10 ml

↓

Pipette 25 ml of the filtrate (V ml) into a 250 ml volumetric flask. Dilute to mark with water (maximum concentration of D (+) gluconate is 400 mg/L)

↓

This is the prepared extract. Pipette into each of 2 photometric cells - 2.5 ml of pH 8 buffer, 0.1 ml NADP, 0.1 ml ATP

↓

Into one of the cells pipette 0.2 ml extract, into the other 0.20 ml water

↓

Pipette 0.05 ml of 6 - PGDH suspension on to a plastic spatula, mix with the contents of one of the cells

↓

Repeat the operation with the second cell. Read the absorbance of each cell against air at 365 nm after 5 minutes. Retain the cells for reaction

A1 = Absorbance of Test Solution A, A1B = Absorbance of Blank

Pipette 0.01 ml of GK (Gluconate Kinase) 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.

A2 = Absorbance (T = 0) of the test solution

A2 B = Absorbance (T = 0) of the blank solution

(Reference: - Pearsons Composition and Analysis of Foods (9th edn) 1991, Pp. 502)

Calculation :

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

Glucono-delta-Lactone % by mass = $15058X\frac{\triangle A}{v \times m}X(100+\frac{M \times m}{100})$

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

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

Alternatively following method can also be used:

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

3.2.5 Estimation of Total Fat

Keeping fat content consistent in sausage products will help to generate repeat sales by ensuring that product quality remains consistent. Many accurate & rapid methods exist to determine fat content.

Reagents Required:

- NH_4OH
- HCl
- Glass beaker
- Steam bath
- Filter paper
- Soxhlet apparatus

Procedure:

Weigh accurately 3-4 g of well mixed sample in a 100 ml beaker

↓

Add a few drops of NH_4OH and warm on a steam bath

↓

Add 10ml of conc. 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

Calculation

Where, W_2 = weight (in g) of the flask with the dried (accumulated) fat;

W_1 = weight (in g) of the empty extraction flask with boiling chips

And, W_0 = weight (in g) of the test portion (sample).

Take the result as the average of the two determinations.

The difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst should not be greater than O-5 g of total fat per 100 g of sample.

(Reference:- ISO 1443- 1973 Codex approved method - Extraction / gravimetric Type I method)

(Please also refer to IS: 5960 (Part 3) 1970 Methods of test for meat and meat products. Determination of total fat content) oi

3.2.6 Estimation of Total Protein

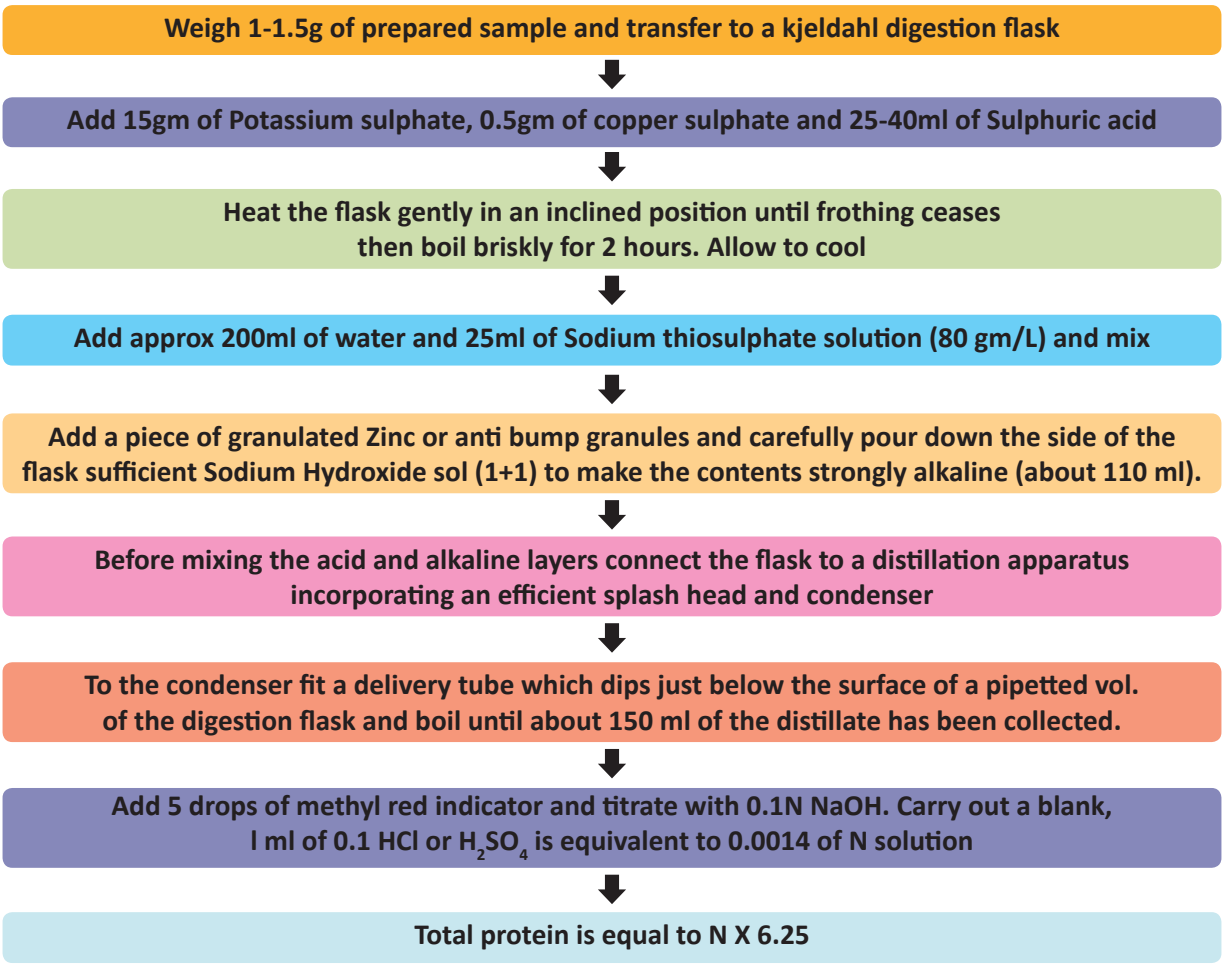
Meat is an excellent source of proteins and essential amino acids, with its contributions to the human dietary needs. Protein is hydrolyzed the digestive system to amino acids, which are used to build proteins specific to the consuming organism. The amino acid profile is important because some amino acids cannot be synthesized by humans and must be obtained from diet. Meat is rich in so-called essential amino acids –lysine, leucine, isoleucine, and sulfur-containing amino acids – and this sense meat is a high-quality protein.

Generally proteins from meat and meat products are 95-100% digestible.

Reagents Required:

- Kjeldahl catalyst:- 15 g Potassium Sulphate + 0.5 g Copper Sulphate
- Sulphuric Acid - Concentrated
- NaOH solution- 50% (1+1). Let stand until clear
- Standard NaOH solution- 0.1 N=0.1 M (4.00 g/litre)
- Standard acid solution- Prepare either HCl or H_2SO_4 solution HCl sol-0.1
- N= 0.1 M (3.646 g/litre)
- H_2SO_4 sol - 0.1N=0.05 M (4.9 g/litre)
- Methyl Red Indicator - 0.5 g in 100 ml ethanol

Procedure:



Note: On the basis of early determinations, the average nitrogen (N) content of proteins was found to be about 16 percent, which led to use of the calculation N x 6.25 (1/0.16 = 6.25) to convert nitrogen content into protein content.

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

IS- 5960 (Part 1) 1996/ISO 937-1978 Meat and Meat Products - Determination of Nitrogen Content.)

3.2.7 Determination of pH

The pH of muscle/meat is a measurement of acidity. Glycogen is broken down to lactic acid when muscle turns into meat. Pale, Soft, and Exudative (PSE) pork commonly results from a rapid breakdown of glycogen into lactic acid after slaughter. Abrupt lowering of pH indicates PSE. The top five attributes that influence consumer decisions are taste, tenderness, food safety, appearance (mostly, colour) and juiciness. In a normal living muscle the pH is approximately 7.2 (ranging from 5.2 to 7.0). Microorganisms, including yeasts, molds and bacteria are sensitive to a food's pH. Very low or high pH values will prevent microbial growth.

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

The pH can be measured by fallowing two methods –

Digital pH meter and Chemical indicator method (Nitrazine yellow)

Test protocol-1: Modern method using Digital pH meter

Materials Required-

- Digital pH meter
- Distilled water
- Beaker and electrolyte solution.

Procedure:

Blend 15 gm meat with 30 ml distilled water at 27-300 C. → 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)

Test protocol-2: Conventional method using Chemical indicator (Nitrazine- Yellow Test):

This test determines the acidity of meat.

Materials Required:

- Nitrazine- Yellow indicator
- Glass rod
- Petri plate

Procedure:

Take a piece of meat free of blood, fat, and connective tissue in a petri dish

↓

Add Nitrazine Yellow indicator (1:10000) sufficient to cover the meat piece

↓

Mix with stirring rod. Note the color change with standard chart provided

↓

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

3.2.8 Determination of Protein Degradation (Tyrosine value)

In the presence of tyrosine, Folin–Ciocalteu reagent (FCR) reagent produces blue colour, the intensity of which is a measure of protein breakdown. Tyrosine value can effectively monitor the meat quality to indicate proteolysis and to measure the amino acids- tyrosine and tryptophan present in a extract of meat. The purpose of the protein assay is to determine the amount or concentration of a specific protein or an array of different proteins a sample. Tyrosine value increases rapidly in meat stored at 7° C than at -10° C.

Reagents Required:

- 0.5N NaOH Solution: 20gm/100ml
- Folin-Ciocalteau reagent(FCR)

Instrument Required:

- UV-VIS Spectrophotometer
- Electronic balance

Procedure (for preparation of Folin-Ciocalteau reagent):

Reflux gently for 10 hours a mixture consisting of 100g sodium tungstate (Na2WoO4.2H2O), 25g sodium molybdate (Na2MoO4.2H2O), 700 ml water, 50 ml of 85% phosphoric acid, and 100 ml of concentrated hydrochloric acid in a 1.5lit flask

↓

Add 150g lithium sulfate, 50ml water and a few drops of bromine water. Boil the mixture for 15min without condenser to remove excess bromine. Cool, dilute to 1L and filter. The reagent should have no greenish tint. (Determine the acid concentration of the reagent by titration with 1N NaOH to a phenolphthalein end-point).

↓

This is commercially available and has to be diluted with equal volume of water just before use.

Standard Tyrosine solution: 5mg/ml Bovine Serum Albumin (commercially available-used as Standard)

Procedure:

Take 0.2, 0.4, 0.6, 0.8 and 1ml into series in five test tubes, add water to bring the volume of one ml in each test tube.

↓

Add 5ml of 0.5N NaOH solution and finally 1.5ml of dilute F/C reagent to each test tube mix well and measure the O.D. with a photoelectric colorimeter at 700nm (or) by using a red filter within 2 to 10 minutes

↓

Prepare a Blank with 1ml of distilled water instead of Tyrosine solution construct the standard curve with micrograms of tyrosine

↓

Tyrosine concentration on the X-axis and OD on Y-axis using a standard curve determine the amount of tyrosine present in a given unknown solution.

Report: The concentration of Tyrosine present in the given solution is _____mg

Sl No.	Volume of Working standard (ml)	Concentration of Tyrosine (micrograms)	Volume of water (ml)	F/C Reagent (ml)	0.5 N NaOH solution (ml)	OD at 700 nm
Blank	0	0	1	1.5	5	
S1	0.2	50	0.8	1.5	5	
S2	0.4	100	0.6	1.5	5	
S3	0.6	150	0.4	1.5	5	
S4	0.8	200	0.2	1.5	5	
S5	1.0	250	----	1.5	5	
Unknown	----	----	----	1.5	5	

Ref:- http://biochemden.in

3.2.9 Determination of Extract Release Volume (ERV)

Extract release volume (ERV) is the volume of extract released by a homogenate of meat when allowed to pass through the filter paper for a given period of time. ERV is of value in determining spoilage of meat as well as in predicting shelf life of meat. It is inversely proportional to the extent of spoilage.

In this method meat of good microbial and good organoleptic qualities releases large volume of extract while meat of poor quality releases smaller volume.

Requirements:

- Beaker
- Distilled water
- Cellulose based qualitative filter paper
- Graduated cylinder
- Mortar and pestle.

Procedure:

Take 25 g meat sample in 100 ml distilled water

↓

Blend it with in pestle and mortar

↓

Filter through cellulose based qualitative filter paper, folded thrice so as to make eight sections

↓

Allow the homogenate to seep between the folds

↓

Collect the extract in 100 ml graduated cylinder for 15 min

↓

Record extracts release volume and interprets results

Interpretation:

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

(Reference:- 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.)

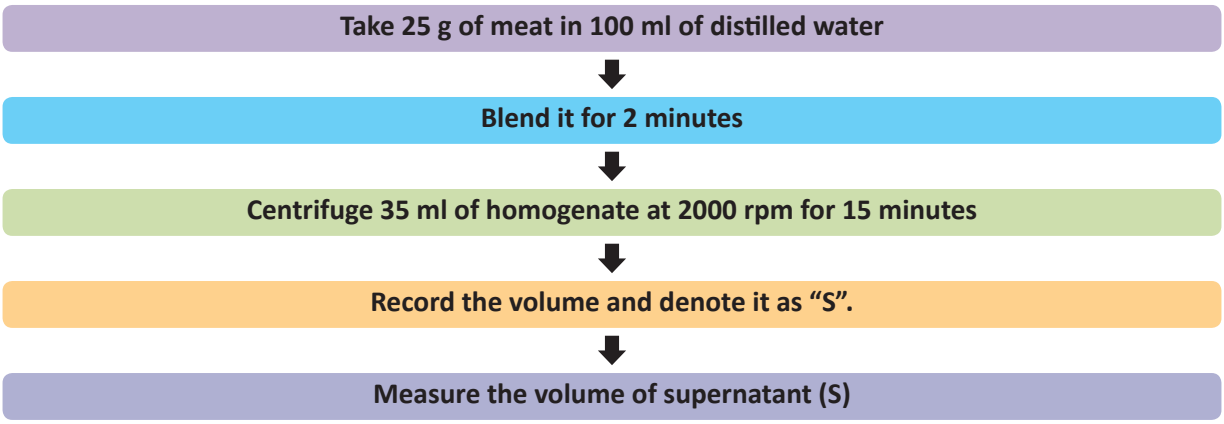
3.2.10 Determination of Meat Swelling Capacity (MSC)

With the microbially spoiled meats, the highest degree of correlation occurred between meat swelling and ERV. This finding indicates that ERV and meat swelling are quite similar and equally reliable in determining meat microbial quality while the relationships between viscosity and ERV, and η & WHC were of lower orders of significance.

Materials Required:

- Distilled water,
- Centrifuge machine
- Blender
- Graduated cylinder

Procedure:



Percent meat swelling can be determined as-

$$\% \text{ Meat Swelling} = (35\text{-}S\text{-}7)/7 \text{ X } 100$$

(Reference:- 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 Vol: 15: Pp. 404.)

3.2.11 Determination of Total Volatile Basic Nitrogen (TVBN)

Total Volatile Basic Nitrogen (TVBN) is a measure to evaluate the degree of spoilage and is one important index of pork's freshness. Also, TVBN refers to the nitrogen contents of various kind of amines and ammonia.

TVBN is a substance produced in the process of pork spoilage, and its content in pork is proportional to the extent of pork spoilage.

Determination of TVBN by Micro diffusion technique:

Reagent Required:

a) 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 color is produced.
- Make the volume up to 500 ml with alcohol.

b) 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.
- Filter through cellulose based qualitative filter paper. The clear TCA extract thus obtained is used to determine TVBN value following the technique of Conway (1947) and Pearson (1968 b).

c) 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.

Procedure:

Add One ml of TVBN reagent in to the inner well of the Conway unit

↓

Deposit one ml of TCA extract in outer wall followed by the addition of one ml saturated potassium carbonate (K2CO3)

↓

Close the Conway unit immediately with an airtight ground glass plate

↓

Rotate clockwise and antilock wise and incubate at room temperature for 3 hours

↓

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

Calculations:

'N' mg/ml of extract= 14 X a X b

C= 100 x N

Where, 14 = Molecular weight of Nitrogen

a = Normality of H2SO4

b = volume of H2SO4 (Titration value)

c = mg% of TVBN value

TVBN values are expressed as mg%.

(Reference:- 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. Vol: 19: Pp. 366-369. 3. Alternatively BIS reference method IS 5960: Part 3: 1970 may be used.)

3.2.12 Determination of Picric Acid Turbidity (PAT)

It is possible practical freshness test of pork

It is to detect presence of reducing sugar

Materials Required:

- 70% Ethanol
- Saturated aqueous Picric acid
- Ashless quantitative Filter paper (20 µm nominal particle retention rating)
- Spectrophotometer.

Procedure:

Blend 5 g of meat with 20 ml of 70 % ethanol for 30 sec

↓

Add 5 ml of saturated aqueous picric acid solution and re-blend the slurry for about 20 sec.

↓

Filter the slurry through ash-less quantitative filter paper.

↓

Measure the optical density of the filtrate at 540 nm wavelength with visible spectrophotometer as a turbidity of the solution

(Reference:- a) Kurtzman and Synder (1960), The picric acid turbidity: A possible practical freshness test for ice shrimps, Food Technology. Vol: 14, No. 7: Pp. 337.)

3.2.13 Determination of Dye Reduction Capacity

A dye reduction method aims for estimation of total aerobic and/or psychrotrophic bacterial (bacteria that are capable of surviving or even thriving in extremely cold environment). counts in ground pork. The method is based on color changes in indicator disks placed on the meat surface.

This test estimates bacterial population in meat sample indirectly.

Materials Required:

- Resazurin dye/tablet
- Filter paper strips
- Polythene bag,
- Nutrient broth,
- Swab and distilled water.

Procedure: This test can be done by the following two procedures-

Test protocol-1:

Four Resazurin tablets are dissolved in 100 ml of water sample

↓

Filter paper strips are dipped in above solution and dried in dark and cool room.

↓

For testing, the strip is moistened and a drop of meat juice to be tested is placed on it for 1 minute

↓

The strip is then placed in polythene bag and kept in dark room (22^0-23^0 C).

↓

Time taken for the blue color to the paper to change to red is noted.

Test protocol-2:

Soak the swab in nutrient broth.

↓

Sample 1 cm2 area of meat.

↓

Break the swab in 10 ml nutrient broth yeast extract medium.

↓

Collect the washings in a sterile glass beaker containing 0.3 ml freshly prepared 0.05% aqueous Resazurin dye.

↓

Incubate the beaker at 30°C in a dark room.

↓

Note the time taken for the change in color 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

3.2.14 Determination of Fat Oxidation

Fat content and composition are of major importance for consumers due to their importance for meat quality and nutritional value. Lipids contain a large number of substances such as essential fatty acids or fat-soluble vitamins that can only be provided by the diet. In addition, lipids impart many desirable characteristics to meats and meat products like- flavor, tenderness and juiciness .

However, lipids undergo degradation by way of oxidation which greatly reduces the nutritional value of meats due to the loss of essential fatty acids and vitamins.

Therefore it seems clear that oxidation products are involved in the development of innumerable diseases .

Reagents Required:

- Acetic acid - chloroform solvent mixture (3:2). Mix 3 volumes of glacial acetic acid with 2 volumes of chloroform.
- Freshly prepared saturated potassium iodide solution.
- 0.1 N and 0.01 N sodium thiosulphate solutions. Weigh 25 g of sodium thiosulphate and dissolve in 1 lit of distilled water.
- Boil and cool, filter if necessary. Standardize against standard potassium dichromate solution.
- Starch solution - 1% water-soluble starch solution.

Procedure:

Weigh 5 g (±50 mg) sample into a 250 ml stoppered conical flask

↓

Add 30 ml acetic acid chloroform solvent mixture and swirl to dissolve

↓

Add 30 ml acetic acid chloro Add 0.5 ml saturated potassium iodide solution with a Mohr pipette form solvent mixture and swirl to dissolve

↓

Add about 0.5 ml starch solution as indicator and continue titration shaking vigorously to release all I_2 from $CHCl_3$ layer until blue colour disappears

↓

Slowly titrate the liberated iodine with 0.1 N Sodium Thiosulphate solution, with vigorous shaking until yellow colour is almost gone.

↓

Let stand for 1min in dark with occasional shaking, and then add about 30 ml of water

↓

If less than 0.5 ml of 0.1 N $Na_2S_2O_3$ is used repeat using 0.01 N $Na_2S_2O_3$

↓

Conduct blank determination (must be less than 0.1 ml 0.1 N $Na_2S_2O_3$).

Calculation:

Peroxide value expressed as mili equivalent of peroxide oxygen per kg sample (meq/kg):

$$\text{Peroxide value} = \frac{\text{Titre X N X 100}}{\text{Weight of the sample}}$$

Where, Titre = ml of Sodium Thiosulphate used (blank corrected)

N = Normality of sodium thiosulphate solution.

Fresh oils usually have peroxide values well below 10 meq/kg. A rancid taste often begins to be noticeable when the peroxide value is above 20 meq/kg (between 20 – 40 meq / Kg). In interpreting such figures, however, it is necessary to take into account the particular oil or fat.

(Reference:- A.O.A.C. 17th edn, 2000,Official Method 965.33 Peroxide Value in Oils and Fats/ Pearsons Composition and Analysis of Foods, 9th edn.Pp. 641)

3.2.15 Determination of Water Holding Capacity

Water-holding capacity (WHC) of meat and meat products determines the visual acceptability, weight loss, and cook yield as well as sensory traits on consumption. Water-holding capacity of meat is defined as the ability of the postmortem muscle (meat) to retain water even though external pressures (e.g. gravity, heating) are applied to it. The characteristic of water-holding capacity is not trivial.

There are two methods for determination of Water Holding Capacity

Press method and Centrifuge method

Test protocol-1: By Press method:

Materials Required:

- Freshly minced pork (300 mg)
- No. one Whatman filter paper
- Glass slide
- 100 g weight block
- Planimeter /Graph paper/Plastic grid

Procedure:

Weigh 300 mg of freshly minced pork

↓

Take a Whatman no. one filter paper on a glass slide and place the minced meat on it

↓

Take another glass slide and place the same over the minced meat

↓

Place a 100 g of weight over the glass slide and keep the weight on the slide for three minutes

↓

After three minutes, remove the weight and slide from the filter paper

↓

Remove the meat from filter paper and the impression formed due to meat juice is to be air dried

↓

Measure the area with a planimeter or graph paper or plastic grid and express the area in square centimeter

Result: Total number of smaller squares covered by the impression smear=332 nos. (say)

100 m =1 sq cm

So, 332 mm = 332/100 sq cm
= 3.32 sq cm

So, the given sample has a water holding capacity of 3.32 sq cm

Test protocol-2: By Centrifuge method:

Materials Required:

- 50 g of already minced pork sample
- 50 ml centrifuge tube
- 0.6 M Sodium chloride solution
- Centrifuge machine

Procedure:

Take about 15 g sample (already minced) in a 50 ml polyurethane centrifuge tube

↓

Add 22.5 ml of 0.6 M Sodium chloride solution

↓

Stir for 15 minutes

↓

Centrifuge for 15 minutes at 3500 rpm

↓

Decant the supernatant fluid (V) and measure the volume

↓

The amount of added solution (ml) retained by 100 g of pork was calculated and reported as Water holding capacity

Water holding capacity (ml/100 g) = 22.5-V x 100/15

Result: V = 12 ml (say)

WHC(ml/100 g)= 22.5-V x 100/15

= 22.5-12 x 100/15

=10.5 x 100/15

= 1050/15

= 70 ml /100 g

So, the WHC of the given pork sample is 70 ml /100 g

3.3 Microbiological Test

3.3.1 Determination of total microbial load

It is of prime importance to minimize the presence of foodborne bacteria in pork because these can cause serious public health problems. Food-borne pathogens are one of the leading causes of illness and death particularly in developing countries. The prevalence of E. coli, Salmonella, and S. aureus in minced meat and contact surface samples are found to be significantly high which correlates to poor personal and work area sanitation.

This test is aimed to determine the total microbial load in the pork sample so that necessary measures could be taken in the event of finding total microbial load higher that the maximum permissible limit.

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.

a) Enumeration of Total Viable Count (TPC) / Aerobic Plate Count (APC)

- For evaluating total viable counts of the microorganisms, standard pour plate technique is used. Take 0.1 ml 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 45-500 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

Compositional Meat and Meat Products

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.

3.3.1.1 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 the 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 an airtight stopper

↓

Carry out analysis as soon as possible

3.3.1.2 Canned meat

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

↓

Dry portions of samples not needed for immediate analysis either 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 (Boiling Point 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:- AOAC 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

3.3.2 Coliform count

Coliform bacteria are commonly found in meat products.. Many strains of *E. coli* have been found to be important zoonotic foodborne pathogens. Because of their ability to cause numerous foodborne disease outbreaks in humans, they have become a significant concern of threat to public health. Severe gastroenteritis can develop within one to seven hours after the consumption food contaminated with coliform bacteria, leading to diarrhoea, vomiting, and dehydration.

Medium and reagents required:

- Violet Red Bile Agar (VRBA)
- Lauryl SulphateTryptose Broth (LST)
- Brilliant – Green Lactose Bile Broth 2% (BGLB)

Procedure: Coliforms in foods may be enumerated by the solid medium method or by the Most Probable Number (MPN) method.

Test Protocol-1: Solid medium method

Preparation of meat homogenate

- Weigh 50+0.1 g of solid or semi-solid sample into a sterile blender jar or into a stomacher bag.
- Add 450 ml of diluents. Blend for 2 minutes at low speed (approximately 8000 rpm) or mix in the stomacher for 30-60 seconds.

Dilution:

- Pipette 1 ml of food homogenate into a tube containing 9 ml of the diluent. From the first dilution transfer 1ml to second dilution tube containing 9ml of the diluent.
- Repeat using a third, fourth or more tubes until the desired dilution is obtained.

Pour plating:

- Pipette 1 ml of the food homogenate (prepared sample) and of each dilution into each of the appropriately marked duplicate petri dishes.
- Pour into each petri-dish 10-12 ml of VRBA (tempered to 48^0 C) and swirl plates to mix. Allow to solidify. Overlay with 3 to 5 ml VRBA and allow to solidify.
- Incubate the dishes, inverted at 35^0C for 18 to 24 hours.

Counting the colonies:

- Following incubation, count all colonies that are purple red in colour, 0.5 mm in diameter or larger and are surrounded by a zone of precipitated bile acids.
- Optimally the plates should have 30 to 100 colonies.

Calculation:

Multiply the total number of colonies per plate with the reciprocal of the dilution used and report as coliforms per g or ml.

Test Protocol-2:

Preparation of meat homogenate:

- Weigh 50+0.1 g of solid or semi-solid sample into a sterile blender jar or into a stomacher bag.
- Add 450 ml of diluent. Blend for 2 minutes at low speed (approximately 8000 rpm) or mix in the stomacher for 30-60 seconds.

Dilution:

- Pipette 1 ml of food homogenate into a tube containing 9 ml of the diluents.
- From the first dilution transfer 1ml to second dilution tube containing 9ml of the diluents.
- Repeat using a third, fourth or more tubes until the desired dilution is obtained.

Inoculation:

- Inoculate each of 3 tubes of LST broth (containing inverted Durham tubes) with 1ml of food homogenate (1:10).
- Carry out the same operation from the first (1 in 100) and the second (1 in 1000) dilution tubes. Using a fresh sterile pipette for each dilution.

Incubation: Incubate the LST tubes at 35 ± 0.5^0 C for 24 and 48 hours.

Presumptive test for Coliforms:

- Record tubes showing gas production after 24 hours and re-incubate negative tubes for further 24 hours.
- Then record tubes showing gas production.

Confirmed test for Coliforms:

- Transfer a loopful from each gas positive tube of LST to a separate tube of BGLB broth.
- Incubate the BGLB broth tubes at $35+0.5^0$ C for 48+2h.
- The formation of gas confirms the presence of coliform bacteria.
- Record the number of positive tubes that were confirmed as positive for coliform.

Calculation:

Note the MPN appropriate to the number of positive tubes from the table. e.g., 3 in 1:10; 1 in 1:100 and 0 in 1:1000.

Coliforms= present/absent per g

3.3.3 Test for *Salmonella*

Salmonella is one of the most important foodborne bacteria and has wide health and socioeconomic impacts worldwide. Fresh pork meat is one of the main sources of *Salmonella*, and efficient and rapid methods for detection are therefore necessary to prevent spread and occurance of salmonellosis among the consumers.

Medium and reagents required:

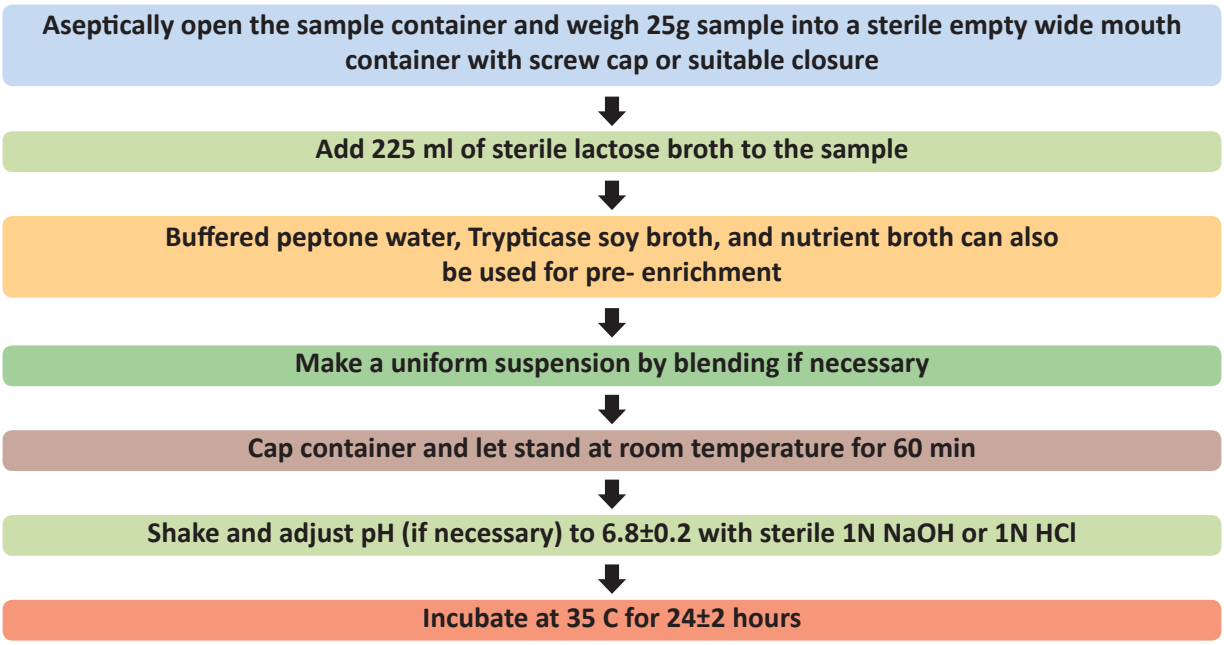
- Lactose broth,
- Trypticase Soy Broth,
- Trypticase Soy Broth Containing Potassium Sulfite at a final concentration of 0.5%,
- Reconstituted Non-Fat Dry Milk,
- 1% aqueous Brilliant Green Dye Solution,
- Selenite Cystine Broth,
- Tetrathionate Broth,
- Xylose Lysine Deoxycholate (XLD) Agar,
- Hektoen Enteric Agar (HEA),
- Bismuth Sulphite Agar (BSA),
- Triple Sugar Iron (TSI) Agar,
- Lysine Iron Agar (LIA),
- Urea Broth,
- Phenol Red Dulcitol Broth,
- Phenol Red Lactose Broth,
- Tryptone Broth,
- KCN Broth,
- Malonate Broth,
- Buffered Glucose (MR-VP) Medium,
- Brain Heart Infusion (BHI) Broth,
- Buffered Peptone Water

Instead of lactose broth the recommended pre-enrichment broth for the following pork samples is as follows:

- Nonfat dry milk and dry whole milk – Sterile distilled water.
- Add 0.45 ml of 1% aqueous brilliant green dye before incubation.
- Dried active yeast – Trypticase soy broth
- Onion-garlic powder – Trypticase soy broth containing potassium sulfite at a final concentration of 0.5%
- Milk Chocolate – Reconstituted non fat dry milk.

Procedure:

a. Preparation of sample and pre-enrichment:



b. Selective enrichment:

Gently shake incubated sample mixture and transfer 1 ml to 10 ml of Selenite Cystine broth and an additional 1 ml to tetrathionate broth. Incubate 24±2 hours at 35^0 C.

c. Selective media plating:

- Vortex – mix and streak 3 mm loopful of incubated selenite cystine broth on selective media plates of XLD, HEA and BSA.
- Repeat with 3mm loopful of incubated tetrathionate broth.
- Incubate plates at 350 C for 24±2 hours and 48±2 hours.

d. Observe plates for typical Salmonella colonies

- On XLD (after 24h) - Pink colonies with or without black centres.
- On HEA (after 24h) - Blue green to blue colonies with or without black centers.
- On BSA (after 24 to 48h) – Brown, grey or black colonies sometimes with metallic sheen.
- Surrounding medium is usually brown at first, turning black with increasing incubation time.

e. Treatment of typical or suspicious colonies

- Pick with needle typical or suspicious colonies (if present) from each XLD, HEA and BSA plates.
- Inoculate portion of each colony first into a TSI agar slant, streaking slant and stabbing butt and then do the same into an LIA slant.
- Incubate TSI and LIA slants at 35^0 C for 24+2 hours and 48+2 hours respectively. Cap tubes loosely to prevent excessive H_2S production.

Typical Salmonella reactions are:

	TSI	LIA
Slant	Alkaline (red)	Alkaline (purple)
Butt	Acid (yellow)	Alkaline (purple)
H2S production (blackening in butt)	+ or -	+

A culture is treated as presumptive positive if the reactions are typical on either or both TSI and LIA slants.

f. Biochemical tests:

- Using sterile needle inoculate a portion of the presumptive positive culture on TSI slant into the following broths.
- Incubate at 35^0C for the specified period of days and read for Salmonella typical reactions.

Broth/ Media	Time of Incubation	Results
Urea broth	24±2h	Negative (no change in yellow colour of medium)
Phenol red lactose broth	48±2h	*Negative for gas and/or acid reaction
Phenol red sucrose broth	48±2h	*Negative for gas and/or acid reaction
Phenol red dulcitol broth	48±2h	*Positive for gas and/or acid reaction
Tryptone broth	24±2h	Negative for Indole test
KCN broth	48±2h	Negative (no turbidity)
Malonate broth	48±2h	*Negative (green colour unchanged)
MR-VP medium	48±2h	Negative for VP test but positive for MR test

(Note: Majority of *S. arizonae* are atypical for these reactions).

Test(s) or substrate(s)	Results
Urease test	Positive (purple red)
Indole test	Positive (red)
Flagellar test (Polyvalent or spicer-Edwards)	Negative (no agglutination)
Lysine decarboxylase test	Negative (yellow)
KCN broth	Positive (growth)
Phenol red sucrose broth	Positive (acid and/or gas)**
Lysine decarboxylase test	Negative (yellow)
KCN broth	Positive (growth)
Voges-Proskauer test	Positive (red)
Methyl red test	Negative (yellow)

*Malonate broth positive cultures are tested further to determine if they are *Salmonella arizonae*

** Do not discard positive broth cultures if corresponding LI agar cultures give typical Salmonella reactions; test further to determine if they are Salmonella sp. (vide 9).

g. Serological Tests:

- To reduce number of presumptive positive cultures (TSI positive and urease negative) carried through biochemical identification tests, the following serological flagellar (H) screening test may be carried out.
- Transfer 3mm loopful of culture into 5ml of BHI broth and incubate at 35^0 C until visible growth

occurs (About 4-6 hours).

- Add about 2.5 ml formalized physiological saline solution.
- Test with Salmonella flagellar (H) antisera. Positive cultures show visible agglutination.
- Further confirmation can be made by using Salmonella Polyvalent (O) antiserum.

Expression of Result:

Salmonella = Present/Absent per 25 g

3.3.4 Test for *Staphylococcus aureus*

Staphylococcus aureus (S. aureus) is a highly pathogenic bacteria that causes a wide variety of infections in humans, including skin and soft tissue infections, bacteremia, pneumonia, and food poisoning. Further complications such as-meningitis, osteomyelitis, and toxic shock syndrome have been associated with staphylococcal infection. It may be carried on the nostrils, skin and hair of animals and humans posing a serious problem at the community level as well as in the food industry. It can develop high degree of resistance to antibiotics and , also produces a variety of potent staphylococcal enterotoxins (SEs), which are resistant to inactivation by gastrointestinal proteases and are consequently responsible for staphylococcal food poisoning.

Medium and reagents:

- Tripticase (tryptic) soy broth with 10% sodium chloride and 1% sodium pyruvate,
- Baird Parker (BP) Medium,
- Brain Heart Infusion (BHI) Broth,
- Desiccated Coagulase Plasma (rabbit) with EDTA,
- Butterfields Buffered Phosphate Diluent,
- Plate Count Agar (PCA)

Procedure:

a. Preparation of food homogenate:

Aseptically weigh 50 g food sample into the sterile blender jar. Add 450ml of diluent (1:10) and homogenize 2 min at high speed (16000-18000 rpm) → Alternately use stomacher for sample preparation

b. Dilution:

Pipette 10 ml of the food homogenate into 90 ml of diluent (or 1ml to 9ml) to make a 1:100 dilutions. Mix well using a vortex-mixer → Transfer 1ml from this dilution to a fresh tube of 9ml to give a 1:1000 dilution. Repeat until the desired dilution is obtained

Test protocol-1: Most probable number method:

This procedure is recommended for testing processed foods likely to contain a small number of *S. aureus*.

c. Inoculation:

- Inoculate each of 3 tubes of tryptose soy broth (with 10% sodium chloride and 1% sodium pyruvate) with 1 ml of food homogenate.
- Carry out the same operation from the first and subsequent dilutions using a fresh sterile pipette each time.
- Maximum dilution of sample must be high enough to yield negative end point. Incubate at 35^0 C for 48 hours.

d. Surface Streaking:

- Vortex mix the tubes from above and then using 3mm loop transfer one loopful from each growth positive tube to dried BP medium plates.

- Streak so as to obtain isolated colonies. Incubate at 35-37^0 C for 48 hours.

Interpretation:

- Colonies of *S. aureus* are typically grey black to jet black, circular, smooth, convex, moist and 2-3 mm diameter on uncrowded plates.
- Frequently there is a light colored (off-white) margin, surrounded by opaque zone (precipitate) and frequently with outer opaque zone (precipitate) and frequently with outer clear zone; colonies have buttery to gummy consistency when touched with the inoculating needle.

Confirmation techniques:

- Using a sterile needle, transfer (noting the dilution) at least one suspected colony from each plate to tubes containing 5ml BHI and to PCA slants.
- Incubate BHI cultures and slants at 35^0 C for 18-24h.
- Perform coagulase test on the BHI cultures. Retain slant cultures for repeat tests.

Reporting:

- Coagulase positive cultures are considered to be *S. aureus*. Now record number of positive tubes (and the respective dilutions) of *S. aureus*.
- Report most probable number (MPN) of *S. aureus* per gram from Table of MPN values.

Test protocol-2: Surface Plating method:

This method is applicable for general purpose use in testing foods expected to contain > 10 cells of *S. aureus* per g.

- Transfer 1ml of the food homogenate (1:10 dilution) and other dilutions to triplicate plates of BP medium and equitably distribute 1ml of inoculum over the triplicate plates.
- Spread inoculum over agar surface using sterile bent glass streaking rods (hockey sticks).
- Incubate plates in upright position in the 35-37^0 C incubator for about 1 hour or until the inoculum is absorbed by medium.
- Then invert plates and incubate 45-48 hours.

Counting colonies:

- Count colonies of typical *S. aureus* appearance.
- Test for coagulase production on suspected colonies.
- Add number of colonies on triplicate plates represented by colonies giving positive coagulate test.
- Multiply the count obtained by inverse of corresponding sample dilution.
- Report as *S. aureus* per g or ml of the sample.

Expression of result:

Staphylococcus aureus = x/g (x= No. of colonies)

3.3.5 Test for *Listeria monocytogenes*

In recent years, ready-to-eat (RTE) meat and poultry products contaminated with *Listeria monocytogenes* (which is so widespread in the environment) have caused hundreds of illnesses and dozens of deaths. *L.monocytogenes* can survives in the digestive systems of at least 37 species of domestic and wild mammals and is very hardy kind of organism that and can survive freezing, drying, high salinity and heat. In general, healthy people are at a low risk of contracting *listeriosis,* but he can be affected on consumption of pork heavily contaminated with *L.monocytogenes.* The high-risk human population includes -newborns, pregnant women, the elderly, and people with weakened immune systems, person with diabetes, cirrhosis, asthma, and ulcerative colitis.

This test aims at detection of this organism in pork.

Preparation of test sample:

Take 25 g of a well-mixed sample in stomacher bag and use 225 ml of Half Frazer broth

↓

Stomach the sample for two minutes

↓

Pour aseptically the contents in to a wide mouth bottle and incubate at 30°C for 24±2h (a black coloration may develop)

↓

Take one ml of the above culture and transfer to 9ml of Frazer broth

↓

Incubate the inoculated tube at 37°C for 48± 2h at 35-37°C

↓

From 24 h culture of Half Frazer broth and 48h Frazer broth streak out culture on Modified Oxford Agar and PALCAM agar so that well separated colonies are obtained

↓

Invert the plates and incubate at 35 or 37°C for 24 h and if required an additional 18 h if growth is slight or no colonies appear. Examine the plates for colonies presumed to be L. monocytogenes

Appearance of colonies:

- On Modified Oxford agar the colonies are small (1mm) greyish surrounded by a black halo.
- After 48 hours the colonies turn darker with a possible green luster and are about 2 mm in diameter with black halos and sunken centres.
- On PALCAM agar after 24 hours the colonies appear 1.5 to 2 mm in diameter greyish green or olive green sometimes with black centre and always surrounded by a black halo and depressed centre.

Confirmation of *Listeria* species:

- Select five typical colonies from one plate of each medium.
- If presumed colonies are less than five on a plate, take all of them.
- Streak the selected colonies from each plate on to the surface of a well dried TSYEA for obtaining well separated colonies.
- Invert the plates and incubate at 35°C or 37°C for 18 to 24 hours or until the growth is satisfactory.
- Typical colonies are 1 to 2 mm in diameter, convex, colourless and opaque with an entire edge.
- Carry out the following tests from colonies of a pure culture on the TSYEA.

Catalase reaction:

- With the help of loop pick up an isolated colony and place it in H_2O_2 solution on a glass slide.
- Immediate production of gas bubbles indicates catalase positive reaction.

Gram staining:

Perform Gram staining on a colony Listeria are Gram positive slim short rods.

Motility Test:

- Take colony from TSYEA plate and suspend it in TSYE borth.
- Incubate at 25°C for 8 to 24 hours until cloudy medium is observed.
- Take a drop of culture and place it on a glass slide.
- Cover the top with a cover slip and observe under a microscope.
- Listeria is seen as slim rods with a tumbling motility (cultures grown above 25°C fail to show this motion.
- Compare them with a known culture – cocci or large rods with rapid motility are not Listeria.
- As an alternative stab motility agar tube with an isolated colony from TSYEA and incubate at 25°C for 48 h. typical umbrella like appearance around the stab indicate motility positive culture.
- If growth in not positive incubate up to five days and observe for the stab again.

Confirmation of *Listeria monocytogenes*:

Haemolysis test:

- Take a colony from TSYEA and stab it on a well dried surface of sheep blood agar plate.
- Simultaneously, stab positive (*L. monocytogenes*) and negative (*L. innocua*) control cultures. Invert the plates and incubate at 35°C or 37°C for 24±2 hours. Examine the plates.
- *L. monocytogenes* show clear light zones of beta haemolysis.
- *L. innocua* does not show any haemolysis.
- Examine the plates in a bright light to compare test cultures with the controls.

Carbohydrate utilization:

- Inoculate each of the carbohydrate utilization broths (rhamnose and xylose) with a culture from TSYE broth and incubate at 35 °C or 37v for upto 5 days.
- Appearance of yellow colour indicates a positive reaction within 24 to 48 hours.

CAMP test:

- On a well dried surface of sheep blood agar streak each of the *Staphylococcus aureus* and *Rhodococcus equi* cultures in single lines and parallel to each other and diametrically opposite, a thin even innoculum is required.
- Streak the test strain separated in a similar manner at right angles to these cultures as that the test strain and *S. aureus* and *R.equi* cultures do not touch but their closest are about 1 mm or 2 mm apart.
- Several test strains can be streaked on the same plate. Simultaneously streak control cultures of *L monocytogenes, L innocua* and *L. ivanovii*.

Species	Haemolysis	Production of acid with Rhamnose	Production of acid with Xylose	CAMP Test	
				S. aureus	*R equi*
L. monocytogenes	+	+	-	+	-
L. innocua	-	V	-	-	-
L. ivanovii	+	-	+	-	+
L. seeligeri	+	-	+	+	-
L. welshmeri	-	V	+	-	-
L. grayi sub species grayi	-	-	-	-	-
L. grayi sub species murrayi	-	V	-	-	-

- Incubate plates at 35 to 37^0 C for 18 to 24 hours.
- Observe plates against bright light. In *L. monocytogenes* case there is enhanced zone of beta haemolysis at the intersection of *S. aureus*.
- *L. innocua* does not show any enhanced zone of haemolysis with *S. aureus* or *R. equi*.
- In case of *L. ivanovii* enhanced beta zone of haemolysis is seen on *R. equi* side.

Interpretation of results:

- All Listeria species are small, Gram positive rods that demonstrate motility and catalase positive reaction.
- *L monocytogenes* are distinguished from other species by the characteristics listed in table given below.

Expression of results:

Based on the observations and interpretation of the results, report presence or absence of *L. monocytogenes* in test portion specifying the mass in grams or mililitres of the sample taken-

L. monocytogenes = present or absent/ g or ml

3.4 Other tests

3.4.1 Determination of Microbial Toxins

Some species of bacteria produce toxins and ingestion of food contaminated with those bacterial toxins can cause severe cases of food poisoning.There are mainly two types of microbial toxins that could be present in pork. These are (i).Shiga toxin produced by *Shigella dysenteriae* and some serotypes **of** *Escherichia coli* and(ii). Clostridium botulinum toxin

This test is aimed to detect presence of such toxins in pork

Test protocol-1; Determination of *Shiga* toxin (produced by *Escherichia coli* and *Shigella dysenteriae* type 1)

Reference method:

USDA (Laboratory Guide)

Test protocol-2; Determination of *Clostridium botulinum* toxin

Reference method:

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

For determination of microbial toxins in pork sample, a number of ELISA kits are available in the market specific for different toxin liberating organisms.

Materials required and procedure is as per the instruction given in the manual along with the kits.

3.4.2 Determination of Antimicrobial Residues

Antimicrobial residue in meat is a serious health concern because of its harmful effects on human health. Antibiotics that are commonly used in veterinary practices persists as residues in food of animal origin and subsequently may lead to adverse health effects on the consumers. When humans get exposed to significant levels of antibiotic residues from animal products, it may aggravate immunological responses in susceptible individuals and negatively affect intestinal micro flora.

Materials Required:

Premi Test : (DSM) kit (a test for screening meat within 4 hours)

Procedure: As per the Premi Test (DSM) kits instruction.

Or

ELSIA Kit test

Materials Required:

- ELSIA Kit,
- Multichannel Pipettes,
- Pipettes tips,
- Tissue sample,
- Mortar and pestle,

Procedure:

- The tissue sample has to be triturated using mortar and pestle prior to performing ELISA test.
- Extraction Procedure for ELISA Analysis for the residue of the antimicrobials is done according to the manufacture's guidelines.
- Cross-reactivity might occur with the secondary antibody, resulting in non-specific signal.

Analytical Procedures for ELISA:

Analysis of all the antibiotics can be carried out as per the manufacturer's instructions. The absorbance is expressed in percentage (%) and calculated using the following formula or as per m:

$$\frac{\text{Absorbance standard or (sample)}}{\text{Absorbance zero standard}} \text{ X } 100 = \%\ \text{Absorbance}$$

3.4.3 Detection of Heavy Metals:

Packaging of foods is a potential source for heavy metals in foods. Elements such as arsenic, tin, zinc, iron, cadmium or lead can migrate into the contents of the can, particularly for acidic canned foods that are in direct contact with the metal in the can.

Arsenic and other heavy metals such as cadmium, lead, and mercury are all considered toxic and have adverse health effects in human metabolism. Accumulation of heavy metals in the food chain can occur by bio-concentration e.g. from water or the food source.

*Requires GC-MS / LC-MS to perform the test.

[**Gas chromatography–mass spectrometry (GC-MS)** is an analytical method that combines the features of gas-chromatography and mass spectrometry to identify different substances within a test sample.

Liquid chromatography–mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (or HPLC) with the mass analysis capabilities of mass spectrometry (MS)]

*Note: Since this test includes high throughput equipment and has to be performed by highly skilled technician, we don't recommend these tests. In the later stage of the project in due course of time, the department may consider this test.

3.4.4 Determination of Insecticide & Pesticide residues

Some pesticides can be used for veterinary purposes. *Regulation (UE) Nº 37/2010* establishes MRLs (maximum residue limit) for residues of pharmacologically active substances in foodstuffs of animal origin. This regulation includes pesticides used as veterinary drug such as Cypermethrin, Deltamethrin, Permethrin, Fenvalerate and Diazinon.

Pest control in intensive agriculture involves treatments with a variety of synthetic chemicals generically known as pesticides. These chemicals can be transferred from plants to animals via the food chain. Furthermore, animals and their accommodations can be sprayed with pesticide solutions to prevent infestations. Consequently, both contamination routes lead to bio-accumulation of pesticides in food products of animal origin as meat, fish, fat, offal, eggs, and milk.

Materials Required: Insecticide / Pesticide Detection kit

Procedure: As per the instructions mentioned in the kit.

3.4.5 Formalin test:

Materials Required: Formalin Detection kit

Procedure: As per the instructions mentioned in the kit.

*Note: Formalin is commonly used in case of fish and not in case of meat. The department may prioritize this test in due course of time if found necessary.

3.4.6 Meat speciation using DNA based molecular technique

Meat species specification is an utmost important field of quality control management in meat industry. These tests are very much important for accurate detection of meat species and their fraudulent substitution in another meat. Fraudulent substitution is a malpractice in meat industry, in which inferior or cheaper quality meat is mixed into superior quality meat. Meat species specification practices are also helpful in implementation of prevention of cow slaughter acts of different states of India, wild life conservation act, PFA acts of India and some other similar acts of the world.

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. 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 Lenth 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

For conducting this test, the laboratory should be well equipped with specialized infrastructure, equipments and manpower. At present, the Deptt. may not be having such kind of facilities in the departmental labs for conducting this test. Therefore, this test is not elaborately presented in this document. The Deptt. may incorporate this test into the labs provided sufficient infrastructure, equipments and manpower are available in the labs.

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Annexure-I

FORM VI

(Refer rule 3.4.3 (7))

Memorandum to Food Analyst

From:

…….. Date: _____

To

Food Analyst/Lab Incharge

……

……

MEMORANDUM

(Refer rule (v)a of 3.4.1(8))

1. The sample described below is sent herewith for analysis under ___ of ___ of section ____ of Food Safety and Standards Act, 2006

- Code Number
- Date and place of collection
- Nature of articles submitted for analysis
- Nature and quantity of preservative, if any, added to the sample.

2. A copy of this memo and specimen impression of the seal used to seal the packet of sample are being sent separately by post/courier/hand delivery (strike out whichever is not applicable)

(Sd/-)

Address:

The International Livestock Research Institute (ILRI)