
GELATIN MANUFACTURERS INSTITUTE OF AMERICA

# STANDARD TESTING METHODS FOR EDIBLE GELATIN

Official Procedure of the Gelatin Manufacturers Institute of America, Inc.

http://www.gelatin-gmia.com

Revised July 2013

# GMIA STANDARD METHODS FOR THE TESTING OF EDIBLE GELATIN

## TABLE OF CONTENTS

# TABLE OF CONTENTS

***Continued***

## Preface

The Standard Methods for the Sampling and Testing of Edible Gelatin contained in this booklet are the results obtained by a co-operative testing program conducted by the Technical Staffs of the entire membership of the Gelatin Manufacturers Institute. These methods have been found to give accurate and consistent results.

## 1. General Information

### 1.1 Definition

Gelatin is the product obtained from the acid, alkaline, or enzymatic treatment of collagen, the chief protein component of the skins, bones, and connective tissues of animals, including fish and poultry. These animal sources shall have not been exposed to pentachlorophenol.

Type A gelatin is produced by the acid processing of collagenous raw materials and exhibits an isoelectric point between pH = 7 and pH = 9. Type B gelatin is produced by the alkaline or lime processing of collagenous raw materials and exhibits an isoelectric point between pH = 4.6 and pH = 5.2. Mixtures of Types A and B as well as gelatins produced by modifications of the above mentioned processes might exhibit isoelectric points outside of the stated ranges. (Food Chemicals Codex).

### 1.2 Description

Gelatin is nearly tasteless and odorless. It is a vitreous, brittle solid that is faintly yellow to light tan. It is supplied in various physical forms such as coarse granules, fine powders and leaves.

### 1.3 Stability

Gelatin is very stable when stored in its original container in ambient humidity with controlled temperature. The shelf life of gelatin is generally recognized as stable for at least 5 years when stored under these conditions.

### 1.4 Functionality in Foods

Gelatin is used in confectionery, water jellies and desserts, dairy products or functional food, for its versatility. Its functionalities include firming agent, formulation

and processing aid, stabilizer and thickener, surface-active agent, and water finishing agent.

## 1.5 Characteristics

When gelatin granules are immersed in cold water, they hydrate into discrete, swollen particles. On being warmed, gelatin disperses into the water, resulting in a stable suspension. Water solutions of gelatin will form a reversible gel if cooled below the specific gel point of gelatin. The gel point is dependent on the source of the raw material. Gelatin extracted from the tissues of warm-blooded animals will have a gel point in the range of 30ºC to 35ºC. Gelatin extracted from the skin of cold-water ocean fish will have a gel point in the range of 5ºC to 10ºC. Gelatin is stable in aqueous solutions of polyhydric alcohols such as glycerine and propylene glycol. It is insoluble in most organic solvents (Food Chemicals Codex).

## 1.6 Identification

### Principle

Gelatin can be identified by a visual, physical state change; formation of a precipitate or turbid solution; and determination of hydroxyproline content.

### Reagents and Solutions

1. Dry, granular gelatin
2. Deionized water
3. Trinitrophenol TS
4. Potassium dichromate solution
5. 3N hydrochloric acid

### Apparatus

1. 100 mL volumetric flask
2. Water bath
3. Refrigerator

### Procedure

1. Gelatin forms a reversible gel.

A. Dissolve 10 g of gelatin in 100 mL hot water. When all gelatins are dissolved, place the solution in a refrigerator (2-10°C) for 4 hours. Gelatin gels.
B. Remove the gelled solution and place the container in 60°C. Within 30 minutes, when stirred, the gel reverts to the original liquid state.

2. To a 1:100 aqueous solution, add trinitrophenol TS or a solution of potassium dichromate (1:15) previously mixed with ¼ its volume of 3N hydrochloric acid. A yellow precipitate forms.
3. For conclusive evidence of identity, test for hydroxyproline

## 1.7 Hydroxyproline Content

### Principle

Gelatin contains a high amount of the amino acid hydroxyproline. Hydroxyproline is liberated through acid hydrolysis, oxidized, and then identified with Erlich's reagent (5% *p*-dimethylaminobenzaldehyde in *n*- propanol).

### Reagents and Solutions

1. 0.05N $CuSO_4$
2. 2.5N NaOH
3. 6% $H_2O_2$
4. 3N $H_2SO_4$
5. Erlich's Reagent – 5% *p*-Dimethylaminobenzaldehyde in *n*-Propanol (make fresh each time).

### Apparatus

1. Oil bath, capable of 145°C.
2. Water bath, 40°C
3. Ice bath
4. Bunsen burner
5. 18 x 150 mm test tubes
6. 500 mL volumetric flask
7. Aluminum foil
8. Distilled/deionized water
9. Concentrated HCl

### Procedure

1. Dissolve 1.0 gram of material in 200 mL of water.
2. Add 3 mL of this solution and 3mL of conc. HCl to an 18 x 150 mm test tube. Seal by melting the top in a Bunsen burner. Hydrolyze at 145°C for 1.5 hours in an agitated oil bath.
3. Cool the hydrolysate, cut off the top of the tube, transfer contents to the volumetric flask and dilute to 500 mL.
4. Transfer 1 mL to an 18 x 150 mm test tube. Add 1 mL 0.05N $CuSO_4$, 1 mL 2.5N NaOH, and place in a 40°C water bath for 5 minutes.
5. Add 1 mL 6% $H_2O_2$ and mix immediately.
6. Keep the sample at 40°C for 10 minutes, ***Shake*** and rotate to remove all excess $H_2O_2$.
7. Cool rapidly in an ice bath. Add 4 mL 3N $H_2SO_4$, mix, then add 2 mL Ehrlich's reagent, mix, cover with aluminum foil and hold at room temperature for 15 minutes. The development of an intense red color shows the presence of Hydroxyproline.

### Quality Control

All chemicals are reagent grade.

## 1.8 Sampling

### Principle

Aseptic gelatin samples are required for all testing. The square root method plus one, should be followed to determine the number of samples per lot.

### Reagents and Solutions

None

### Apparatus

1. Sterile gloves
2. Scoop
3. Air - tight container

4. U. S. standard sieve (8 mesh)
5. Table top blender

**Procedure**

1. Take aseptic sample by scooping out a cone several inches below surface of the gelatin in the container.
2. Pull the scoop up, across the vertical surface of the cone to obtain a representative sample.
3. Place samples as drawn in clean airtight containers.
4. Proportion the amount taken from each container selected to at least 120g of sample per container.
5. Blend or mix samples thoroughly.
6. Withdraw and retain at least 500g as the final sample.

**Note**: *Gelatin coarser than 8 mesh U.S. standard sieve should be ground so that all particles pass the 8-mesh sieve. The entire final sample should be re-blended.*

## *2.* Physical / Chemical Testing

### 2.1 Gel Strength

**Principle**

The gel strength of gelatin is a measure of the rigidity of a gel formed from a 6.67% solution and prepared according to certain arbitrary prescribed conditions. Bloom is a measure of force (weight) required to depress a prescribed area of the surface of the sample a distance of 4 mm.

**Apparatus**

1. TA.XT2 (Texture Technologies) or LFRA (Brookfield Engineering) Texture Analyzer
   AOAC plunger, 0.5" diameter, sharp edge (not beveled).
2. Automatic pipette, water capacity 105.0 ± 0.2 g at 25 ± 2°C. The delivery rate should be 105 ± 0.2 g of distilled/deionized water in 15 seconds.
3. Stir rods, stainless steel or brass, approximately 3 mm diameter and 15 cm in length and tapered at one end.

4. Bloom jar (available from Schott or Brookfield Engineering): capacity 150 mL, overall height 85 mm, inside diameter 59 mm, outside diameter 66 mm, neck inside diameter 41 mm, shoulder height 65 mm. All linear dimensions are ± 1.0 mm. Bottle must have flat bottom to ensure it does not rock on a flat surface. Uneven bottles should be corrected by grinding or rejected.
5. Appropriate size single hole stoppers.
6. Analytical balance with 0.01g sensitivity.
7. Thermostatic water bath capable of uniform temperature at 65 ± 1°C with agitation. The bath should be provided with a false bottom at such height that the water level is approximately 1 cm above the surface of the test gelatin solution in the Bloom bottle.
8. Thermostatic water bath, same as #7, held at 45 ± 2°C with agitation
9. Refrigerated water bath with agitation, with heating and cooling units, capable of maintaining water at 10 ± 0.1°C throughout the bath. The bath should be provided with a false bottom at such height that the water level is approximately 1 cm above the surface of the test gelatin solution in the Bloom bottle. The false bottom must be level ensure sample test surface is level. *Note: the design of the chill bath should be such that it will recover to 10 ± 0.1°C within one hour after the bloom samples are placed in the bath.*
10. Dummy bloom strip device available from Brookfield Engineering or internal gelatin standards.

**Procedure**

1. Weigh 7.50 ± 0.01 g of gelatin into the bloom bottles using an analytical balance.
2. Add 105.0 ± 0.2 g of distilled water at 25 ± 2°C from an automatic pipette. Stir with a stirring rod while adding the water, using the pointed end to remove all gelatin in the groove at the bottom of the bottle. Wash any gelatin adhering to the rod into the bottle with the last portion of water.
3. Insert a perforated stopper and allow the sample to hydrate 1-3 hours at room temperature.
4. Place the sample bottle in the 65°C bath. Stir or swirl periodically, and after 8-10 minutes remove the bottle from the bath. Swirl the bottles several times to be sure that all of the gelatin is in solution. Replace the bottle in the bath, insert a thermometer into the sample and stir just enough to effect thorough mixing until the temperature of the sample is 61°C. The total time in the 65°C bath shall not exceed 15 minutes.
5. When the temperature of the solution reaches 61°C and the sample is completely dissolved and thoroughly mixed, remove the thermometer and

transfer to a viscometer and determine the viscosity according to the designated procedure (see Viscosity Procedure). Collect all of the effluent from the viscometer in the original bottle and immediately replace the stopper.

6. Temper the sample by placing the bottle in the 45°C bath for 30-45 minutes or letting it stand for 15-20 minutes at room temperature. Note: If the viscosity is not to be determined or if the viscosity is to be determined after the jelly strength, insert the stopper and temper immediately after the 65°C bath as above.
7. After tempering, gently swirl the solution in the Bloom bottle to remove condensation from the sides of the bottle. Remove the stopper and gently remove all foam from the center of the gelatin solution surface with a spoon or other convenient means. Replace the stopper and place the sample bottle in the 10.0 ± 0.1°C water bath for 16-18 hours.
8. Set up Stevens LFRA and TAXT2 according to Annex 1.
9. Remove the jelled sample from the 10°C bath and quickly wipe the water from the exterior of the bottle. Remove the stopper and center the bottle on the texture analyzer platform so that the plunger contacts the sample as close its midpoint as possible. (If there is any foam at this area, start the determination over). The determination is to be done immediately after removal of the bottle from the chill bath.
10. Press “Start” (LFRA) or “Run” (TA.XT2) to run the determination. If using the LFRA, check the zero weight often. Repeat as needed until all bloom determinations are complete.

**Quality Control**

1. Check calibration with standard weights at least monthly per manufacturer’s instructions.
2. Monitor daily operation with the use of the Dummy Bloom strip or internal gelatin standards of determined bloom strength. The Dummy Bloom Strip must read ± 1 g to accept test results. Internal gelatin standards must fall within acceptable SPC ranges determined during development of the standard(s) to accept test results.
3. Repeatability within one lab of 1.5% standard deviation is realistic.
4. Reproducibility between labs using the same procedure: Ring test results indicate 3% or better standard deviation is achievable.

**Texture Analyzer Set-Up**

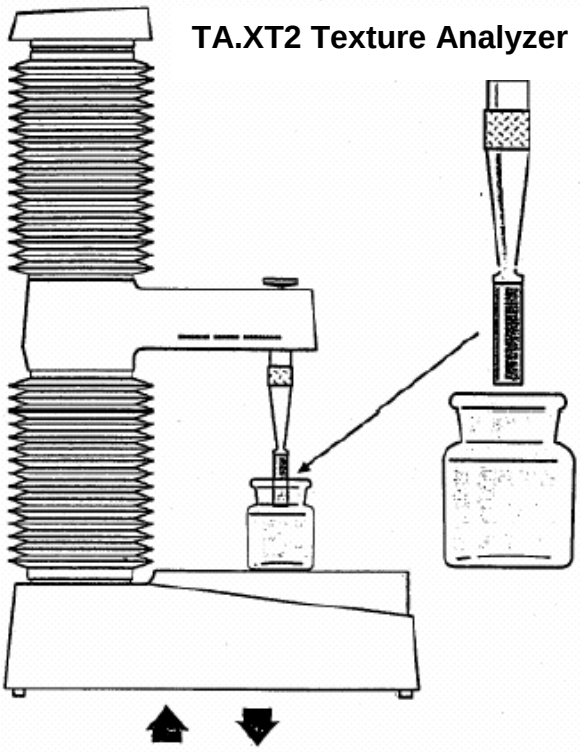
TA.XT2
Speed = 1 mm/sec
Distance = 4 mm
Trigger = 5
Pre = 2.0
Post = 5.0
Penet = 1.0
PHT = 1.0
Test Output = Final

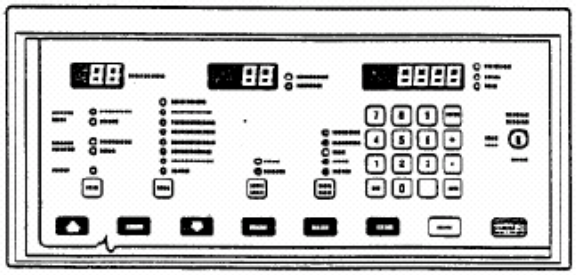
LFRA (Power on – allow 15 minute warm-up).
Penetration Speed = 1 mm/sec
Penetration Distance = 4 mm
Cycle = Normal
Adjust the test platform , assuring it is level and the probe is at least 10 mm above the surface of the sample.
Set the digital readout to “0”

*The AOAC plunger is to be used for either texture analyzer.*


**LFRA Texture Analyzer.**

**TA.XT2 Texture Analyzer**





| | TA-XT2/5 | TA-XT2/25 |
|---|---|---|
| Maximum Force | 5Kg | 25Kg |
| Force Resolution | 0.1gm | 1gm |
| Force Accuracy | 0.025% | 0.025% |
| Speed Range | 0.1 - 10mm/sec | |
| Return Speed Range | 0.1 - 10mm/sec | |
| Speed Accuracy | 0.01% (0.1-5mm/s)<br>0.1% (5-10mm/s) | |
| Position Range Setting | 0.1 - 295mm | |
| Range Resolution | 0.01mm | |
| No. of Cycles | 1 - 999.999 repeat cycles | |
| Data Acquisition Rate | 500Hz. Force and distance readings taken simultaneously. | |
| Time Delay | 0.1 - 999.999 sec | |
| Displays | Separate displays for speed, distance and force. Distance can be displayed directly as strain. | |

## 2.2 Viscosity

### Principle

The viscosity of a 6.67% gelatin solution is determined at 60ºC by measuring the flow time of 100 mL of the solution through a standard pipette.

### Apparatus

1. PIPETTE: Calibrated 100 mL pipette with a precision capillary outlet and and upper and lower mark on the glass
2. THERMOSTATIC BATH: such as is available from Lurex Scientific (see Manufacturers of Equipment: Viscometer) equipped with thermostatic device, such as a heating circulator, to maintain 60.00 ± 0.05ºC.
3. PRECISION THERMOMETER: graduated in 0.01ºC with a long slim stem for measuring the temperature inside the pipette.
4. STOP WATCH: accurate to 0.01 seconds.
5. BALANCE: with 0.01 g sensitivity
6. WATER BATH: constant temperature at 65 ± 0.5ºC

### Procedure

1. Weigh 7.50 ± 0.01 g gelatin into a bloom jar or 150 mL beaker.
2. Add 105.0 ± 0.2 g deionized water, stirring often to suspend all gelatin particles
3. Cover and let stand 1 – 3 hours at room temperature
4. Dissolve the sample in a 65ºC water bath for 10 – 15 minutes, stirring or swirling as required.
5. When the temperature of the solution reaches 61ºC, and the sample is completely dissolved and thoroughly mixed, transfer the solution to the viscosity pipette and proceed with the viscosity determination.
6. Using a finger of the free hand cover the capillary end of the pipette and pour enough solution into the pipette to bring the level approximately 1 cm above the upper mark.
7. Place the thermometer inside the pipette and slowly raise and lower it until a constant temperature of 60.00 ± 0.05ºC is maintained.
8. Remove the thermometer from the pipette.
9. Read and record the time required for 100 mL of solution to pass through the capillary tube of the pipette by draining the gelatin solution and starting the stopwatch as soon as the meniscus of the liquid hits the top line of the pipette. Stop the stopwatch when the meniscus hits the lower line of the pipette.

10. Record the time obtained to the nearest tenth of a second; this value is the efflux time.

**Calculation of the Viscosity**

The viscosity (to the nearest millipoise) at 60ºC of any sample with efflux time ***t*** (in seconds) may be calculated from the following equation:

$$V = ( A\boldsymbol{t} - {}^{B}/_{\boldsymbol{t}} ) \times d$$

V = Viscosity, in millipoises (mP)
A, B = A and B pipette constants (Refer to Annex I, Calibration of Viscosity Pipette, to obtain A and B constants, if not available)
***t*** = efflux time, in seconds
d = solution density

Refer to Section D, Calibration, to obtain A and B constants, if not available.

For a 6.67% gelatin solution at 60ºC d = 1.001

**Pipette Calibration**

1. Pipettes can be calibrated using two standard oils of different viscosities. The pipette must be thoroughly cleaned before the calibration and dried with reagent grade acetone.
2. Preheat both oils in a constant temperature bath set at 63-64ºC.
3. Obtain the efflux time (***t***), in triplicate, for each standard at 60ºC. Clean the pipette thoroughly between different oils using a suitable organic solvent for removing the oil and acetone to remove residual solvent and dry.
4. Calculation of the A and B constants:

$$B = \frac{\boldsymbol{t_1 t_2} (V_2 \boldsymbol{t_1} - V_1 \boldsymbol{t_2})}{\boldsymbol{t_2^2 - t_1^2}}$$

$$A = \frac{V_1 + {}^{B}/_{\boldsymbol{t_1}}}{\boldsymbol{t_1}} = \frac{V_2 + {}^{B}/_{\boldsymbol{t_2}}}{\boldsymbol{t_2}}$$

$V_1$ = kinematic viscosity of lower viscosity oil, in millistokes
$V_2$ = kinematic viscosity of higher viscosity oil, in millistokes
$\boldsymbol{t_1}$ = average efflux time of lower viscosity oil, in seconds

$t_2$ = average efflux time of higher viscosity oil, in seconds

## 2.3 Determination of Moisture Content of Gelatin

### Principle

A weighed sample of gelatin is maintained for 16 to 18 hours at 105 ± 2ºC and is then reweighed. The moisture content is defined as the percentage loss in weight of the sample.

### References

**GME Monograph, June 2005, Version 4**

### Reagents and Solutions

None

### Apparatus

1. Pyrex evaporating dishes, 45 mm in diameter and 30 mm high, or other suitable moisture pan
2. Drying oven, set at 105 ± 2ºC.
3. Dessicator
4. Analytical balance, capable of weight to 0.001 grams

### Procedure

### Sample Preparation

1. Wash the evaporating dish very carefully in hot water.
2. Place the dish in the drying oven at 105ºC for at least one hour.
3. Cool dish in the dessicator until room temperature is reached.
4. Weigh approximately 5.0 g of gelatin to the nearest milligram and note the weight of the test sample ($m_0$) and the weight of the sample together with the evaporating dish ($m_1$).

### Determination

1. Place the evaporating dish containing the sample in the drying oven at 105 ± 2ºC for 16 to 18 hours.
2. Cool the dish in the dessicator until room temperature is reached and weigh to the nearest milligram ($m_2$), weigh and calculate the percentage of residue

### Result

The moisture content, expressed as a percentage by weight, is equal to:

$$\% \ Moisture = [(m_1) - m_2) / m_0] \times 100\%$$

where: $m_0$ is the weight in grams of the test sample
$m_1$ is the weight in grams of the test sample and the evaporating dish, before drying $M_2$ is the weight in grams of the test sample and the evaporating dish, after drying

## 2.4 pH

### Principle

The pH of a 1.5 % gelatin solution is determined by potentiometry at a temperature of 35 ± 1°C using a pH meter.

### References

GME Monograph, June 2005, Version 4

### Reagents and Solutions

1. pH 4 Buffer Solution
2. pH 7 Buffer Solution
3. Deionized Water

### Apparatus

1. BALANCE: with 0.01 g sensitivity
2. WATER BATH: constant temperature at 65 ± 0.5ºC
3. WATER BATH: constant temperature at 35 ± 0.5ºC
4. pH Meter: conventional pH meter with at least two decimal place display
5. pH Electrode: combination pH electrode with temperature compensation

### Procedure

1. Weigh 1.60 ± 0.01 g gelatin into a bloom jar or 150 mL beaker.
2. Add 105.0 ± 0.2 g deionized water, stirring often to suspend all gelatin particles
3. Cover and let stand 1 – 3 hours at room temperature
4. Dissolve the sample in a 65ºC water bath for 10 – 15 minutes, stirring or swirling as required.
5. Transfer the sample to the 35ºC water bath and temper to 35ºC.
6. Perform a two-point calibration on the pH meter, using pH 4 and pH 7 buffers, at 35ºC.
7. Determine the pH of the gelatin solution according the pH-meter instructions.
8. Swirl the solution well using the pH probe to ensure the electrode is sufficiently saturated.
9. Rinse the electrode with warm distilled water once testing is complete.

## 2.5 Granulation

### Principle

A weighed sample of gelatin is placed on a sieve shaker to determine the particle size distribution of the sample.

### References

GMIA Official Procedures of Gelatin,1986

### Apparatus

1. Ro-Tap testing sieve shaker (W.S. Tyler Company)
2. Assorted Sieves
   - 8 mesh (0.0937 in., W.S. Tyler Co.)
   - 10 mesh (0.0787 in., W.S. Tyler Co.)
   - 16 mesh (0.0469 in., W.S. Tyler Co.)
   - 20 mesh (0.0335 in., W.S. Tyler Co.)
   - 30 mesh (0.0234 in., W.S. Tyler Co.)
   - 40 mesh (0.0167 in., W.S. Tyler Co.)
   - 60 mesh (0.0098 in., W.S. Tyler Co.)
   - 80 mesh (0.0070 in., W.S. Tyler Co.)
   - 100 mesh (0.0059 in., Fisher Scientific Co.)

3. Sieve Cover
4. Sieve Receiver
5. Balance (readability to 0.1g)
6. Brushes (bristle and steel)
7. Large tray or basin, plastic or non-stick freezer paper

**Procedure**

1. Weigh a homogeneous sample of gelatin to be tested.
2. Select the sieves to be used. The selection should cover the full range of expected particle sizes.
3. Arrange the sieves with the coarsest screen on top and a catch tray on the bottom.
4. Weigh out 100 ± 0.1 grams of gelatin.
5. Pour gelatin sample into the top screen, place the cover on, position in the shaker, and place hammer on top of the cover.
6. Turn the shaker on for five minutes.
7. When the shaking is completed, remove from the shaker; brush out all granules from each sieve.
8. Use the freezer paper to capture all gelatin from each screen. Weigh the sample retained on each screen and that which passed through the finest screen (material in catch tray).
9. Record the actual weight retained on each screen.
10. Calculate the percentages of gelatin retained and/or passed through the sieves.

## 2.6 Determination of Ash Content in Gelatin

**Principle**

Gelatin is ashed in a crucible at 550ºC using a muffle furnace. The residue is determined by differential weighing and the result expressed as a weight percentage of the original sample.

**References**

USP 23 NF 18, 1995

## Reagents and Solutions

1. Paraffin

## Apparatus

1. Muffle furnace, capable of heating to 550 + 25ºC
2. Analytical balance, capable of weight to 0.001 grams

## Procedure

### Sample Preparation

1. Weigh approximately 5.0 g of gelatin to 0.001 g in a suitable crucible that has previously has been ignited, cooled and weighed.
2. Add 1.5 to 2.0 g of paraffin to avoid loss due to swelling.
3. Heat, gently at first on an electric hot plate or in a muffle furnace, until the substance is thoroughly charred.
4. Finish ashing in a muffle furnace at 550ºC for 15 to 20 hours.

### Determination

1. Cool the sample in a dessicator.
2. Weigh the sample and calculate the percentage of residue

*Note: do not handle dried crucibles without gloves or crucible tongs*

### Result

The ash content, expressed as % ash, is equal to:

$$\% \ ash = [\text{weight of ash}] / [\text{weight of sample}] \ X \ 100\%$$

## 2.7 Sulfur Dioxide

### Principle

Sulfur dioxide content can be determined using the Monier-Williams test method.

### References

AOAC Method 962.16 as stated in Food Chemicals Codex, General Tests and Assays, Appendix X.

### Reagents and Solutions

Stated in Sulfur Dioxide Determination in Food Chemicals Codex, General Tests and Assays, Appendix X

### Apparatus

Monier-Williams apparatus as per AOAC Method 962.16

### Procedure

Performed by contact laboratories as per AOAC Method 962.16.

## 2.8 Clarity

### Principle

The clarity of a 6.67% gelatin solution is determined at 45ºC by measuring the percent transmittance through a 1 cm cuvette at 640 nm.

### Apparatus

1. Spectrophotometer
2. 1 cm cuvettes, optically matched
3. Automatic pipette, capacity 105.0 ± 0.2

4. Bloom jar or 150 mL beaker
5. Balance with 0.01 g sensitivity
6. Constant temperature water baths at 65ºC and 45ºC

**Procedure**

1. Weigh 7.50 ± 0.01 g gelatin into bloom jar or 150 mL beaker
2. Add 105.0 ± 0.2 g deionized water, stirring often to suspend all gelatin particles
3. Cover and let stand 1 – 3 hours at room temperature
4. Dissolve the sample in a 65ºC water bath for 10 – 15 minutes, stirring or swirling as required.
5. Remove the sample to a 45ºC water bath and hold until sample temperature is 45 ± 1ºC.
6. Calibrate the spectrophotometer to 100% transmittance with deionized water blank at 640 nm according to the manufacturer's instructions.
7. Transfer an aliquot of the sample solution to the cuvette and record the percent transmittance value at 640 nm.

*Note: Sample preparation for the clarity test is the same as for gel strength, viscosity and colour tests; those samples may be utilized. The clarity determination may be conducted on samples after the gel strength test by melting down and tempering to 45ºC as previously described.*

## 3. Microbiological Testing

### 3.1 Total Aerobic Count

**Principle**

Use of plate-count agar media to enumerate in 48 hours at 35ºC the level of microorganisms, including mesophilic bacteria, yeasts and moulds, by counting the colony forming units (CFU) per 1 g of gelatin.

**References**

U.S. Food and Drug Administration Center for Food Safety & Applied Nutrition (USDA CFSAN) Bacteriological Analytical Manual (BAM) Aerobic Plate Count.

**Apparatus**

1. Petri dishes at least 15 x 90 mm.
2. Sterile pipettes with pipetting aids.
3. Butterfield's phosphate buffer, 90 ± 1 mL, sterile dilution bottles.
4. Plate Count agar or tryptic soy agar.
5. Circulating Water Bath thermostatically controlled to 45 ± 1ºC.
6. Incubator ± 1ºC.
7. Colony counter, dark-field, Quebec, or equivalent, with suitable light source and grid plate.
8. BALANCE: with 0.1 g sensitivity.

**Procedure**

**1/10 Solution preparation**

1. Under aseptic conditions, weigh out 10 ± 0.1 g of gelatin into a sterile Butterfield's phosphate buffer dilution bottle
2. Leave the gelatin at room temperature to absorb the buffer for approximately 1 hour.
3. Transfer the bottle to the 45ºC water bath.
4. Shake gently to ensure proper gelatin dissolution over a period of, maximum, one hour.

**Inoculation**

1. Aseptically transfer 1 mL of the 1/10 gelatin solution into each of two sterile Petri dishes.
2. Immediately pour approximately 15 mL of tempered plate count agar (45 ±1ºC) onto each dish.
3. Homogenize each plated sample and leave the agar solidify at room temperature.

**Incubation**

1. Invert the solidified agar plates and incubate at 35ºC for 48 ± 2 hrs.

**Aerobic plate count (apc) determination**

1. Following incubation count the number of colonies on each plate. For accurate enumeration the number of colonies counted per plate should be between 30 and 300.


2. Calculate the average of the counts and multiply by 10. Report as colony forming units per gram of gelatin (CFU/g). Report the absence of any colonies as <10 CFU/g.

### 3.2 E. coli

#### Principle

The USP Method is a conventional cultural method that is applicable to the detection of viable *E. coli* in raw materials, finished products and environmental swabs. The entire detection testing protocol may take up to 3 days to complete. The FDA-BAM method is also applicable.

#### References

Current USP Microbiological Tests.
(Also applicable is the current BAM method of Enumeration of E. coli)

#### Reagents and Solutions

1. Lactose Broth
2. MacConkey Agar Medium
3. Levine Eosin-Methylene Blue Agar Medium
4. 1 N NaOH

#### Apparatus

1. 500 mL Erlenmeyer flask
2. Sterile 1 mL pipette
3. Sterile Inoculating Loop
4. Water bath at 42ºC
5. Refrigerator at 4ºC
6. Incubator at 35ºC
7. Balance
8. Bunsen Burner

#### Procedure

#### Sample Preparation

1. Aseptically transfer 10 grams of sample into a sterile 500 ml Erlenmeyer flask

containing 90 mL of sterile Lactose Broth Medium.

2. Swirl for 5 seconds to evenly distribute the enrichment broth through the sample.
3. For a flask of raw gelatin, swell at 25ºC for 1 hour.
4. Transfer the flask to a water bath at 42ºC. Warm until completely dissolved. Swirl occasionally for 1 hour to ensure even distribution of the microorganisms. (A shaking water bath may be used).
5. Adjust pH using sterile 1 N NaOH or HCl, if necessary, to 6.8 ± 0.2. Verify the final pH after addition of chemical (NaOH or HCl), using pH paper.
6. Incubate the broth solution for 24 ± 2 hour at 35ºC.
7. Warm the broth solution in a 42ºC in order to melt the gelatin.

**Isolation**

1. Warm the broth solution in a 42ºC in order to melt the gelatin.
2. Examine the medium for growth, and if growth is present, mix by gently shaking and proceed with procedure.
3. Transfer a sterile loopful to the surface of MacConkey Agar Medium. Cover, invert, and incubate at 35ºC for 24 ± 2 hours.
4. Upon examination, if none of the colonies conforms to the description given below, then the sample meets the requirements for absence of Escherichia coli.

Table – Morphologic Characteristics of Eschericia coli on MacConkey Agar Medium

| Gram Stain | Characteristic Colonial Morphology |
|---|---|
| Negative Rods | Brick Red; may have surrounding zone of precipitated bile. |

5. If colonies match the description given above, then proceed by transferring the suspect colonies, individually, by means of an inoculating loop, to the surface of Levine Eosin-Methylene Blue Agar Medium, plated on petri plates.
6. Cover, invert, and incubate the plates at 35ºC for 24 ± 2 hours.
7. Upon examination, if none of the colonies exhibits both a characteristic metallic sheen under reflected light and blue-black appearance under transmitted light, the sample meets the requirements for the absence of E. coli.
8. The presence of E. coli can be confirmed by the API 20E, Enterotube method or other approved identification kit.

## 3.3 Salmonella

### Principle

The AOAC/BAM Method is a conventional cultural method that is applicable to the detection of viable *Salmonella* in raw materials, finished products and environmental swabs. The entire detection testing protocol may take up to 5 days to complete.

### References:

FDA Bacteriological Analytical Manual (BAM), online. Updated June 2006 Chapter 5.

Note: There are rapid methods for the detection of Salmonella in food and the environment, please select the link below for more information regarding rapid methods.

Rapid Methods for Detecting Food borne Pathogens - BAM Manual Appendix 2

### Reagents and Solutions

| | |
|---|---|
| 1. Lactose Broth | (BD/Difco 266520) |
| 2. Tetrathionate Broth (TT) | (BD/Difco 249120) |
| 3. Rappaport-Vassiliadis (RV) | (BD/Difco 218581) |
| 4. Xylose Lysine Desoxycholate (XLD) Medium | (BD/Difco 278830) |
| 5. Hektoen Enteric (HE) Agar | (BD/Difco 285340) |
| 6. Bismuth Sulfite (BS) Agar | (BD/Difco 273300) |
| 7. Triple Sugar Iron (TSI) Agar | (BD/Difco 226540) |
| 8. Lysine Iron Agar (LIA) | (BD/Difco 284920) |
| 9. 1 N NaOH | (Aldrich 319511) |
| 10. 1 N HCl | (Aldrich 318949) |
| 11. Papain Solution, 5% | (ABL-Coralase 7089) |

### Apparatus

1. Incubator at 35ºC
2. Incubator at 42ºC)
3. 500 mL wide-mouth, screw-cap jars, sterile (autoclavable)

4. 4 L volume autoclavable nalgene screw cap jars
5. Sterile Petri dishes 5.
6. 16x150mm culture tubes with caps
7. pH Paper Test Strips
8. 3 mm transfer loops (10 ul)

**Sample Preparation for Pre-enrichment of Gelatin:**

**Gelatin**. Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap jar (500 mL) or other appropriate container. Add 225 mL sterile lactose broth and 5 mL 5% aqueous papain solution and mix well. For the U.S., a composite of up to 375g (15 x 25g samples) may be tested. Weigh the composited samples into a sterile, wide-mouth 4 L screw cap container and add approximately 3375 mL of Lactose broth and 75 mL of the 5% aqueous papain solution. For Europe, a composite of up to 250g (10 x 25g samples) may be tested. Weigh the composited samples into a sterile, wide-mouth 4L screw cap container and add approximately 2225 mL of Lactose broth and 50 mL of the 5% aqueous papain solution. Cap jar securely and incubate at 35°C for 60 ± 5 min. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 using the 1N NaOH or 1N HCl. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°C.

**Selective Enrichment**

1. Tighten lid and gently swirl incubated sample mixture; transfer 1 mL mixture to 10 mL Rappaport-Vassiliadis (RV) medium and another 1 mL of mixture to 10 mL Tetrathionate (TT) broth.
2. Incubate the RV medium for 24 ±2 hours at 42 ºC and the TT broths 24± 2 h at 35ºC.

**Selective Plating / isolation**

1. Mix (vortex, if tube) and streak a 3 mm loopful of incubated TT broth on Bismuth Sulfite (BS) agar, Xylose Lysine Desoxycholate (XLD) agar, and Hektoen Enteric (HE) agar.
2. Prepare BS plates the day before streaking and store in dark at room temperature until streaked.
3. Repeat with a 3 mm loopful of SC broth.
4. Incubate plates 24 ± 2 h at 35ºC.
5. Examine plates for presence of colonies suspected to be *Salmonella*.

HEKTOEN ENTERIC (HE) AGAR
Typical *Salmonella* colonies may appear blue-green to blue colonies with or without black centers. Many cultures of *Salmonella* may produce colonies with large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* species produce yellow colonies with or without black centers
BISMUTH SULFITE (BS) AGAR
Typical *Salmonella* colonies may appear brown, gray, or black; sometimes they have a metallic sheen. Surrounding medium is usually left brown at first, but may turn black in time with increased incubation, producing the so-called halo effect. Some strains may produce green colonies with little or no darkening of surrounding medium
XYLOSE LYSINE DESOXYCHOLATE (XLD) AGAR
Typical Salmonella colonies may appear pink with or without black centers. Many cultures of *Salmonella* may have large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* species produce yellow colonies with or without black centers.

**Atypical *Salmonella* Colony Morphology**

In the absence of typical or suspicious *Salmonella* colonies, search for atypical *Salmonella* colonies as follows:

1. **HE and XLD agars**. Atypically a few *Salmonella* cultures produce yellow colonies with or without black centers on HE and XLD agars. In the absence of typical *Salmonella* colonies on HE or XLD agars after 24 ± 2 h incubation, then pick 2 or more atypical *Salmonella* colonies.
2. **BS agar.** Atypically some strains produce green colonies with little or no darkening of the surrounding medium. If typical or suspicious colonies are not present on BS agar after 24 ± 2 h, then do not pick any colonies but reincubate an additional 24 ± 2 h. If typical or suspicious colonies are not present after 48 ± 2 h incubation, then pick 2 or more atypical colonies.

**Biochemical Media /screening and identification**

1. Select 2 or more colonies typical of (or suspected to be) *Salmonella* from each selective agar. If no typical colonies are present, select representatives of atypical colony types to confirm the absence of atypical Salmonella. Inoculate into Triple Sugar Iron (TSI) agar and Lysine Iron Agar (LIA). If BS plates have no colonies typical of (or suspected to be) *Salmonella* or no growth


whatsoever, incubate them an additional 24 ± 2 h. ***If no growth is detected on any of the selective agar plates, results may be reported as negative per sample size tested.***

2. Lightly touch the very center of the colony to be picked with sterile inoculating needle and inoculate TSI agar slant by streaking agar slant and stabbing agar butt. Without flaming, inoculate LIA by stabbing agar butt twice and then streaking slant. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have a deep butt (4 cm). Store picked selective agar plates at 5-8ºC.
3. Incubate TSI agar and LIA slants at 35ºC for 24 ± 2 h.
4. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive $H_2S$ production. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of $H_2S$ (blackening of agar) in TSI agar. In LIA, *Salmonella* typically produces alkaline (purple) reaction in butt of tube. Consider only distinct yellow in butt of tube as acidic (negative) reaction. Do not eliminate cultures that produce discoloration in butt of tube solely on this basis. Most *Salmonella* cultures produce $H_2S$ in LIA. Some non-*Salmonella* cultures produce a brick red reaction on LIA slants.
5. Suspect cultures should be streaked onto Brain Heart Infusion Agar plates and incubated overnight at 35ºC. Micro Id (Rummel), API 20e (bioMerieux) or other approved identification kit may be run to get a quick identification as Salmonella. Always do an oxidase test before running Micro ID. Only oxidase negative cultures are run on Micro ID.

   All cultures that give an alkaline butt in LIA, regardless of TSI reaction, should be retained as potential *Salmonella* isolates and submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an alkaline slant and acid butt in TSI should also be considered potential *Salmonella* isolates and should be submitted for biochemical and serological tests. Cultures that give an acid butt in LIA and an acid slant and acid butt in TSI may be discarded as not being *Salmonella*.