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BELIZE NATIONAL STANDARD

SPECIFICATION FOR BOTTLED/ PACKAGED WATER

Committee Representation

The preparation of this standard for the Standard Advisory Council established under the Standards Act of 1992, was carried out under the supervision of the Bureau's Technical Committee for Food and Food Related Products, which at the time comprised of the following members:

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Mrs. Helen Reynolds Arana
Belize Bureau of Standards

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0 FOREWORD

0.1 This Standard has been prepared to set levels of quality and safety for bottled/packaged water imported, produced and traded in Belize.

0.2 Uncarbonated bottled/packaged water is quite possibly the fastest growing segment of the beverage industry. In addition to local production, bottled/packaged water is imported in copious quantities to satisfy the growing demand for this product. This standard sets the guidelines for the production and promotion of mineral water, spring water and purified water.

0.3 This standard will be reviewed and may be revised from time to time to allow for changes in manufacturing technology or consumer preference within Belize.

0.4 In drafting this standard assistance was derived from the following:

SLNS 29: 1992	Specification for Bottled Water (Mineral Water, Spring Water, Purified Water), Saint Lucia National Standard
IS 13428: 1992	Mineral Water - Specification, Bureau of Indian Standard
CODEX STAN: 227-2001	General Standard for Bottled/Packaged Drinking Waters (other than Natural Mineral waters)

0.5 This standard is intended to be compulsory.

1.0 SCOPE

1.1 This standard specifies requirements for the purity, treatment, bacteriological acceptability, packaging and labelling of spring water, mineral water and purified waters that are prepackaged for sale and used as beverages or in foods.

1.2 This standard does not apply to water distributed by the public water supply system, to carbonated water, soda water or to bottled water sold for purposes other than as a beverage.

1.3 This standard should be used in conjunction with the Belize National Code of Hygienic Practice for the Collecting, Processing and Marketing of Packaged Water.

2.0 DEFINITIONS

2.1 **Fortified Mineral Water** is water derived from any source of potable water, which may be blended/treated/fortified with mineral salts for achieving the requirement of this standard.

2.2 **Natural Mineral Water** is potable water that is obtained directly from an underground source, which is characterized by containing concentrations of inorganic substances that may confer flavour or that may produce beneficial physiological effects when used regularly. The level of total dissolved material salts shall be greater than 250 ppm.

2.3 **Packaged Water** is fortified mineral water, natural mineral water, purified water or spring water supplied in containers for human consumption and conforms to the requirements of the standard.

2.4 **Potable Water** is water that is suitable for regular use for human consumption.

2.5 **A Public Water Supply System** is a source of potable water operated by a public utility, a company or other body, using distribution through pipelines or tank-wagons**.**

2.6 **Prepared Waters** do not comply with all the provisions set for waters defined by origin, they may originate from any type of water supply.

2.7 **Purified Water** is potable water derived from a public water supply system, a river, or stream, or a reservoir, with or without the addition of mineral salts or mineral water, that has been treated and purified and conforms to the requirements of this standard.

2.8 **Spring water shall** be water from an underground source from which there is a natural flow to the surface. This water shall be collected only at the spring or through a bore hole trapping the underground formation feeding the spring. Spring water collected with the use of an external force shall be from the same underground stratum as the spring as shown by a measurable hydraulic connection using a hydro-geologically valid method between the bore hole and the natural spring. Such water shall have the same physical and chemical properties before treatment as the water that flows naturally to the surface of the earth**.** If spring water is collected with the use of external force, water must continue to flow naturally to the surface of the earth through the spring's natural orifice. The level of total dissolved mineral salts shall be less than 500 ppm.

3.0 REQUIREMENTS FOR PROCESSING NATURAL MINERAL WATER AND SPRING WATER

3.1 Natural mineral water or spring water shall be obtained from an underground aquifer that is not polluted by agricultural, domestic, industrial, or other wastes, and may be treated by the following processes:

(a) decantation, to remove solids;

(b) filtration, to remove particles of suspended matters;

(c) aeration with clean, filtered air;

(d) ozonation, using ozonized oxygen (ozone); and

(e) ultra-violet radiation.

3.2 Natural mineral water or fortified mineral water is characterized by containing concentrations of inorganic substances that may confer flavour or that may produce beneficial physiological effects when regularly used.

4.0 REQUIREMENTS FOR PROCESSING PURIFIED WATER

4.1 Purified water shall be decanted, filtered, and clarified using chemical agents or other approved clarification methods and shall be treated with chlorine or a source of chlorine (with excess chlorine being removed by aeration), or other approved method of disinfecting; or be distilled, and may:

(a) have added fluoride or ozone;

(b) be demineralized, so that inorganic substances are reduced below 10 parts per million;

(c) be treated by reverse osmosis; and

(d) ozonation using ozone oxygen.

5.0 MICROBIOLOGICAL REQUIREMENTS

5.1 Mineral Water, spring water and purified water, when sampled and tested within 12 hours of packaging, as in section 12 and 13, shall contain:

(1) no coliform bacteria in 250 mL at 37^{o}C and 44.5^{o}C
(2) no faecal *Streptococci* in 250 mL

(3) no *Pseudomonas aeruginosa* in 250 mL

(4) (i) no more than 100 aerobic bacteria per millitre at 22°C in 72 hours
(ii) no more than 20 aerobic bacteria per mL at 37°C in 24 hours.

(5) no Salmonella or Shigella; and

(6) no Vibro Cholerae and V Parahaemolyticus.

6.0 LIMITS ON CERTAIN SUBSTANCES

6.1 Fortified mineral water or natural mineral water or spring water, when sampled as in Section 12 and tested by the methods mentioned in Section 13, shall not contain the substances named in Column 1 of Table 1 in concentration exceeding the limits specified in Column 2 of that Table.

6.2 Purified water, when sampled as in Section 12 and tested by the methods
mentioned in Section 13 shall not contain the substance named in Column 1 of Table 2 in concentration exceeding the limits specified in Column 2 of that Table.

TABLE 1
MAXIMUM CONCENTRATIONS OF CERTAIN SUBSTANCES IN FORTIFIED OR NATURALMINERAL WATER OR SPRING WATER IN mg/L (ppm)

(1) **SUBSTANCE**	(2) **MAXIMUM CONCENTRATION**
Aluminium	0.2
Antimony	0.005
Arsenic	0.05
Barium	0.7
Cadmium	0.003
Chromium (VI)	0.05

1 Any increase in the total viable colony count of the water between 12 hours after packaging and the time of sale shall not be greater than that normally expected.

TABLE 1 CONTINUE

(1) **SUBSTANCE**	(2) **MAXIMUM CONCENTRATION**
Copper	2.0
Lead	0.01
Manganese	2.0
Mercury	0.005
Nickel	0.001
Selenium	0.01
Thallium	0.002
Zinc	5.0
Borate	30 calculated as H_3BO_3
Fluoride	1.5 calculated as F^-
Chloride	250
Nitrate	50 calculated as NO_3
Sulphide	0.05 calculated as H_2S
Organic matter	3 calculated as $\mathbf{O}_2$
Ra228	15pCi/L

TABLE 2
MAXIMUM CONCENTRATIONS OF CERTAIN SUBSTANCES IN PURIFIED WATER IN mg/L (ppm)

(1) **SUBSTANCE**	(2) **MAXIMUM CONCENTRATION**
Aluminium	0.2
Antimony	0.005
Arsenic	0.05
Barium	1.0
Cadmium	0.003
Chromium (VI)	0.005

TABLE 2 CONTINUE

(1) **SUBSTANCE**	(2) **MAXIMUM CONCENTRATION**
Copper	1.0
Iron	0.4
Lead	0.01
Manganese	0.05
Mercury	0.001
Nickel	0.02
Thallium	0.002
Selenium	0.01
Borate	30 calculated as H_3BO_3
Fluoride	1.5 calculated as F^-
Organic Matter	3 calculated as O_2
Zinc	3.0
Chloride	400
Nitrate	50
Sulphate	500

7.0 CONTAMINANTS

7.1 Natural mineral water, fortified mineral water, spring water and purified water shall not contain:

(a) cyanide ion;

(b) any detectable residues of pesticides, such as:

(i) Organochlorines e.g. endrin, lindane, toxaphene, 2-4-D, 2, 4, 5 – TP;

(ii) Organophosphates e.g. pirimiphos – ethyl, ethoprop, diazinon, Malathion, glyphosate;

(iii) Carbamates e.g – carbofuran, oxamyl, propoxur;
(iv) Bipyridinium salts e.g – paraquat, diquat; and

(c) Trihalomethanes and Bromate from ozonation.

7.2 Spring water shall not contain calcium (as $CaCO_3$), in excess of 150 mg/L.

8.0 REQUIREMENTS FOR HYGIENE IN COLLECTING, PROCESSING AND MARKETING OF BOTTLED/ PACKAGED WATERS

8.1 The products covered by the provisions of this standard shall be prepared in accordance with the Belize Code of Hygiene Practice for the Collecting, Processing and Marketing of Bottle/Packaged Water.

8.2 **Construction and maintenance of Buildings**

8.2.1 The room used for bottling water shall be separated from other areas of the plant by self-closing doors, and have tight ceiling and floors to prevent contamination of the product. Conduits for utilities and openings for conveyors shall be no larger than necessary. Floors shall be of non-skid impervious materials, graded to drains, and wall surfaces shall be smooth and impervious to water.

8.2.2 Ventilation in the bottling rooms and other rooms used in processing shall be sufficient to prevent condensation.

8.2.3 Washing and sanitizing of containers shall be done in a closed room next to the bottling room to prevent contamination after cleaning.

8.2.4 Rooms in which the water is stored, processed, packaged or bottled and rooms where containers and closures are stored, washed or sanitized, shall not open directly into rooms used as offices or into rooms for staff lockers, lunch, recreation, washing, or toilet facilities.

8.3 **Sanitation**

8.3.1 Potable water shall be used in cleaning and sanitizing containers and equipment. Such water may be derived from a source different to that used for the plant through a separate supply system which has no connection with the pipe system used for the product.

8.3.2 Sanitizing of equipment and containers may be done using steam or chemical agents which have adequate bactericidal action. Residues of chemical agents shall be rinsed from equipment and containers using steam or potable water free from pathogenic bacteria.

8.3.3 Any compressed air used in the plant, which may come in contact with the product, or equipment surfaces in contact with product, or containers, shall be free from oil, dirt, dust, and rust and shall not affect the odour, colour, flavour or bacteriological quality of the product.

8.3.4 The surfaces of all equipment, utensils, pipe-lines and valves that come in contact with the product, and are used in transport, handling, processing and storage of the product, shall be kept clean and sanitized, and shall be regularly inspected and cleaned to maintain sanitary conditions.

8.3.5 Retail containers that are returned for re-use shall be cleaned, washed, and sanitized as in 8.2.3, and inspected, and any defective containers shall be removed from production.

8.3.6 After cleaning all equipment, utensils, dis-assembled pipelines and valves and re-usable retail containers shall be drained and protected from contamination until used.

8.3.7 Retailed containers intended for single use and closures shall be stored under sanitary conditions and inspected before use; if necessary they shall be washed and sanitized.

8.4 **Equipment**

All plant equipment shall be suitably designed for proper use, and all surfaces that will come into contact with the product shall be of non-toxic, non-absorbent materials that will not contaminate the product, and can withstand the cleaning and sanitizing procedures.

Storage tanks that hold the product before, during, or after processing shall be adequately vented and capable of excluding foreign matter.

If it is found during production that the product or the source is contaminated, all production shall cease until the cause of contamination is traced and eliminated, and all equipment, pipelines and valves be cleaned and sanitized before production is resumed.

9.0 PACKAGING

9.1 Mineral water, spring water and purified water shall be packed in hermetically sealed retail containers/packages, which are suitable for preventing the possible adulteration of the water. Retail containers/packaging materials and closures shall be made of non-toxic materials that will not contaminate the water or affect its flavour, and shall be designed to withstand stresses that may be experienced in bottling, handling transport and storage.

9.2 The packages used for containing water for sale shall be made of glass, polyetheneterephthalate (PET) or other suitable food grade plastic material.

9.3 At regular intervals, samples of unfilled containers and closures shall be sampled at the point of filling, and tested bacteriologically for coliform organisms. At least four containers and four closures shall be taken, and the packaging process shall be deemed acceptable if

(a) no coliform organisms are found; and

(b) not more than 1 aerobic bacterium/ml of container capacity, or not more than 1 bacterium/cm^2 of container surface is found.

9.4 Retail containers shall be protected during transport by suitable shipping cartons or crates. If crates are reusable they shall be inspected before re-use and cleaned as may be necessary to minimise risk of contamination of the product.

9.5 Closures shall be so designed as to prevent contamination and to show signs Of any tampering after filling and sealing the containers.

10.0 LABELLING

10.1 The labelling of retail packages of bottled water shall conform to the requirements of the **Belize National Standard Specification for Labelling Part 3: Labelling of Prepackaged Food – BZS 1: Part 3: 1998**.

10.2 **Purified Water**

10.2.1 Labels on retail containers of purified water shall include the following information in addition to that requires by 10.1:-

(a) the product name, “Purified Water” or “Water”, which may be modified by the words

(i) “Distilled”, when treated by distillation;
(ii) “Demineralised”. Where the mineral content has been reduced by other means than distillation;

(iii) “Carbonated”, where carbon dioxide has been added;

(b) a statement of the total content of fluoride ion in mg/L or parts per million (ppm);

(c) an indication of the method used in treatment except where the water has been:

(i) Chlorinated, followed by removal of chlorine and chlorinating agent;

(ii) decanted;

(iii) filtered;

(iv) clarified; or

(v) an ingredient declared on the label has been added.

10.3 **Spring Water**

10.3.1 Labels on retail containers of spring water shall carry the following information in addition to that required by 10.1:-

(a) the geographical location of the source;

(b) a statement of the total dissolved mineral salt content in mg/L or parts per million (ppm);

(c) the total fluoride content in mg/L or parts per million (ppm); and

(d) a declaration of the addition of any fluoride or of ozone.

10.3.2 Labels on retail containers of spring water may also include:

(a) a statement of the results of chemical analysis of the water of the source, or as bottled in the container; and

(b) “low sodium”, if the sodium content is less than 20 mg/L (ppm).

10.4 **Natural Mineral Water**

10.4.1 Labels on retail containers of natural mineral water shall carry the following information in addition to that required by 10.1:-

(a) the geographical location of the source;

(b) a statement of the total dissolved mineral salt content in mg/L or parts per million (ppm); and

(c) the total fluoride content in mg/L or part per million (ppm).

10.4.2 Labels on retail containers of natural mineral water may also include:-

(a) a statement of the process used in treatment, as in 3.1;

(b) the word “alkaline”, where the content of bicarbonate ion, HCO_3, exceeds 600 ppm;

(c) the word “saline” where the content of sodium chloride, NaC1, exceeds 1000 ppm;

(d) the words “contain fluoride” where the content of fluoride ion, F^-, exceeds 1 ppm,

(e) the words “contains iron” where the content of iron, Fe^{2+} & Fe^{3+} exceeds 1 ppm,

(f) the words “contains iodine” where the content of the iodine ion, I^-, exceeds 1 ppm,

(g) the words “may be diuretic” where the contents of total dissolved solids exceeds 1000 ppm; and

(h) the words “low in sodium” where the content of the sodium ion, Na^+, is less than 20 ppm.

10.4.3 Where a source of mineral water has been inspected, sampled, tested and approved by an official agency that is concerned with public health, a statement of such approval may be included on the label.

10.5 **Fortified Mineral Water**

10.5.1 Labels on retail containers of fortified mineral water may carry the following information in addition to that required by 10.1 and 10.4.1:

(a) the word “alkaline” where the content of bicarbonate ion, HCO_3, exceeds 600 ppm;

(b) the word “saline” where the content of sodium chloride, NaC1, exceeds 1000 ppm;

(c) the words “contains fluoride” where the content of fluoride, F, exceeds 1 ppm;

(d) the words “contains iron” where the content of iron, Fe^{2+}& Fe^{3+}, exceeds 5 ppm; and

(e) a statement of the result of chemical analysis of the water as bottled in the container.

10.6 **Statements Not to Be Used in Labelling or Advertising**

10.6.1 No statement or pictorial device shall be used on a label of a retail container of natural mineral water, fortified mineral water, spring water, or purified water which may mislead the consumer as to its nature, origin, composition, or properties.

10.6.2 Trade or brand names referring to natural mineral water, fortified mineral water or spring water shall not include a name of a location or community unless the source is located within that location or community.

10.6.3 The trade or brand name referring to purified water shall not include a reference to a geographical feature, location or community.

10.6.4 No claims for medicinal effects (whether preventive, nutritive, alleviative, or curative) shall be made in labels or advertisements of natural mineral water, fortified mineral water, spring water or purified water, other than those allowed by 10.4.2 and 10.6.1.

11.0 QUALITY ASSURANCE

11.1 To be eligible for a licence to use the Belize Standard Mark, the manufacturer shall operate as quality assurance system conforming to the general requirements of international standard ISO 9002 - 1994, using adequate staff, sampling procedures, testing equipment and record keeping as approved by the Belize Bureau of Standards.

11.2 An approved quality assurance system may include sampling or test procedures suited to routine or continuous production that differs from those mentioned in section 12 and 13.

11.3 It is recommended that a Hazard Analysis Critical Control Point (HACCP) System be used as part of the quality assurance procedures.

12.0 SAMPLING

12.1 Where a lot or shipment of bottled water is to be examined for conformity to this standard, it is recommended that a sample including a number **(n)** of retail containers should be taken at random from the total number **(N)** of retail containers in the lot or shipment in accordance with columns 1, 2 and 3 of Table 3.

TABLE 3
Number of Containers to be Selected for Testing

(1) Number in the Lot N	(2) For Bacteriological Tests n	(3) For Chemical Tests n
Up to 1300	3	9
1301 to 3200	6	12
3201 and over	9	15

12.2 The contents of retail containers sampled for chemical testing may be combined in a clean dry container, and shall be sealed and marked with identifying information (place, date and time of sampling, marking on the lot, name of sampler).

12.3 Retail containers sampled for bacteriological testing shall be kept separate and each sealed and marked with identifying information.

12.4 The samples shall be delivered and tested as soon as possible after collection, with precaution against contamination or deterioration.

13.0 TEST METHODS

13.1 It is recommended that the following test methods be used in connection with this standard. These methods are based on that published by the International Organization for Standardization (ISO), ISO Standard Compendium, Environmental – Water Quality, Vol. 3 – Physical, Biological and Microbiological Methods.

13.2 ***Basic Materials***

For the preparation of the medium, use ingredients of uniform quality and chemicals of analytical; grade; alternatively, use an equivalent

dehydrated complete medium and follow the manufacturer's instructions.

For making media, use glass-distilled or deionized water free from substances which might inhabit growth under the conditions of the tests.

13.3 ***Dilutions***

For making the dilutions use one of the diluents given in ISO 8199.

13.4 ***Sampling***

Take the samples of water in accordance with the instructions given in Section 12 of this document.

13.5 ***Colony Count by Inoculation in or a Nutrient Agar Culture Medium***

13.5.1 **Culture Medium and Diluents**

13.5.2 **Yeast extract agar**

Tryptone…….	6 g
Dehydrated yeast extract……	3 g
Agar, power or in pellets…… (according to gel strength)	12 g
Water……..	1000 ml

Add the ingredients, or the complete dehydrated medium, to the water and dissolve by heating. Adjust the pH if necessary so that after sterilization it will be 7.2 ± 0.2 at 25^0C.

Distribute volumes of 15 ml in tubes, bottles or other containers. For storage in large volumes, use containers up to 500 ml capacity. Sterilize in the autoclave at 121^0C for 15 min.
For use, melt the medium, allow to cool and maintain it at 45 ± 1^0C using the water bath.

13.5.3 **Apparatus and glassware**

Usual microbiological laboratory equipment, and in particular.

13.5.4 **Apparatus for sterilization by steam (autoclave)**

13.5.5 **Incubators** capable of maintaining a temperature of 37 ± 1^0C .

13.5.6 **Glass or plastics Petri dishes** with a diameter of 90 mm or 100mm.

13.5.7. **Water-bath**, or similar apparatus capable of maintaining a temperature of 45 ± 1^{0}C.

13.5.8 **Colony-**counting equipment with a method of illumination against a dark background, a hand-lens (optional) and preferably a mechanical or electronic digital counter.

13.5.9 **Procedure**

13.5.10 **Preparation and Inoculation**

Carry out preparation of the sample, make dilutions and inoculation of culture media, in accordance with ISO 8199.

For pour plates, place the test volume in the Petri Dish, add the molten medium and mix carefully by gentle rotation; allow the medium to set. For spread plates, place the test volume on the dry surface of the agar medium and distribute it over the surface with a sterile glass rod; allow the inoculum to be absorbed.
Inoculate at least two plates for each test volume at each temperature.

13.5.11 **Incubation and examination**

Invert the plates and incubate one set at 37 ± 1^{0}C for 24 ± 1 h or 48 ± 4 h; include the other set of plates at 22 ± 1^{0}C for 72 ± 4 h. Examine the plates as soon as they are removed from the incubators; if this is not possible, store them at 4^{0}C and examine them within 24 h. Reject any plates with confluent growth.

13.5.12 **Counting of Colonies**

Count the colonies present in or on each plate, if necessary with magnification and the aid of a counting device.
Determine the average number of colonies from the pairs of plates from each dilution, each plate ideally containing between 24 and 300 colonies. For each temperature of incubation, calculate the estimated number of colony-forming units

present in 1 ml of the sample.
Alternatively, if more than one pair of dilutions yields counts of between 25 and 300 colonies, then determine the weighted mean according to the formula given in 8.4 of ISO 8199. From these values estimate for each temperature of incubation the number of colony-forming units present in 1 ml of the sample.

13.5.13 **Expression of results**

Express the results as the number of colony-forming units per millilitre of the sample for each temperature of incubation.

If there are no colonies in or on the plates inoculated with test volumes of the undiluted sample, express the results as less than 1 colony-forming unit per millilitre. If there are more than 300 colonies on the plates inoculated with the highest dilutions used, express the results as approximate only.

13.6 ***Detection and Enumeration of Faecal Streptococci***

Part 1: Method of enrichment in a liquid medium

13.6.1 **Culture media and reagent**

Warning – All selective media described in this part of the document contain sodium azide. As this substance is highly toxic and mutagenic, precautions should be taken to avoid contact with it, especially by the inhalation of fine dust during the preparation of commercially available dehydrated complete media. Azide- containing media should not be mixed with strong inorganic acids, as toxic hydrogen azide (HN_3) may be produced. Solutions containing azide can also from explosive compounds when in contact with metal pipe-work, for example from sinks.

Sodium azide deteriorates with time so that dehydrated media have a limited shelf-life.

13.6.2 **Culture media**

13.6.2 Azide glucose broth (single strength)

Beef extract	4.5g
Tryptone	15.0g
glucose	7.5g
sodium chloride (NaC1)	7.5g
sodium azide (NaN_3)	0.2g
bromocresol purple (ethanolic solution 15g/1)	1 ml
water	up to 1000 ml

Dissolve the ingredients in the water by boiling.

Adjust the pH so that after sterilization it will be 7.2 ± 0.1 at 25^0C.

Distribute in tubes in 10 ml volumes.

Sterilize the medium for 15 min at 121 ± 1^0C.

NOTE – ***For the examination of samples of water of more than 1 ml, double strength broth should be prepared in volumes equal to those of the sample to be examined.***

13.6.4 **Bile-aesculin-azide agar**

tryptone	17.0 g
peptone	3.0 g
yeast extract	5.0 g
ox-bile, dehydrated	10.0 g
sodium choride (NaC1)	5.0 g
aesculin	1.0 g
ammonium iron (III) citrate	0.5 g
sodium azide (NaN_3)	0.15g
agar	12 to 20g[2]
water	up to 1000 ml

Dissolve the ingredients in the water by boiling.
Adjust the pH so that after sterilization it will be 7.2 ± 0.1 at 25^0C.

Distribute in suitable containers.

Sterilize for 15 mins at 121 ± 1^0C.

Cool to 50 to 60^0C and pour into Petri dishes to a depth of at least 3 mm and allow to set on a cool, horizontal surface.

[2] According to manufacturer's instruction

13.6.5 **Hydrogen peroxide,** solution, 30g/1.

13.6.6 **Apparatus**

Usual microbiological laboratory equipment and:

13.6.7 Incubator, capable of being maintained at 35 ± 1^{0}C or 37 ± 1^{0}C.

13.6.8 Incubator, capable of being maintained at 44 ± 0.5^{0}C.

13.6.9 Autoclave, capable of being maintained at 121 ± 1^{0}C.

13.6.10 **Procedure**

13.6.11 **Enrichment**

Add 1 ml of sample (or diluted sample) to 10 ml of azide glucose broth (13.6.3.) and mix thoroughly.

Volumes greater than 1 ml should be added to the same volume of double strength broth.

Incubate at 35 ± 1^{0}C or 37 ± 1^{0}C for 22 ± 2 h.

Consider all tubes showing a yellow colour (throughout the whole tube or in the lower part of the tube only) as giving a positive reaction. Reincubate negative tubes for an additional 22 ± 2 h.

After this incubation even a faint colour change to reddish purple should be considered indicative of acid production. In order to improve the interpretation, the colour of the inoculated tube should be compared with the colour of an uninoculated tube.

For quantitative results the most probable number (MPN) method should be used.

13.6.12 **Confirmation**

Confirm each enrichment culture showing acid production as follows.

Streak a loopful of the resuspended enrichment broth on a plate of bile-aesculin-azide agar (13.6.4).

Incubate at 44 ± 0.5^{0}C for 44 ± 4 h.

Regard all plates showing a tan to black colour in the colonies and/or in the surrounding medium as giving a positive reaction.

13.6.13 **Catalase test**

Place a drop of hydrogen peroxide solution (13.6.5) on colonies on bile-aesculin-azide agar. Evolution of bubbles of oxygen indicates catalase-positive organisms. Only catalase-negative colonies should be considered as faecal streptococci.

NOTE – ***To elimate errors due to false negative catalase reactions which may occur on the bile-aesculin-azide agar, the test may be repreated on a subculture on a non-selective medium.***

13.7 ***Detection and Enumeration of Faecal Streptococci***

Part 2: Method by Membrane Filtration

13.7.1 **Culture media**

13.7.2 **KF – streptococcus agar** (Kenner)

13.7.3 **Basal medium**

Proteose peptone	10.0 g
yeast extract	10.0 g
sodium chloride (NaC1)	5.0 g
sodium glycerophosphate	10.0 g
maltose	20.0 g
lactose	1.0 g
sodium azide (NaN_3)	0.4 g
bromocresol purple (ethanolic solution 15/gl)	1 ml
agar	12 to 20 g
water	up to 1000 ml

13.7.4 Dissolve the ingredients in the water by heating in a boiling water-bath.

After dissolution is complete, heat for an additional 5 min.

Allow to cool to 50 to 60^{0}C.

13.7.5 **TTC solution** (2, 3, 5 – triphenyltetrazolium chloride water).

Dissolve the dye in the water by stirring.

Sterilize by filtration (0.22 µm).

The solution should be protected against the action of light.

13.7.6 **Complete medium**

Basal medium (13.7.3)	1000 ml
TTC solution (13.7.5)	10 ml

Add the TTC solution to the basal medium cooled to 50 to 60^{0}C. TTC is thermolabile, so that overheating must be avoided.

Adjust the pH if necessary to 7.2 with a sterile solution of sodium carbonate (100g/l).

Pour the medium into Petri dishes to depth of at least 3 mm and allow to set on a cool, horizontal surface.

Poured plates may be stored in the dark for up 30 days at 4 ± 2^{0}C.

13.7.7 **m-eneterococcus agar (Slanetz and Barley)**

13.7.8 **Basal medium**

Tryptose	20.0 g
yeast extract	5.0 g
glucose	2.0 g
dipotassium hydrogenorthophosphate (K_2HPO_4)	4.0 g
sodium azide (NaN_3)	0.4 g
agar	15.0 g
water	up to 1000 ml

Dissolve the ingredients in the water by heating in a boiling water-bath.

After dissolution is complete, heat for an additional 5 min.
Cool to 50 to 60^{0}C.

13.7.9 **TTC solution** (see 13.7.5)

13.7.10 **Complete medium**

Basal medium (13.7.3)	1000 ml
TTC solution (13.7.5)	10 ml

Add the TTC solution to the basal medium cooled to 50 to 60^{0}C.

Adjust the pH if necessary to 7.2 with a solution of sodium of sodium carbonate (100g/l).
Pour 20 ml into Petri dishes of 9 cm diameter (or an equivalent amount in a dish of another size) and allow to set on a cool, horizontal surface.

Poured plates may be stored in the dark for up to 30 days at 4 ± 2^{0}C.

13.7.11 **Bile-aesculin-azide agar**

Tryptone	17.0 g
peptone	3.0 g
yeast extract	5.0 g
ox-bile, dehydrated	10.0 g
sodium chloride	5.0 g
aesculin	1.0 g
ammonium iron (III) citrate	0.5 g
sodium azide (NaN_3)	0.15 g
agar	12 to 20 g[2]
water	up to 1000ml

Dissolve the ingredients in the water by boiling.

Adjust the pH so that after sterilization it will be 7.1 ± 0.1 at 25^{0}C.

Distribute in volumes of 250 ml in screw-capped bottles of 500 ml capacity.

Sterilize for 15 min at 121 ± 1^{0}C.

Cool to 50 to 60^{0}C and pour into Petri dishes to a depth of at least 3 mm and allow to set on a cool, horizontal surface.

13.7.12 **Hydrogen peroxide,** solution, 30g/l.

[2] According to the manufacturer's instruction

13.7.13 **Apparatus**

Usual microbiological laboratory equipment and:

13.7.14 **Membrane filtration apparatus**

13.7.15 **Sterile membrane filters,** with a normal pore size of 4.45 µm.

The quality of membrane filters may vary from brand to brand or even from batch to batch. It is therefore advisable to check the quality on a regular basis, according to ISO 7704.

13.7.16 **Incubator,** capable of being maintained at 35 ± 1^0C of 37 ± 1^0C.

13.7.17 **Incubator,** capable of being maintained at 44 ± 0.5^0C.

13.7.18 **Autoclave,** capable of being maintained at 121 ± 1^0C.

13.7.19 **Procedure**

Filter a suitable volume of water.

Place the membrane filter on either KF-streptococcus agar (13.7.2) or m-enterococcus agar (13.7.7).

Incubate the plates at 35 ± 1^0C or 37 ± 1^0C for 44 ± 4 h.

13.7.20 **Enumeration**

After incubation, count all raised colonies which show a red, maroon or pink colour, either in the centre or throughout the colony. Consider these colonies as presumptive faecal streptococci.

NOTE – ***Occasionally bacteria other than group D streptococci may produce this type of colony. Elevation of the incubation temperature to 44 ± 0.5^0C, after an initial incubation for 5 ± 1 h at 37 ± 1^0C, may prevent the growth of these organisms.***

13.7.21 **Confirmation**

Subculture a representative sample of typical colonies on a plate of bile-aesculin-azide agar (13.6.4).

Incubate at 44 ± 0.5^0C for 48 h.

Regard all plates showing a tan to black colour in the colonies and/or in the surrounding medium as given a positive reaction.

13.7.22 **Catalase test**

Place a drop of hydrogen peroxide solution (13.6.5) on colonies on bile-aesculin-azide agar (13.6.4). Evolution of bubbles of oxygen indicates catalase-positive organisms. Only catalase-negative colonies should be considered as faecal streptococci.

NOTE – ***To eliminate errors due to false negative catalase reactions which may occur on the vile-aesculine-azide agar, the test may be repeated on a subculture on a non-selective medium.***

13.8 ***Detection and Enumeration of Pseudomonas Aeruginosa***

Part 1: Method by Enrichment in Liquid Medium

13.8.1 **Dilution fluids**

Use one of the diluents given in ISO 8199.

13.8.2 **Culture media**

It is essential that the culture medium used be suited for the type of water to be analysed and the purpose of the analysis. Use the following medium for the determination of presumed *Pseudomonas aeruginosa.*

13.8.3 **Asparagine broth with ethanol**

(Drakes' medium 10)

13.8.4 Composition

	Single Strength	**Concentrated**
DL-asparagine	2 g	3.2g
L-proline	1 g	1.6 g
Anhydrous dispotassium hydrogen phosphate	1 g	1.6 g
Magnesium sulfate heptahydrate	0.5 g	0.8 g
Anhydrous potassium sulfate	10 g	16 g
Ethanol	25 ml	40 ml

Water	to 1000 ml	to 1000 ml

13.8.5 **Preparation**

Dissolve all the constituents in the water and proceed in either of the following ways.

Add the ethanol and distribute in sterile screw-capped bottles. Tighten the caps on the bottles to the point where the seal in the lid just begins to engage with the lip of the bottle. Autoclave at $121^0C \pm 1^0C$ for 15 min. Tighten the caps on each bottle, immediately after removal from the autoclave, to prevent loss of ethanol by evaporation do not use poly-propylene caps without seals.

Alternatively, sterilize the ethanol by filteration through a cellulose acetate or nitrate membrane of average pore size 0.22 µm and then add it aseptically to the medium after autoclaving and cooling. Adjust the pH 7.2 ± 0.2. Store in screw-capped bottles in the dark at room temperature for up to a maximum of 3 months.

13.8.6 **Confirmatory medium**

13.8.7 **Milk agar with cetrimide**

13.8.8 **Composition**

Skim milk powder	100 g
Yeast extract broth (see below)	250 ml
Agar	15 g
Hexadecyltrimethlammonium bromide (cetrimide)	0.3 g
Water	to 750 ml

Yeast extract broth:

Bacteriological yeast extract	3g
Bacteriological peptone	10 g
Sodium chloride	5 g
Water	up to 1000 ml

13.8.9 **Preparation of medium**

Prepare the yeast extract broth by dissolving all the constituents in the distilled water by steaming. Adjust the pH to between 7.2 and 7.4. Sterilize by autoclaving at $121^0C \pm 1^0C$ for 20 min.

Mix the sterile yeast extract broth, cetrimide and agar, and steam this mixture until the agar has dissolved. In a separate glass container, add the skim milk powder to the distilled water and mix, preferably with a magnetic stirrer, until the powder has completely dissolved. Autoclave both solutions separately at $121^0C \pm 1^0C$ for 5 min. To prevent caramelization of the milk, take care to follow these instructions. Cool the solutions to 50^0C to 55^0C, aseptically add the milk solution to the agar medium and mix well.

13.8.10 **Preparation of agar plates**

Distribute 15 ml portions of the final agar medium into sterile Petri dishes (see 13.8.1). Allow the medium to solidify in the plates. Dry the plates. Store at $4^0C \pm 1^0C$ for a maximum of 1 month.

13.8.11 **Apparatus and glassware**

Usual microbiological laboratory equipment, and:

13.8.12 **Glassware**

All glassware shall be sterilized at $170^0C \pm 5^0C$ for 1 h in a dry oven or at $121^0C \pm 1^0C$ for 15 min in an autoclave before use.

Use sterile Petri dishes with a diameter of either 90 mm or 100 mm.

13.8.13 **Incubators,** capable of being maintained at $37^0C \pm 1^0C$ and $42^0C \pm 5^0C$.

13.8.14 Ultraviolet lamp emitting light of wavelength 360 nm ± 20 nm.

NOTE – ***Sterile square plastics Repli dishes may be used as an alternative to glass bottles or tubes when the volume of sample or dilution of sample under examinations is 1 ml or less.***

Plastics Repli dishes are square divided into 25 identical compartments which can hold 1 ml of medium together with 1 ml of sample or sample dilution. The use of these dishes allows five replicates from each of five serial dilutions of the sample to be tested simultaneously. The dishes can be obtained presterilized.

13.8.15 **Procedure**

Carry out the preparation of dilutions and the most probable number technique in accordance with ISO 8199 and ISO 6887.

13.8.16 **Dilutions**

Prepare 10-fold serial dilutions of the sample in a pre-sterilized diluent (13.3) in accordance with ISO 8199.

13.8.17 **Inoculation**

Add 1 ml from each sample, or dilution of the sample, to 4 ml portions of the medium (13.8.1) in bottles or tubes. If larger portions of the sample (10 ml, 50 ml) or Repli dishes are to be used, add the sample to an equal volume of the concentrated medium.

13.8.18 **Incubation**

Incubate the containers at 37^0C ± 1^0C for 48 h. Examine for growth and fluorescence under an ultraviolet lamp in either a darkened room or apparatus designed to exclude visible light.

NOTE – ***Incubation at 38^0C to 39^0C may be used if the water samples are likely to contain large numbers of other bacteria. The possible adverse effect of this procedure on the numbers of organisms recovered should be considered.***

13.8.19 **Confirmation**

13.8.20 **Milk agar**

Subculture a loopful of culture medium from each container showing either fluorescence or growth onto a milk agar plate (13.8.7). Incubate the milk agar plates at 42^0C ± 0.5^0C for 24 h. Examine the plates for growth, pigment production, and casein hydrolysis (cleaning of the milk medium around the colonies) and record the reactions as shown in table 1.

TABLE 1

***Pseudomonas aeruginosa* reactions**

Reaction mode	Typical	Atypical*)	
	(1)	(2)	(3)
Casein hydrolysis	+	+	+
Growth at $42^0C \pm 0.5^0C$	+	+	+
Fluorescence (under UV irradiation only)	+	+	-
Pyocyanine (blue-green) pigment	+	-	-

\+ = positive reaction
\- = negative reaction
*) Other bacteria can sometimes give atypical reactions (2) and (3). In each instances the procedure described in 13.8.22 should be followed.

NOTE – *Pigment production in the culture medium may be inhibited by the growth of bacteria other than Pseudomonas aeruginosa. In such cases, the milk agar plates should be exposed to daylight at room temperature before they are examined for pigment production.*

13.8.21 **Enumeration**

All containers of the culture medium exhibiting either growth or fluorescence, which yield colonies (after subculture on milk agar plates) that produce either reaction (1) or (2) (see table 1 in 13.8.20) shall be regarded as positive for the presence of ***Pseudomonas aeruginosa.***

NOTE – *Others identified as non-pigmented or atypical Pseudomonas aeruginosa by the procedure in 13.8.22 may be included also.*

13.8.22 **Non-pigmented strains**

NOTE – *as a further step, it is possible to obtain confirmation of non-pigmented strains. If required, a suitable method is to take a loopful of culture medium and transfer it to a milk agar plate. The plate is incubated at a temperature of $37^0C \pm 1^0C$ for 24 h. A well-isolated colony is selected and final confirmation is obtained by testing for certain biochemical characteristics. Commercially available identification kits may be used.*

13.8.23 **Expression of results**

From the number of containers of culture by reference to statistical tables in ISO 8199 the most probable number of *Pseudomonas aeruginosa* present in 100 ml

of water sample in accordance with ISO 8199.
Alternatively, express the results qualitatively e.g. by stating the *Pseudomonas aeruginosa* were present or absent in 100 ml of water sample.

Where larger volumes are examined, e.g. bottled waters, express the results qualitatively specifying the appropriate volume.

13.9 ***Detection and Enumeration of Pseudomonas aeruginosa***

Part 2: Membrane Filtration Method

13.9.1 **Drake medium 19**

13.9.2 **Confirmatory medium**

13.9.3 **Milk agar with cetrimide**

Refer to section 13.8.7

13.9.4 **Preparation of medium**

Prepare the yeast extract broth by dissolving all the constituents in the distilled water by steaming. Adjust the pH between 7.2 and 7.4. Sterilize by autoclaving $121^0C \pm 1^0C$ for 20 min.

Mix the sterile yeast extract, cetrimide and agar, and steam this mixture until the agar has dissolved. In a separate glass container, add the skim milk powder to the distill water and mix, preferably with a magnetic stirrer, until the powder has completely dissolved. Autoclave both solution separately at $121^0C \pm 1^0C$ for 5 min. To prevent caramelization of the milk, take care to follow these instructions. Cool the solution to 50^0C to 55^0C, aseptically add the milk solution to the agar medium and mix well.

13.9.5 **Preparation of agar plates**

Distribute 15 ml portions of the final agar medium into sterile Petri dishes. Allow the medium to solidify in the plates. Store at $4^0C \pm 1^0C$ for a maximum of one month.

13.9.6 **Apparatus and glassware**

Usual microbiological laboratory equipment, and:

13.9.7 **Glassware**

All glassware shall be sterilized at $170^0C \pm 5^0C$ for 1 h in a dry oven or at $121^0C \pm 1^0C$ for 15 min in an autoclave before use. Use sterile Petri dishes with a diameter of either 90 mm or 100 mm.

13.9.8 **Incubators,** capable of being maintained at $37^0C \pm 1^0C$ and $42^0C \pm 0.5^0C$.

13.9.9 **Ultraviolet lamp** emitting light of wavelength 360 nm ± 20 nm.

13.9.10 **Dilutions**

Prepare 10-fold serial dilutions of the sample in a pre-sterilized diluent.

13.9.11 **Membrane Filtration**

Filter volumes of the water sample or portions of the dilution through a sterile membrane filter with a rated pore diameter equivalent to 0.45 µm. In accordance with ISO 8199, place each membrane on a sterile filter pad saturated with Drake's medium 19, ensuring that no air is trapped beneath.

NOTE – ***Excess Drake's medium 19 should be removed from the Petri dish prior to placing the membrane on the filter pad.***

13.9.12 **Incubation of membranes**

Incubate the Petri dishes at $37^0C \pm 1^0C$ for 48 h in containers that prevent moisture loss. Examine the membrane for blue-green or greenish-brown colonies, or colonies which exhibit florescence under exposure to ultraviolet light in either a darkened room or apparatus which exclude visible light.

NOTE – ***Incubation at $42^0C \pm 0.5^0C$ for up to 48 h may be used if the water samples are likely to contain large numbers of other aquatic bacteria.***

The possible adverse effect of this procedure on the number of organisms recovered should be considered.

13.9.13 **Confirmation**

13.9.14 **Milk agar**

Subculture the characteristic colonies from 13.9.12 onto the surface of milk agar plates. Incubate the milk agar plates at $42^0C \pm 0.5^0C$ for 24 h. Examine the plates for growth, pigment production and casein hydrolysis cleaning of the milk medium around the colonies and record the reactions as shown in table 1.

13.9.15 **Enumeration**

Count as confirmed *Pseudomonas aeruginosa* all colonies which exhibit the reaction (1) and (2) (see table 1 13.8.20).
Count as presumed *Pseudomonas aeruginosa* all colonies which show, after incubation, the following characteristics:

Blue-green or greenish-brown coloration or exhibit fluorescence when exposed to ultraviolet light.

NOTE – *Other identified as non-pigment or atypical Pseudomonas aeruginosa by the procedure in 13.8.22 may be included also.*

13.9.16 **Non-pigmented strains**

NOTE – *As a further step, it is possible to obtain confirmation of non-pigmented strains. If required, a suitable method is to take a loopful of culture medium and transfer it to milk agar plate. The plate is incubated at a temperature of $37^0C \pm 1^0C$ for 24 h. A well-isolated colony is selected and final confirmation is obtained by testing for certain biochemical characteristics. Commercial available identification kits may be used.*

13.9.17 **Expression of results**

From the number of characteristic colonies counted on the membranes, and taking account of the confirmatory tests performed, calculate the number of confirmed *Pseudomonas aeruginosa* present in 100 ml of water sample in accordance with ISO 8199.

Alternatively, express the result qualitatively by stating that *Pseudomonas aeruginosa* were present or absent in 100 ml of water sample.

13.10 ***Detection and Enumeration of Coliform Organisms, Thermotolerant Coliform Organisms and Presumptive Escherichia COLI***

13.10.1 **Part 1 Membrane filtration method**

13.10.2 **Isolation media**

Use one or more of the following culture media either in solid form with agar or as a broth for saturating absorbent pads.

13.10.3 **Lactose TTC agar with Tergitol 7**[2)]

13.10.4 **Lactose agar with Tergitol 7**[2)]

13.10.5 **Membrane enriched Teepol broth**[2)]

13.10.6 **Membrane lauryl sulfate broth**[2)]

13.10.7 **Endo medium**

13.10.8 **LES Endo agar**

13.10.9 **mFC medium**

13.10.10 **Confirmatory media**

Use one or more of the following.

13.10.11 **Medium for gas production**

Lactose peptone water.

13.10.12 **Medium for Indole production**

Tryptone water.

13.10.13 **Single Tube medium for both gas and indole production**

Lauryl tryptose mannitol broth with tryptophan.

13.10.14 **Reagents**

13.10.15 Kovacs' reagent for Indole

13.10.16 Oxidase reagent for the oxidadase test.

13.10.17 **Apparatus**

Usual microbiological laboratory equipment, including:

13.10.18 **Hot-air oven for dry-heat sterilization and an autoclave**

Apart from apparatus supplied sterile, glassware and other equipment shall be sterilized according to the instructions given in ISO 8199.

13.10.19 **Incubator or water bath,** thermostatically control at $30^0C \pm 0.5^0C$.

13.10.20 **Incubator or water bath,** thermostatically control at either $44^0C \pm 0.25^0C$ or $44.5^0C \pm 0.25^0C$.

13.10.21 **pH meter**

13.10.22 **Apparatus for membrane filtration.**

13.10.23 Membrane filters, usually about 47 mm or 50 mm in diameter, with filtration characteristics equivalent to a rated nominal pore diameter of 0.45 µm. If not obtained sterile, they shall be sterilized according to the manufacturer's instructions.

13.10.24 **Forceps,** for handling membranes

13.10.25 **Procedure**

13.10.26 **Preparation of the sample, filtration and Inoculation of media**

For preparation of the sample, making dilutions, filtration and inoculation of isolation media, follow the instructions given in ISO 8199 and ISO 6887.

13.10.27 For coliform organisms, filter the required volume of the sample, or a dilution of it, through one membrane. Place on the medium chosen, ensuring that no air is trapped underneath it.

13.10.28 For thermotolerant coliform organisms, filter the required volume of the sample, or a dilution of it, through one membrane. Place on the medium chosen, ensuring that no air is trapped underneath it.

NOTE – ***the volume of sample filtered should be the same as in 1310.27.***

13.10.29 **Incubation of membranes**

13.10.30 For coliform organisms, incubate the membrane for 18 h to 24 h at either $35^0C \pm 0.5^0C$ or $37^0C \pm 0.5^0C$.

13.10.31 For thermological coliform organisms, incubate the membrane for 18 h to 24 h at either $44^0C \pm 0\ .25^0C$ or $44.5^0C \pm 0.25^0C$.

NOTES – ***The same medium can generally be used for both coilform organisms and thermotolerant coliform organisms, but mFC medium should be used only at 44^0C, and Endo and LES Endo media should be used at 35^0 C or 37^0C.***

A preliminary period at a lower temperature such as 30 C or the first 4 h of incubation is recommended to resuscitate stressed organisms, especially in the examination of drinking water.

13.10.32 **Examination of membranes**

13.10.33 **Coliform organisms**

Examine the membranes and count as presumptive coliform organisms all colonies, irrespective of size, which show, after incubation at 35^0C or 37^0C, the following characteristics:

- On lactose TTC agar with Tergitol (13.10.3): a yellow, orange or brick red colouration with a yellow central halo in the medium under the membrane.

- On lactose agar with Tergitol 7 (13.10.4): a yellow central halo in the medium under the membrane.

- On membrane enrich Teepol broth (13.10.5): a yellow colour extend on to the membrane.

- On membrane lauryl sulfate broth (13.10.6): a yellow low colour extending on to the membrane.

- On Endo agar or broth (13.10.7): a dark red colour with a golden-green metallic sheen.

- On LES Endo agar (13.10.8): a dark red colour with a golden-green metallic sheen.

13.10.34 **Thermotolerant coliform organisms**

Regard as presumptive thermotolerant coliform organisms all colonies which show, after incubation at 44.5± 0.2 ^{0}C, the same colonial characteristics as those described in 13.10.37 with mFC medium (13.10.9), such colonies are blue in colour.

13.10.35 **Confirmatory tests**

It is important to note that the counts of colonies on membranes at 30^{0}C or 37^{0}C and at 44^{0}C are only presumptive coliform results. Since gas production is not detected, there is also an additional presumption that the organisms forming colonies can also produce gas from lactose. For the examination of raw or partly-treated waters, this may be sufficient, but for potable supplies and other circumstances, it is important to carry out confirmatory tests, preferably on pure subcultures.

13.10.36 **Coliform organisms**

To confirm the membrane results, subculture each colony (13.10.33) or a representative number of them to tubes of lactose peptone water (13.10.11) and incubate at 35^{0}C or 37^{0}C for 48 h: gas production within this period confirms the presence of coliform organisms.

13.10.37 **Thermotolerant coliform organisms and presumptive** *E. coli*

For thermotolerant coliform organisms and presumptive *E. coli* on membranes, whether incubated at 44.5^{0}C± 0.2 ^{o}C or at 35^{0}C, subculture each colony (13.10.34) or a representative number of them, to tubes of lactose peptone water and tryptone water and incubate at 44.5^{0}C±0.2 ^{o}C for 24 h. Gas production in lactose peptone water confirms the presence of thermotolerant coliform organisms, and development of a red colour at the surface of the tryptone water culture after the addition of 0.2 ml to 0.3 ml of Kovacs' reagent (13.10.15) confirms the presence of presumptive *E. coli.*

NOTE – The use of lauryl tryptone mannitol broth with tryptophan allows both gas and indole production to be demonstrated in a single tube.

The detection of presumptive *E. coli* is regarded as satisfactory evidence of faecal pollution. However, further tests for the confirmation of *E. coli* may be carried out if considered necessary (13.10.38).

When subculturing from colonies on the membrane to tubes of confirmatory media, it is preferable to subculture also to a plate of nutrient agar medium for the oxidase test.

13.10.38 **Oxidase test**

Some bacteria found in water may conform to the definition of coliform organisms in most respect, but are able to produce gas from lactose only at temperature below 37^0C. They therefore give negative results in the standard confimatory tests for coliform organisms and their presence in water is not usually regarded as significant. *Aeromonas* species, which occur naturally in water, have an optimum growth temperature in the range 30^0C to 35^0C but may nevertheless produce acid and gas from lactose at 37^0C. They are distinguishable from the coliform group by a positive oxidase reaction.

13.10.39 Carry out the oxidase test with pure subcultures of lactose-fermenting organisms, grown on nutrient agar medium, as follows:

- place 2 to 3 drops of freshly prepared oxidase reagent (13.10.16) on a filter paper in a Petri dish;

- with a glass rod, swab stick or platinum (not nichrome) wire loop, smear some of the growth on the prepared filter paper (see note).

- Regard the appearance of a deep blue-purple colour within 10 s as a positive reaction.

NOTE – *On each occasion that the oxidase reagent is used, control tests should be conducted with cultures of an organism known to give a positive reaction (Pseudomonas aeruginosa) and a negative reaction (E. coli).*

13.10.40 **Expression of results**

From the numbers of characteristic colonies counted on the membranes and taking account of the results of the confirmatory tests performed, calculate the numbers of

coliform organisms, thermotolerant coliform organisms and presumptive *E. coli* present in 100 ml of the sample in accordance with ISO 8199, 13.36, according to the following equation:

$$C = \frac{A \times N \times V_s \times F}{B \times V_t}$$

Where

C is the confirmed colony count per 100 ml;

A is the number of colonies actually confirmed;

B is the number of colonies subcultured for confirmation;

N is the number of characteristic colonies on the membrane (13.10.33 and 13.10.34);

V_t Is the test volume of water sample filtered (13.10.27 and 13.10.28);

V_s Is the reference volume for expression of results (100 ml); and

F is the dilution factor.

13.11 ***Detection and Enumeration of coliform organisms, thermotolerant coliform organisms and presumptive escherichia coli.***

13.11.1 **Part 2 Multiple tube (most probable number) method**

13.11.2 **Isolation media**

Use one of the following culture media.

13.11.3 **Lactose broth**

13.11.4 **MacConkey broth**

13.11.5 **Improved formate lactose glutamate medium**[2)]

13.11.6 **Lauryl tryptose (lactose) broth**

13.11.7 **Confimatory media**

Use one or more of the following:

13.11.8 **Media for gas production**

13.11.9 **Brilliant-green lactose (bile) broth**

13.11.10 **EC medium**

13.11.11 **Medium for indole production**

Tryptone water.

13.11.12 **Single-tube medium for both gas and indole production**

Lauryl tryptose mannitol broth with tryptophan.

13.11.13 **Reagents**

13.11.14 **Kovacs' reagent for indole**

13.11.15 **Oxidase reagent for the oxidase test**

13.11.16 **Apparatus**

Usual microbiological laboratory equipment, including

13.11.17 **Hot-air oven for dry-heat sterilization and an autoclave**

Apart from apparatus supplied sterile, glassware and other equipment shall be sterilized according to the instruction given in ISO 8199.

13.11.18 **Incubator or water bath,** thermostatically controlled at either $35^0C \pm 0.5^0C$ or $37^0C \pm 0.5^0C$.

13.11.19 **Incubator or water bath,** thermostatically controlled at either $44^0C \pm o.25^0C$ or $44.\ 5^0C \pm 0.25^0C$.

13.11.20 **pH meter.**

13.11.21 **Procedure**

13.11.22 **Preparation of the sample and inoculation of media**

For preparation of the sample, making dilutions and inoculation of isolation medium with test portions, follow the instructions given in ISO 8199. For test portions of volume greater than 5 ml, use tubes containing "double strength" isolation medium.

13.11.23 **Incubation of tubes**

Incubate the inoculated tubes for 48 h at either $35^0C \pm 0.5^0C$ or $37^0C \pm 0.5^0C$.

13.11.24 **Examination of tubes**

Examine the tube-cultures after incubation for 18 h to 24 h and regard as positive reactions those which show turbidity due to bacterial growth and gas formation in the inner inverted (Durham) tubes, together with acid production if the isolation medium contains a pH indicator. Reincubate those tubes, which do not show any or all of these changes and examine them again for positive reactions after 48 h.

13.11.25 **Confirmatory Tests**

It is important to note that positive reactions in tubes of isolation medium are only presumptive coliform results. It is therefore important to carry out confirmatory tests, preferably on pure subcultures.

13.11.26 **Subculture, incubation and examination**

Subculture from each tube of isolation medium giving a positive result into one or more tubes of the confirmatory media (13.11.7) for gas and indole production.

NOTE 1 – ***if the least inhibitory medium (lactose broth) is used for isolation, subculture to either or the two more selective confirmatory media [[brilliant-green lactose (bile) broth or EC broth] for confirmation is recommended.***

13.11.27 **Coliform organisms**

To confirm the presence of coliform organisms, incubate one tube of brilliant-green lactose (bile) broth (13.11.9) at either 35^0C for or 37^0C, and examine for gas production within 48 h.

13.11.28 **Thermotolerant coliform organisms and presumptive** *E. coli*

To confirm the presence of thermotolerant coliform organisms, incubate another tube of EC medium (13.11.10) at 44.5^0C± 0.2 ^{o}C for 24 h, and examine for gas production.

To confirm the presence of presumptive *E. coli*, incubate a tube of tryptone water (13.11.11) for indole formation at 44.5^0C± 0.2 ^{o}C for 24 h. Then add 0.2 ml to 0.3 ml of Kovacs' reagent (13.11.14) to the tube of tryptone water: development of a red colour after gentle agitation denotes the presence of indole.

NOTE 2 - T*he use of lauryl trytose mannitol broth with trytophan allows both gas and indole production by presumptive E. coli to be demonstrated in a single tube.*

NOTE 3 – T*he detection of presumptive E. coli is regarded as satisfactory evidence of faecal pollution. However, further tests for the confirmation of E. coli may be carried out if considered necessary (13.11.30).*

NOTE 4 – ***When subculturing from colonies on the membrane to tubes of confirmatory media, it is preferable to subculture also to a plate of nutrient agar medium for the oxidase test.***

13.11.29 **Oxidase test** (Refer to section 13.10.38)

13.11.30 **Expression of results**

From the number of tubes of isolation medium and confimatory tests giving positive reactions, calculate by reference to the statistical tables in ISO 8199, the most probable numbers of coliform organisms, thermotolerant coliform organisms and presumptive *E. coli* in 100 ml of the sample.

13.11.31 It is recommended that the references listed in Appendix 1 be used as guides for the chemical and physical analyses of bottled water.

14.0 CONFORMITY

14.1 The lot or shipment sampled as in 12.1 shall be deemed to conform to this standard if the test results satisfy the requirements of Section 5, 6, and 7, and if inspection shows that the labelling satisfies the requirements of Section 10, and the average net contents of the containers is found to be not less than that declared on the label.

14.2 Bottled water shall be deemed to conform this standard if produced under an approved quality assurance system. The quality assurance system must provide test results obtained from routine samples taken during production. The test results must satisfy the requirements specified for the relevant characteristics and there must be adequate evidence on file from testing in plant or by certification by suppliers that the material used (for example: containers, closures, cleaning and sanitizing chemicals and chlorinating chemicals, carbon dioxide) meet the other requirements.

APPENDIX I

RECOMMENDED METHODS OF TEST FOR BOTTLED WATERS

1. *"Official Methods of Analysis", 14th Edition, 1980, AOAC*

   Chapter 13 of this publication covers the analysis of water, and includes procedures for the following components or other characteristics:-

   PH; acidity; alkalinity; solids in water;

   Solids in solution; nitrate ion*; chloride ion*;

   Fluoride ion; carbonate and bicarbonate ions;

   Silicate ion, calcium, barium; potassium*; sulphate ion; manganese; iodine; arsenic; bromide; lead.

   Atomic absorption spectrophotometry is used for the estimation of:-

   Cadmium; chromium; copper; iron; lead; magnesium; Manganese; mercury; sliver and zinc.

2. "Methods of Chemical Analysis of Water and Wastes", 1993 US Environmental Proctection Agency.

3. "Analysis of Trihalomethanes in Drinking Water", issued as Appendix C, Part 141, 40 Code of Federal Regulations, USA.

4. "Methods of Organochlorine Pesticides in Industrial Effluents" issued by the US Environmental Protection Agency, 1973.

5. "Standard methods for the Examination of Water and Waste Water", American Public Health Association, 16th edition 1985.

   This includes bacteriological methods, (multiple or membrane filter), and for colour, turbidity, odour.

6. Official Methods MFO-9 and MFO-15 for the microbiological examination of mineral water, and of water in sealed containers (other than mineral water), Health Protection Branch, Canada.

7. Schwaller, P and Schmidt-Lorenz, W in Zbl. Bakt. Hyg. Abt. C, 1981 - - Method for PS aeruginosa and mesophilics at 42 ^{o}C.