DPNS 601:2008

Carrageenan – Food grade - Specification

1 Scope

This standard specifies the quality requirement and methods of physico-chemical and microbial tests for Carrageenan and Processed Eucheuma Seaweed (PES) obtained from seaweeds of the class *Rhodophyceae* (red seaweeds) commonly used as emulsifier, binder, thickener, gelling agent or stabilizer.

2 References

The references referred to in this standard are listed in the back cover.

3 Definitions

3.1 Carrageenan or Traditionally Refined Carrageenan (TRC) INS No. 407 or E 407

A refined hydrocolloid prepared by water or dilute alkali extraction from the following members of the families *Gigartinaceae* and *Solieriaceae* of the class *Rhodophyceae* (red seaweed); *Eucheuma cottonii, E. spinosum, Chondrus crispus, C. ocellatus, Gigartina acicularis, G. stellata, G. pistillata and G. radula, Kappaphycus spp., Iridaea, Hypnea, Furcellaria fastigiata*.

NOTE[1] – *Eucheuma cottonii* (known in commerce as "cottonii" type) now refers to *Kappaphycus* spp,,*Eucheuma spinosum* (the "spinosum" type) is correctly termed as *Eucheuma denticulatum*

3.2 Processed Eucheuma Seaweed *(*PES) INS No. 407a or E407a

A semi-refined hydrocolloid from red seaweeds as indicated in clause 3.1 treated with alkali at a certain concentration and temperature; for food and non-food applications known also as Philippine Natural Grade (PNG), Semi-Refined Carrageenan (SRC), Alternatively Refined Carrageenan (ARC), Alkali-Modified Flour (AMF), Processed Seaweed Flour (PSF) and Alkali-Treated Carrageenophyte (ATC).

4 Technical Description

Carrageenan is a hydrocolloid consisting mainly of the ammonium, calcium, magnesium, potassium and sodium sulfate esters of galactose and 3,6-anhydrogalactose copolymers. These hexoses are alternately linked α-1,3 and ß-1,4 in the copolymer. The relative proportions of cations existing in carrageenan may be changed during the processing to the extent that one may become predominant.

The prevalent copolymers in the hydrocolloid are designated as kappa-, iota-, and lambda-carrageenan. Kappa-carrageeenan is mostly the alternating polymer of D-galactose-4-sulfate and 3,6-anhydro-D-galactose; iota-carrageenan is similar, except that the 3,6-anhydrogalactose is sulfated at carbon 2. Between kappa-carrageenan and iota-carrageenan there is a continuum of intermediate compositions differing in degree of sulfation at carbon 2. In lambda-carrageenan, the alternating monomeric units are mostly D-galactose-2-sulfate (1,3-linked) and D-galactose-2,6 disulfate (1,4-linked).

Carrageenan may be produced by commercially available process detailed in Annex A.

Articles of commerce may be diluted with sugars for standardization purposes, mixed with salts to obtain specific gelling or thickening characteristics or may contain emulsifiers carried over from drum drying processes. Carrageenan is a yellowish or tan to white, coarse to fine powder that is practically odorless. Kappa and Iota are soluble in water at a temperature of 80ºC while Lambda is soluble in water at room temperature.

PES is a hydrocolloid that may contain up to 15% algal cellulose and minor amounts of other insoluble matter in addition to the above description.

5 Requirements

5.1 Identification

Carrageenan and PES shall be identified for the characteristics specified in Table 1 when determined with the test methods specified therein.

Table 1 Characteristics/Identification for Carrageenan and PES

Characteristics	Test Method
Solubility in Water	Annex B
Insolubility in ethanol	
Identification of hydrocolloid and predominant type of copolymer	Annex C
Infrared absorption spectra	Annex D_1 (Carrageenan)
	Annex D_2 (PES)

5.2 Quality Requirements

Carrageenan and PES shall conform to the requirements specified in Table 2 and Table 3 when tested with the methods specified therein.

6 Conditions of Manufacture

Carrageenan and PES shall be manufactured under the hygienic conditions in accordance with BFAD AO 153 s. 2004.

Table 2 - Physico-Chemical Requirements for Carrageenan and PES

Properties/Contaminants	Limits		Test method
	PES	TRC	
Loss on drying (105ºC to constant weight) For powder, max. For chips, max.	 12% 15%	 12% -	Annex E
Residual alcohol, max	0.1 %	0.1 %	Annex F
pH (for 1% concentration)	8 – 11	8 – 11	Annex G
Viscosity of 1.5% (solution at 75ºC), min.	5 mPa/s	5 mPa/s	Annex H_1(Carrageenan) Annex H_2(PES)
Sulfate (as SO_4)	15% - 40%	15% - 40%	Annex I
Total ash (on dry weight basis)	15% - 30%	15% - 40%	Annex J
Acid-insoluble ash, %, max	1.0%	1.0 %	Annex K
Acid Insoluble Matter	8% - 15%	2 %, max	Annex L
Arsenic, max	3 mg/kg	3 mg/kg	Annex M
Lead, max	5 mg/kg	5 mg/kg	Annex N
Mercury, max	1 mg/kg	1 mg/kg	Annex O
Cadmium, max	2 mg/kg	2 mg/kg	Annex P

7 Packaging

Carrageenan and PES shall be packed in inert material to prevent contamination and provide protection.

8 Sampling

Carrageenan and PES shall be sampled in accordance with Annex U.

Table 3 - Microbiological Requirements

Microorganisms	Limits		Test method
	PES	TRC	
Total Plate count (Aerobic plate count), max	5,000 cfu/g	5,000 cfu/g	Annex Q
Yeast and mold, max	300 cfu/g	300 cfu/g	Annex R
E. Coli	Negative in 1g	Negative in 1g	Annex S
Salmonella Spp.	Negative in 25g	Negative in 25g	Annex T

Note – Colony forming unit (CFU) is equivalent to colonies per gram.

9 Labelling

9.1 Each package of carrageenan shall be labeled with the following information:
9.1.1 Name of product
9.1.2 Name and address of manufacturer/processor
9.1.3 Lot/batch number
9.1.4 Net mass in kilogram
9.2 The following information are recommended to be included on the label:
9.2.1 Precautionary measure such as:
9.2.2 “Store in a cool and dry place”
9.2.3 “Use no hook”
9.3 Type or code

Annex A

(Informative)

Processes for Carrageenan and PES

C1 Carrageenan is obtained by extraction from seaweed into water or aqueous dilute alkali. Carrageenan may be recovered by alcohol precipitation, by drum drying, or by precipitation in aqueous potassium chloride and subsequent freezing. The alcohols used during recovery and purification are restricted to methanol, ethanol and isopropanol. Articles of commerce may include sugars for standardization purposes, salts to obtain specific gelling or thickening characteristics, or emulsifiers carried over from drum drying processes.

C2 PES is obtained by soaking the clean seaweed in alkali for a period of time at controlled temperatures. The material is then thoroughly washed with water to remove residual salts and excess alkali followed by drying and milling.

Annex B

Solubility in Water & Insolubility in ethanol (Carrageenan)

Insoluble in ethanol; soluble in water at a temperature of about 80º, forming a viscous clear or slightly opalescent solution that flows readily; disperses in water more readily if first moistened with alcohol, glycerol, or a saturated solution of glucose or sucrose in water.

Solubility in Water & Insolubility in ethanol (PES)

Forms cloudy viscous suspensions in water; insoluble in ethanol.
A 1 g sample disperses and partially dissolves in 100 ml of water at 80^{o} giving a cloudy opalescent solution. (The sample disperses in water more readily if first moistened with alcohol, glycerol, or a saturated solution of glucose or sucrose in water).

Annex C

Identification of Hydrocolloid and Predominant Type of Copolymer

Add 4 g of sample to 200 ml of water, and heat the mixture in a water bath at 80^{o}, with constant stirring until dissolved. Replace any water lost by evaporation, and allow the solution to cool to room temperature. The solution becomes viscous and may form a gel. To 50 ml of the solution or gel, add 200 mg of potassium chloride, then reheat, mix well, and cool. A short-textured ("brittle") gel indicates a carrageenan of a predominantly kappa-type. A compliant ("elastic") gel indicates a predominantly iota-type. If the solution does not gel, the carrageenan is of predominantly lambda type.

Annex D_1

Infrared Absorption Spectra Test for Carrageenan

Obtain infrared absorption spectra on the gelling and non-gelling fractions of the sample by the following procedure:

Disperse 2 g of the sample in 200 ml of 2.5% potassium chloride solution, and stir for 1 h. Let stand overnight, stir again for 1 h, and transfer into a centrifuge tube. (If the transfer cannot be made because the dispersion is too viscous, dilute with up to 200 ml of the potassium chloride solution.) Centrifuge for 15 min at approximately 1000 x g.

Remove the clear supernatant, resuspend the residue in 200 ml of 2.5% potassium chloride solution, and centrifuge again. Coagulate the combined supernatants by adding 2 volumes of 85% ethanol or isopropanol (NOTE: Retain the sediment for use as directed below). Recover the coagulum, and wash it with 250 ml of the alcohol. Press the excess liquid from the coagulum, and dry it at 60^{o} for 2 h. The product obtained is the non-gelling fraction (lambda-carrageenan).

Disperse the sediment (retained above) in 250 ml of cold water, heat at 90° for 10 min, and cool to 60^{o}. Coagulate the mixture, and then recover, wash, and dry the coagulum as described above. The product obtained is the gelling fraction (kappa- and iota-carrageenan).

Prepare a 0.2% aqueous solution of each fraction, cast films 0.5 mm thick (when dry) on a suitable non-sticking surface such as Teflon, and obtain the infrared absorption spectrum of each film. (Alternatively, the spectra may be obtained using films cast on potassium bromide plates, if care is taken to avoid moisture).

Carrageenan has strong, broad absorption bands, typical of all polysaccharides, in the 1000 to 1100 cm^{-1} region. Absorption maxima are 1065 and 1020 cm^{-1} for gelling and non-gelling types, respectively. Other characteristic absorption bands and their intensities relative to the absorbance at 1050 cm^{-1} are as follows:

Wave number (cm^{-1})	**Molecular Assignment**	**Absorbance relative to 1050 cm^{-1}**		
		Kappa	**Iota**	**Lambda**
1220 – 1260	ester sulfate	0.2 – 1.2	1.2 – 1.6	1.4 – 2.0
928 – 933	3,6 anhydrogalactose	0.2 – 0.6	0.2 – 0.4	0 – 0.2
840 – 850	galactose – 4-sulfate	0.1 – 0.5	0.2 – 0.4	-
825 – 830	galactose – 2-sulfate	-	-	0.2 – 0.4
810 – 820	galactose – 6-sulfate	-	-	0.1 – 0.3
800 - 805	3,6- anhydrogalactose-2-sulfate	0 – 0.2	0.2 – 0.4	-

Annex D_2

Infrared Absorption Spectra Test for PES

Prepare a 0.2% aqueous solution of the sample. Cast films of 0.5 mm thickness (when dry) on a suitable non-sticking surface such as Teflon and obtain the infrared absorption spectrum of each film. (Alternatively, the spectra may be obtained using films cast on potassium bromide plates if care is taken to avoid moisture).

Iota- and kappa-carrageenan have strong, broad absorption bands, typical of all polysaccharides, in the 1000 to 1100 cm^{-1} region. Other characteristic absorption bands and their intensities relative to the absorbance at 1050 cm^{-1} are as follows:

Wave number (cm^{-1})	**Molecular Assignment**	**Absorbance relative to 1050 cm^{-1}**	
		Kappa	**Iota**
1220 -1260	ester sulfate	0.6-1.0	0.9-1.2
928 - 933	3,6 anhydrogalactose	0.3-0.6	0.2-0.6
840 - 850	galactose – 4-sulfate	0.3-0.5	0.2-0.4
800 - 805	3,6- anhydrogalactose-2-sulfate	0.0-0.2	0.2-0.4

Annex E

Loss on Drying

Loss on drying (105°C to constant weight)

Loss on drying is the amount of volatile matter expelled under the conditions specified in the monograph. Because the volatile matter may include materials other than water, this test is designed for compounds in which the loss on drying may not definitely be attributable to water alone. The water content may be determined by a method such as *Karl Fischer* titration method

Note: *Suitable precautionary steps should be taken when weighing hygroscopic or deliquescent samples to ensure that they do not absorb moisture.*

Unless otherwise directed in the individual monograph, conduct the determination on 1 to 2 g of the substance, previously well mixed and accurately weighed. Reduce the sample to a fine powder when it occurs as crystals. Tare a glass-stoppered, shallow weighing bottle that has been dried for 30 min under the same conditions as will be employed in the determination. Transfer the sample into the bottle, replace the cover, and weigh the bottle and the sample. Distribute the sample as evenly as practicable to a depth of about 5 mm, and not over 10 mm in the case of bulky materials. Place the bottle with its contents in the drying chamber, removing the stopper and leaving it also in the chamber, and dry the sample at the temperature and for the time specified in the monograph. Upon opening the chamber, close the bottle promptly and allow it to come to room temperature in a desiccator before weighing.

If the substance melts at a lower temperature than that specified for the determination of *Loss on Drying*, prepare the sample as described above, then place it in a vacuum desiccator containing sulfuric acid. Evacuate the desiccator to 130 Pa (1 mm of mercury), maintain this vacuum for 24 h, and then weigh the dried sample.

Annex F

Residual Alcohol

D1 Standard Alcohol Solution

Transfer 500 mg each of chromatographic quality methanol, ethanol, and isopropanol into a 50 ml volumetric flask, dilute to volume with water, and mix. Pipet 10 ml of this solution into a 100-ml volumetric flask, dilute to volume with water and mix.

D2 TBA Standard Solution

Transfer 500 mg of chromatographic quality tertiary-butyl alcohol into a 50-ml volumetric flask, dilute to volume with water, and mix. Pipet 10 ml of this solution into a 100-ml volumetric flask, dilute to volume with water and mix.

D3 Mixed Standard Solution

Pipet 4 ml each of the Standard Alcohol Solution and of the TBA Standard Solution into a 125-ml graduated Erlenmeyer flask, dilute to about 100 ml with water, and mix. This solution contains approximately 40 µg of each alcohol per ml.

D4 Sample Preparation

Disperse 1 ml of a suitable antifoam emulsion, such as Dow-Corning G-10 or equivalent, in 200 ml of water contained in a 1000-ml 24/40 round-bottom distilling flask. Add about 5 g of the sample, accurately weighed, and shake for 1 h on a wrist-action mechanical shaker. Connect the flask to a fractionating column, and distil about 100 ml, adjusting the heat so that foam does not enter the column. Add 4.0 ml of TBA Standard Solution to the distillate to obtain the Sample Preparation.

D5 Procedure

Inject 5 µl of the Mixed Standard Solution into a suitable gas chromatograph equipped with a flame-ionization detector and a 1.8-m x 3.2-mm stainless steel column packed with 80/100-mesh Porapak QS or equivalent. The carrier is helium flowing at 80 ml per min. The injection port temperature is 200°; the column temperature is 165°; and the detector temperature is 200°. The retention time of isopropanol is about 2 min, and that of tertiary-butyl alcohol about 3 min.

Measure the areas of the methanol, ethanol, isopropanol, and TBA peaks. Calculate each response factor, f_i, by the formula A_i / A_{TBA}, in which A_i is the area of each alcohol peak (I = methanol, ethanol, or isopropanol).
Similarly, inject 5 µl of the Sample Preparation, and measure the peak areas, recording the area of each alcohol peak as A_i, and that of the tertiary-butyl alcohol peak as A_{TBA}.

Calculate each alcohol content, in mg/kg, in the sample taken by the formula:

$$A_i \cdot 4000 / f_i \cdot A_{TBA} \cdot W$$

where W is the weight of the sample taken (grams).

Annex G

Determination of pH (Potentiometric Method)

The pH of an aqueous solution may be determined accurately by potentiometry using a pH meter. The practical definition of pH in water may be given by the equation:
$pH = pHo + [(E - Eo)/0.0591]$, where pH is the value for the solution being measured, pH_o is the value for a standard buffer, E is the potential value for the solution being measured, E_o is the potential value for the standard buffer, and 0.0591 is the value at 25° of the Nernstian constant. The equation does not apply to solvents other than water, or to mixed solvents that include water. However, the pH meter gives reproducible readings in other solvent systems, on the basis of calibration with aqueous buffers, and while the pH readings lack thermodynamic significance they are useful in setting specifications.

The measurement of pH using a pH meter is a matter of comparing the meter reading of an unknown solution with the meter readings of standard buffers whose pH values are accurately known. Standard buffer solutions are described in compendia, such as the Merck Index. Routine measurement uses only one buffer and an approximation of the electrode slope, usually made by a temperature compensator, pH measurement accurate to ± 0.05 pH unit or better requires the use of two buffers that bracket, if possible, the expected pH range. All samples and buffer should be at the same temperature.

The choice and care of glass and reference electrodes must be carefully considered. The ordinary glass electrode begins to be sensitive to alkali metal cations at pH values above about 9, leading to the so-called alkaline error. Electrodes with a greatly reduced alkaline error should be used for readings in the alkaline range. Store the electrodes in distilled water when not in use, in order to avoid dehydration. "Flow-type" electrodes may be used if evidence of validity of pH measurement with the electrode is demonstrated.

The measurement of the pH of "highly buffered solutions" (distilled water or solutions of non-ionic organic compounds in distilled water) is a particularly difficult measurement. The addition of 0.3 ml of a saturated solution of potassium chloride per 100 ml of distilled water helps by providing a small amount of electrolyte. However, it will usually be necessary to protect the solution being measured from the carbon dioxide in air by use of a blanket of nitrogen during the measurement. Measure the pH of successive portions of the distilled water or test solutions, with vigorous agitation, until the observed results for two successive portions agree within 0.1 pH unit.

Procedure

Use a suitable pH meter and follow the manufacturer's instructions. Each time the electrodes are used, rinse them with distilled or deionised water and carefully blot them dry with clean absorbent tissue. Form a fresh reference electrode liquid junction. Rinse the sample vessel three times with each new solution to be introduced.

Choose two standard buffers (standard buffer solutions are described in compendia, such as the Merck Index) to bracket, if possible, the anticipated pH of the unknown. Warm or cool these standards as necessary to match within 2° the temperature of the unknown, and initially set the

temperature compensator to that temperature. Immerse the electrodes in a portion of the first standard buffer, and following the manufacturer's instructions adjust the appropriate standardization control (knob, switch, or button) until the pH reading is that of the buffer. Repeat this procedure with fresh portions of the first standard buffer until two successive readings are within ± 0.02 pH unit without an adjustment of the standardization control.

Rinse the electrodes, blot dry, and immerse them in a portion of the second standard buffer of lower pH. Do not change the setting of the standardization control. Following the manufacturer's instructions, adjust the slope control (thumbwheel switch, knob, or temperature compensator) until the exact buffer pH is displayed.

Repeat the sequence of standardization with both buffers until the pH readings are within ± 0.02 pH unit for both buffers without any adjustment of either control (the amount of sample to be used in sample preparation is given where applicable in the individual specification.). The pH of the unknown solution may then be measured (The difference between the results of two pH determinations when carried out simultaneously on in rapid succession by the same analyst, under the same conditions, should not exceed 0.05 pH unit.).

Always re-standardize the instrument after even a short period during which the amplifier is turned off.

Annex H_1

Viscosity Test for Carrageenan

Transfer 7.5 g of the sample into a tared, 600-ml tall-form (Berzelius) beaker, and disperse with agitation for 10 to 20 min in 450 ml of deionized water. Add sufficient water to bring the final weight to 500 g, and heat in a water bath with continuous agitation, until a temperature of 80° is reached (20 - 30 min). Add water to adjust for loss by evaporation, cool to 76-77°, and heat in a constant temperature bath at 75°. Pre-heat the bob and guard of a Brookfield LVF or LVT viscometer to approximately 75º in water. Dry the bob and guard, and attach them to the viscometer, which should be equipped with a No. 1 spindle (19 mm in diameter, approximately 65 mm in length) and capable of rotating at 30 rpm. Adjust the height of the bob in the sample solution, start the viscometer rotating at 30 rpm and, after six complete revolutions of the viscometer, take the viscometer reading on the 0-100 scale.

If the viscosity is very low, increased precision may be obtained by using the Brookfield UL (ultra low) adapter or equivalent. (Note. Samples of some types of carrageenan may be too viscous to read when a No. 1 spindle is used. Such samples obviously pass the specification, but if a viscosity reading is desired for other reasons, use a No. 2 spindle and take the reading on the 0-100 scale or on the 0-500 scale.)

Record the results in centipoises, obtained by multiplying the reading on the scale by the factor given by the Brookfield manufacturer.

Annex H_2

Viscosity Test for PES

Transfer 7.5 g of the sample into a tared, 600-ml tall-form (Berzelius) beaker, and disperse with agitation for 10 to 20 min in 450 ml of deionized water. Add sufficient water to bring the final weight to 500 g and heat in a water bath, with continuous agitation, until a temperature of 80° is reached (20-30 min). Add 7.5 g of diatomaceous earth or perlite filter aid.

Stir for two minutes. Add water to adjust for loss by evaporation. Filter the solution through a Büchner funnel (pre-heated with hot water to 80°) equipped with a coarse filter paper. Place the filter assembly in a vacuum receiver bottle.

Filter 200 ml of solution. Cool to 76-77°, and heat in a constant temperature bath at 75°. Pre-heat the bob and guard of a Brookfield LVF viscometer to approximately 75° in water. Dry the bob and guard and attach them to the viscometer, which should be equipped with a No. 1 spindle (19 mm in diameter, approximately 65 mm in length) and capable of rotating at 30 rpm. Adjust the height of the bob in the sample solution, start the viscometer rotating at 30 rpm and, after six complete revolutions of the viscometer, take the viscometer reading on the 0-100 scale.

If the viscosity is very low, increased precision may be obtained by using the Brookfield UL (ultra low) adapter or equivalent.

Record the results in centipoises, obtained by multiplying the reading on the scale by the factor given by the Brookfield manufacturer.

Annex I

Sulfate (as SO_4)

Principle

Hydrolysed sulfate groups are precipitated as barium sulfate.

Procedure

(a) Disperse an accurately weighed 8 g sample of commercial product into 400 ml of 60% w/w isopropanol/water at room temperature. Stir gently for 4 h. Filter through ash-free filter paper. Discard the filtrate. Wash the material remaining on the filter paper with two 10-ml portions of 60% isopropanol/water. Dry the material at 105° to constant weight.
Approximately 1 g of the dried matter is to be used for part (b). The remainder should be retained for determination of Total ash and Acid-insoluble matter.

(b) Accurately weigh a 1 g sample (W_1) obtained from part (a). Transfer the sample to a 100-ml long-neck round-bottom flask. Add 50 ml of 0.2 N hydrochloric acid. Fit a condenser, preferably one with at least 5 condensing bulbs, to the flask and reflux for 1 h. Add 25 ml of a 10% (by volume) hydrogen peroxide solution and resume refluxing for about 5 h or until the solution becomes completely clear. Transfer the solution to a 600-ml beaker, bring to a boil, and add dropwise 10 ml of a 10% barium chloride solution. Heat the reaction mixture for 2 h on a boiling water bath. Filter the mixture through ash-free slow-filtration filter paper. Wash with boiling distilled water until the filtrate is free from chloride. Dry the filter paper and contents in a drying oven. Gently burn and ash the paper at 800° in a tared porcelain or silica crucible until the ash is white. Cool in a desiccator.

Weigh the crucible containing the ash. Calculate the percentage sulfate from the weight in g (W_2) of the ash (barium sulfate) using the formula:

$$(W_2/W_1) \times 100 \times 0.4116$$

Annex J

Total Ash

Accurately weigh 2 g of the dried sample (W_1) obtained from part (a) under the procedure for sulfate determination in Annex I. Transfer to a previously ignited, tared silica or platinum crucible. Heat the sample with a suitable infrared lamp, increasing the intensity gradually, until the sample is completely charred; continue heating for an additional 30 min. Transfer the crucible with the charred sample into a muffle furnace and ignite at about 550º for 1 h. Cool in a desiccator and weigh. Repeat the ignition in the muffle furnace until a constant weight (W_2) is obtained. If a carbon-free ash is not obtained after the first ignition, moisten the charred spot with a 1-in-10 solution of ammonium nitrate and dry under an infrared lamp. Repeat the ignition step.

Calculate the percentage of total ash of the sample:

$$(W_2/W_1) \times 100$$

Retain the ash for the Acid-insoluble ash test.

Annex K

Acid-insoluble ash

Boil the ash obtained as directed under Ash (Total) in Annex J, with 25 ml of dilute hydrochloric acid TS for 5 min, collect the insoluble matter on a suitable ash-less filter, wash with hot water, ignite at 800 ± 25°, cool, and weigh. Calculate the percentage of acid-insoluble ash from the weight of the sample taken.

Annex L

Acid Insoluble Matter

Transfer 2 g of the sample, accurately weighed, into a 250-ml beaker containing 150 ml of water and 1.5 ml of sulfuric acid TS. Cover the beaker with a watch glass and heat the mixture on a steam bath for 6 h rubbing down the wall of the beaker frequently with a rubbertipped stirring rod and replacing any water lost by evaporation. Weigh 500 mg of a suitable acid washed filter aid, pre-dried at 105° for 1 h, to the nearest 0.1 mg, add this to the sample solution and filter through a tared Gooch crucible provided with an asbestos pad. Wash the residue several times with hot water, dry the crucible and its contents at 105° for 3 h, cool in a desiccator and weigh. The difference between the total weight and the weight of the filter aid plus crucible and pad is the weight of the Acid-insoluble matter. Calculate as percentage.

Annex M

Determination of Arsenic

Measurement of Arsenic and Antimony by Atomic Absorption Hydride Technique

Arsenic and antimony are determined after preparation of their volatile hydrides which are collected either in the generation vessel itself or, in some designs, in a rubber balloon attached to the vessel. The gases are then expelled with Argon into a hydrogen flame.

Preparation of standard curve solution
Into a series of 100-ml volumetric flasks add from a burette, 0, 1, 2, 3, 4 and 5 ml of standard arsenic or antimony solution [Standards (g) and (d)] and dilute to about 50 ml with distilled water. Add 8 ml 98% sulfuric acid TS and 10 ml hydrochloric acid [1.18 specific gravity]. Shake to dissolve, and when solution is complete, dilute to the mark with distilled water.

Instrumental conditions
Using the atomic absorption spectrophotometer with the appropriate hollow cathode or electrode-less discharge lamp, select the wavelength for either arsenic (193.7 nm) or antimony (217.6 nm).

Procedure
Measure 5.0 ml of the strongest standard into the generation vessel, add 25 ml of water and 2 ml 5 N hydrochloric acid. Stopper the vessel and expel any air as described in the maker's instructions, filling the apparatus with Argon. Isolate the vessel from the atomizer using the by-pass valve. Remove the atomizer and then quickly add 1 pellet (about 0.2 g) of sodium borohydride and replace the stopper. Ensure that all the joints are secure.

When the reaction slows (20 - 30 sec) open the appropriate taps to allow the Argon to drive the generated hydride into the flame. When the hydride has all been expelled as shown by the recorder trace, return the taps to their original position and empty the vessel.

Optimize the instrument settings to give full scale deflection for the strongest standard. Measure the other standards, the sample and the blank solution using the same procedure.

Plot a graph relating peak height on the recorder to concentration of the arsenic or antimony in the standards. Using the net absorbance of the sample, read the concentration of arsenic or antimony in the solution from the graph .

Calculation
Arsenic or antimony in the sample (mg/kg) =

[Concentration of arsenic or antimony (µg/ml) x 50] / [Weight of sample taken (g)]

Annex N

Determination of Lead

Principle

The sample is wet-ashed with nitric and perchloric acids and analysed using flame atomic absorption spectrophotometry.

Equipment

Atomic absorption spectrophotometer

Reagents

Nitric acid, concentrated, Reagent Grade
Perchloric acid, concentrated, Reagent Grade
Hydrochloric acid, concentrated, Reagent Grade
Lead standard solution (certified)

Solutions

Stock solution (1 mg/ml): Dilute an appropriate volume of certified reagent lead standard solution with distilled and deionized water (D/D water) to make one liter.

Intermediate solutions: (a) 100 µg/ml. Pipet 10 ml of the stock solution into a 100-ml volumetric flask and dilute to volume with D/D water. (b) 10 µg/ml. Pipet 10 ml of the 100 µg/ml solution into a 100-ml volumetric flask and dilute to volume with D/D water.

Working solutions: Assemble four 100-ml volumetric flasks and transfer to them (pipet), respectively, 1, 5, 10, and 20 ml of intermediate lead solution (b). Dilute to volume with D/D water to make solutions containing 0.1, 0.5, 1, and 2 µg Pb/ml.

Sample preparation

(CAUTION: This procedure employs concentrated oxidizing acids and results in evolution of noxious gases. Perform operations in a fume hood.)

Accurately weigh 7.5 grams of a representative dry powdered test sample into a 250-ml Erlenmeyer flask. Set up a reagent blank and carry through the same operations as performed on the test sample. Wet the test sample with ca. 10 ml of D/D water and add 25 ml of nitric acid. Heat gently on a hot plate (100^{o} - 150^{o}) until most of the dark fumes are evolved (ca. one hour); swirl the flask occasionally. Cool and add 5 ml of perchloric acid; particles become visible at this stage. Heat gently (hot plate, 100^{o} - 150^{o}) to concentrate until the solution turns yellowish or colourless (ca. one hour). Midway during the heating, if the solution darkens, slowly add 2-3 ml portions of nitric acid as necessary until the desired colour is achieved; do not let the solution go

to dryness. Cool the digest and wash the sides of the flask with ca. 5 ml of D/D water and swirl. Add 2 ml of hydrochloric acid. Heat again until all brown fumes are evolved and the solution is white to yellowish in colour; do not let the solution go to dryness. Cool the solution and wash the sides of the flask with ca. 10 ml of D/D water. Transfer the slightly viscous solution to a 50-ml volumetric flask and dilute to volume with D/D water. Filter using two layers of filter paper (Whatman no. 5 or equivalent).

Determination

Set the spectrophotometer to previously established optimum conditions at 283.3 nm using an air/acetylene oxidizing flame. Measure the absorbance of the sample, blank, and working solutions. Prepare a standard curve by plotting absorbance against μg Pb/ml for the blank and working solutions. Determine the concentration of lead in the sample solution from the standard curve.

The concentration of lead in the test sample (mg Pb/kg) is:

$$[Pb] = F \times A/B$$

where A is the concentration of lead in the sample solution (μg/ml), B is the weight of the test sample (grams), and F is the dilution factor (50 ml).

Annex O

Determination of Mercury

Principle

The sample is wet-ashed with nitric and perchloric acids and analysed using hydride-generation atomic absorption spectrophotometry.

Equipment

Atomic absorption spectrophotometer equipped with a hydride vapour generator. Integral to the generator is a reactor tube or coil and a peristaltic pump with dual tubing channels: one channel for the sample solution and one for the two reagent solution tubes. Flow control is determined by tubing size and tubing clamps. Flow rates are measured at the exit of the hydride generator.

Reagents

Nitric acid, concentrated, Reagent Grade
Perchloric acid, concentrated, Reagent Grade
Hydrochloric acid, concentrated, Reagent Grade
Sodium borohydride, >98%
Sodium hydroxide, Reagent grade
Mercury standard solution (certified)

Solutions

Nitric acid-perchloric acid (1:1): Mix equal volumes of the two acids.

Hydrochloric acid, 5M: Dilute 417 ml concentrated hydrochloric acid to 1 liter with deionized water.

Sodium borohydride solution, 0.4% (Prepare immediately before use.): First, dissolve 2.5 g sodium hydroxide in deionized water. Then, add and dissolve 2.0 g sodium borohydride. Dilute to 500 ml.

Stock solution (1 mg/ml): Dilute an appropriate volume of certified reagent mercury standard solution with distilled and deionized water (D/D water) to make one liter.

Intermediate solutions: (a) 10,000 µg/l. Pipet 1 ml of the stock solution into a 100-ml volumetric flask and dilute to volume with D/D water. (b) 100 µg/l. Pipet 1 ml of the 10,000 µg/l solution into a 100-ml volumetric flask and dilute to volume with D/D water.
Working solutions: Assemble five 100-ml volumetric flasks and transfer to them (pipet), respectively, 1, 5, 10, 15, and 20 ml of intermediate solution (b). To each, add 10 ml of 1:1 nitric

acid-perchloric acid and dilute to volume with D/D water to make solutions containing 1, 5, 10, 15, and 20 µg Hg/l.

Sample preparation

(CAUTION: This procedure employs concentrated oxidizing acids and results in evolution of noxious gases. Perform operations in a fume hood.)

Accurately weigh 5 grams of a representative dry powdered test sample into a 250 ml Erlenmeyer flask. Set up a reagent blank and carry through the same operations as performed on the test sample. Wet the test sample with 5 ml of D/D water and then add 10 ml of 1:1 nitric acid-perchloric acid. Heat gently on a hot plate (100°-150°) until all of the dark fumes are evolved and the solution turns yellowish or colourless; swirl the flasks occasionally. Do not let the solution go to dryness. Cool and wash the sides of the flask with a small amount of D/D water. (Some particles may be visible.) Cover the flask lightly and let the slightly viscous solution stand overnight. Transfer the solution to a 50-ml volumetric flask and dilute to volume with D/D water. Filter using 2 layers of Whatman no. 5 (or equivalent) filter paper into a 100-ml Erlenmeyer flask. Immerse the flask in an ultrasonic bath and sonicate it for 10 minutes or until bubbles no longer form on the surface of the solution.

Determination

Calibrate (using water) the peristaltic pump to provide a flow rate of the sample solution of 8 ml/min and a combined flow rate for the two reagent solutions (sodium borohydride and 5M hydrochloric acid) of 2 ml/min. (The combined flow rate is achieved with a single pump setting.) Set the spectrophotometer to previously established optimum conditions at the mercury lamp wavelength of 253.7 nm.

Transfer suitable quantities of the two reagent solutions into separate graduated cylinders. Insert separate aspirator tubing leading from the peristaltic pump into each reagent solution and into the sample flask.
Start the flow of argon carrier gas (tank outlet pressure: 3.2±0.2 kg/cm2) through the hydride vapour generator of the spectrophotometer. Start the pump to initiate flow of the three solutions into the hydride generator manifold where they are mixed and pass into the reactor coil to generate atomic mercury, which is carried into the absorbance cell of the spectrophotometer. Measure the absorbance for the sample. Repeat for the blank solution and each of the working standards.
Prepare a standard curve by plotting absorbance against µg Hg/l for the blank and working solutions. Determine the concentration of mercury in the sample solution from the standard curve.

The concentration of mercury in the test sample (mg Hg/kg) is:

$$[Hg] = FxA/1000B$$

where A is the concentration of mercury in the sample solution (µg /l), B is the weight of the test sample (grams), and F is the dilution factor (50 ml).

Annex P

Determination of Cadmium

Proceed as directed in Annex N for the determination of lead, using 228.8 nm as the analysis wavelength. Intermediate and working solutions are prepared from certified reagent cadmium standard solution as follows:

Intermediate solutions: (a) 100 μg/ml. Pipet 10 ml of the stock solution (1mg/ml) into a 100-ml volumetric flask and dilute to volume with distilled and deionized (D/D) water. (b) 10 μg/ml. Pipet 10 ml of solution (a) into a 100-ml volumetric flask; dilute to volume with D/D water. (c) 1 μg/ml. Pipet 1 ml of solution (a) into a 100-ml volumetric flask; dilute to volume with D/D water.

Working solutions: Assemble five 50-ml volumetric flasks and transfer to them (pipet), respectively, 0.5, 2.5, 5.0, 10, and 20 ml of intermediate solution (c). Dilute to volume with D/D water to make solutions containing 0.01, 0.05, 0.1, 0.20, and 0.40 μg Cd/ml.

The concentration of cadmium in the test sample (mg Cd/kg) is:

$$[Cd] = F \times A/B$$

where A is the concentration of cadmium in the sample solution (μg/ml), B is the weight of the test sample (grams), and F is the dilution factor (50 ml).

Annex Q

Total (Aerobic) Plate Count

Equipment and materials

1. Work area, level table with ample surface in clean, well-lighted (100 foot-candles at working surface) and well-ventilated room that is reasonably free of dust and drafts. The microbial density, measured in fallout pour plates taken during plating, of air in working area should not exceed 15 colonies /per plate during 15 min exposure.
2. Petri dishes, glass (15 x 100 mm) or plastic (15 x 90 mm).
3. Pipets, 1, 5, and 10 ml, graduated in 0.1 ml units.
4. Dilution bottles, 6 oz (160 ml), borosilicate-resistant glass, with rubber stoppers or plastic screw caps.
5. Water bath, for tempering agar, thermostatically controlled to 45 ± 1°.
6. Incubator, 35 ± 1°.
7. Colony counter, dark-field, Quebec, or equivalent, with suitable light source and grid plate.
8. Tally register.
9. Thermometers appropriate range; accuracy checked.
10. Dilution blanks, 90 ± 1 ml Butterfield's phosphate-buffered dilution water

Media and reagents

1. Butterfield's phosphate-buffered dilution water.
2. Plate count agar.

Procedure

Using separate sterile pipets, prepare decimal dilutions of 10^{-2}, 10^{-3}, 10^{-4}, and others as appropriate, of sample homogenate by transferring 10 ml of previous dilution to 90 ml of diluent. Avoid sampling foam. Shake all dilutions 25 times in 30 cm (1 ft) arc within 7 sec. Pipet 1 ml of each dilution into separate, duplicate, appropriately marked petri dishes. Reshake dilution bottle 25 times in 30 cm arc within 7 sec if dilution stands more than 3 min before pipeting test portion into petri dish. Add 12-15 ml plate count agar (cooled to 44-46°) to each plate within 15 min of original dilution. Add agar immediately to petri dishes when sample diluent contains hygroscopic materials. Pour agar and dilution water control plates for each series of samples. Immediately mix sample dilutions and agar medium thoroughly and uniformly by alternate rotation and back-and-forth motion of plates on flat level surface. Let agar solidify, invert petri dishes, and incubate promptly for 48 ± 2 h at 35°.

After incubation, count duplicate plates in suitable range (25-250 colonies), using colony counter and tally register; record results per dilution plate counted. Duplicate plates of at least 1 of 3 dilutions should be in 25-250 colony range. When only 1 dilution is in appropriate range, compute average count per g for dilution and report as total plate count per g (see Table 1, Sample No. 1). When 2 dilutions are in appropriate range, determine average count per dilution before averaging 2 dilution counts to obtain total plate count per g (see Table 1, Sample No. 2). If none or only one of duplicate plates of required dilution yields 25-250 colonies, proceed as in

"Guidelines", below. Round off counts to two significant figures only at time of conversion to total plate counts. When rounding off numbers, raise second digit to next higher number only when third digit from left is 5 or greater, and replace dropped digit with zero. If third digit is 4 or less, replace third digit with zero and leave second digit the same.

Guidelines for calculating and reporting total plate counts in uncommon cases

Report all total plate counts computed from duplicate plates containing less than 25 or more than 250 colonies as estimated counts. Use the following as a guide:

- *Plates with fewer than 25 colonies*. When duplicate plates of lowest dilution have fewer than 25 colonies, count actual number on each duplicate of that dilution, average the number of colonies per plate, and multiply by dilution factor to obtain estimated total plate count. Mark total plate count with asterisk to denote that it was estimated from counts outside 25-250 per plate range (see Table 1, Sample No. 3).

- *Plates with more than 250 colonies*. When number of colonies per plate exceeds 250, count colonies in those portions of plate that are representative of colony distribution. Mark calculated total plate count with asterisk to denote that it was estimated from counts outside 25-250 per plate range (see Table 1, Sample No. 4).

- *Spreaders*. Spreading colonies are usually of 3 distinct types: 1) a chain of colonies, not too distinctly separated, that appears to be caused by disintegration of a bacterial clump; 2) one that develops in film of water between agar and bottom of dish; and 3) one that forms in film of water at edge or on surface of agar. If plates prepared from sample have excessive spreader growth such that (a) area covered by spreaders, including total area of repressed growth, exceeds 50% of plate area, or (b) area of repressed growth exceeds 25% of plate area, report plates as spreader. Determine average count for each dilution; report arithmetic average of these values as total plate count. (See Table 1, Sample No. 5). When it is necessary to count plates containing spreaders not eliminated by (a) or (b) above, count each of the 3 distinct spreader types as one source. For the first type, if only one chain exists, count it as a single colony. If one or more chains appear to originate from separate sources, count each source as one colony. Do not count each individual growth in such chains as a separate colony. Types 2 and 3 usually result in distinct colonies and are counted as such. Combine the spreader count and the colony count to compute the total plate count.

- *Duplicate plates, one with 25-250 colonies, the other with more than 250 colonies*. When one plate contains 25-250 colonies and the duplicate contains more than 250 colonies, count both plates and include the plate with more than 250 colonies in computing total plate count (see Table 1, Sample No. 6).

- *Duplicate plates, one plate of each dilution with 25-250 colonies*. When one plate of each dilution contains 25-250 colonies and the duplicate contains more than 250 colonies or fewer than 25 colonies, count all 4 plates and include plates with more than 250 or fewer than 25 colonies in computing the total plate count (see Table 1, Sample No. 7).

- *Duplicate plates, both plates of one dilution with 25-250 colonies and only one duplicate of the other dilution with 25-250 colonies*. When both plates of one dilution contain 25-250 colonies and only one duplicate of the other dilution contains 25-250 colonies, count all 4 plates and include the plate with fewer than 25 or the plate with more than 250 colonies in computing aerobic plate count (see Table 1, Sample No. 8). *Plates with no CFU*. When plates from all dilutions have no colonies, report APC as less than 1 times the corresponding lowest dilution used. Mark calculated APC with asterisk to denote that it was estimated from counts outside the 25-250 per plate range. When plate(s) from a sample are known to be contaminated or otherwise unsatisfactory, record the result(s) as laboratory accident (LA).

Table 1. Examples of computation of total plate count (2 plates/dilution poured)

Sample No.	**Colonies counted**			
	1:100	**1:1,000**	**1:1,000**	**Aerobic plate count/g**
1	TNTC	175	16	190,000
	TNTC	208	17	
2	TNTC	224	25	250,000
	TNTC	245	30	
3	18	2	0	1,600*
	14	0	0	
4	TNTC	TNTC	523	5,200,000*
	TNTC	TNTC	487	
5	TNTC	245	35	290,000
	TNTC	230	Spreader	
6	TNTC	245	23	260,000
	TNTC	278	20	
7	TNTC	225	21	270,000
	TNTC	255	40	
8	TNTC	210	18	230,000
	TNTC	240	28	
	TNTC	260	30	270,000
	TNTC	230	28	

*(Asterisk) : estimated count
TNTC : Too numerous to count. Colony count is significantly beyond count range of 250 colonies.

Underlined numbers are used to calculate aerobic plate count.

Annex R

Enumeration of Yeast and Molds

Equipment and materials

1. Basic equipment (and appropriate techniques) for preparation of sample homogenate
2. Equipment for plating samples
3. Incubator, 25°
4. Arnold steam chest
5. pH meter
6. Water bath, 45 ± 1°

Media and reagents

1. Potato dextrose agar (PDA)
2. Malt extract agar
3. Malt agar (MA)
4. Plate count agar (PCA), standard methods; add 100 mg chloramphenicol/liter when this medium is used for yeast and mould enumeration. This medium is not efficient when "spreader" moulds are present.
5. Antibiotic solutions
6. Dichloran rose bengal chloramphenicol (DRBC) agar
7. Dichloran 18% glycerol (DG18) agar

Procedures

Antibiotics

Antibiotics are added to mycological media to inhibit bacterial growth. Chloramphenicol is the antibiotic of choice, because it is stable under autoclave conditions. Therefore, media preparation is easier and faster due to the elimination of the filtration step. The recommended concentration of this antibiotic is 100 mg/liter medium. If bacterial overgrowth is apparent, prepare media by adding 50 mg/liter chloramphenicol before autoclaving and 50 mg/liter filter-sterilized chlortetracycline when the media have been tempered, right before pouring plates.

Prepare stock solution by dissolving 0.1 g chloramphenicol in 40 ml distilled water; add this solution to 960 ml medium mixture before autoclaving. When both chloramphenicol and chlortetracycline are used, add 20 ml of the above chloramphenicol stock solution to 970 ml medium before autoclaving. Then, prepare chlortetracycline stock solution by dissolving 0.5 g antibiotic in 100 ml distilled water and filter sterilize. Use 10 ml of this solution for each 990 ml of autoclaved and tempered medium. Refrigerate in the dark and re-use remaining stock solutions for up to a month. Stock solutions should be brought to room temperature before adding to tempered medium.

Sample Preparation

Analyze 25-50 g from each subsample; generally, larger sample sizes increase reproducibility and lower variance compared with small samples. Add appropriate amount of 0.1% peptone water to the weighed sample to achieve 10-1 dilution, then homogenize in a stomacher for two

min. Alternatively, blending for 30-60 sec can be used but is less effective. Make appropriate 1:10 (1+9) dilutions in 0.1% peptone water. Dilutions of 10-6 should suffice.

Plating and incubation of samples

Spread-plate method

Aseptically pipet 0.1 ml of each dilution on pre- poured, solidified DRBC agar plates and spread inoculum with a sterile, bent glass rod. DG18 is preferred when the water activity of the analyzed sample is less than 0.95. Plate each dilution in triplicate.

Pour-plate method

Use sterile cotton-plugged pipet to place 1 ml portions of sample dilutions into prelabelled 15 x 100 mm petri plates (plastic or glass), and immediately add 20-25 ml tempered DG18 agar medium. Mix contents by gently swirling plates clockwise then counter clockwise, taking care to avoid spillage on dish lid. Add agar within 1-2 min after adding dilution. Otherwise, dilution may begin to adhere to dish bottom (especially if sample is high in starch content and dishes are plastic) and may not mix uniformly. Plate each dilution in triplicate, using wide bore pipets. From preparation of first sample dilution to pouring of final plate, no more than 20 min, preferably 10 min, should elapse. Incubate plates in dark at 25°. Do not stack plates higher than 3 and do not invert. Let plates remain undisturbed until counting.

Counting of plates

Count plates after 5 days of incubation. If there is no growth at 5 days, re-incubate for another 48 h. Do not count colonies before the end of the incubation period because handling of plates could result in secondary growth from dislodged spores, making final counts invalid. Count plates containing 10-150 colonies. If mainly yeasts are present, plates with 150 colonies are usually countable. However, if substantial amounts of mould are present, depending on the type of mold, the upper countable limit may have to be lowered at the discretion of the analyst. Report results in colony forming units (CFU)/g or CFU/ml based on average count of triplicate set. Round off counts to two significant figures. If third digit is 6 or above, round off to digit above (e.g., 456 = 460); if 4 or below, round off to digit below (e.g., 454 = 450). If third digit is 5, round off to digit below if first two digits are an even number (e.g., 445 = 440); round off to digit above if last two digits are an odd number (e.g., 455 = 460). When plates from all dilutions have no colonies, report mould and yeast counts (MYC) as less than 1 times the lowest dilution used

Annex S

Coliforms and E. Coli

Equipment and materials

1. Covered water bath, with circulating system to maintain temperature of 45.5 ± 0.2°. Water level should be above that of medium in immersed tubes
2. Immersion-type thermometer, 1-55°, about 55 cm long, with 0.1° subdivisions, National Bureau of Standards certified, or equivalent
3. Incubator, 35 ± 1°
4. Balance with capacity of >= 2 kg and sensitivity of 0.1 g
5. Blender and blender jar
6. Sterile graduated pipets, 1.0 and 10.0 ml
7. Sterile utensils for sample handling
8. Dilution bottles made of borosilicate glass, with stopper or polyethylene screw caps equipped with Teflon liners. Commercially prepared dilution bottles containing sterile Butterfield's phosphate buffer can also be used.
9. Colony counter
10. pH meter

Media and reagents

1. Brilliant green lactose bile (BGLB) broth, 2%
2. Lauryl tryptose (LST) broth
3. EC broth
4. Levine's eosin-methylene blue (L-EMB) agar
5. Tryptone (tryptophane) broth
6. MR-VP broth
7. Koser's citrate broth
8. Plate count agar (PCA)
9. Butterfield's phosphate-buffered dilution water
10. Kovacs' reagent
11. Voges-Proskauer (VP) reagents
12. Gram stain reagents
13. Methyl red indicator

Presumptive test for coliform bacteria

Aseptically weigh 10 g sample into sterile, screw-cap jar. Add 90 ml diluent and shake vigorously (50 times through 30 cm arc) to obtain 10-1 dilution. Let stand 3-5 min and shake to re-suspend (5 times through 30 cm arc) just before making serial dilutions and inoculations.

Prepare all decimal dilutions with 90 ml sterile dilution water plus 10 ml from previous dilution unless otherwise specified. The dilutions to be prepared depend on the anticipated coliform density. Shake all suspensions 25 times in 30 cm arc for 7 sec. Do not use pipets to deliver <10% of their total volume. Transfer 1 ml portions to 3 LST tubes for each dilution for 3 consecutive dilutions. Hold pipet at angle so that its lower edge rests against tube. Let pipet drain 2-3 sec.

Not more than 15 min should elapse from time sample is blended until all dilutions are in appropriate media.

Incubate tubes 48 ± 2 h at 35°. Examine tubes at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate negative tubes for additional 24 h. Examine a second time for gas. Perform a confirmation test on all presumptive positive (gassing) tubes.

Confirmation test for coliforms

Gently agitate each gassing LST tube. Hold the LST tube at angle and insert a loop to avoid transfer of pellicle (if present). Transfer one loopful of suspension to a tube of BGLB broth. Incubate BGLB tubes 48 ± 2 h at 35°. Examine for gas production and record. Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for three consecutive dilutions.

Confirmation test for *E. coli*

Gently agitate each gassing LST tube and transfer a loopful of each suspension to tube of EC broth. Incubate EC tubes 48 ± 2 h at 45.5 ± 0.2°. Examine for gas production at 24 ± 2 h; if negative, examine again at 48 ± 2 h. Streak a loopful of suspension from each gassing tube to L-EMB agar. It is essential that 1 portion of plate exhibit well-separated colonies. Incubate 18-24 h at 35°. Examine plates for suspicious *E. coli* colonies, i.e., dark centered with or without metallic sheen. Pick two suspicious colonies from each L-EMB plate and transfer them to PCA agar slants for morphological and biochemical tests. Incubate PCA slants 18-24 h at 35°. If typical colonies are not present, pick 5-10 or more colonies deemed most likely to be *E. coli* , from every plate.

Perform gram stain. Examine all cultures appearing as gram-negative short rods or cocci for the following biochemical activities (the first four tests are collectively termed IMViC):

- *Indole production*. Inoculate tube of tryptone broth and incubate 24 ± 2 h at 35°. Test for indole by adding 0.2-0.3 ml Kovacs' reagent. Appearance of distinct red colour in the upper layer is positive test.
- *Voges-Proskauer-reactive compounds*. Inoculate tube of MR-VP broth and incubate 48 ± 2 h at 35°. Transfer 1 ml to 13 x 100 mm tube. Add 0.6 ml alpha-naphthol solution and 0.2 ml 40% KOH, and shake. Add a few crystal of creatine. Shake and let stand 2 h. Test is positive if eosin pink colour develops.
- *Methyl red-reactive compounds*. Incubate MR-VP tube additional 48 ± 2 h at 35° after Voges-Proskauer test. Add 5 drops methyl red solution to each tube. A distinct red colour is a positive test. Yellow is a negative reaction.
- *Use of citrate*. Lightly inoculate tube of Koser's citrate broth; avoid detectable turbidity. Incubate 96 ± 2 h at 35°. Development of distinct turbidity is positive reaction.
- *Production of gas from lactose*. Inoculate tube of LST broth and incubate 48 ± 2 h at 35°. Displacement of medium from inner vial or effervescence after gentle agitation is a positive reaction.

Interpretation
All cultures that (a) ferment lactose with production of gas within 48 h at 35°, (b) appear as Gram-negative non-sporeforming rods or cocci, and (c) give IMViC (the first four tests.) patterns of ++-- (biotype 1) or -+-- (biotype 2) are considered to be *E. coli*.

Alternatively, MPN determination of *E. coli*, fecal coliforms and coliforms protocols are given below.

Note: *Alternatively, instead of performing the IMViC tests, use commercially prepared biochemical strip tests. Use growth from PCA slants to perform these assays.*

MPN method (Alternative Method)

MPN - Presumptive test for coliforms, fecal coliforms and *E. coli*

Weigh 50 g into sterile high-speed blender jar. Add 450 ml of Butterfield's phosphate-buffered water and blend for 2 min. If <50 g of sample are available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades.

Prepare decimal dilutions with sterile Butterfield's phosphate diluent. The number of dilutions to be prepared depends on anticipated coliform density. Shake all suspensions 25 times in 30 cm arc or vortex mix for 7 s. Do not use pipets to deliver <10% of their total volume. Transfer 1 ml portions to three LST tubes for each dilution for at least three consecutive dilutions. Hold pipet at angle so that its lower edge rests against the tube. Let pipet drain 2-3 s. Not more than 15 min should elapse from time the sample is blended until all dilutions are inoculated in appropriate media.

Incubate LST tubes at 35°. Examine tubes and record reactions at 24 ± 2 h for gas, i.e., displacement of medium in fermentation vial or effervescence when tubes are gently agitated. Re-incubate gas-negative tubes for an additional 24 h and examine and record reactions again at 48 ± 2 h. Perform confirmed test on all presumptive positive (gas) tubes.

MPN - Confirmed test for coliforms

From each gassing LST tube, transfer a loopful of suspension to a tube of BGLB broth, avoiding pellicle if present. Incubate BGLB tubes at 35° and examine for gas production at 48 ± 2 h. Calculate most probable number (MPN) of coliforms based on proportion of confirmed gassing LST tubes for 3 consecutive dilutions.

MPN - Confirmed test for fecal coliforms and *E. coli*

From each gassing LST tube from the Presumptive test, transfer a loopful of suspension to a tube of EC broth (a sterile wooden applicator stick may also be used for these transfers). Incubate EC tubes 24 ± 2 h at 45.5° and examine for gas production. If negative, re-incubate and examine again at 48 ± 2 h. Use results of this test to calculate fecal coliform MPN. To continue with *E. coli* analysis, follow protocol for Completed test for *E. coli* (below).

Note: *Fecal coliform analyses are done at 45.5± 0.2°.*

MPN - Completed test for *E. coli*.

Gently agitate each gassing EC tube and streak for isolation, a loopful to a L-EMB agar plate and incubate for 18-24 h at 35°. Examine plates for suspicious *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Transfer up to five suspicious colonies from each L-EMB plate to PCA slants incubate for 18-24 h at 35° and use for further testing.

Note: *Identification of any one of the five colonies as E. coli is sufficient to regard that EC tube as positive; hence, not all five isolates may need to be tested.*

Perform Gram stain. All cultures appearing as Gram-negative short rods, should be tested for the IMViC reactions above and also re-inoculated back into LST to confirm gas production.

Annex T

Determination of Salmonella

Equipment and materials

1. Blender and sterile blender jars
2. Sterile, 16 oz (500 ml) wide-mouth, screw-cap jars, sterile 500 ml Erlenmeyer flasks, sterile 250 ml beakers, sterile glass or paper funnels of appropriate size.
3. Sterile, bent glass or plastic spreader rods
4. Balance, with weights; 2000 g capacity, sensitivity of 0.1 g
5. Balance, with weights; 120 g capacity, sensitivity of 5 mg
6. Incubator, 35 ± 2°
7. Refrigerated incubator or laboratory refrigerator, 4 ± 2°
8. Water bath, 49 ± 1°
9. Water bath, circulating, thermostatically-controlled, 43 ± 0.2°
10. Water bath, circulating, thermostatically-controlled,42 ± 0.2°
11. Sterile spoons or other appropriate instruments for transferring food samples
12. Sterile culture dishes, 15 x 100 mm, glass or plastic
13. Sterile pipets, 1 ml, with 0.01 ml graduations; 5 and 10 ml, with 0.1 ml graduations
14. Inoculating needle and inoculating loop (about 3 mm id or 10 5l), nichrome, platinum-iridium, chromel wire, or sterile plastic
15. Sterile test or culture tubes, 16 x 150 mm and 20 x 150 mm; serological tubes, 10 x 75 mm or 13 x 100 mm
16. Test or culture tube racks
17. Vortex mixer
18. Sterile shears, large scissors, scalpel, and forceps
19. Lamp (for observing serological reactions)
20. Fisher or Bunsen burner
21. pH test paper (pH range 6-8) with maximum graduations of 0.4 pH units per color change
22. pH meter

Media and reagents

1. Lactose broth
2. Nonfat dry milk (reconstituted)
3. Selenite cystine (SC) broth
4. Tetrathionate (TT) broth
5. Rappaport-Vassiliadis (RV) medium
6. Xylose lysine desoxycholate (XLD) agar
7. Hektoen enteric (HE) agar
8. Bismuth sulfite (BS) agar
9. Triple sugar iron agar (TSI)
10. Tryptone (tryptophane) broth
11. Trypticase (tryptic) soy broth
12. Trypticase soy broth with ferrous sulfate

13. Lauryl tryptose (LST) broth
14. Trypticase soy-tryptose broth
15. MR-VP broth
16. Simmons citrate agar
17. Urea broth
18. Urea broth (rapid)
19. Malonate broth
20. Lysine iron agar (LIA) (Edwards and Fife)
21. Lysine decarboxylase broth
22. Motility test medium (semisolid)
23. Potassium cyanide (KCN) broth
24. Phenol red carbohydrate broth
25. Purple carbohydrate broth
26. MacConkey agar
27. Nutrient broth
28. Brain heart infusion (BHI) broth
29. Papain solution, 5%
30. Cellulase solution, 1%
31. Tryptose blood agar base
32. Universal preenrichment broth
33. Buffered peptone water
34. Potassium sulfite powder, anhydrous
35. Chlorine solution, 200 mg/kg, containing 0.1% sodium dodecyl sulfate
36. Ethanol, 70%
37. Kovacs' reagent
38. Voges-Proskauer (VP) test reagents
39. Creatine phosphate crystals
40. Potassium hydroxide solution, 40%
41. 1 N Sodium hydroxide solution
42. 1 N Hydrochloric acid
43. Brilliant green dye solution, 1%
44. Bromcresol purple dye solution, 0.2%
45. Methyl red indicator
46. Sterile distilled water
47. Tergitol Anionic 7
48. Triton X-100
49. Physiological saline solution, 0.85% (sterile)
50. Formalinized physiological saline solution
51. *Salmonella* polyvalent somatic (O) antiserum
52. *Salmonella* polyvalent flagellar (H) antiserum
53. *Salmonella* somatic group (O) antisera: A, B, C_1, C_2, C_3, D_1, D_2, E_1, E_2, E_3, E_4, F, G, H, I, Vi, and other groups, as appropriate
54. *Salmonella* Spicer-Edwards flagellar (H) antisera

Procedure

Pre-enrichment

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 mix well, essentially preparing a 1:9 sample/broth ratio. Cap jar securely and let stand 60 ± 5 min at room temperature. Mix well by swirling and determine pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°.

Special cases

In some cases, the analysis of samples may be hampered by the viscosity of thickening agents. Additional treatment may be required.

- For gum ghatti, 0.1% of NaCl (final concentration) in lactose broth pre-enrichment medium, adjusted to pH 6.5, is added.
- For the analysis of gelatin, add 5 ml of a 5% papain solution (final concentration of 0.1%) in lactose broth pre-enrichment medium, mix well. Cap jar securely and incubate at 35° for 60 ± 5 min. Mix by swirling and adjust to pH 6.8, if necessary. Loosen jar cap about 1/4 turn and incubate 24 ± 2 h at 35°.
- For carob bean gum and guar gum, aseptically weigh 25 g sample into sterile beaker (250 ml) or other appropriate container. Prepare a 1.0%mannan endo-1,4- betamannosidase (EC 3.2.178) solution (add 1 g mannosidase to 99 ml sterile distilled water). Dispense into 150 ml bottles. Mannosidase solution may be stored at 2-5°C for up to 2 weeks. Add 225 ml sterile lactose broth and 2.25 ml sterile 1 % mannosidase solution to sterile, wide-mouth, screw-cap jar (500 ml) or other appropriate container. While vigorously stirring the mannosidase/lactose broth with magnetic stirrer, pour 25 g analytical unit quickly through sterile glass funnel into the mannosidase/lactose broth. Cap jar securely and let stand 60 ± 5 min at room temperature. Incubate loosely capped container without pH adjustment, for 24 ± 2 h at 35°.

Enrichment

For Carob Bean gum and Guar gum. Transfer 1 ml mixture to 10 ml selenite cystine (SC) broth and another 1 ml mixture to 10 ml TT broth. Vortex. Incubate SC and TT broths 24 ± 2 h at 35°.

For all other samples. Transfer 0.1 ml mixture to 10 ml Rappaport-Vassiliadis (RV) medium and another 1 ml mixture to 10 ml tetrathionate (TT) broth. Vortex.

1. Incubate selective enrichment media as follows:

   High microbial load. Incubate RV medium 24 ± 2 h at 42 ± 0.2° (circulating, thermostatically-controlled, water bath). Incubate TT broth 24 ± 2 h at 43 ± 0.2° (circulating, thermostatically-controlled, water bath).

Low microbial load (except carob bean gum and guar gum). Incubate RV medium 24 ± 2 h at 42 ± 0.2°C (circulating, thermostatically controlled, water bath). Incubate TT broth 24 ± 2 h at 35 ± 2.0°.

2. Mix (vortex, if tube) and streak 3 mm loopful (10 µl) incubated TT broth on bismuth sulfite (BS) agar (prepare BS plates the day before streaking and store in the dark at room temperature), xylose lysine desoxycholate (XLD) agar, and Hektoen enteric (HE) agar.
3. Repeat with 3 mm loopful (10 µl) of RV medium (for samples of high and low microbial load foods) and of SC broth (for guar gum).
4. For options of refrigerating incubated sample pre-enrichments and incubated sample selective enrichments (SC and TT broths only) of low moisture foods, see 994.04 in *Official Methods of Analysis,* AOAC International.
5. Incubate plates 24 ± 2 h at 35°.
6. Examine plates for presence of colonies that may be *Salmonella.*

Colony screening

Examine plates as follows:

1. *Typical Salmonella colony morphology*

- Hektoen enteric (HE) agar: 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, grey, or black; sometimes they have a metallic sheen. Surrounding medium is usually 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: Pink colonies 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.

2. *Atypical Salmonella colony morphology*

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

- 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 two or more atypical *Salmonella* colonies.
- 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 re-incubate an additional 24 ± 2 h. If typical or suspicious colonies are not present after 48 ± 2 h incubation, then pick two or more atypical colonies.

3. *Suggested control cultures*

In addition to the positive control cultures (typical *Salmonella),* three additional *Salmonella* cultures are recommended to assist in the selection of atypical *Salmonella* colony morphology on selective agars. These cultures are a lactose-positive, H2S-positive *S. diarizonae* (ATCC 12325) and a lactose-negative, H2S-negative *S. abortus equi* (ATCC 9842); OR a lactose-positive, H_2S-negative *S. diarizonae* (ATCC 29934). These cultures may be obtained from the American Type Culture Collection, 10801 University Boulevard, Manassas, VA 20110-2209.

Agar slant analysis

1. Select two or more colonies typical or suspected to be *Salmonella* from each selective agar. Inoculate into triple sugar iron (TSI) agar and lysine iron agar (LIA). If BS agar plates have no colonies typical or suspected to be *Salmonella* or no growth whatsoever, incubate them an additional 24 h. Lightly touch the very center of the colony to be picked with sterile inoculating needle and inoculate TSI agar slant by streaking slant and stabbing butt. Without flaming, inoculate LIA by stabbing butt twice and then streaking slant. Since lysine decarboxylation reaction is strictly anaerobic, the LIA slants must have deep butt (4 cm). Store picked selective agar plates at 5-8°.
2. Incubate TSI agar and LIA slants at 35° for 24 ± 2 h. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive H2S production. *Salmonella* in culture typically produces alkaline (red) slant and acid (yellow) butt, with or without production of H2S (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 H2S in LIA. Some *non-Salmonella* cultures produce a brick-red reaction in LIA slants.
3. If typical colonies are present on the BS agar after 24 ± 2 h incubation, then pick two or more colonies. Irrespective of whether or not BS agar plates are picked at 24 ± 2 h, re-incubate BS agar plates an additional 24 ± 2 h. After 48 ± 2 h incubation, pick two or more typical colonies, if present, from the BS agar plates, only if colonies picked from the BS agar plates incubated for 24 ± 2 h give atypical reactions in triple sugar iron agar (TSI) and lysine iron agar (LIA) that result in culture being discarded as not being *Salmonella.*
4. 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.* Test retained, presumed-positive TSI cultures as directed below, to determine if they are *Salmonella* species, including *S. arizonae.* If TSI cultures fail to give typical reactions for *Salmonella*

(alkaline slant and acid butt) pick additional suspicious colonies from selective medium plate not giving presumed-positive culture and inoculate TSI and LIA slants as described above.

5. Apply biochemical tests to:

- Three presumptive TSI agar cultures recovered from set of plates streaked from RV medium, if present, and presumptive TSI agar cultures recovered from plates streaked from tetrathionate broth, if present.

- If three presumptive-positive TSI cultures are not isolated from one set of agar plates, test other presumptive-positive TSI agar cultures, if isolated, by biochemical and serological tests. Examine a minimum of six TSI cultures for each 25 g analytical unit.

Biochemical and Serological Testing for Salmonella

1. *Mixed cultures*.

Streak TSI agar cultures that appear to be mixed on MacConkey agar, HE agar, or XLD agar. Incubate plates 24 ± 2 h at 35°. Examine plates for presence of colonies suspected to be *Salmonella,* as follows:

a. MacConkey agar. Typical colonies appear transparent and colourless, sometimes with dark center. Colonies of *Salmonella* will clear areas of precipitated bile caused by other organisms sometimes present.
b. Hektoen enteric (HE) agar. See *'Typical Salmonella colony morphology',* above, for procedure.
c. Xylose lysine desoxycholate (XLD) agar. See *'Typical Salmonella colony morphology',* above, for procedure.

Transfer at least two colonies suspected to be *Salmonella* to TSI agar and LIA slants as described above, and continue as under *'Agar slant analysis'.*

2. Pure cultures:

a. Urease test (conventional). With sterile needle, inoculate growth from each presumed-positive TSI agar slant culture into tubes of urea broth. Since occasional, uninoculated tubes of urea broth turn purple-red (positive test) on standing, include uninoculated tube of this broth as control. Incubate 24 ± 2 h at 35°.
b. Optional urease test (rapid). Transfer two 3 mm loopfuls of growth from each presumed-positive TSI agar slant culture into tubes of rapid urea broth. Incubate 2 h in 37 ± 0.5° water bath. Discard all cultures giving positive test. Retain for further study all cultures that give negative test (no change in colour of medium).

3. Serological polyvalent flagellar (H) test:

a. Perform the polyvalent flagellar (H) test at this point, or later, as described below. Inoculate growth from each urease-negative TSI agar slant into 1) brain heart infusion broth and incubate 4-6 h at 35° until visible growth occurs (to test on same day); or 2) trypticase soy-tryptose broth and incubate 24 ± 2 h at 35° (to test on following day). Add 2.5 ml formalinized physiological saline solution to 5 ml of either broth culture.
b. Select two formalinized broth cultures and test with *Salmonella* polyvalent flagellar (H) antisera. Place 0.5 ml of appropriately diluted *Salmonella* polyvalent flagellar (H) antiserum in 10 x 75 mm or 13 x 100 mm serological test tube. Add 0.5 ml antigen to be tested. Prepare saline control by mixing 0.5 ml formalinized physiological saline solution with 0.5 ml formalinized antigen. Incubate mixtures in 48-50° water bath. Observe at 15 min intervals and read final results in 1 h.

Positive - agglutination in test mixture and no agglutination in control. Negative - no agglutination in test mixture and no agglutination in control. Nonspecific - agglutination in both test mixture and control. Test the cultures giving such results with Spicer-Edwards antisera, below.

4. *Spicer-Edwards serological test*:

Use this test as an alternative to the polyvalent flagellar (H) test. It may also be used with cultures giving non-specific agglutination in polyvalent flagellar (H) test. Perform Spicer-Edwards flagellar (H) antisera test as described above. Perform additional biochemical tests (below) on cultures giving positive flagellar test results. If both formalinized broth cultures are negative, perform serological tests on four additional broth cultures (above). If possible, obtain two positive cultures for additional biochemical testing. If all urease-negative TSI cultures from sample give negative serological flagellar (H) test results, perform additional biochemical tests.

5. Testing of urease-negative cultures:

a. Lysine decarboxylase broth. If LIA test was satisfactory, it need not be repeated. Use lysine decarboxylase broth for final determination of lysine decarboxylase if culture gives doubtful LIA reaction. Inoculate broth with small amount of growth from TSI agar slant suspicious for *Salmonella.* Replace cap tightly and incubate 48 ± 2 h at 35° but examine at 24 h intervals. *Salmonella* species cause alkaline reaction indicated by purple colour throughout medium. Negative test is indicated by yellow colour throughout medium. If medium appears discoloured (neither purple nor yellow) add a few drops of 0.2% bromcresol purple dye and re-read tube reactions.
b. Phenol red dulcitol broth or purple broth base with 0.5% dulcitol. Inoculate broth with small amount of growth from TSI agar culture. Replace cap loosely and incubate 48 ± 2 h at 35°, but examine after 24 h. Most *Salmonella* species give positive test, indicated by gas formation in inner fermentation vial and acid pH (yellow) of medium. Production of acid should be interpreted as a positive reaction. Negative test is indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromcresol purple as indicator) colour throughout medium.

c. Tryptone (or tryptophane) broth. Inoculate broth with small amount of growth from TSI agar culture. Incubate 24 ± 2 h at 35° and proceed as follows:

1. Potassium cyanide (KCN) broth. Transfer 3 mm loopful of 24 h tryptophane broth culture to KCN broth. Heat rim of tube so that good seal is formed when tube is stoppered with wax-coated cork. Incubate 48 ± 2 h at 35° but examine after 24 h. Interpret growth (indicated by turbidity) as positive. Most *Salmonella* species do not grow in this medium, as indicated by lack of turbidity.
2. Malonate broth. Transfer 3 mm loopful of 24 h tryptone broth culture to malonate broth. Since occasional uninoculated tubes of malonate broth turn blue (positive test) on standing, include uninoculated tube of this broth as control. Incubate 48 ± 2 h at 35°, but examine after 24 h. Most *Salmonella* species cultures give negative test (green or unchanged colour) in this broth.
3. Indole test. Transfer 5 ml of 24 h tryptophane broth culture to empty test tube. Add 0.2-0.3 ml Kovacs' reagent. Most *Salmonella* cultures give negative test (lack of deep red colour at surface of broth). Record intermediate, varying shades of orange and pink as ±.
4. Serological flagellar (H) tests for *Salmonella*. *If either polyvalent flagellar (H) test (above) or the Spicer-Edwards flagellar (H) test tube test (above) has not already been performed, either test may be performed here.*
5. Discard as not *Salmonella* any culture that shows either positive indole test and negative serological flagellar (H) test, or positive KCN test and negative lysine decarboxylase test.

6. *Serological somatic (O) tests for Salmonella*.

Note: *Pre-test all antisera to Salmonella with known cultures.*

a. Polyvalent somatic (O) test.

Using wax pencil, mark off two sections about 1x2 cm each on inside of glass or plastic petri dish (15 x 100 mm). Commercially available sectioned slides may be used. Emulsify 3 mm loopful of culture from 24-48 h TSI slant or, preferably, tryptose blood agar base (without blood) with 2 ml 0.85% saline. Add 1 drop of culture suspension to upper portion of each rectangular crayon-marked section. Add 1 drop of saline solution to lower part of one section only. Add 1 drop of *Salmonella* polyvalent somatic (O) antiserum to other section only. With clean sterile transfer loop or needle, mix culture suspension with saline solution for one section and repeat for other section containing antiserum. Tilt mixtures in back-and-forth motion for 1 min and observe against dark background in good illumination. Consider any degree of agglutination a positive reaction. Classify polyvalent somatic (O) test results as follows:

Positive - agglutination in test mixture; no agglutination in saline control. Negative - no agglutination in test mixture; no agglutination in saline control. Nonspecific - agglutination in test and in control mixtures. Perform further biochemical and serological tests as described in *Edwards and Ewing's Identification of Enterobacteriaceae*

(Ewing, W.H. 1986. Edwards and Ewing's Identification of Enterobacteriacae, 4th ed. Elsevier, New York).

b. Somatic (O) group tests:

Test as in 6a, above, using individual group somatic (O) antisera including Vi, if available, in place of *Salmonella* polyvalent somatic (O) antiserum. For special treatment of cultures giving positive Vi agglutination reaction, refer to sec. 967.28B in *Official Methods of Analysis* (AOAC International). Record cultures that give positive agglutination with individual somatic (O) antiserum as positive for that group. Record cultures that do not react with individual somatic (O) antiserum as negative for that group.

Table 2. Summary of biochemical and serological reactions of *Salmonella*

Test or substrate	Positive	Negative	species reactions [a]
1. Glucose (TSI)	yellow butt	red butt	+
2. Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3.H_2S(TSIandLIA)	blackening	no blackening	+
4. Urease	purple-red colour	no colour change	-
5. Lysine decarboxylase broth	purple colour	yellow colour	+
6. Phenol red dulcitol broth	yellow colour and/or gas	no gas; no colour change	+[b]
7. KCN broth	growth	no growth	-
8. Malonate broth	blue colour at surface	no colour change	-[c]
9. Indole test	deep red colour at surface	yellow colour at surface	-
10. Polyvalent flagellar test	agglutination	no agglutination	+
11. Polyvalent somatic test	agglutination	no agglutination	+
12. Phenol red lactose broth	yellow colour and/or gas	no gas; no colour change	-[c]
13. Phenol red sucrose broth	yellow colour and/or gas	no gas; no colour change	-
14. Voges-Proskauer test	pink-to-red colour	no colour change	-
15. Methyl red test	diffuse red colour	diffuse yellow colour	+
16. Simmons citrate	growth; blue colour	no growth; no colour change	v

NOTE:

[a] + is 90% or more positive in 1 or 2 days; - is 90% or more negative in 1 or 2 days; v is variable.
[b] Majority of *S. arizonae* cultures are negative.
[c] Majority of *S. arizonae* cultures are positive.

Classify as *Salmonella* those cultures which exhibit typical *Salmonella* reactions for test Nos. 1-11, shown in Table 2, above. If one TSI culture from 25 g sample is classified as *Salmonella,* further testing of other TSI cultures from the same 25 g sample is unnecessary. Cultures that contain demonstrable *Salmonella* antigens as shown by positive *Salmonella* flagellar (H) test but do not have biochemical characteristics of *Salmonella* should be purified and retested.

Table 3. Criteria for discarding *non-Salmonella* cultures

Test or substrate	Results
1. Urease	positive (purple-red colour)
2. Indole test and Polyvalent flagellar (H) test or Spicer-Edwards flagellar test	positive (violet colour at surface) negative (no agglutination)
3. Lysine decarboxylaseKCN broth	negative (yellow colour) positive (growth)
4. Phenol red lactose broth	positive (yellow colour and/or gas) [a,b]
5. Phenol red sucrose broth	positive (yellow colour and/or gas) [b]
6. KCN broth Voges-Proskauer test Methyl red test	positive (growth) positive (pink-to-red colour) negative (diffuse yellow colour)

NOTE:

[a]Test malonate broth positive cultures further to determine if they are *Salmonella arizonae.*

[b]Do not discard positive broth cultures if corresponding LIA cultures give typical *Salmonella* reactions; test further to determine if they are *Salmonella* species.

Atypical Salmonella Colony Testing

Perform the following additional biochemical tests on cultures that do not give typical *Salmonella* reactions for test Nos. 1-11 in Table 12, above, and that consequently do not classify as *Salmonella* (see Table 23, also above).

a. Phenol red lactose broth or purple lactose broth

1. Inoculate broth with small amount of growth from unclassified 24-48 h TSI agar slant. Incubate 48 ± 2h at 35°, but examine after 24 h. Positive—-acid production (yellow colour) and gas production in inner fermentation vial. Consider production of acid only as positive reaction. Most cultures of *Salmonella* give negative test result, indicated by no gas formation in inner fermentation vial and red (with phenol red as indicator) or purple (with bromcresol purple as indicator) colour throughout medium.
2. Discard as not *Salmonella* cultures that give positive lactose tests, except cultures that give acid slants in TSI and positive reactions in LIA or cultures that give positive malonate broth reactions. Perform further tests on these cultures to determine if they are *S. arizonae.*

b. Phenol red sucrose broth or purple sucrose broth

Follow procedure described as directly above. Discard as not *Salmonella,* cultures that give positive sucrose tests, except those that give acid slants in TSI and positive reactions in LIA.

c. MR-VP broth

Inoculate medium with small amount of growth from each unclassified TSI slant suspected to contain *Salmonella.* Incubate 48 ± 2 h at 35°.

1. Perform Voges-Proskauer (VP) test at room temperature as follows: Transfer 1 ml 48 h culture to test tube and incubate remainder of MR-VP broth an additional 48 h at 35°. Add 0.6 ml α-naphthol and shake well. Add 0.2 ml 40% KOH solution and shake. To intensify and speed reaction, add a few crystals of creatine. Read results after 4 h; development of pink-to-ruby red color throughout medium is positive test. Most cultures of *Salmonella* are VP-negative, indicated by absence of development of pink-to-red color throughout broth.
2. Perform methyl red test as follows: To 5 ml of 96 h MR-VP broth, add 5-6 drops of methyl red indicator. Read results immediately. Most *Salmonella* cultures give positive test, indicated by diffuse red color in medium. A distinct yellow color is negative test. Discard, as not *Salmonella,* cultures that give positive KCN and VP tests and negative methyl red test.

d. Simmons citrate agar

Inoculate this agar, using needle containing growth from unclassified TSI agar slant. Inoculate by streaking slant and stabbing butt. Incubate 96 ± 2 h at 35°C. Read results as follows:

Positive - presence of growth, usually accompanied by color change from green to blue. Most cultures *of Salmonella* are citrate-positive.

Negative - no growth or very little growth and no color change.

Alternative Method for Identification of Salmonella

As alternative to conventional biochemical tube system, use any of 5 commercial biochemical systems (API 20E, Enterotube II, *Enterobacteriaceae* II, MICRO-ID, or Vitek GNI) for presumptive generic identification of *Salmonella.* Choose a commercial system based on a demonstration in the analyst's own laboratory of adequate correlation between commercial system and the biochemical tube system outlined in this identification section.

Commercial biochemical kits should not be used as a substitute for serological tests.

Assemble supplies and prepare reagents required for the kit. Inoculate each unit according to Method 978.24 (API 20E, Enterotube II, and Enterobacteriaceae II), sec. 989.12 (MICRO-ID), and Method 991.13 (Vitek GNI) in *Official Methods of Analysis,* incubating for time and temperature specified. Add reagents, observe, and record results. For presumptive identification, classify cultures, according to *Official Methods of Analysis* (AOAC International) as *Salmonella* or not *Salmonella.*

For confirmation of cultures presumptively identified as *Salmonella,* perform the *Salmonella* serological somatic (O) test and the *Salmonella* serological flagellar (H) test or the Spicer-Edwards flagellar (H) test and classify cultures according to the following guidelines:

a. Report as *Salmonella* those cultures classified as presumptive *Salmonella* with commercial biochemical kits when the culture demonstrates positive *Salmonella* somatic (O) test and positive *Salmonella* (H) test.
b. Discard cultures presumptively classified as not *Salmonella* with commercial biochemical kits when cultures conform to AOAC criteria for classifying cultures as not *Salmonella.*
c. For cultures that do not conform to a or b, classify according to additional tests specified above, or additional tests as specified by Ewing, or send to a reference typing laboratory for definitive serotyping and identification.

Treatment of cultures giving negative flagellar (H) test.

If biochemical reactions of certain flagellar (H)-negative culture strongly suggest that it is *Salmonella,* the negative flagellar agglutination may be the result of non-motile organisms or insufficient development of flagellar antigen. Proceed as follows: Inoculate motility test medium in petri dish, using small amount of growth from TSI slant. Inoculate by stabbing medium once about 10 mm from edge of plate to depth of 2-3 mm. Do not stab to bottom of plate or inoculate any other portion. Incubate 24 h at 35°C. If organisms have migrated 40 mm or more, retest as follows: Transfer 3 mm loopful of growth that migrated farthest to trypticase soy-tryptose broth. Repeat either polyvalent flagellar (H) or Spicer-Edwards serological tests. If cultures are not motile after the first 24 h, incubate an additional 24 h at 35°C; if still not motile, incubate up to 5 days at 25°C. Classify culture as non-motile if above tests are still negative.

Annex U

Sampling

B1 Definitions

For the purpose of this sampling method, the following definitions shall apply:

B1.1 Consignment – the quantity of goods duplicated or received at one time and covered by a particular contract or shipping document. The consignment may be made up of one or more lots or parts of lots.

B1.2 Lot – a lot shall consist of products of the same material and manufactured under similar conditions or a state quantity of the consignment, presumed to be of uniform characteristics, taken from the consignment.

B1.3 Primary sample – a small quantity of carrageenan, taken at one time from point in a single container.

B1.4 Bulk sample – the quantity of carrageenan sample obtained by bringing together the primary sample from different positions in the lot.

B1.5 Laboratory sample – a prescribed quantity of carrageenan taken from the bulk after thoroughly mixing and quartering the primary samples. It is representative of the quality of the lot.

B2 General

B2.1 Sampling shall be carried out by a person appointed by agreement between buyer and seller and, if desired by either of them, in the presence of the buyer (or his representative) and of the seller (or his representative).

B2.2 In taking, preparing, storing and handling the samples, care shall be taken that the properties of the product are not affected. The following precautions and directions shall be observed.

B2.2.1 Samples shall be taken in a protected place not exposed to damp air, dust or soot.

B2.2.2 The sampling apparatus shall be clean and dry.

B2.2.3 Precautions shall be taken to protect the samples, the product being sampled and the sample containers from adventitious contamination.

B3 Apparatus

B3.1 Taking samples – spoons, scoop, borers or other instruments suitable for taking samples from the interior of containers.

B3.2 Mixing and dividing – apparatus suitable for the purpose of mixing and quartering the bulk samples to obtain the laboratory samples.

B4 Constitution of lots

All the containers in a single production or consignment of carrageenan.

B5 Method of Taking Primary Sample

B5.1 Sampling from bags during packaging or (before sealing).

Take with the appropriate apparatus mentioned in B3, 50 g sample from every fifth bag filled from the bin and before sealing.

B5.2 Sampling from lot/stationary stock.

Sampling from stationary stock depends on the size of the lot and shall be in accordance with Table A.

Table A – Sampling from stationary stock

Lot Size (N)	**Number of containers to be taken for sampling**
1 to 5 containers	All containers
6 to 49 containers	5 containers
50 to 100 containers	10% of the containers
Over 100 containers	The square root of the number of containers, rounded to the nearest whole number

These containers should, as far as possible, be taken at random from the lot and, in order to achieve this, a random number table, agreed upon between the buyer and seller, should be used. If such a table is not available, the following procedure shall be adopted:

Starting from any container, count the containers as 1, 2, 3, …etc up to r and so on. Withdraw from the lot every rth container thus counted for sampling; the value of r is equal to :

$$r = \frac{N}{n}$$

where:

N is the total number of containers in the lot;

n is the number of containers to be taken (see table).

If r is a fractional number, its value shall be taken as equal to the integral part of it.

B5.3 When the product is in movement, samples may be taken at the time of loading or unloading of the containers. For this purpose, the number of containers to be taken shall also be in accordance with the table. The value of r shall be calculated as indicated above, and every rth container counted during loading or unloading shall be removed for sampling.

B5.4 Take primary samples by means of an appropriate sampling instrument, from different parts of each container selected.

B5.5 A series of primary sample is taken from different positions in the lot.

B6 Bulk sample

B6.1 Thoroughly mix all the primary sample taken as described above to form the bulk sample.

B6.2 The size of the bulk sample shall be four times or more of the quantity of sample required to carry out all the tests required in the specification.

B7 Laboratory samples

Divide the sample into four equal parts, according to the number of laboratory samples required. Laboratory sample shall be 200g – 500 g. Each part thus obtained constitute a laboratory sample: one of these samples is intended for the buyer and another for the seller. The third sample, bearing the seals of the buyer and of the seller (or of their representatives) if they were present at the time of sampling or of the person who sampled the lot, shall constitute the reference sample to be used in case of dispute between the buyer and seller; it shall be kept at a place acceptable to both parties.

B8 Packaging and labeling of samples

B8.1 Packaging of samples

The laboratory samples shall be placed in clean, dry, airtight glass containers or other suitable containers which do not react with the product. The sample container shall be of such a size that they are almost completely filled by the sample. Each sample container, after filling, shall be made airtight by means of a stopper or other suitable closure, and sealed in such a way that it cannot be opened and re-sealed without detection.

B8.2 Labeling of samples

B8.2.1 The laboratory samples shall be labeled to give information concerning the sample and full details of the sampling i.e.

B8.2.1.1 The date of sampling

B8.2.1.2 The name and address of the person taking the sample;

B8.2.1.3 The name of the product;

B8.2.2 If any defect is found at the time of sampling, a record of this shall be included in the details of sampling given on the sample container.

B8.3 Storage and dispatch of samples

B8.3.1 The laboratory samples shall be stored in such manner that the temperature of the product does not vary unduly from the normal atmospheric temperature. Samples which are required to be kept for a long time shall be stored in a cool and dark place.

B8.3.2 Laboratory samples on which an analysis is to be carried out shall be dispatched to the laboratory as soon as possible.

B9 Sampling report

If a sampling report is prepared, besides giving the usual information, it shall make reference to the condition of the carrageenan sampled to the technique applied if this is other than that described in this standard and to any circumstances that may have influenced the sampling.

References

BFAD AO 153 s. 2004 - Guidelines, Current good Mfg. Practice in Manufacturing, Packing, Repacking or Holding Food

Joint FAO/WHO Expert Committee on Food Additives. Combined Compendium of Food Additive Specifications. Volume 4. Analytical methods, test procedures and laboratory solutions used by and referenced in the food additive specifications. 2006. Food and Agriculture Organization of the United Nations, Rome

Joint FAO/WHO Expert Committee on Food Additives. 57th Session. 2001. Compendium of Food Additive Specifications Food and Nutrition Paper 52 Addendum 9. Food and Agriculture Organization of the United Nations, Italy