Doc.: AYD 07(27181) WC
January 2025

BUREAU OF INDIAN STANDARDS

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भारतीय मानक मसौदा

पारम्परिक औषधियों में प्रयोग हेतु पिलोकार्पस जैबोरैंडी होम्स पत्तियां – विशिष्टि

Draft Indian Standard

Pilocarpus Jaborandi Holmes Leaves for Use in Traditional Medicine – Specification

Homeopathic Sectional Committee, AYD 07 **Last Date of Comments: 24 March 2025**

FOREWORD

(Formal clauses would be added later)

Pilocarpus jaborandi Holmes is a small shrub, with about 30-45 cm long leaves with 3 pairs of leaflets. It is native to northeast Brazil, Central America and West Indies. It is commonly known as Jaborandi with synonym *Pilocarpus cearensis* Rizzini and common Pilocarpus *(*English); Jaborandi (French); Jaborandi latter (German).

It contains imidazole alkaloids among, which pilocarpine is most important. Other alkaloids isopilocarpine, pilocarpidine, pilosine, pseudopilocarpine and isopilosine. The range of total alkaloids in different species is between 0.5 percent to 1 percent. It is used for making Mother tincture, dilutions or potencies in Homoeopathy and as an ingredient for formulations in traditional systems of medicine.

The standard is one of the series of standards being brought out on ingredients used in formulations of traditional medicine for the advantage of researchers, academicians, students, clinical practitioners and drug manufacturers.

In formulating this standard, significant assistance has been derived from Homeopathic Pharmacopoeia of India, Vol. 2, 1974; Vol. 10, 2013 published by Ministry of Ayush, Government of India, Inputs have also been derived from the information available in the public domain in print and electronic media, including authoritative books

Also, due consideration has been given to the provisions of the Drug and Cosmetics Act, 1940 and the Rules 1945, framed thereunder, including the latest amendments. In case of any disparity, this standard is subject to the restrictions imposed under these Rules and Regulations, wherever applicable.

For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of a test or analysis, shall be rounded off in accordance with IS 2 : 2022 ‘Rules for rounding off numerical values (second

revision)'. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

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PILOCARPUS JABORANDI HOLMES LEAVES FOR USE IN TRADITIONAL MEDICINE – SPECIFICATION

1 SCOPE

This standard prescribes the specific requirements and methods of testing for *Pilocarpus jaborandi* Holmes leaves; syn. *Pilocarpus cearensis* Rizzini (Family Rutaceae).

2 REFERENCES

The standards listed in Annexure A contain provisions which, through reference in this text, constitute provision of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards.

3 REQUIREMENTS

3.1 Description

3.1.1 *Macroscopic Examination of Jaborandi leaves*

Dried entire or broken pieces of leaflets (Fig 1A), 3 - 4.5 × 2-3 cm, oblong, elliptical, apex emarginated, base symmetric or unequal, with exception of terminal leaflet, margin entire, faded green to light brown and glabrous above, midrib depressed, greenish-brown to brown and slightly hairy below; with minute pellucid gland, petiolules short; odour characteristic aromatic, taste pungent bitter.

3.1.2 *Microscopic Examination of Jaborandi leaves*

Vertical section of leaflets (Fig 1B) shows single layered epidermis of rectangular or slightly tangentially elongated cells, covered with thick, yellow cuticle; trichomes non-glandular, unicellular, thick-walled, bent or curved and few uniseriate, multicellular; stomata anomocytic; mesophyll differentiated into 2-4 layers of palisade and 4-6 layers of spongy parenchyma, containing rosette crystals of calcium oxalate, tannin and secretory cavities with oil globules; midrib consists of arc-shaped, conjoint, collateral vascular bundle; surrounded by 4-5 layer of thick-walled, lignified, pericyclic fibres; stomatal index on lower surface 10.73; palisade ratio 7.8; vein islet 18.78 per square mm.(Fig 1 E, 1 F)

Transection of Petiole (Fig 1C, 1D) shows crescent-shaped in outline; epidermal single layer of rectangular, thick-walled cells, covered with thick cuticle; trichomes as on leaf; hypodermis 1-3 layers of tanniferous cells; ground tissue parenchymatous, having rosette aggregates of calcium oxalate and secretory cavities; vascular bundle arc-shaped, conjoint, collateral, surrounded by interrupted layers of pericyclic fibers; two subsidiary vascular strands present in lateral projections. Petiolule description is same as for petiole.

3.1.3 *Powder*

Dark green, smooth, with aromatic odourand bitter taste; microscopy shows fragments of epidermal cells with stomata, thin –walled parenchymatous cell with rosette crystals of calcium oxalate, a few fragments of thick-walled sclerenchymatous cells; broken or entire unicellular or multicellular trichomes; fragments of tracheary elements with annular thickening and a few palisade cells with chloroplasts and occasional secretory cavities with oil globules.

Fig 1 A Raw leaves

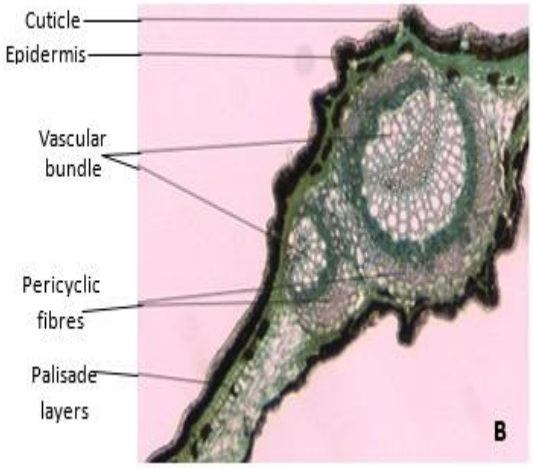

Fig 1 B Section of leaf blade through midrib

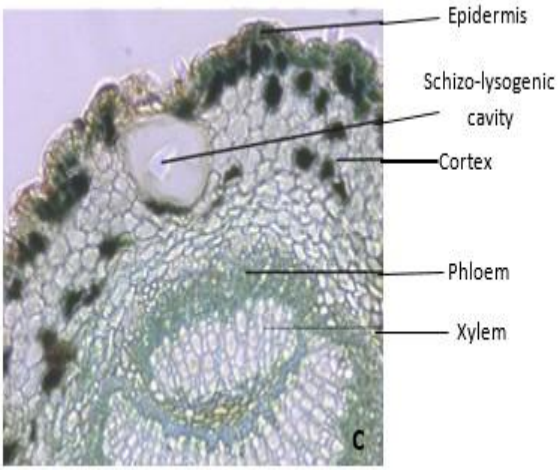

Fig 1 C Enlarged view of petiolue section

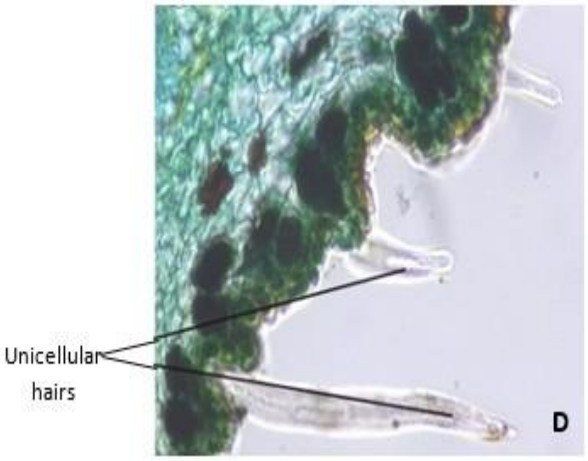

Fig 1 D Enlarged view of petiolue section showing hairs

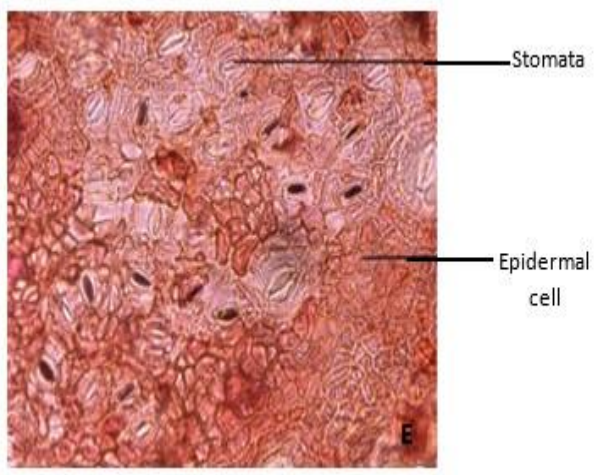

Fig 1 E Leaf peel showing stomata

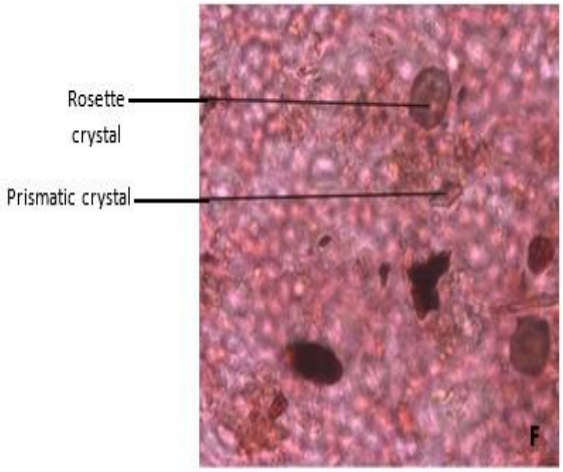

Fig 1 F Leaf peel showing crystals

3.2 General Specifications

3.2.1 *Jaborandi* leaves shall be free from extraneous/artificial flavours.

3.2.2 *Jaborandi* leaves shall comply with physical, chemical and microbiological requirements given in Table 1.

3.2.3 If any pesticide other than those for which minimum requirements are given in Table 1 have been applied to the herb before or after harvesting, those should also be tested.

Table 1 Requirements for *Jaborandi* leaves

(Clauses 3.2.2, 3.2.3 and 5.2)

SI No. (1)	**Characteristic** (2)	**Requirement** (3)	**Method of Test, Ref. to** (4)
i)	Foreign matter, percent by mass, *Max.*	NMT 2 percent	6.2 of IS 4333 (Part 1)
ii)	Loss on drying, percent by mass, *Max.*	NMT 12 percent	IS 13859
iii)	Total ash, percent by mass, *Max.*	NMT 10 percent	6 of IS 1797
iv)	Acid insoluble ash, percent by mass, *Max.*	NMT 0.41 percent	8 of IS 1797
v)	Alcohol Soluble Extractive, percent by mass, *Min.*	NLT 3 percent	10 of IS 1797
vi)	Water soluble Extractive, percent by mass, *Min.*	NLT 24 percent	11 of IS 1797
vii)	Thin layer chromatography identification	Should comply	Annex B
viii)	Quantitative analysis	Should comply	Annex B
ix)	Aflatoxin B1 ng/kg, *Max*	Less than 2ng/kg	IS 16287

x)	Total Aflatoxin (B1+B2+G1+G2) ng/kg, *Max*	Less than 5ng/kg	IS 16287
xi)	Lead (as Pb) mg/kg, *Max*	10.0	IS 16913
xii)	Arsenic (as As) mg/kg, *Max*	3.0	IS 16913
xiii)	Cadmium (as Cd) mg/kg, *Max*	0.3	IS 16913
xiv)	Mercury (as Hg) mg/kg, *Max*	1.0	IS 16913
xv)	*Staphylococcus aureus*, per g	Absent	Annex C
xvi)	*Pseudomonas aeruginosa*, per g	Absent	Annex C
xvii)	*Salmonella Spp.*, per g	Absent	Annex C
xviii)	*Escherichia coli*, per g	Absent	Annex C
xix)	Total microbial plate count per g, *Max*	10^5 for extract and powder 10^7 for plant material	Annex C
xx)	Total yeast and mould per g, *Max*	10^3 for extract and powder 10^5 for plant material	Annex C
xxi)	**Pesticide residues**		
1.	Alachlor, mg/kg, *Max*	0.02	IS 17924
2.	Aldrin, dieldrin (sum of), mg/kg, *Max*	0.05	IS 17924
3.	Azinphos-methyl, mg/kg, *Max*	1.0	IS 17924
4.	Bromopropylate, mg/kg, *Max*	3.0	IS 17924
5.	Chlordane (sum of cis- trans- and oxythlordane), mg/kg, *Max*	0.05	IS 17924
6.	Chlorfenvinphos, mg/kg, *Max*	0.5	IS 17924
7.	Chlorpyrifos, mg/kg, *Max*	0.2	IS 17924
8.	Chlorpyrifos-methyl, mg/kg, *Max*	0.1	IS 17924
9.	Cypermethrin and isomers, mg/kg, *Max*	1.0	IS 17924
10.	DDT (sum of p, p'-DDT, o, p' DDT, p,p'-DDE and p, p'-TDE) , mg/kg, *Max*	1.0	IS 17924
11.	Deltamethrin, mg/kg, *Max*	0.5	IS 17924
12.	Diazinon, mg/kg, *Max*	0.5	IS 17924
13.	Dichlorvos, mg/kg, *Max*	1.0	IS 17924
14.	Dithiocarbamates (as CS_2), mg/kg, *Max*	2.0	IS 17924
15.	Endosulfan (sum of isomers and endosulfan sulphate, mg/kg, *Max*	3.0	IS 17924
16.	Endrin, mg/kg, *Max*	0.05	IS 17924
17.	Ethion, mg/kg, *Max*	2.0	IS 17924
18.	Fenitrothion, mg/kg, *Max*	0.5	IS 17924
19.	Fenvalerate, mg/kg, *Max*	1.5	IS 17924
20.	Fonofos, mg/kg, *Max*	0.05	IS 17924
21.	Heptachlor (combined residues of heptachlor and its epoxide to be determined and expressed Milled as Heptachlor), mg/kg, *Max*	0.05	IS 17924
22.	Hexachlorobenzene, mg/kg, *Max*	0.1	IS 17924

23.	Hexachlorocyclohexane isomers (other than γ), mg/kg, *Max*	0.3	IS 17924
24.	Lindane (γ Hexachlorocyclohexane), mg/kg, *Max*	0.6	IS 17924
25.	Malathion, mg/kg, *Max*	1.0	IS 17924
26.	Methidathion, mg/kg, *Max*	0.2	IS 17924
27.	Parathion, mg/kg, *Max*	0.5	IS 17924
28.	Parathion Methyl, mg/kg, *Max*	0.2	IS 17924
29.	Permethrin, mg/kg, *Max*	1.0	IS 17924
30.	Phosalone, mg/kg, *Max*	0.1	IS 17924
31.	Piperonyl butoxide, mg/kg, *Max*	3.0	IS 17924
32.	Pirimiphos- methyl, mg/kg, *Max*	4.0	IS 17924
33.	Pyrethrin (sum of), mg/kg, *Max*	3.0	IS 17924
34.	Quintozene (sum of quintozene, pentachloroaniline, and methyl pentachlorophenyl sulphide), mg/kg, *Max*	1.0	IS 17924

NOTES — Quality of reagents

1 Reagents, including pure chemicals used, shall be of analytical grade.
2 Reagent grade water for laboratory use shall be as per IS 1070.
3 ‘Pure chemicals’ shall mean chemicals that do not contain impurities that affect the analysis results.

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4 PACKING, STORAGE AND MARKING

4.1 Packing

Jaborandi leaves shall be packed in clean, sound and dry containers made of metal, glass, food-grade polymers, wood, or jute bags. The wooden boxes or jute bags shall be suitably lined with moisture-proof lining, which does not impart any foreign smell to the product. The packing material shall be free from any fungal or insect infestation and should not impart any foreign smell. Each container shall be securely closed and sealed.

4.2 Storage

Jaborandi leaves, shall be stored under conditions that prevent contamination and as far as possible, deterioration. The storage area shall be clean, well-ventilated and protected from direct sunlight, moisture, insects and rodents. A leaflet containing instructions for storage shall be enclosed with each packing.

4.3 Marking

The following particulars shall be legibly and indelibly marked or labeled on each pack of *Jaborandi* leaves:

a) Name of the material, including part of the plant, botanical name and trade name or brand name, if any;
b) Name and address of the producer or packer, including contact details;

c) State and country of production (if known);
d) Batch number;
e) Net quantity;
f) Date of packing (MM/YYYY);
g) Date of expiry or use before (MM/YYYY);
h) QR code for authentication (optional);
j) Instructions for storage; and
k) Any other information requested by the buyer, such as the date of harvesting (MM/YYYY) (if known).

5 SAMPLING

5.1 Representative samples of the material shall be drawn and tested for conformity to this specification as prescribed in IS 13145.

5.2 The samples of *Jaborandi* leaves shall be tested to ascertain conformity of the material to the requirements in accordance with the relevant clauses given in col (4) of Table 1.

6. BIS Certification Marking

The product(s) conforming to the requirements of this standard may be certified as per the conformity assessment schemes under the provisions of the Bureau of Indian Standards Act, 2016 and the Rules and Regulations framed there under and the product(s) may be marked with the Standard Mark.

ANNEX A

LIST OF REFERRED STANDARDS

(Clause 2)

IS No.	Title
IS 1070 : 2023	Reagent grade water — Specification (*fourth revision*)
IS 1797 : 2017	Spices and condiments — Methods of test (third revision)
IS 4333 (Part 1) : 2018	Methods of analysis for foodgrains: Part 1 Refractions (third revision)
IS 13859 : 1993/ ISO 7513 : 1990	Instant tea in solid form — Determination of moisture content (loss in mass at 103 °C)
IS 13145 : 2014	Spices and condiments — Methods of sampling (second revision)
IS 16287: 2015/ ISO 16050 : 2003	Foodstuffs — Determination of aflatoxin B1, and the total content of aflatoxins B1, B2, G1 and G2 in cereals, nuts and derived products — High performance liquid chromatographic method
IS 16913 : 2018	Methods of test for cosmetics — Determination of heavy metals (arsenic, cadmium, lead, and mercury) by atomic absorption spectrometry (AAS)
IS 17924 : 2022	Determination of pesticide residue in herbal materials

ANNEX B

IDENTIFICATION OF JABORANDI LEAVES

[Table 1, SI No. (vii)(viii)]

B-1: GENERAL IDENTIFICATION (REACTION TEST):

Solution S-I: Stir 0.5 g of the powdered herbal drug with 5 ml of alcohol 70 percent R for 2h, then filter.

Solution S-II: Moisten 1 g of the powdered herbal drug with I ml of dilute ammonia R2, then add 10 ml of alcohol R. Stir for 30 min, then filter. Evaporate the filtrate almost to dryness under reduced pressure and dissolve the residue in 3 ml of alcohol R.

a. To 1 ml of solution S-I add 5 ml of alcohol 70 per cent R and 0.5 ml of dilute sodium hydroxide solution R. A dark yellow colour is produced. Leave to stand. A gelatinous precipitate is produced within about 30 min.

b. To 1 ml of solution S-II add 0.3 ml of potassium tetraiodomercurate solution R. Turbidity is produced

B- 2 THIN LAYER CHROMATOGRAPHY (TLC)

B-2.1 Apparatus

B-2.1.1 Thin Layer chromatography with a Pre-coated Silica Gel 60 F 254 Plate

B-2.1.2 Analytical Balance

B-2.1.3 Standard Glassware

B-2.1.4 Water Bath

B-2.2 Reagents

B-2.2.1 Ethanol – AR or Equivalent Grade

B-2.2.2 Water

B-2.2.3 Chloroform

B-2.2.4 Acetone

B-2.2.5 formic acid

B-2.2.6 Dragendorff’s reagent

B-3.3 Procedure

B-3.3.1 Preparation of Hydro-alcoholic extract

To 5g of the substance being examined, add 50 ml of alcohol. Allow to stand for twenty-four hours, shake & filter. Evaporate 25 ml of Mother Tincture on water bath to remove *alcohol* and extract with 3x20 ml of *chloroform*. Combine and concentrate *chloroform* layer to 2 ml. Carry out TLC of *chloroform extract* in silica gel 60 F_{254} *pre-coated* plate.

B-3.3.2 Mobile phase

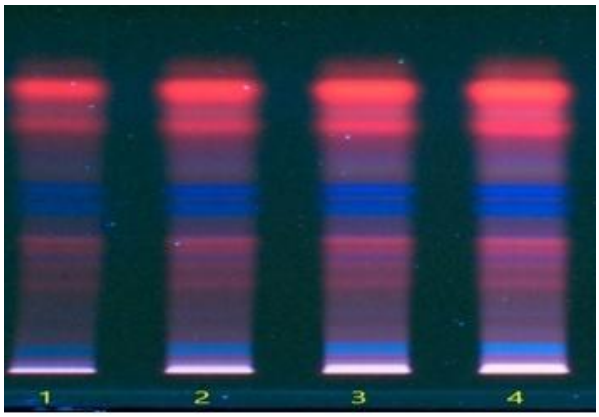
Chloroform: acetone: formic acid (7.5:1.6:0.86v/v/v)

B-3.3.3 Estimation

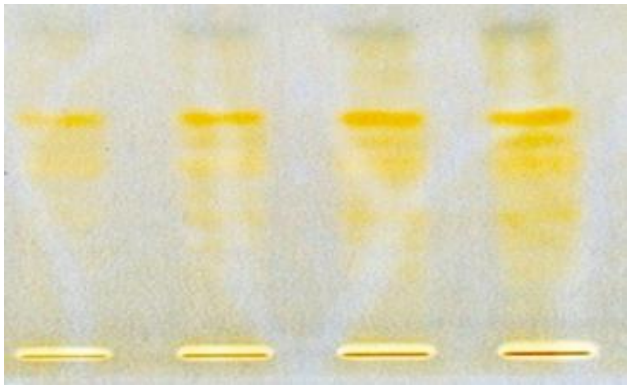
Apply 1-5µl each test solution on a TLC plate as bands of 10 mm. Develop the plate to a distance of 80 mm from the line of application. Dry the plate in air and examine under UV light 254 nm no spots appear.

Under UV light 366 nm, seven spots appear at R_f. 0.25 (Red), 0.33 (Red), 0.41 (Red), 0.49 (Blue), 0.57 (Blue), 0.75 (Red), 0.85 (Red)

After derivatization with Dragendorff's reagent three spots appear at R_f.0.64, 0.68, 0.79 (all yellowish).



Under UV 366 nm
Plate 2. TLC profile of *Jaborandi* Leaves



After derivatization with Dragendorff's reagent
Plate 3. TLC profile of *Jaborandi* Leaves

ANNEX-C

[*Table* 1, *Sl No.* (XIV to XX), *Col* 4]

MICROBIAL LIMIT TESTS

C-1 GENERAL

The tests are designed for the estimation of the number of viable aerobic microorganisms present and for detecting the presence of designated microbial species in the extract. The term 'growth' is used to designate the presence and presumed proliferation of viable microorganisms.

C-2 APPARATUS

C-2.1 Oven for Dry Sterilization
C-2.2 Autoclave for Wet Sterilization
C-2.3 Incubator
C-2.4 Water Bath
C-2.5 pH-meter
C-2.6 Sterile Membrane Filters, 50 mm in Diameter
C-2.7 Colony Counting Equipment
C-2.8 Analytical Balance
C-2.9 Standard Glassware

C-3 MEDIA

Culture media may be prepared as given below or dehydrated culture media may be used provided that, when reconstituted as directed by the manufacturer, they have similar ingredients and / or yield media comparable to those obtained from the formulae given below.

Where agar is specified in a formula, use agar that has a moisture content of not more than 15 percent. Where water is called for in a formula, use purified water. Unless otherwise indicated, the media should be sterilized by heating in an autoclave (15 psi) at 121 °C for 15 min. In preparing media by the formulas given below, dissolve the soluble solids in the water, using heat if necessary, to effect complete solution, add solutions of 0.1N hydrochloric acid or 0.1N sodium hydroxide in quantities sufficient to yield the required *p*H in the medium when it is ready for use. Determine the *p*H at 25°C± 2°C.

C-3.1 Baird Parker Agar Medium

C-3.1.1 Composition

Pancreatic digest of casein	10.0 g
Beef extract	5.0 g
Yeast extract	1.0 g
Lithium chloride	5.0 g
Agar	20.0 g
Glycine	12.0 g

Sodium pyruvate	10.0 g
Water	1000 ml

C-3.1.2 Preparation

Suspend the components in 1000 ml of water, heat with frequent agitation and boil for 1 min. Sterilize, cool in between 45 °C to 50 °C, add 10 ml of a one percent w/v solution of sterile potassium tellurite and 50 ml of egg yolk emulsion. Mix thoroughly, but gently and pour into plates. (Prepare the egg yolk emulsion by disinfecting the surface of whole shell eggs, aseptically cracking the eggs, and separating out intact yolks into a sterile graduated cylinder. Add sterile saline solution, get a 3 to 7 ratio of egg yolk to saline. Add to a sterile blender cup and mix at high speed for 5 seconds). Adjust the pH after sterilization to 6.8 ± 0.2.

A-3.2 Bismuth Sulphite Agar Medium

C-3.2.1 Composition

Solution (1)

Beef extract	6 g
Peptone	10 g
Agar	24 g
Ferric citrate	0.4 g
Brilliant green	10 mg
Water	1 000 ml

Solution (2)

Ammonium bismuth citrate	3 g
Sodium sulphite	10 g
Anhydrous disodium hydrogen phosphate	5 g
Dextrose monohydrate	5 g
Water	100 ml

C-3.2.2 Preparation

Suspend the components of Solution 1 in 1000 ml of water. Heat to boiling to dissolve the medium completely. Sterilize by maintaining at 115 °C for 30 min. Suspend the components of Solution 2 in 100 ml of water. Heat to boiling to dissolve the medium completely. Do not autoclave. Add 1 volume of solution 2 to 10 volumes of solution 1 previously melted and cooled to a temperature of 55 °C. Bismuth Sulphite Agar Medium should be stored at 2°Cto 8°C for 5 days before use.

C-3.3 Brilliant Green Agar Medium

C-3.3.1 Composition

Peptone	10.0 g
Yeast extract	3.0 g

Lactose	10.0 g
Sucrose	10.0 g
Sodium chloride	5.0 g
Phenol red	80.0 g
Brilliant green	12.5 mg
Agar	12.0 g
Water	1 000 ml

C-3.3.2 Preparation

Mix the components, allow to stand for 15 min, sterilize by maintaining at 115 °C for 30 min and mix before pouring.

C-3.4 Buffered Sodium Chloride Peptone Solution pH 7.0

C-3.4.1 Composition

Potassium dihydrogen phosphate	3.56 g
Disodium hydrogen phosphate	7.23 g
Sodium chloride	4.30 g
Peptone (meat or casein)	1.0 g
Water	1 000 ml

C-3.4.2 Preparation

Mix the components and heat if necessary to dissolve the medium completely. 0.1 to 1.0 percent w/v Polysorbate 20 or polysorbate 80 may be added. Sterilize by heating in an autoclave at 121°C for 15 min.

C-3.5 Casein Soyabean Digest Agar Medium

C-3.5.1 Composition

Pancreatic digest of casein	15.0 g
Papaic digest of soyabean meal	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Water	1 000 ml

C-3.5.2 Preparation

Suspend the components in water. Heat to boiling to dissolve the medium completely. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.3 ± 0.2.

C-3.6 Cetrimide Agar Medium

C-3.6.1 Composition

Pancreatic digest of gelatin	20.0 g
Magnesium chloride	1.4 g

Potassium sulphate	10.0 g
Cetrimide	0.3 g
Agar	13.6 g
Glycerin	10.0 g
Water	1 000 ml

C-3.6.2 Preparation

Dissolve the components in 1000 ml of water. Heat to boiling for 1 min with shaking. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.0 to 7.4.

C-3.7 Deoxycholate Citrate Agar Medium

C-3.7.1 Composition

Beef extract	5.0 g
Peptone	5.0 g
Lactose	10.0 g
Trisodium citrate	8.5 g
Sodium thiosulphate	5.4 g
Ferric citrate	1.0 g
Sodium deoxycholate	5.0 g
Neutral red	0.02 g
Agar	12.0 g
Water	1 000 ml

C-3.7.2 Preparation

Mix the components and allow to stand for 15 min. Gently boil with continuous stirring and continue boiling until solution is complete. Cool to 80 °C, mix, pour and cool rapidly.

Care should be taken not to overheat Deoxycholate Citrate Agar during preparation. It should not be re-melted and the surface of the plates should be dried before use.

C-3.8 Fluid Casein Digest Soya Lecithin Polysorbate 20 Medium

C-3.8.1 Composition

Pancreatic digest of casein	20 g
Soya lecithin	5 g
Polysorbate 20	40 ml
Water	1 000 ml

C-3.8.2 Preparation

Dissolve the pancreatic digest of casein and soya lecithin in water, heating in a water bath at 48 °C to 50 °C for about 30 min to effect solution. Add polysorbate 20, mix and dispense as desired. Sterilize at 121 °C for 15 min in an autoclave.

C-3.9 Fluid Lactose Medium

C-3.9.1 Composition

Beef extract	3.0 g
Pancreatic digest of gelatin	5.0 g
Lactose	5.0 g
Water	1 000 ml

C-3.9.2 Preparation

Suspend the components in 1000 ml water. Heat if necessary to dissolve the medium completely. Sterilize at 121 °C for 15 min in an autoclave. Cool as quickly as possible after sterilization. Adjust the pH after sterilization to 6.9 ± 0.2.

C-3.10 Lactose Broth Medium

C-3.10.1 Composition

Beef extract	3.0 g
Pancreatic digest of gelatin	5.0 g
Lactose	5.0 g
Water	1 000 ml

C-3.10.2 Preparation

Suspend the components in water and heat if necessary to dissolve the medium completely. Sterilize at 121 °C for 15 min in an autoclave. Adjust the pH after sterilization to 6.9 ± 0.2.

C-3.11 Levine Eosin Methylene Blue Agar Medium

C-3.11.1 Composition

Pancreatic digest of gelatin	10.0 g
Dibasic potassium phosphate	2.0 g
Agar	15.0 g
Lactose	10.0 g
Eosin Y	400 mg
Methylene blue	65 mg
Water	1 000 ml

C-3.11.2 Preparation

Dissolve the pancreatic digest of gelatin, dibasic potassium phosphate and agar in water with warming and allow to cool. Just prior to use, liquify the gelled agar solution and the remaining ingredients, as solutions, in the following amounts and mix. For each 100 ml of the liquified agar solution use 5 ml of a 20 percent w/v solution of lactose, 2 ml of a 2 percent w/v solution of eosin Y and 2 ml of a 0.33 percent w/v solution of methylene blue. The finished medium may not be clear. Adjust the pH after sterilization to 7.1±0.2.

C-3.12 MacConkey Agar Medium

C-3.12.1 Composition

Pancreatic digest of gelatin	17.0 g
Peptone (meat and casein, equal parts)	3.0 g
Lactose	10.0 g
Sodium chloride	5.0 g
Bile salts	1.5 g
Agar	13.5 g
Neutral red	30 mg
Crystal violet	1 mg
Water	1000 ml

C-3.12.2 Preparation

Boil the mixture of solids and water for 1 min to effect solution. Sterilize at 121 °C for 15 min in an autoclave. Adjust the pH after sterilization to 7.1 ± 0.2.

C-3.13 MacConkey Broth Medium

C-3.13.1 Composition

Pancreatic digest of gelatin	20.0 g
Lactose	10.0 g
Dehydrated ox bile	5.0 g
Bromocresol purple	10 mg
Water	1000 ml

C-3.13.2 Preparation

Suspend the components in 1000 ml of water and heat if necessary to dissolve the medium completely. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.3 ± 0.2.

C-3.14 Mannitol Salt Agar Medium

C-3.14.1 Composition

Pancreatic digest of gelatin	5.0 g
Peptic digest of animal tissue	5.0 g
Beef extract	1.0 g
D-Mannitol	10.0 g
Sodium chloride	75.0 g
Agar	15.0 g
Phenol red	25 mg
Water 1000 ml	

C-3.14.2 Preparation

Mix the components, heat with frequent agitation and boil for 1 min to effect solution. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.4 ± 0.2.

C-3.15 Nutrient Broth Medium

C-3.15.1 Composition

Beef extract	10.0 g
Peptone	10.0 g
Sodium chloride	5 mg
Water	1000 ml

C-3.15.2 Preparation

Dissolve the components with the aid of heat. Adjust the pH to 8.0 to 8.4 with 5M sodium hydroxide and boil for 10 min. Filter and sterilize by maintaining at 115 °C for 30 min and adjust pH to 7.3 ± 0.1.

C-3.16 Nutrient Agar Medium

Nutrient broth gelled by the addition of 1 to 2 percent w/v of agar.

C-3.17 Pseudomonas Agar Medium for Detection of Flourescein

C-3.17.1 Composition

Pancreatic digest of casein	10.0 g
Peptic digest of animal tissue	10.0 g
Anhydrous dibasic potassium phosphate	1.5 g
Magnesium sulphatehepta hydrate	1.5 g
Glycerin	10.0 ml
Agar	15.0 g
Water	1 000 ml

A-3.17.2 Preparation

Dissolve the solid components in water before adding glycerin. Heat with frequent agitation and boil for 1 min to effect solution. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.2 ± 0.2.

C-3.18 Pseudomonas Agar Medium for Detection of Pyocyanin

C-3.18.1 Composition

Pancreatic digest of gelatin	20.0 g
Anhydrous magnesium chloride	1.4 g
Anhydrous potassium sulphate	10.0 g
Agar	15.0 g
Glycerin	10.0 ml
Water	1 000 ml

C-3.18.2 Preparation

Dissolve the solid components in water before adding glycerin. Heat with frequent agitation and boil for 1 min to effect solution. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 7.2 ± 0.2.

C-3.19 Sabouraud Dextrose Agar Medium

C-3.19.1 Composition

Dextrose	40 g
Peptic digest of animal tissue and Pancreatic digest of casein (1:1)	10 g
Agar	15 g
Water	1000 ml

C-3.19.2 Preparation

Mix the components and heat to boiling to dissolve completely. Sterilize at 121 °C for 15 min in an autoclave and adjust the pH after sterilization to 5.6 ± 0.2.

C-3.20 Sabouraud Dextrose Agar Medium with Antibiotics

To 1000 ml of Sabouraud Dextrose Agar Medium, add 0.1 g of benzylpenicillin sodium and 0.1 g of tetracycline HCL or alternatively add 50 mg of chloramphenicol immediately before use.

C-3.21 Selenite F Broth

C-3.21.1 Composition

Peptone	5 g
Lactose	4 g
Disodium hydrogen phosphate	10 g
Sodium hydrogen selenite	4 g
Water	1 000 ml

C-3.21.2 Preparation

Suspend the components in water and mix well. Warm to dissolve the medium completely. Distribute in sterile containers and sterilize by maintaining at 100 °C for 30 min.

C-3.22 Fluid Selenite Cystine Medium

C-3.22.1 Composition

Pancreatic digest of casein	5.0 g
Lactose	4.0 g
Sodium phosphate	10.0 g
Sodium hydrogen selenite	4.0 g
l-Cystine	10.0 mg

Water	1 000 ml

C-3.22.2 Preparation

Suspend the components in water and heat in flowing steam for 15 min. Adjust the final pH to 7.0 ± 0.2. Do not sterilize.

C-3.23 Tetrathionate Broth Medium

C-3.23.1 Composition

Beef extract	0.9 g
Peptone	4.5 g
Yeast extract	1.8 g
Sodium chloride	4.5 g
Calcium carbonate	25.0 g
Sodium thiosulphate	40.7 g
Water	1000 ml

C-3.23.2 Preparation

Dissolve the solids in 1000 ml of water and heat the solution to boil. On the day of use, add a solution prepared by dissolving 5 g of potassium iodide and 6 g of iodine in 20 ml of water.

C-3.24 Tetrathionate Bile Brilliant Green Broth Medium

C-3.24.1 Composition

Peptone	8.6 g
Dehydrated ox bile	8.0 g
Sodium chloride	6.4 g
Calcium carbonate	20.0 g
Potassium tetrathionate	20.0 g
Brilliant green	70 mg
Water	1000 ml

C-3.24.2 Preparation

Suspend the components in 1000 ml of water. Heat just to boiling. Do not autoclave or reheat. Adjust the pH so that after heating it is 7.0 ± 0.2.

C-3.25 Triple Sugar Iron Agar Medium

C-3.25.1 Composition

Beef extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Lactose	10.0 g

Sucrose	10.0 g
Dextrose monohydrate	1.0 g
Ferrous sulphate	0.2 g
Sodium chloride	5.0 g
Sodium thiosulphate	0.3 g
Phenol red	24 mg
Water	1 000 ml

C-3.25.2 Preparation

Mix the components in 1000 ml of water, allow standing for 15 min. Heat to boiling to dissolve the medium completely. Mix well and distribute into test tubes and Sterilize by maintaining at 121 °C for 15 min. Allow the medium to stand in a sloped form with a butt about 2.5 cm long.

C-3.26 Urea Broth Medium

C-3.26.1 Composition

Potassium dihydrogen orthophosphate	9.1 g
Anhydrous disodium hydrogen phosphate	9.5 g
Urea	20.0 g
Yeast extract	0.1 g
Phenol red	10 mg
Water	1 000 ml

C-3.26.2 Preparation

Mix the components, sterilize by filtration and distribute aseptically in sterile containers.

C-3.27 Vogel Johnson Agar Medium

C-3.27.1 Composition

Pancreatic digest of casein	10.0 g
Yeast extract	5.0 g
Mannitol	10.0 g
Dibasic potassium phosphate	5.0 g
Lithium chloride	5.0 g
Glycerin	10.0 g
Agar	16.0 g
Phenol red	25.0 mg
Water	1 000 ml

C-3.27.2 Preparation

Suspend the components in 1000 ml of water. Boil the solution of solids for 1 min. Sterilize at 121 °C for 15 min in an autoclave. Cool to 45 °C to 50 °C and add 20 ml of 1 percent w/v sterile solution of potassium tellurite. Adjust the pH after sterilization to 7.0 ± 0.2.

C-3.28 Xylose Lysine Deoxycholate Agar Medium

C-3.28.1 Composition

Xylose	3.5 g
l-Lysine	5.0 g
Lactose	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	80 mg
Agar	13.5 g
Sodium deoxycholate	2.5 g
Sodium thiosulphate	6.8 g
Ferric ammonium citrate	800 mg
Water	1000 ml

C-3.28.2 Preparation

Suspend the components in 1000 ml of water. Heat with frequent agitation until the medium boils. Do not overheat or sterilize. Transfer at once to a water bath maintained at about 50 °C and pour into plates as soon as the medium has cooled. Adjust the final pH to 7.4 ± 0.2.

C-4 SAMPLING

Use 10 ml or 10 g specimens for each of the tests specified in the Indian Standard.

C-5 PRECAUTIONS

The microbial limit tests should be carried out under conditions designed to avoid accidental contamination during the test. The precautions taken to avoid contamination must be such that they do not adversely affect any microorganisms that should be revealed in the test.

C-6 PRELIMINARY TESTING

Prior to doing the tests, inoculate diluted extracts being examined with separate viable cultures of Escherichia coli, Salmonella species, Pseudomonas aeruginosa and Staphylococcus aureus. This is done by adding 1 ml of 24 h broth culture containing not less than 1000 microorganisms to the first dilution (in buffer solution pH 7.2, fluid soyabean casein digest medium or fluid lactose medium) of the test material and following the test procedure. If the organisms fail to grow in the relevant medium the procedure should be modified by (a) increasing the volume of diluent with the quantity of test material remaining the same, or (b) incorporating a sufficient quantity of a suitable inactivating agent in the diluents, or (c) combining the afore mentioned modifications so as to permit growth of the organisms in the media. If inhibitory substances are present in the extracts, 0.5 percent of soya lecithin and 4 percent of polysorbate 20 may be added to the culture medium. Alternatively, repeat the test as described in the previous paragraph, using fluid casein digest soya lecithin polysorbate 20 medium, to demonstrate neutralization of preservatives or other antimicrobial agents in the test material. Where

inhibitory substances are contained in the extracts and the latter is soluble, the membrane filtration method described under Total Aerobic Microbial Count may be used. If in spite of incorporation of suitable inactivating agents and a substantial increase in the volume of diluent it is still not possible to recover the viable cultures described above and where the article is not suitable for applying the membrane filtration method, it can be assumed that the failure to isolate the inoculated organism may be due to the bactericidal activity of the product. This may indicate that the article is not likely to be contaminated with the given species of microorganisms. However, monitoring should be continued to establish the spectrum of inhibition and bactericidal activity of the article.

C-7 TOTAL AEROBIC MICROBIAL COUNT

Pre-treat the extracts and raw materials being examined as described below.
NOTE –The raw materials need to be ground as a coarse powder before analysis.

C-7.1 Water Soluble Products

Dissolve 10 g or dilute 10 ml of the extract preparation being examined, unless otherwise specified, in buffered sodium chloride peptone solution pH 7.0 or any other suitable medium shown to have no antimicrobial activity under the conditions of test and adjust the volume to 100 ml with the same medium. If necessary, adjust the pH to about 7.

C-7.2 Products Insoluble in Water (Non Fatty)

Suspend 10 g or 10 ml of the extract preparation being examined, unless otherwise specified, in buffered sodium chloride peptone solution pH 7.0 or any other suitable medium shown not to have antimicrobial activity under the conditions of the test and dilute to 100 ml with the same medium. If necessary, divide the preparation being examined and homogenize the suspension mechanically. A suitable surface active agent such as 0.1 percent w/v of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust pH of the suspension to about 7.

C-7.3 Fatty Products

Homogenise 10 g or 10 ml of the extract preparation being examined, unless otherwise specified, with 5 g of polysorbate 20 or polysorbate 80. If necessary, heat to not more than 40 °C. Mix carefully while maintaining the temperature in the water bath or in an oven. Add 85 ml of buffered sodium chloride peptone solution pH 7.0 or any other suitable medium shown to have no antimicrobial activity under the conditions of the test, heated to not more than 40 °C if necessary. Maintain this temperature for the shortest time necessary for formation of an emulsion and in any case for not more than 30 min. If necessary, adjust the pH to about 7.

C-7.4 Examination of the Sample

Determine the total aerobic microbial count in the extract being examined by any of the following methods.

C-7.5 Membrane Filtration

Use membrane filters 50 mm in diameter and having a nominal pore size not greater than 0.45 μm the effectiveness of which in retaining bacteria has been established for the type of preparation being examined. Transfer 10 ml or a quantity of each dilution containing 1g of the preparation being examined to each of two membrane filters and filter immediately. If necessary, dilute the pretreated extract preparation so that a colony count of 10 to 100 may be expected. Wash each membrane by filtering through it three or more successive quantities, each of about 100 ml, of a suitable liquid such as buffered sodium chloride peptone solution pH 7.0.For fatty substances add to the liquid polysorbate 20 or polysorbate 80.Transfer one of the membrane filters, intended for the enumeration of bacteria, to the surface of a plate of casein soyabean digest agar and the other, intended for the enumeration of fungi, to the surface of a plate of Sabouraud dextrose agar with antibiotics.

Incubate the plates for 5 days, unless a more reliable count is obtained in shorter time, at 30 °C to 35 °C in the test for bacteria and 20 °C to 25 °C in the test for fungi. Count the number of colonies that are formed. Calculate the number of microorganisms per g or per ml of the extract preparation being examined, if necessary count bacteria and fungi separately.

C-7.6 Plate Count for Bacteria

Using Petri dishes 9 to 10 cm in diameter, add to each dish a mixture of 1 ml of the pretreated extract preparation and about 15 ml of liquified casein soyabean digest agar at not more than 45 °C.

Alternatively, spread the pretreated extract preparation on the surface of the solidified medium in a petri dish of the same diameter. If necessary, dilute the pretreated extract preparation as described above so that a colony count of not more than 300 may be expected. Prepare at least two such petri dishes using the same dilution and incubate at 30 °C to 35 °C for 5 days, unless a more reliable count is obtained in a shorter time. Count the number of colonies that are formed. Calculate the results using plates with the greatest number of colonies but taking 300 colonies per plate as the maximum consistent with good evaluation.

C-7.7 Plate Count for Fungi

Proceed as described in the test for bacteria but use Sabouraud dextrose agar with antibiotics in place of casein soyabean digest agar and incubate the plates at 20 °C to 25°Cfor 5 days, unless a more reliable count is obtained in a shorter time. Calculate the results using plates with not more than 100 colonies.

C-7.8 Multiple Tube or Serial Dilution Method

In each of fourteen test tubes of similar size place 9.0 ml of sterile fluid soyabean casein digest medium. Arrange twelve of the tubes in four sets of three tubes each. Put aside one set of three tubes to serve as controls. Into each of three tubes of one set (“100”) and into fourth tube (A) pipette 1 ml of the solution of suspension of the test specimen (extract) and mix. From tube A pipette 1 ml of its contents into the one remaining tube (B) not included in the set and mix. These two tubes contain 100 mg (or 100 μl) and 10 mg (or 10 μl) of the specimen respectively. Into each of the second set (“10”) of three tubes pipette 1 ml from tube A, and into each tube

of the third set ("1") pipette 1 ml from tube B. Discard the unused contents of tube A and B. Close well and incubate all of the tubes.

Following the incubation period, examine the tubes for growth. The three control tubes remain clear. Observations in the tubes containing the test specimen, when interpreted by reference to Table 2, indicate the most probable number of microorganisms per g or per ml of the test specimen.

Table 2
Most probable Total Count by Multiple Tube or Serial Dilution Method
(Clause C-7.8)

S.No.	**Observed Combination of Numbers of Tubes Showing Growth in Each Set**			**Most Probable Number of Microorganisms per g or per ml**
	Number of mg (or ml) of Specimen per Tube			
	100 (100 µl)	10 (10 µl)	1 (1 µl)	
(1)	(2)	(3)	(4)	(5)
i.	3	3	3	>1100
ii.	3	3	2	1100
iii.	3	3	1	500
iv.	3	3	0	200
v.	3	2	3	290
vi.	3	2	2	210
vii.	3	2	1	150
viii.	3	2	0	90
ix.	3	1	3	160
x.	3	1	2	120
xi.	3	1	1	70
xii.	3	1	0	40
xiii.	3	0	3	95
xiv.	3	0	2	60
xv.	3	0	1	40
xvi.	3	0	0	23

C-8 TESTS FOR SPECIFIED MICROORGANISMS

C-8.1 Pre-treatment of the Extract Sample Being Examined

Proceed as described under the test for total aerobic microbial count but using lactose broth or any other suitable medium shown to have no antimicrobial activity under the conditions of test in place of buffered sodium chloride peptone solution pH 7.0.

C-8.2 Escherichia coli

Place the prescribed quantity in a sterile screw capped container, add 50 ml of nutrient broth, shake, allow to stand for 1 h (4 h for gelatin) and shake again. Loosen the cap and incubate at 37 °C for 18 to 24 h.

C-8.2.1 Primary Test

Add 1 ml of the enrichment culture to a tube containing to 10 ml Mac Conkey broth and a Durham tube. Incubate at 42 to 44°C for 24-48 hours. If the contents of the tube show acid and gas production in Durham tube, carry out the secondary test.

C-8.2.2 Secondary Test

Add 0.1 ml of the contents of the tubes containing (a) 5 ml of Mac Conkey broth and (b) 5 ml of peptone water. Incubate in a water bath at 43.5 °C to 44.5 °C for 24 h and examine tube (a) for acid and gas and tube (b) for indole. To test for indole, add 0.5 ml of Kovac's reagent, shake well and allow to stand for 1 min. If a red colour is produced in the reagent layer indole is present. The presence of acid and gas and of indole in the secondary test indicates the presence of Escherichia coli.

Carry out a control test by repeating the primary and secondary tests, adding 1.0 ml of the enrichment culture and a volume of broth containing 10 to 50 Escherichia coli (NCTC 9002) organisms, prepared from a 24 h culture in nutrient broth, to 5 ml of Mac Conkey broth. The test is not valid unless the results indicate that the control contains Escherichia coli.

C-8.2.3 Alternative Test

By means of an inoculating loop, streak a portion from the enrichment culture (obtained in the previous test) on the surface of Mac Conkey agar medium. Cover and invert the dishes and incubate.

Upon examination, if none of the colonies are brick red in colour and have a surrounding zone of precipitated bile the sample meets the requirements of the test for the absence of Escherichia coli. If the colonies described above are found, transfer the suspect colonies individually to the surface of Levine eosin methylene blue agar medium, plated on petri dishes. Cover and invert the plates and incubate. Upon examination, if none of the colonies exhibits both a characteristic metallic sheen under reflected light and a blue black appearance under transmitted light, the sample meets the requirements of the test for the absence of Escherichia coli. The presence of Escherichia coli may be confirmed by further suitable cultural and biochemical tests.

C-8.3 Salmonella

Transfer a quantity of the pretreated preparation being examined containing 10 g or 10 ml of the product to 100 ml of nutrient broth in a sterile screw capped jar, shake, allow to stand for 4 h and shake again. Loosen the cap and incubate at 35 °C to 37 °C for 24 h.

C-8.3.1 Primary Test

Add 1.0 ml of the enrichment culture to each of the two tubes containing (a) 10 ml of selenite F broth and (b) tetrathionate bile brilliant green broth and incubate at 36 °C to 38 °C for 48 h.

From each of these two cultures subculture on at least two of the following four agar media: bismuth sulphate agar, brilliant green agar, deoxycholate citrate agar and xylose lysine deoxycholate agar. Incubate the plates at 36 °C to 38 °C for 18 to 24 h. Upon examination, if none of the colonies conforms to the description given in Table 3, the sample meets the requirements of the test for the absence of the genus Salmonella. If any colonies conforming to the description in Table 3 are produced, carry out the secondary test.

Table 3

Interpretation of the Confirmatory Tests for Salmonella
(Clause C-8.3.1)

S.No.	Medium	Description of Colony
(1)	**(2)**	**(3)**
i.	Bismuth sulphite agar	Black or green
ii.	Brilliant green agar	Small, transparent and colorless, or opaque, pinkish or white (frequently surrounded by a pink or red zone)
iii.	Deoxycholate citrate agar	Colorless and opaque, with or without black centers
iv.	Xylose lysine deoxycholate agar	Red with or without black centres

A-8.3.2 Secondary Test

Subculture any colonies showing the characteristics given in Table 3 in triple sugar iron agar by first inoculating the surface of the slope and then making a stab culture with the same inoculating needle, and at the same time inoculate a tube of urea broth. Incubate at 36 °C to 38 °C for 18 to 24 h. The formation of acid and gas in the stab culture (with or without concomitant blackening) and the absence of acidity from the surface growth in the triple sugar iron agar, together with the absence of a red color in the urea broth, indicate the presence of Salmonella. If acid but no gas is produced in the stab culture, the identity of the organisms should be confirmed by agglutination tests.

Carry out the control test by repeating the primary and secondary tests using 1.0 ml of the enrichment culture and a volume of broth containing 10 to 50 Salmonella abony (NCTC 6017) organisms, prepared from a 24 h culture in nutrient broth, for the inoculation of the tubes (a) and (b). The test is invalid unless the results indicate that the control contains Salmonella.

A-8.4 Pseudomonas aeruginosa

Pretreat the preparation being examined as described above and inoculate 100 ml of fluid soyabean casein digest medium with a quantity of the solution, suspension or emulsion thus obtained containing 1 g or 1 ml of the preparation being examined. Mix and incubate at 35 °C to 37 °C for 24 to 48 h. Examine the medium for growth and if growth is present, streak a portion of the medium on the surface of cetrimide agar medium, each plated on petri dishes. Cover and incubate at 35 °C to 37 °C for 18 to 24 h.

If, upon examination, none of the plates contains colonies having the characteristics listed in Table 4for the media used, the sample meets the requirement for freedom from Pseudomonas aeruginosa. If any colonies conforming to the description in Table 4are produced, carry out the oxidase and pigment tests. Streak representative suspect colonies from the agar surface of cetrimide agar on the surfaces of Pseudomonas agar medium for detection of fluorescein and Pseudomonas agar medium for detection of pyocyanin contained in petri dishes. Cover and invert the inoculated media and incubate at 33 °C to 37 °C for not less than 3 days. Examine the streaked surfaces under ultra violet light. Examine the plates to determine whether colonies conforming to the description in Table 4 are present. If growth of suspect colonies occurs, place 2 or 3 drops of a freshly prepared 1 percent w/v solution of N,N,N1,N1-tetramethyl-4-phenylenediamine dihydrochloride on filter paper and smear with the colony. If there is no development of a pink color, changing to purple, the sample meets the requirements of the test for the absence of Pseudomonas aeruginosa.

Table 4
Interpretation of the Confirmatory Tests for Pseudomonas aeruginosa
(Clause C-8.4)

S.No.	Selective Medium	Characteristic Colonial Morphology	Fluorescence in UV Light	Oxidase Test	Gram Stain
(1)	**(2)**	**(3)**	**(4)**	**(5)**	**(6)**
i.	Cetrimide agar	Generally greenish	Greenish	Positive	Negative rods
ii.	Pseudomonas agar medium for detection of fluorescein	Generally colorless to yellowish	Yellowish	Positive	Negative rods
iii.	Pseudomonas agar for detection of pyocyanin	Generally greenish	Blue	Positive	Negative rods

C-8.5 Staphylococcus aureus

Proceed as described under Pseudomonas aeruginosa, if upon examination of the incubated plates, none of them contains colonies having the characteristics listed in for the media used, the sample meets the requirements for the absence of Staphylococcus aureus. If growth occurs, carry out the coagulase test. Transfer representative suspect colonies from the agar surface of any of the media listed in Table 5 to individual tubes, each containing 0.5 ml of mammalian, preferably rabbit or horse, plasma with or without additives

Table 5

Interpretation of the Confirmatory Tests for Staphylococcus aureus
(Clause C-8.5)

S.No.	Selective Medium	Characteristic Colonial Morphology	Gram Stain
(1)	**(2)**	**(3)**	**(4)**
i.	Vogel johnson agar	Black surrounded by yellow zones	Positive cocci (in clusters)
ii.	Mannitol salt agar	Yellow colonies with yellow zones	Positive cocci (in clusters)
iii.	Baird parker agar	Black, shiny, surrounded by clear zones of 2 to 5 mm	Positive cocci (in clusters)

C-8.6 Validity of the Tests for Total Aerobic Microbial Count

Grow the following test strains separately in tubes containing fluid soyabean casein digest medium at 30 °C to 35 °C for 18 to 24 h or, for Candida albicans, at 20°C for 48 h.

Staphylococcus aureus (ATCC 6538; NCTC 10788)
Bacillus subtilis (ATCC 6633; NCIB 8054)
Escherichia coli (ATCC 8739; NCIB 8545)
Candida albicans (ATCC 2091; ATCC 10231)

Dilute portions of each of the cultures using buffered sodium chloride peptone solution pH 7.0 to make test suspensions containing about 100 viable microorganisms per ml. Use the suspension of each of the microorganisms separately as a control of the counting methods, in the presence and absence of the preparation being examined, if necessary.

A count for any of the test organisms differing by not more than a factor of 10 from the calculated value for the inoculum should be obtained. To test the sterility of the medium and of the diluent and the aseptic performance of the test, carry out the total aerobic microbial preparation. There should be no growth of microorganisms.

C-8.7 Validity of the Tests for Specified Microorganisms

Grow separately the test strains of Staphylococcus aureus and Pseudomonas aeruginosa in fluid soyabean casein digest medium and Escherichia coli and Salmonella typhimurium at 30 °C to 35 °C for 18 to 24 h. Dilute portions of each of the cultures using buffered sodium chloride peptone solution pH 7.0 to make test suspensions containing about 103 viable microorganisms per ml. Mix equal volume of each suspension and use 0.4 ml (approximately 102 microorganisms of each strain) as an inoculum in the test for *E. coli, S. typhimurium, P. aeruginosa* and *S. aureus,* in the presence and absence of the extract preparation being examined, if necessary. A positive result for the respective strain of microorganism should be obtained.