



# MANUAL OF METHODS OF ANALYSIS OF FOODS

**WATER**



**FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA  
MINISTRY OF HEALTH AND FAMILY WELFARE  
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**MANUAL FOR ANALYSIS OF WATER**  
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*Note:*

- 1. The test methods given in the manual are validated/ standardized test methods. However, it would be the responsibility of the respective testing laboratory to confirm that the methods in the manual are validated/verified in their laboratory for fitness for the purpose.*
- 2. Approved International Standard Test Methods from Organizations like ISO/APHA/ASTM/AOAC/EPA/ EN may also be followed after proper verification of the method in lab to establish fitness for the purpose.*
- 3. In case of dispute methods referred in respective IS standards of IS 13428, IS 14543, IS 4251 and IS 10500 shall be the referee methods.*

**ABBREVIATIONS**

<b>Sr. No.</b>	<b>Abbreviation</b>	<b>Expanded Form</b>
1.	%	Percentage
2.	µm	Micro meter
3.	°C	Degree Celsius
4.	Hr	hour
5.	L	Liter
6.	min	Minutes
7.	mL	Milliliter
8.	mm	millimeter
9.	µL	Microliter
10.	IS	Indian Standard
11.	ppb	Parts per billion
12.	ppm	Parts per million
13.	ISO	International Organization for Standardization
14.	gm	Gram
15.	mg	Milligram
16.	Cfu	Colony forming units
17.	sp.	species
18.	µg	Microgram
19.	UV	Ultra Violet

## SCOPE

This manual covers methods of analysis of following types of water for the parameters as mentioned in Food Safety and Standards Regulations 2011 as well as current Indian Standards for respective category of Water:

1. Natural Mineral Water- Food Safety & Standard Regulations - 2011 and IS 13428:2005 Reaff. 2009 requirements
2. Packaged Drinking Water other than Natural Mineral Water- Food Safety & Standard Regulations - 2011 and IS 14543:2004 Reaff. 2009 requirements
3. Water for processed Food Industry- IS 4251: 1967 Reaff. 1992 requirements
4. Drinking water- IS 10500 : 2012

**SECTION A**

**ORGANOLEPTIC AND PHYSICO-CHEMICAL PARAMETERS**

## CHAPTER I: ORGANOLEPTIC & PHYSICAL PARAMETERS

### 1. COLOUR

#### Introduction:

Colour in water may be due to inorganic ions, such as iron & manganese, humus & peat materials, plankton, weeds and industrial wastes. The term 'colour' is used to mean true colour that is the colour of water from which turbidity has been removed. The term apparent colour includes not only the colour due to substances in solution, but, also that due to suspended matter. Apparent colour is determined on the original sample without filtration or centrifugation.

#### A. Platinum cobalt (visual comparison) method

**1. Principle** - Colour is measured by visual comparison of the sample with platinum-cobalt standards. One unit of color/Hazen is, that produced by 1 mg platinum per liter in the form of chloroplatinate ion.

#### 2. Interferences

**2.1.** Slight amount of turbidity interfere with the determination. Therefore sample showing visible turbidity should be clarified by centrifugation.

**2.2.** The method is pH dependant. Colour of water normally increases with increase in pH value unless the coloured ion precipitates.

**2.3.** Use of filter paper may result in removal of some of the color, leading to erroneous results. Therefore filter paper should not be used for determination of true color

**3. Sample Handling and preservation** - Representative samples shall be taken in clean glassware. Color should be determined as early as possible after the collection of samples as biological activity or physical changes occurring during storage may affect the colour. Refrigeration of water samples at 4°C is recommended.

#### 4. Apparatus

4.1. Nessler cylinders 50 mL capacity.

4.2. Centrifuge or filter assemblies with glass fiber filters or membrane filters with functional pore sizes of approximately 0.45µm.

#### 5. Reagents:

5.1. **Standard chloroplatinate solution** - Dissolve 1.246 gm potassium chloroplatinate ( $K_2PtCl_6$ ) (equivalent to 500 mg metallic platinum) and 1.0 gm crystalline cobaltous chloride ( $CoCl_3 \cdot 6H_2O$ ) (equivalent to 250 mg metallic cobalt) in distilled water containing 100 mL concentrated hydrochloric acid. Dilute to 1000mL with distilled water. This standard solution is equivalent to 500 colour units.

#### 5.2. Preparation of standards

5.2.1. Prepare standards having color units of 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60 and 70 by diluting 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0 and 7.0 mL standard chloroplatinate solution with distilled water to 50 mL. Use distilled water as 0 unit standard/Control.

5.2.2. Protect these standards against evaporation and contamination by use of clean inert stoppers. The standards should also be protected against absorption of ammonia which causes increase in colour.

#### 6. Procedure

6.1. **Apparent color** - Observe the color of the sample by filling a matched Nessler cylinder to the 50 mL mark with water and compare with standards. Compare by looking vertically downward through the cylinder towards a white surface placed at such an angle that light is reflected upwards through the column of liquid. If turbidity has not been removed, report the colour as 'apparent colour'. If the colour exceeds 70 units, dilute the sample with distilled water until the colour is in the range of the standards.

**6.2. True color-** Remove turbidity by centrifuging the sample until the supernatant liquid is clear. Compare the centrifuged sample with distilled water to ensure that turbidity has been removed. If the sample is clear, then compare with the standards.

**6.3. Calculation** - Calculate the color units as follows

$$\text{Color units/Hazen Units} = \frac{50A}{V}$$

Where

A= Estimated color of diluted sample

V= Volume in ml of sample taken for dilution

**6.4. Report** – Report the results in whole numbers as follows:

Sr. No.	Color units/Hazen Units	Record to nearest
1.	1 to50	1
2.	51 to100	5
3.	101 to250	10
4.	251 to500	20

**7. Reference:**

**7.1.** IS:3025 (part 4) - 1983 (Reaffirmed 2002) - Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : Colour

## B. Spectrophotometric Method

**1. Principal** - Colour characteristics are measured at pH 7.6 and original pH of the sample by obtaining the visible absorption spectrum of the sample on a spectrophotometer. The percent transmission at certain wavelengths is used to calculate the results which are expressed in terms of dominant wavelength, hue, luminance and purity.

## 2. Apparatus-

**2.1. Spectrophotometer** - Having 10 mm absorption cells a narrow (10 mm or less) spectral band and an effective operating range from 400 to 700 nm

**2.2. Filtration system** - Consisting of following

2.2.1. Filtration flasks 250 mL with side tubes

2.2.2. Crucible holder

2.2.3. Micrometallic filter crucible average pore size 40  $\mu\text{m}$

2.2.4. Calcined filter aid (celite 505 equivalent )

2.2.5. Vacuum system

**2.3. Sample Handling and preservation** – Since biological activity may change the colour characteristics of a sample, the determination should be made as soon as possible. Refrigeration to 4°C recommended.

## 3. Procedure

**3.1.** Take two 50 mL samples and bring to room temperature use one sample at original pH value and adjust pH of other sample to 7.6 by use of suitable volume of concentrated sulphuric acid or sodium hydroxide so that not more than 0.5 mL acid or alkali is used. Remove suspended material by centrifuging. Treat each sample separately by thoroughly mixing 0.1 gm filter aid in a 10mL portion of centrifuged sample and filtering the slurry to form a precoat in the filter crucible. Direct the filtrate to waste flask of filtration system. Mix 40 mg filter aid in a 35 mL portion of the centrifuged sample. With the vacuum still on, filter through the precoat and pass the filtrate to waste flask until clear, and then direct the clear filtrate flow to clean flask by means of three way stop cock. Collect 25 mL sample for measurement of transmittance.

**3.2.** For determination of light transmittance characteristic clean 10 mm absorption cells with detergent, rinse with distilled, filtered water and fill the cell with filtered water. Determine the transmittance values (in percent) for the sample at each of the

visible wavelength values given in **Table-1**. For fairly accurate work take readings at 10 ordinates marked with as asterisk, and for increases accuracy at all 30 ordinates. Set the instrument to read 100 percent transmittance on the distilled water blank. Make all determinations with a narrow spectral band.

### 3.3. Calculation

3.3.1. Tabulate the transmittance values corresponding to wavelength shown in col X, Y and Z. Add each of transmittance columns and multiply the tables by the appropriate factors (for 10 or 30 ordinates) shown at the bottom of the table to obtain tristimulus values X, Y and Z. The tristimulus value Y is the percent luminance of the waste.

3.3.2. Calculate the trichromatic coefficients X and Y from Tristimulus values X, Y and Z by the equations

$$X = \frac{X}{X + Y + Z}$$

$$Y = \frac{Y}{X + Y + Z}$$

Locate the Point(X, Y) on one of the chromaticity diagrams shown in **Fig-1** and determine the dominant wavelength and purity from this diagram. Determine the hue values from dominant wavelength value according to ranges given in **Table-2**.

**3.4. Report** – Report the colour characteristic at pH 7.6 at original pH in terms of dominant wavelength, hue, Luminance and purity. Mention the type of instrument (i.e Spectrophotometer). The number of selected ordinate (10 or 30) and the spectral bandwidth.

**Table-1 Selected Ordinates for Spectrophotometric colour Determinations:**

Ordinate No.	X	Y	Z
	Wavelength (nm)		
1	424.4	465.9	414.1
2*	435.5*	489.5*	422.2
3	443.9	500.4	426.3

4	452.1	508.7	429.4
5*	461.2*	515.2*	432.0
6	474.0	520.6	434.3
7	531.2	525.4	436.5
8*	544.3*	529.8*	438.6
9	552.4	533.9	440.6
10	558.7	537.7	442.5
11*	564.1*	541.4*	444.4
12	568.9	544.9	446.3
13	573.2	548.4	448.2
14*	577.4*	551.8*	450.1
15	581.3	555.1	452.1
16	585	558.5	454
17*	588.7*	561.9*	455.9
18	592.4	565.3	457.9
19	596.0	568.9	459.9
20*	599.6*	572.5*	462.0
21	603.3	576.4	464.1
22	607.0	580.4	466.3
23*	610.9*	584.8*	468.7
24	615.0	589.6	471.4
25	619.4	594.8	474.3
26*	624.2*	600.8*	477.7
27	629.8	607.7	481.8
28	636.6	616.1	487.2
29*	645.9*	627.3*	495.2
30	663.0	647.4	511.2
	Factors when 30 Ordinates used		
	0.03269	0.03333	0.03938
	Factors when 10 Ordinates used		
	0.09806	0.10000	0.11814
*Insert in each column the transmittance value in percent corresponding to the			

given wavelength. Where limited accuracy is sufficient, only the ordinates marked with an asterisk may be used.

**Table 2: Colour Hues for Dominant Wavelength ranges:**

<b>Dominant wavelength range(nm)</b>	<b>Colour hue</b>
400-465	Violet
465-482	Blue
482-497	Blue green
497-530	Green
530-575	Greenish yellow
575-580	Yellow
580-587	Yellowish orange
587-598	Orange
598-620	Orange red
620-700	Red
400-530 C	Blue purple
530 C-700	Red Purple
Note: See Fig 2 for significance of C	

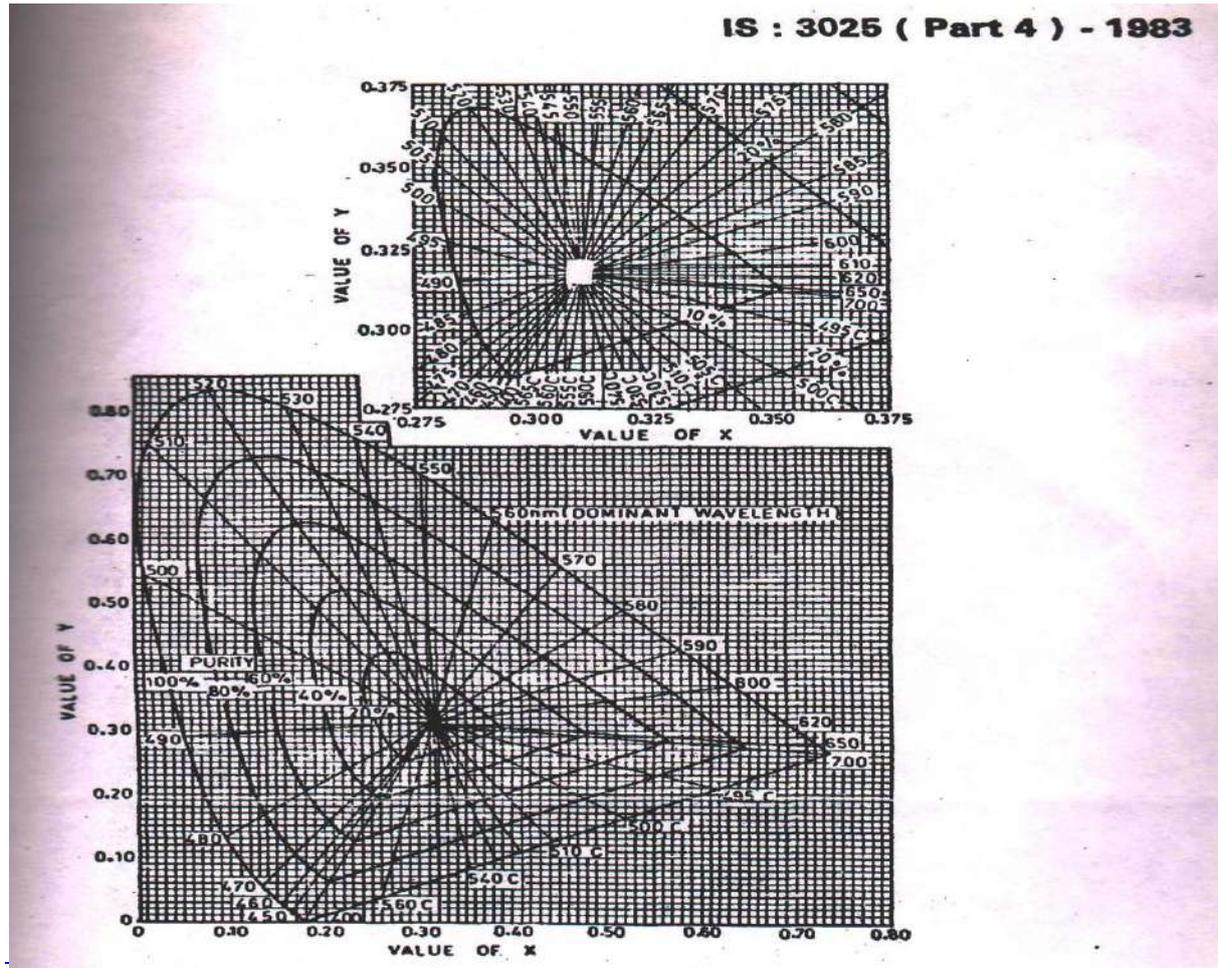


Fig. 1 Chromatcity Diagram

## 2. ODOUR

### Introduction:

Odour is recognized as a quality factor affecting acceptability of drinking water and food prepared from it, tainting of fish and other aquatic organisms & aesthetes of recreational waters. Most organic and some inorganic chemicals contribute taste or odour. These chemicals may originate from municipal and industrial waste discharges, natural sources, such as decomposition of vegetable matter or from associated microbial activity.

Odour of water, though very important, cannot be determined in absolute units. Olfactory sense, which is the most sensitive means of detecting small concentrations of odoriferous substances, lacks precision and mathematical expression nevertheless a qualitative test is prescribed. In case of doubt as to the intensity or character of odour, a majority opinion of several observers should be recorded

**1. Preparation of apparatus** – Thoroughly clean the requisite number of wide-mouth glass stoppered bottles of about one liter capacity. Rinse them with hydrochloric acid and render them completely odour-free by repeated washing with odour-less distilled water, which can be prepared by passing distilled water through a column of granulated activated carbon

**2. Procedure:**

2.1. As soon as possible after collection of sample, fill a cleaned bottle half full of sample, insert the stopper, shake vigorously for 2 to 3 seconds and then quickly observe the odour. The sample taken for observation of odour shall be at a room temperature.

2.2. When it is desired to record the odour at an elevated temperature, make the observation after warming the sample to about 60°C in a clean stoppered bottle.

**3. Report**

3.1. Report the true odour of the sample at the mouth of the bottle as rotten egg, burnt, sugar, soapy, fishy, septic, aromatic, chlorinous, alcoholic odour or any other specific odour. In case it is not possible to specify the exact odour, report as agreeable or disagreeable.

**4. Reference:**

4.1. IS:3025 (part 5) : 1983 (Reaffirmed 2002) - Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : Odour

### 3. pH

#### Introduction:

pH value is the logarithm of reciprocal of hydrogen ion activity in moles per liter. In water solution, variations in pH value from 7 are mainly due to hydrolysis of salts of strong bases and weak acids or vice versa. Dissolved gases such as carbon dioxide, hydrogen sulphide and ammonia also affect pH value of water. The overall pH value range of natural water is generally between 6 and 8. In case of alkaline thermal spring waters pH value may be more than 9 while for acidic thermal spring waters, the pH may be 4 or even less than 4. Industrial wastes may be strongly acidic or basic and their effect on pH value of receiving water depends on the buffering capacity of water. The pH value of water obtained in the laboratory may not be the same as that the time of collection of water samples, due to loss or absorption of gases, reactions with sediments, hydrolysis and oxidation or reduction taking place within the same sample bottle. pH value should preferably be determined at the time of collection of sample.

Methods for determination of pH in water are prescribed:-

- A. Electrometric Method
- B. Colorimetric Method

#### A. Electrometric method

**1.1. Principle** – The pH value is determined by measurement of the electromotive force of a cell consisting of an indicator electrode immersed into the test solution and a reference electrode. Contact between the test solution and the reference electrode is usually achieved by means of a liquid junction which forms part of the reference electrode. The electromotive force is measured with a pH meter i.e. a high impedance voltmeter calibrated in terms of pH.

1.1.1. Several types of electrodes have been suggested for electrometric determination of pH value. Although the hydrogen gas electrode is recognized as primary standard, the glass electrode in combination with calomel electrode is generally used with reference potential provided by saturated calomel electrode. The glass electrode system is based on the fact that a change of 1 pH unit produces an electrical change of 59.1 mV at 25°C.

The active element of glass electrode is membrane of a special glass. The membrane forms a partition between two liquids of differing hydrogen ion concentration and a potential is produced between the two sides of the membrane which is proportional to the difference in pH between the liquids.

## 1.2. Interference

1.2.1. At pH value above 10, high sodium concentrations interfere with the measurement. Correction for the sodium error may be made by consulting the chart supplied by the manufactures of electrodes being used. Sodium errors at pH value levels greater than 10 can be reduced or eliminated by using a low sodium error electrode.

1.2.2. Oil and grease may interfere by coating the pH electrode and causing a sluggish response. These coatings can usually be removed by gentle wiping or detergent washing, followed by distilled water rinsing. An additional treatment with hydrochloric acid (1%) may be necessary to remove any remaining film.

1.2.3. Temperature affects the pH values in two ways. The first is covered by the change in electrode output at various temperatures. This interference can be controlled with instruments having temperature compensation or by calibrating the electrode instrument system at the temperature of the samples. The second source is the change of pH inherent in the sample at various temperatures. This error is sample dependent and cannot be controlled. Therefore the temperature at the time of analysis should be reported.

## 1.3. Apparatus

1.3.1. pH Meter with glass and reference electrode (saturated calomel) preferably with temperature compensation.

1.3.2. Magnetic stirrer with polytetrafluoroethylene coated stirring bar.

1.3.3. Thermometer with least count of 0.5°C.

**1.4. Reagents** - Standard pH buffer solutions be prepared using commercially available tablets or powder with NIST traceability or known amount of chemicals.

Procedures for the preparation of some standard pH buffer solutions are given below and Table 1 shows the pH value of these buffers at different temperatures.

1.4.1. **Borax buffer** - 0.01 M solution, pH 9.18 at 25°C: Dissolve 3.814 gm borax ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ) in deionized or distilled water and dilute to 1 lt. Fresh borax may be used or it may be recrystallized, but, it should not be over dried. For preparation of dilution water, freshly boil and cool deionized or distilled water to expel carbon dioxide gas. Specific conductance of dilution water should be less than  $2 \mu\text{S}$  at 25°C and pH value 5.6 to 6.0 for preparation of all standard solutions.

1.4.2. **Phosphate buffer** - 1:1 solution, pH 6.865 at 25°C: For preparing 0.025M potassium dihydrogen phosphate and 0.025 M disodium hydrogen phosphate, dry potassium dihydrogen phosphate and sodium dihydrogen phosphate in an oven at 130°C for 2 hr and cool in a desiccator. Dissolve 3.388 gm potassium dihydrogen phosphate and 3.533gm sodium dihydrogen phosphate in deionized or distilled water and make up to 1 lt.

1.4.3. **Tartrate buffer** – 0.034M solution, pH 3.56 at 25°C: Prepare a saturated solution of potassium hydrogen tartrate in deionized or distilled water.

1.4.4. **Phthalate buffer** - 0.05M solution, pH 4.008 at 25°C: Dissolve 10.12 gm potassium hydrogen phthalate in deionized water and dilute to 1 lt.

1.4.5. **Tetraoxalate buffer**- 0.05M solution, pH 1.68 at 25°C: Dissolve 12.61 gm potassium tetraoxalate dihydrate in deionized water and dilute to 1 lt.

**Table 1: pH value of buffers at different temperatures:**

S. No.	Temperature °C	Potassium Tetraoxalate(0.05M)	Potassium Hydrogen Tartarate (Saturated) (0.034M)	Potassium Hydrogen Phthalate (0.05M)	Potassium Dihydrogen Phosphate & Disodium Hydrogen Phosphate (0.025M)	Sodium Borate Decahydrate (Borax) (0.01M)	Calcium Hydroxide Saturated (0.0203 M)
1.	0	1.67	-	4.01	6.98	9.46	13.43

2.	5	1.67	-	4.01	6.95	9.39	13.21
3.	10	1.67	-	4.00	6.92	9.33	13.00
4.	15	1.67	-	4.00	6.90	9.27	12.31
5.	20	1.67	-	4.00	6.88	9.23	12.63
6.	25	1.68	3.56	4.01	6.86	9.18	12.45
7.	30	1.68	3.55	4.02	6.85	9.14	12.30
8.	35	1.69	3.55	4.03	6.84	9.10	12.04
9.	40	1.69	3.55	4.04	6.84	9.07	11.99
10.	50	1.71	3.55	4.06	6.83	9.01	11.70
11.	60	1.72	3.56	4.09	6.85	8.96	11.45

**1.4.6 Calcium Hydroxide Buffer** - 0.0203M solution, pH 12.45 at 25°C: Ignite well washed calcium carbonate ( $\text{CaCO}_3$ ) of low alkali grade in a platinum dish at 1000°C for 1hr. Hydrate the cooled calcium oxide by adding deionized water slowly with stirring and heat to boiling. Filter the cooled suspension and collect the solid calcium hydroxide on fritted glass filter of medium porosity. Dry the collected calcium hydroxide in an oven at 110°C, cool and pulverize to uniformly fine granules. Vigorously shake an excess amount of this product in polyethylene bottle with distilled or demineralized water. Allow the gross excess to settle and filter by suction through a fritted glass funnel. Keep the securely stoppered bottle to prevent ingress of carbon dioxide.

## 1.5. Sample handling /preservation

1.5.1. Samples should be analyzed as soon as possible preferably in the field at the time of sampling.

1.5.2. High purity waters and waters not at equilibrium with the atmosphere (ground waters or lake waters collected at depth) are subject to changes when exposed to the atmosphere. Therefore the sample containers should be filled completely and kept sealed prior to analysis.

**1.6. Procedure**— Follow the manufacturer's instructions for operation of pH meter. After required warm-up period, standardize the instrument with a buffer solution of pH near that of the sample and check electrode against at least one additional buffer of different pH value. Measure the temperature of the water and if temperature compensation is available in the instrument adjust it accordingly. Rinse and gently wipe the electrodes with solution. If field measurements are being made, the electrodes may be immersed directly in the sample stream to an adequate depth and moved in a manner to ensure sufficient sample movement across the electrode, the sensing element as indicated by drift free readings (<0.1 pH unit). If necessary, immerse them into the sample beaker or sample stream and stir at a constant rate to provide homogeneity and suspension of solids. Rate of stirring should minimize the air transfer rate at the air-water interface of the sample. Note and record sample pH and temperature. However, if there is a continuous drift, take a second reading with the fresh aliquot of sample without stirring and report it as the pH value.

**1.7. Calculation** — Report pH to the nearest coefficient or 0.01 unit (if instrument reads up to 2 decimal places) and temperature to the nearest °C.

## B. Colorimetric Method

**2.1 Principle** — A series of indicators and buffer solutions are used for determination of pH value by visual comparison.

### 2.2 Reagents

**2.2.1 Indicators** - Prepare universal Indicator by dissolving 0.05 gm of methyl orange, 0.15 gm of methyl red, 0.3 gm of bromethymol blue and 0.35 gm of phenolphthalein in one liter of alcohol (66 percent). The color changes are:

pH	Color
Upto 3	Red
4	Orange Red
5	Orange
6	Yellow
7	Yellowish green

8	Greenish Blue
9	Blue
10	Violet
11	Reddish Violet

2.2.2 Prepare indicator solution as given below:

**Table 1:** Preparation of indicator solution:

S. No	Name of Indicator	pH Range	Color Change	Method of Preparation
1.	Thymol blue (acid range)	1.2 to 2.8	Red to yellow	Weigh 0.10 gm, add 10.75 mL of N/50 sodium hydroxide solution and dilute with water to 250 mL
2.	Bromophenol blue	3.0 to 4.5	Yellow to blue violet	Weigh 0.10 gm, add 7.45 mL of N/50 sodium hydroxide solution and dilute with water to 250 mL
3.	Bromocresol green	3.8 to 5.4	Yellow to blue	Weigh 0.10 gm, add 7.15 mL of N/50 sodium hydroxide solution and dilute with water to 250 mL
4.	Methyl red	4.2 to 6.3	Red to yellow	Weigh 0.10 gm, add 18.60 mL of N/50 sodium hydroxide solution and dilute with water to 250 mL
5.	Bromocresol purple	5.2 to 6.8	Yellow to blue violet	Weigh 0.10 gm, add 9.25 mL of N/50 sodium hydroxide solution and dilute with water to 250 mL

6.	Bromothymol blue	6.0 to 7.8	Yellow to blue	Weigh 0.10 gm, add 8.00 mL of N/50 sodium hydroxide solution and dilute with water to 250 mL
7.	Phenol red	6.8 to 8.4	Yellow to red	Weigh 0.10 gm, add 14.20 mL of N/50 sodium hydroxide solution and dilute with water to 250 mL
8.	Cresol red	7.2 to 8.8	Yellow to red	Weigh 0.10 gm, add 13.10 mL of N/50 sodium hydroxide solution and dilute with water to 250 mL
9.	Thymol blue (alkaline range)	8 to 9.5	Yellow to blue	Weigh 0.10 gm, add 10.75 mL of N/50 sodium hydroxide solution and dilute with water to 250 mL
10.	Thymolphthalein	9.3 to 10.5	Colourless to blue	Dissolve 0.10gm in 100mL of rectified spirit [see IS : 323-1959 Specifications for rectified spirit (revised)]
11.	Thymol violet	9.0 to 13.0	Yellow to green to violet	Dissolve 0.10gm of tropaeolin O in 100mL of water. Dissolve 0.04gm of thymolphthalein in a mixture of 50mL of water. Mix one part of tropaeolin O solution with 4 parts of thymolphthalein solution.

**2.3 Procedure** — Take 100 mL of the sample in a hard glass tube and determine the approximate pH by using the universal indicators. Repeat using a solution of the indicator (about 1/20 of the volume of the liquid being tested) which corresponds to

the approximate pH found above. Compare the color produced with a series of buffer solutions of known pH each containing the same proportion of the indicators.

**2.4 Report** — Report the pH of that buffer solution which matches with that of the sample to the nearest 0.1 unit.

### **3 Reference:**

**3.1** IS 3025 (part 11) - 1983 (Reaffirmed 2002)- Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : pH Value

## **4. TASTE**

**1. Principle** – Each panelist (tester) is presented with a list of nine statements about the water, ranging from very favorable to very unfavorable. The tester selects a statement that best expresses his opinion. The scored rating is the scale number of the statement selected. The panel rating is the arithmetic mean of the scale numbers of all testers.

### **2. Apparatus**

**2.1. Tasting** – Present each sample to the observer in a clean 50 mL beaker filled to the 30mL level.

**2.2. Temperature control** – Temperature of the samples shall be such that the observers find it pleasant for drinking. Maintain this by using a controlled temperature water bath. A temperature of 15°C is recommended but in any case do not allow it to exceed 27°C.

### **3. Reagents**

Taste and odour-free water and 2000 mg/L solution of sodium chloride prepared with taste and odour -free water as reference sample.

## 4. Procedure

**4.1.** For test efficiency a single rating session may contain up to 10 samples including the reference samples. Observers should work separately after receiving thorough instructions and trial or orientation sessions followed by questions and discussion on procedures. Select panel members on the basis of performance in trial sessions.

**4.2.** Rate the samples as follows:

4.2.1. Initially taste about half of the sample by taking the water into the mouth, holding it for several seconds and leaving off it without swallowing.

4.2.2. Form an initial judgment on the rating scale

4.2.3. Make a second tasting the same manner as the first.

4.2.4. Make a final rating for the sample and record the results on the appropriate data form

4.2.5. Rinse the mouth with taste and odour free water.

4.2.6. Give a gap of one minute before repeating steps 4.2.1 to 4.2.5 on the next sample

**4.3.** Independently randomize sample order for each judge. Allow 30 min gap between repeated rating sessions. The observers should not know the composition or source of specific samples. Use the scale given in 4.4 for rating and record ratings as integers ranging from one to nine with one given the highest quality rating.

**4.4. Rating scale :** Action tendency scale

- a. I would be very happy to accept this water as my every day drinking water.
- b. I would be happy to accept this water as my every day drinking water.
- c. I am sure that I could accept this water as my everyday drinking water.
- d. I could accept this water as my everyday drinking water.
- e. May be I could accept this water as my everyday drinking water.
- f. I do not think I could accept this water as my everyday drinking water.
- g. I cannot accept this water as my everyday drinking water.
- h. I could never drink this water.
- i. I cannot stand this water in my mouth and I could never drink it.

## 5. Precautions

5.1. Make taste tests only on samples known to be safe for ingestion.

5.2. Do not use samples that may be contaminated with bacteria, virus, parasites or toxic chemicals such as arsenic, dichlorinating agents or that derived from an unaesthetic source.

5.3. Observe all sanitary and esthetic precautions with regard to apparatus and containers containing the sample. Practice hospital level sanitation of these items.

5.4. Make analysis in a laboratory free from interfering background odours. If possible, provide carbon filtered air at constant temperature and humidity because without such precautions the test measures flavor and not taste.

6. **Calculations** – Calculate the mean and standard variation of all rating given to each sample. Report the temperature at which the sample is tested.

## 7. Reference :

7.1. IS 3025 part-8:1984 (Reaffirmed 2002)- Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : Taste Rating

## 5. TURBIDITY

### Introduction:

The turbidity of sample is the reduction of transparency due to the presence of particulate matter such as clay or slit, finely divided organic matter, plankton or other microscopic organisms. These cause light to be scattered and absorbed rather than transmitted in straight lines through the sample. The values are expressed in nephelometric turbidity units (NTU). The method is applicable to drinking, surface and saline waters in the range of turbidity 0-40 NTU. Higher values may be obtained by dilution of the sample.

### Nephelometric method

**1. Principle** - It is based on comparison of the intensity of light scattered by the sample under defined conditions with the intensity of light scattered by a standard reference suspension under the same conditions.

**2. Interferences** - Coloured solutes cause lowering of turbidity values.

### **3. Apparatus**

3.1. Sample tubes - The sample tubes should be of clear and colourless glass.

3.2. Turbidimeter - The turbidimeter shall consist of a nephelometer with a light source for illuminating the sample and one or more photoelectric detectors with a readout device to indicate the intensity of light scattered at right angles to the path of the incident light. The turbidimeter should be so designed that little stray light reaches the detector in the absence of turbidity and should be free from significant drift after a short warm up period.

### **4. Reagents**

4.1. Turbidity free water - Pass distilled water through membrane filter having a pore size not greater than 0.45  $\mu\text{m}$ . If such filtered water shows a lower turbidity than the distilled water, discard the first 200mL collected water. Other wise use distilled water.

4.2. Hexamethylene tertramine solution - Dissolve 10.0gm hexamethylene tetramine in demineralized water and dilute to 100mL.

4.3. Hydrazine sulphate solution - Dissolve 1.0 gm hydrazine sulphate  $[(\text{NH}_2)_2\text{H}_2\text{SO}_4]$  in demineralized water and dilute to 100mL

4.4. Turbidity standard suspension-I (Formazin) - In a 100mL volumetric flask mix 5.0mL hydrazine sulphate solution with 5.0mL hexamethylene tetramine solution. After 24 hr standing at  $25\pm 3^\circ\text{C}$ , dilute to 100mL with demineralized water and mix well. Prepare fresh suspension monthly.

4.5. Turbidity standard suspension-II -Dilute 10mL turbidity standard suspension 1 to 100mL with demineralized water. The turbidity of this suspension is defined as 40

Jackson Turbidity Units (JTU). Prepare fresh suspension weekly. This suspension may be diluted as required to prepare more dilute turbidity standards.

**5. Sample Handling and Preservation** - Preservation of sample is not practical. Analysis should begin as soon as possible. Refrigeration or chilling to 4°C is recommended to minimize microbiological activity.

**6. Procedure** -

6.1. Turbidimeter calibration - Follow the manufacturer's operating instructions. Measure the standards on turbidimeter covering the range of interest. If the instrument is already calibrated in standard turbidity units, this procedure will check the accuracy of calibration.

6.2. Turbidity less than 40 units - Shake the sample to disperse the solids. Wait until air bubbles disappear. Pour the sample into turbidimeter tube and read turbidity directly from the instrument scale or from calibration curve.

6.3. Turbidity greater than 40 units - In case turbidity values are greater than 40 units, dilute the sample with turbidity free water to bring the values within range. Take reading of diluted sample. Compute the turbidity of the original sample from the turbidity of the diluted sample and the dilution factor.

6.4. Calculation- Calculate the turbidity of diluted samples using the following equation

$$\text{Turbidity Units} = \frac{A \times (B + C)}{C}$$

Where

A= Turbidity units of diluted sample

B= Volume in mL of dilution water used

C= Volume of sample in mL taken for dilution

**7. Report** - Report turbidity as follows:

Turbidity range in unit	Record to the Nearest
0-1	0.05

1-10	0.1
10-40	1
40-100	5
100-400	10
400-1000	50
Greater than 1000	100

## 8. Reference :

8.1. IS : 3025 Part-10 1984 (Reaffirmed 2002)- Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : Turbidity

## 6. TOTAL DISSOLVED SOLIDS (TDS)

The following two methods are applicable for TDS measurement

- A. Gravimetric Method
- B. Determination of TDS based on conductivity

### A. Gravimetric method

**1. Principle** – The sample is filtered and the filtrate evaporated in a tarred dish on steam bath. The residue after evaporation is dried to constant mass at 103-105°C or 179-181°C

### 2. Interferences –

**2.1.** Highly mineralized waters containing significant concentration of calcium, magnesium, chloride and sulphate may be hygroscopic. These may require prolonged drying, desiccation and rapid weighing. However, prolonged drying may also cause loss of constituents, particularly nitrates and chlorides.

**2.2.** A large amount of residue in the evaporating basin may crust over and entrap water preventing its evaporation during drying. For this reason, the volume of the

sample should be adjusted so that the residue left after drying should be about 100-200mg.

### **3. Apparatus -**

**3.1.** Filter - Any one of the following filter may be used.

3.1.1. Glass fiber filter disc - (Whatman GF/C or equivalent) 2.1 to 5.5 cm in diameter, pore size 1.2  $\mu\text{m}$

3.1.2. Paper – Acid washed ashless hard filter finish; filter paper sufficiently retentive for the fine particles (Pore size 2-2.5  $\mu\text{m}$  equivalent to Whatman filter no.542).

3.1.3. Gooch crucible-30mL capacity with 2.1 or 2.4 cm diameter glass fibre filter disc (Whatman or equivalent).

3.1.4. Sintered disc-G-5 or its equivalent with pore size 1 to 2  $\mu\text{m}$ .

3.1.5. Membrane filters 0.45  $\mu\text{m}$  membrane.

**3.2.** Filtering assembly depending upon the type of filter selected.

**3.3.** Drying oven with thermostatic control for maintaining temperature up to  $180 \pm 2^\circ\text{C}$ .

**3.4.** Desiccators provided with a colour indicating desiccant.

**3.5.** Analytical Balance 200gm capacity and capable of weighing to nearest 0.1 mg.

**3.6.** Magnetic stirrer with Teflon coated stirring bars.

**4. Sample handling and preservation** – Preservation of the samples is not practical. Analysis should begin as soon as possible. Refrigeration or chilling to  $4^\circ\text{C}$  to minimize microbiological decomposition of solids is recommended.

### **5. Procedure**

**5.1.** Heat the clean evaporating dish to  $180^\circ\text{C}$  for 1 hr. Cool in the desiccator, Weigh and store in the desiccators until ready for use.

**5.2.** Filter a portion of the sample through any of the filter mentioned in 4.1. Select volume of the sample which has residue between 25 and 250mg preferably between 100 to 200mg. This volume may be estimated from values of specific conductance to

obtain a measurable residue, successive aliquots of filtered sample may be added to the sample dish.

**5.3.** Stir volume of sample with a magnetic stirrer or shake it vigorously. Pipette this volume to a weighed evaporating dish placed on a steam bath. Evaporation may also be performed in a drying oven. The temperature of drying oven shall be lowered to approximately 98°C to prevent boiling and splattering of the sample. After complete evaporation of water from the residue, transfer this dish to an oven at 103-105°C or 179-181°C and dry to constant mass i.e. till the difference in the successive weighing is less than 0.5 mg. Drying for a long duration (usually 1-2 hr) is done to eliminate necessity of checking for constant mass. The time for drying to constant mass with a given type of sample when a number of samples of nearly same type are to be analyzed has to be determined by trial.

**5.4.** Weigh the dish as soon as it has cooled avoiding residue to stay for long time as some residues are hygroscopic and may absorb water form desiccant that is not absolutely dry.

**5.5.** Calculation - Calculate filterable residue from the following equation

$$\text{Filterable residue, mg/L} = \frac{1000M}{V}$$

Where

M = Mass in mg of filterable residue

V = volume in mL of the sample

**6. Report** - Report in whole numbers for less than 100 mg/L and to three significant figures for values above 100mg/L. Report the temperature of determination.

**7. Reference:**

**7.1.** IS : 3025 part 16 – 1984 (Reaffirmed 2002)- Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : Filterable Residue (Total Dissolved Solids)

## **B. Determination of TDS based on conductivity**

This method involves two steps:

1. Determination of Conductivity
2. Calculation of TDS by conductivity

### **1. Determination of Conductivity**

**1.0 Principle-** Specific conductance is determined by using a wheatstone bridge in which a variable resistance is adjusted so that it is equal to the resistance of the unknown solution between platinized electrodes of a standard conductivity cell. The cell constant is determined by the following relationship:

Specific conductance = Conductance × Cell constant, or

$$\text{Specific conductance} = \frac{\text{Cell constant}}{\text{Resistance}}$$

The cell constant is determined experimentally with a standard solution of known conductance.

### **2.0 Interference**

**2.1** Temperature affects conductivity, which varies by about 2% per degree Celsius. The temperature of 25<sup>0</sup>C is taken as standard. It is desirable to observe the conductivity at 25<sup>0</sup>C or as near to this temperature as possible, although compensation for variations from it can be made. In some instruments, this is made automatically.

**2.2** Dissolved carbon dioxide increases conductivity without increasing the mineral salt content. However, the effect is not large and it is usual to ignore it. In low pH water,

H<sup>+</sup> ions and in high pH water OH<sup>-</sup> ions, may contribute substantially to conductivity owing to high equivalent conductivity of these ions. Water with high silica (SiO<sub>2</sub>) content give relatively low values of electrical conductivity to total dissolved solids ratio as SiO<sub>2</sub> (H<sub>4</sub>SiO<sub>4</sub>) does not contribute significantly to electrical conductance values.

**2.3** It is not convenient to use water containing large amount of suspended matter. It should be settled or filtered. High suspended matter also affects electrical conductance values.

**2.4** Samples containing fat, grease, oil, tar, etc, may contaminate the electrodes causing erratic results.

### 3. Apparatus

**3.1. Conductivity Meter-** Wheatstone bridge type or equivalent direct reading meter

**3.2. Conductivity Cells-** Cells of at least two different cell constants, for measurement of wide range of conductivities. Specific conductance ranges and corresponding values of cell constants are given below

SPECIFIC CONDUCTANCE μS/cm at 25°C	CELL CONSTANT
20 - 1000	0.2
40 - 2000	0.5
100 - 4000	1.0
200 - 10000	2.0
400 - 20000	5.0
10000 - 40000	10.0

**3.3 Thermometer-** 0 to 50°C, graduated in 0.1°C.

**Note-** some direct reading conductivity meters have automatic compensation built into the instrument.

#### 4. Reagents

4.1. **Standard Potassium Chloride Solution-** Dissolve 0.5232gm potassium chloride dried at 180°C for 1 hr in demineralized water and dilute to 1000mL. The distilled water used for preparing standard solutions should have a very low conductivity. The specific conductance of this solution at 25°C is 1000µs/cm and the concentration of this solution is 0.00702 N. Alternatively, dissolve 0.7456 gm of anhydrous potassium chloride, dried at 180°C for 1 hour in distilled water and make up to 1000 mL at 25°C . The specific conductance of this solution at 25°C is 1408µs/cm and the concentration of this solution is 0.01N.

#### 5. Procedure

5.1 **Platinizing of cell-** Platinization of cell is required when readings become erratic. For platinizing, clean the cell in chromic acid solution once and rinse several times with distilled water. Place the cell in a commercial platinizing solution or dissolve 3 gm of chloroplatinic acid (H<sub>2</sub>PtCl<sub>6</sub>) in 10 mL water to which 20 mg lead acetate has been added. Connect it with two dry cells of 1.5 volts each in parallel and reverse the direction of the current once a minute for 6 minutes or till the shining platinum surface is covered. Repeat the electrolytic process using 10% sulphuric acid to remove chlorine. Wash with distilled water and keep the cell immersed in distilled water when not in use.

5.2 Set the instrument according to manufacturer's instruction. In some instruments correction for cell constant and temperature factor is provided. If this arrangement is not there, cell constant may be separately determined and values of specific conductance should be converted to 25° C by multiplying with the factor given in table1.

$$\text{Cell Constant, } L = \frac{K_1 + K_2}{K_x \times f}$$

Where

K1 = conductivity in µs /cm of the potassium chloride solution at 25°C;

$K_2$  = Conductivity in  $\mu\text{s}/\text{cm}$  of distilled water at  $25^\circ\text{C}$  used for preparing the reference solution;

$K_x$  = measured conductance in  $\mu\text{s}/\text{cm}$ ; and

$f$  = temperature factor for converting specific conductance value to that at  $25^\circ\text{C}$  (see table 1)

**Note-** if  $K_2$  is very low, it may be ignored.

**5.3** Determine conductivity of 0.00702 N potassium chloride or 0.01 N. Potassium chloride solution by use of instrument in accordance with manufacturer's instructions. Measure the temperature of the solution before and after the test and take the mean value ( $t^\circ\text{C}$ ).

**5.4** Because the cell constants are subject to slow change even under ideal conditions and sometimes to more rapid change under adverse conditions, it is recommended that cell constant be periodically established.

**5.5** Determine conductance of the unknown sample.

**6. Calculation** – Calculate specific conductance as follows:

$$\text{Specific conductance at } 25^\circ\text{C, } \mu\text{s}/\text{cm} = KLf$$

Where

$K$  = conductivity,  $\mu\text{s}/\text{cm}$ ;

$L$  = Cell Constant; and

$f$  = factor for converting specific conductance value to that at  $25^\circ\text{C}$

Temperature °C	Factor <i>f</i>	Temperature °C	Factor <i>f</i>	Temperature °C	Factor <i>f</i>
15.0	1.247	23.0	1.043	30.2	0.904
16.0	1.218	23.2	1.038	30.4	0.901
16.2	1.212	23.4	1.034	30.6	0.897
16.4	1.206	23.6	1.029	30.8	0.894
16.6	1.200	23.8	1.025	31.0	0.890
16.8	1.194	24.0	1.020	31.2	0.887
17.0	1.189	24.2	1.016	31.4	0.884
17.2	1.184	24.4	1.012	31.6	0.880
17.4	1.179	24.6	1.008	31.8	0.877
17.6	1.174	24.8	1.004	32.0	0.873
17.8	1.169	25.0	1.000	32.2	0.870
18.0	1.163	25.2	0.996	32.6	0.864
18.2	1.157	25.4	0.992	32.8	0.861
18.4	1.152	25.6	0.988	33.0	0.858
18.6	1.147	25.8	0.983	33.2	0.855
18.8	1.142	26.0	0.979	33.4	0.852
19.0	1.136	26.2	0.975	33.6	0.849
19.2	1.131	26.4	0.971	33.8	0.846
19.4	1.127	26.6	0.967	34.0	0.843
19.6	1.122	26.8	0.964	35.0	0.829
19.8	1.117	27.0	0.960	36.0	0.815
20.0	1.112	27.2	0.956	37.0	0.801
20.2	1.107	27.4	0.953	38.0	0.788
20.4	1.102	27.6	0.950	39.0	0.775
20.6	1.097	27.8	0.947	40.0	0.763
20.8	1.092	28.0	0.943	41.0	0.750
21.0	1.087	28.2	0.940	42.0	0.739
21.2	1.082	28.4	0.936	43.0	0.727

21.4	1.076	28.6	0.932	44.0	0.715
21.6	1.073	28.8	0.929	45.0	0.705
21.8	1.068	29.0	0.925	46.0	0.694
22.0	1.064	29.2	0.921	47.0	0.683
22.2	1.060	29.4	0.918		
22.4	1.055	29.6	0.914		
22.6	1.051	29.8	0.911		
22.8	1.047	30.0	0.907		

**7. Precision and Accuracy** – Precision and accuracy depend on the instrument used. Generally a precision and accuracy of about  $\pm 3$  percent or less are possible with good quality instruments.

## 2. Calculation of TDS by conductivity

The ability of a solution to conduct an electric current is the functioning of the concentration and charge of ions in the solution and also depends on ionic mobility. Ionic mobility decreases with increase in number of ions per unit volume of solution due to interionic effect and other factors. Broadly, the relationship between conductivity and dissolved solids and conductivity and soluble cations is given by the following equations:

$$A K = S \text{ and,}$$

$$K = 100 C$$

Where

$A$  = multiplication factor for converting conductivity values to total dissolved solids;

$K$  = conductivity in  $\mu\text{s}/\text{cm}$ ,

$S$  = total dissolved solids in  $\text{mg}/\text{L}$ , and

$C$  = total soluble cations in  $\text{meq}/\text{L}$ .

**Note 1**- the value of A varies from 0.54 to 0.96 depending on the nature of ion present in water, and is usually taken as 0.65.

**Note 2** -The relationship given above is approximate and is used for broad checking only and should not be used for accurate calculations. Types of ions present in solution effect these relationships. A pure solution of sodium bicarbonate with total dissolved solids 980 mg/L will have a conductivity of 1000 $\mu$ s/cm and a solution of sodium chloride with total dissolved solids 500 mg/L will have the same conductivity. Presence of relatively low conductivity particles or molecules like silicic acid and the presence of H<sup>+</sup> and OH<sup>-</sup> ions effect the ratio between conductivity and total dissolved solids.

## CHAPTER II: GENERAL CHEMICAL PARAMETERS

### 1. AMMONIACAL NITROGEN

#### Introduction:

In water nitrogen is present in different forms namely Nitrate, Nitrite Ammonia & organic Nitrogen. All these forms are biochemically interconvertible and are component of nitrogen cycle. In water ammonia is present in surface water. Its concentration is generally low in ground water because it is absorbed in soil particles and clays and is not leached readily from soils.

Methods for determination of Ammoniacal Nitrogen in water are prescribed:-

- A. By titration Method
- B. Nesslerization Method
- C. Phenate Method
- D. Ammonia Selective Electrode Method
- E. Flow Injection Analysis

#### A. By Titration Method

**1. Principle** – The sample is buffered & distilled and Ammonia absorbed in distillate is titrated with standard Sulphuric acid. The following table values are used for selecting sample volume for distillation.

NH <sub>3</sub> -N in Sample (mg/L)	Sample Volume (mL)
5-10	250
10-20	100
20-50	50
50-100	25

## 2. Apparatus

2.1. Distillation assembly- borosilicate glass flask of 800 to 2000 mL capacity attached to a vertical condenser so that the outlet tip may be submerged below the surface of the receiving acid solution.

## 3. Reagents

3.1. **Mixed indicator solution** - Dissolve 200 mg of methyl red indicator in 100 mL 95% ethyl or isopropyl alcohol. Dissolve 100 mg of methylene blue in 50 mL of 95 % ethyl or isopropyl alcohol. Combine these two solutions. Prepare the indicator solution monthly.

3.2. **Indicating boric acid solution** - Dissolve 20 gm hypoboric acid in ammonia free water, add 10 mL of mixed indicator solution and dilute to 1 liter.

3.3. **Borate Buffer** - Add 88 mL of 0.1 N sodium Hydroxide to 500 mL of 0.025 M sodium tetraborate (50 gm  $\text{Na}_2\text{B}_4\text{O}_7$  or 9.5 gm  $\text{Na}_2\text{B}_4\text{O}_7 \cdot \text{H}_2\text{O}$ ) and make up to 1000 mL

3.4. **Standard sulphuric acid titrant** - 0.02 N (1mL=280  $\mu\text{g}$  of nitrogen)

## 4. Procedure

### 4.1. Preliminary Distillation Step

4.1.1. The two major factors that influence selection of the method for determination of ammonia are concentration and presence of interference. Where interferences are present and greater precision is necessary a preliminary distillation step is necessary.

4.1.2. Add 500 mL water and 20 mL of borate buffer solution to a distillation flask and adjust pH to 9.5 with 6 N sodium hydroxide solution. Add a few glass beads and use this mixture to steam out the distillation apparatus until distillate shows no trace of ammonia.

4.1.3. Use 500 mL water of dechlorinated sample or a portion diluted to 500mL with water. Remove residual chlorine by adding, dechlorinating agent equivalent to chlorine residual at the time of collection. If necessary, neutralize to pH 7 with dilute acid or

alkali. Add 25 mL of borate buffer and adjust pH to 9.5 with 6 N sodium hydroxide solutions using a pH meter.

4.1.4. To minimize contamination leave distillation apparatus assembled after steaming out and until just before starting the sample distillation. Disconnect steaming out flask and immediately transfer sample flask to distillation apparatus. Distil at the rate of 6 to 10 mL/min with the tip of the delivery tube below the surface of acid receiving solution. Collect distillate in 500 mL Erlenmeyer flask containing 50 mL indicating boric acid solution. Collect at least 200 mL of distillate. Lower the collected distillate free of contact with the delivery tube and continue distillation during the last minute or two to cleanse condenser and delivery tube. Dilute to 500 mL with water.

4.2. Titrate ammonia in distillate against standard sulphuric acid until indicator turns pale lavender. Carry a blank through all steps of the procedure and apply the necessary correction to the results.

#### 4.3. Calculation

$$\text{Ammonical nitrogen mg/l} = \frac{(A-B) \times 280}{V}$$

Where

A= Volume in mL of sulphuric acid used for sample,

B= Volume in mL of sulphuric acid used for blank, and

V= Volume in mL of sample taken for test.

## B. Nesslerization Method

1. **Principle-** The sample is buffered and distilled. The ammonia in the distillate or in the sample is treated with Nessler's reagent and the colour developed is matched with that of a series of standard ammonia solutions or measured photometrically at 400 to 425 nm.

### 2. Apparatus

2.1 Spectrophotometer- for use at 400 to 500 nm.

**2.2** Filter photometer- equipped with violet filter and having maximum absorbance at 400- 425 nm.

**2.3** Nessler tubes

**2.4** pH meter

### **3. Reagents**

**3.1** Zinc sulphate solution- Dissolve 100 gm of zinc sulphate  $ZnSO_4 \cdot 7H_2O$  and dilute to 1 liter with water.

**3.2** Stabilizer reagent- Use EDTA or Rochelle salt to prevent calcium or magnesium precipitation in undistilled samples:

EDTA reagent- Dissolve 50 gm of EDTA in 60 mL water containing 10 gm of sodium hydroxide. Heat gently to complete dissolution. Cool to room temperature and dilute to 100 mL

Rochella salt solution- Dissolve 50 gm of potassium sodium Tartrate tetrahydrate in 100 mL of water

**3.3** Nessler's reagent- Dissolve 100 gm of mercuric iodide and 70 gm of potassium iodide in a small quantity of water and add this mixture slowly with stirring to a cool solution of 160 gm of sodium hydroxide dissolved in 500 mL of water. Dilute to 1 litre. Store in brown rubber stopper glass bottle. Reagent is stable up to one year. It is toxic and so avoid ingestion.

**3.4** Stock ammonia solution- Dissolve 3.819 gm of anhydrous ammonium chloride in water and dilute to 1 litre (1.00 mL = 1.00 mg of nitrogen = 1.22 mg of ammonia).

**3.5** Standard ammonia solution- Dilute 10.00 mL of stock solution to 1000 mL with water (1.00 mL = 12.2 µg of ammonia = 10.0 µg of N).

### 3.6 Permanent colour solutions

3.6.1 Potassium chloroplatinate solution- Dissolve 2.0 gm of potassium chloroplatinate in 300 to 400 mL of water, add 100 mL of concentrated hydrochloric acid and dilute to 1 liter.

3.6.2 Cobaltous Chloride solution – Dissolve 12 gm of cobaltous chloride ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) in 200 mL of water. Add 100ml of concentrated hydrochloric acid and dilute to 1 liter.

## 4. Procedure

4.1 Treatment of undistilled samples- if necessary, remove residual chlorine. Add 1mL of zinc sulphate solution to 100 mL of sample and mix thoroughly. Add 0.4 to 0.5 mL of 6 N sodium hydroxide solution to obtain a pH of 10.5 and mix well. Let treated sample stand for a few minutes, whereupon a heavy flocculent precipitate should fall, leaving a clear and colorless supernate. Clarify by centrifuging or filtering. Pretest any filter paper used to be sure no ammonia is present as a contaminant. Do this by running water through the filter and testing the filtrate by nesslerization. Filter sample, discarding first 25 mL of filtrate.

### 4.2 Colour development

4.2.1 Undistilled samples- Use 50 mL of sample or a portion diluted to 50 mL with water. If undistilled portion contains sufficient concentrations of calcium, magnesium or other ions that produce turbidity or precipitate with nessler reagent add 1 drop of EDTA reagent or 1 to 2 drops of Rochelle salt solution. Mix well. Add 2.0 mL of nessler reagent if EDTA is used or 1.0 mL of nessler reagent if Rochelle salt is used.

4.2.2 Distilled samples- Neutralize the boric acid used for absorbing ammonia distillate by adding either 2 mL of nessler's reagent, an excess that raises the pH to the desired high level of alternatively, neutralizing the boric acid with sodium hydroxide before adding 1 mL nessler's reagent.

4.2.3 Mix samples by capping tubes with clean rubber stoppers and then inverting the tubes at least 6 times. Keep such conditions as temperature and reaction time the same in blank, samples and standards. Let reaction proceed for at least 10 minutes after adding nessler reagent. Measure colour in sample and standards. If ammoniacal nitrogen is very low use a 30 minute contact time for sample, blank and standards. Measure colour either by photometry or visually as given below.

4.2.4 Photometric measurement – Measure absorbance or transmittance with spectrophotometer or filter photometer. Prepare calibration curve at the same temperature and reaction time used for samples. Measure transmittance readings against a reagent blank and run parallel checks frequently against standards in the nitrogen range of the samples. Redetermine complete calibration curve for each new batch of Nessler's reagent.

4.2.5 For distilled samples prepare standard curve under the same condition as the samples. Distill reagent blank and appropriate standards, each diluted to 500 mL in the same manner as the samples. Dilute 300 mL of distillate plus 50mL of boric acid absorbent to 500 mL with water and take a 50 mL portion for nesslerization.

4.2.6 Visual Comparison- Compare colors produced in sample against those of ammonia standards. Prepare temporary or permanent standards as follows:

4.2.6.1 Temporary standards- Prepare a series of visual standards in Nessler tubes by adding the following volumes of standard ammonium chloride solution and diluting to 50 mL with water: 0,0.2,0.4,0.7,1.0,1.4,1.7,2.0,2.5,3.0,3.5,4.0,4.5,5.0, and 6.0 mL. Nesslerize standards and portions of distillate by adding 1.0 mL Nessler's reagent to each tube and mixing well.

4.2.6.2 Permanent standards- Measure into 50 mL Nessler tube, the volume of potassium chloroplatinate and cobaltous chloride solutions indicated in Table 1 dilute to mark and mix well. The values given in the table are approximate; actual equivalents of the ammonium standards will differ with the quantity of Nessler's reagent, the kind of illumination used and the colour sensitivity of analyst eye. Therefore compare colour

standards with nesslerized temporary ammonia standards and modify the tints as necessary. Make such comparisons for each newly prepared Nessler reagent and satisfy each analyst as to the aptness of the colour match. Protect standards from dust to extend their usefulness for several months. Compare either 10 or 30 minutes after nesslerization, depending upon reaction time used in preparing nesslerized ammonium standards against which they were matched.

## 5. Calculation

**5.1** Deduct the amount of ammoniacal nitrogen in water used for diluting original sample before computing final nitrogen value.

**5.2** Deduct also reagent blank for volume of borate buffer and 6 N sodium hydroxide solutions used with sample.

**5.3** Compute total ammoniacal nitrogen by the following equation:

$$\text{Nitrogen ammoniacal mg/L (51 mL of final volume)} = \frac{A \times B}{V \ C}$$

Where

A =  $\mu\text{g}$  of ammoniacal nitrogen (51 mL of final volume);

B = total volume of distillate collected, in mL, including acid absorbent;

C = volume distillate taken for nesslerization in mL, and

V = volume in mL of sample taken

*Note- The ratio B/C applies only to distilled samples, and should be ignored in direct nesslerization*

Table 1

**PREPARATION OF PERMANENT COLOR STANDARDS FOR VISUAL DETERMINATION  
OF AMMONIACAL NITROGEN**

<i>VALUE IN AMMONIACAL NITROGEN <math>\mu\text{g}</math></i>	<i>APPROXIMATE VOLUME OF PLATINUM SOLUTION, mL (in matched 50 mL nessler tubes)</i>	<i>APPROXIMATE VOLUME OF COBALT SOLUTION, mL (in matched 50 mL nessler tubes)</i>
0	1.2	0.0
2	2.8	0.0
4	4.7	0.1
7	5.9	0.2
10	7.7	0.5
14	9.9	1.1
17	11.4	1.7
20	12.7	2.2
25	15.0	3.3
30	17.3	4.5
35	19.0	5.7
40	19.7	7.1
45	19.9	8.7
50	20.0	10.4
60	20.0	15.0

### C. Phenate Method

**1 Principle-** An intensely blue compound, indophenols, is formed by the reaction of ammonia, hypochlorite and phenol catalyzed by a manganous salt.

**2 Interference-** alkalinity over 500 mg/L as calcium carbonate or acidity over 100mg/L as calcium carbonate or turbidity interfere. Remove these by preliminary distillation.

### **3 Apparatus-**

**3.1** Spectrophotometer or filter photometer- for use at 630 nm. The photometer is equipped with a red-orange filter. The light path of these photometers should be 1 cm approximately.

**3.2** Magnetic stirrer

### **4 Reagents**

**4.1** Ammonia- free- water- Prepare ammonia free water by passing distilled water through an ion exchange column containing a strongly acidic cation exchange resin mixed with a strongly basic anion exchange resin. Alternatively redistill distilled water by adding 0.1 mL of concentrated sulphuric acid to 1 litre of water. It may also be made by treating distilled water with sufficient bromine or chlorine water to produce a free halogen residual of 2 to 5 mg/L. Redistill after standing at least for 1 hour. Discard the first 100 mL distillate.

**4.2** Hypochlorous acid reagent- To 40 mL of water, add 10 mL of 5 percent solution of sodium hypochlorite prepared from commercial bleaching powder. Adjust pH to 6.5 to 7.0 with hydrochloric acid.

**4.3** Manganese sulphate solution- 0.006 N. Dissolve 50 mg of manganous sulphate monohydrate in 100 mL of water.

**4.4** Phenate reagent- Dissolve 2.5gm of sodium hydroxide and 10 gm of phenol in 100 mL of water. Prepare weekly. Since phenol; is corrosive handle with care.

**4.5** Stock ammonium solution- dissolve 381.9 mg of anhydrous ammonium chloride in water and dilute to 1000 mL (1.00 mL = 122 µg as NH<sub>3</sub> or 100 µg as N)

**4.6** Standard ammonia solution- Dilute 5.00 mL of stock solution to 1000 mL with water (1.00 mL = 0.500 µg of nitrogen or 0.607 µg of ammonia)

## 5 Procedure

**5.1** Treatment of sample- To a 10 mL of sample in a 50 mL beaker, add 1 drop of manganous sulphate solution. Place on a magnetic stirrer and add 0.5 mL of hypochlorous acid reagent. Immediately add a drop at a time 0.6 mL of Phenate reagent. Add reagent without delay using a bulb pipette or burette for convenient delivery. Mark pipette for hypochlorous acid at the 0.5 mL level and deliver the Phenate reagent from a pipette or burette that has been calibrated by counting the drops previously found to be equivalent to 0.6 mL. Stir vigorously during addition of reagents. Because colour intensity is affected by the age of reagents, carry a blank and standard a standard with each batch of samples. Measure absorbance to zero in the spectrophotometer. Colour formation is complete in 10 minutes and is stable for at least 24 hours. Although the blue colour has a maximum absorbance at 630 nm, satisfactory measurements can be made in 600 to 660 nm region.

**5.2** Preparation of standards- Prepare a calibration curve in the ammoniacal nitrogen range of 0.1 to 5 µg, treating standards exactly as the sample.

## 6 Calculation

**6.1** Calculate ammonia concentration as follows:

$$\text{Ammoniacal nitrogen, mg/L} = \frac{A \times B}{C \times S} \times \frac{D}{E}$$

Where

A= absorbance of sample;

B= ammoniacal nitrogen in the standard, µg

C= absorbance of standard

S= volume in mL of sample

D= volume in mL of total distillate collected including the acid absorbents, neutralizing agents and ammonia free water added and

E = volume in mL of distillate used for colour development.

*Note- The ratio D/E applies only to distilled samples.*

## D. Ammonia Selective Electrode Method

**1. Principle** – It uses a hydrophobic gas permeable membrane to separate the sample solution from an electrode internal solution of ammonium chloride. Dissolved ammonia is converted into  $\text{NH}_3$  (aq) by raising pH to above 11 with a strong base.  $\text{NH}_3$  (aq) diffuses through the membrane and changes the internal solution pH that is sensed by a pH electrode. The fixed level of chloride in the internal solution is sensed by a chloride ion selective electrode that serves as the reference electrode. Potentiometric measurements are made with a pH meter having an expanded millivolt scale or with a specific ion meter.

**2. Interference-** Amines are a positive interference. Mercury & silver interfere by complexing with ammonia.

### 3. Apparatus

**3.1 Electrometer-** A pH meter with expanded millivolt scale capable of 0.1 mV resolution between – 700 mV and + 700 mV or a specific ion meter.

**3.2 Ammonia-** selective electrode

**3.3 Magnetic stirrer**

### 4 Reagents

**4.1 Ammonia-** free- water- Prepare ammonia free water by passing distilled water through an ion exchange column containing a strongly acidic cation exchange resin

mixed with a strongly basic anion exchange resin. Alternatively, redistill distilled water by adding 0.1 mL of concentrated sulphuric acid to 1 liter of water. It may also be made by treating distilled water with sufficient bromine or chlorine water to produce a free halogen residual of 2 to 5 mg/L. Redistill after standing at least for 1 hour. Discard the first 100 mL distillate.

**4.2** Sodium Hydroxide solution- 10 N

**4.3** Stock ammonium chloride solution- Dissolve 381.9 mg of anhydrous ammonium chloride in water and dilute to 1000 mL (1.00 mL = 122 µg as NH<sub>3</sub> or 100 µg as N)

**4.4** Standard ammonium chloride solution- Dilute 5.00 mL of stock solution to 1000 mL with water (1.00 mL = 0.500 µg of nitrogen or 0.607 µg of ammonia)

## **5 Procedure**

**5.1 Preparation of standards-** Prepare a series of standard solutions covering the concentrations of 1000, 100, 10, 1 and 0.1 mg of nitrogen (ammoniacal) by making decimal dilutions of stock ammoniacal chloride solution with water.

**5.2 Electrometer calibration-** Place 100 mL of each standard solution in a 150 mL beaker immerse electrode in standards of lowest concentration and mix with a magnetic stirrer. Do not stir so rapidly that air bubbles are sucked into the solution because they will get trapped on the electrode membrane. Maintain the same stirring rate and temperature of about 25°C throughout calibration and testing procedures. Add sufficient volume of 10 N sodium hydroxide solution to raise pH above 11. Keep electrode in solution until a stable millivolt reading is obtained. Do not add sodium hydroxide solution before immersing electrode because ammonia may be lost from a basic solution. Repeat procedures with remaining standards, proceeding from lowest to highest concentration. Wait for at least 5 minutes before recording millivolts for standards and sample containing ≤1 mg of nitrogen (ammoniacal) per litre.

**5.3 Preservation of standard curve-** Using semilogarithmic graph paper, plot ammonia concentration in milligrams of nitrogen (ammoniacal) per litre on the log axis vs potential in millivolts on the linear axis starting with the lowest concentration at the bottom of the scale. If the electrode is functioning properly a tenfold change of ammoniacal nitrogen concentration produces a potential change of 59 mV.

**5.4 Calibration of specific ion meter-** Following manufacturer's instructions, follow steps given in 5.1 and 5.2.

**5.5 Measurement of samples-** Dilute, if necessary to bring ammoniacal nitrogen to within calibration curve range. Place 100 mL sample in 150mL beaker and follow procedure given in 5.2. Record the volume of 10N of sodium hydroxide added in excess of 1 mL. Read ammoniacal nitrogen concentration from standard curve.

## 6. Calculation

$$\text{Ammoniacal nitrogen, mg/L} = A \times B \times \left[ \frac{101+C}{101} \right]$$

Where

A= dilution factor;

B = concentration of ammoniacal nitrogen per litre, mg/L from calibration curve; and

C = volume in mL of added 10 N sodium hydroxide in excess of 1 mL.

## E. Flow Injection Analysis

### 1. General Discussion

**1.1 Principle:** A water sample containing ammonia or ammonium cation is injected into an FIA carrier stream to which a complexing buffer, alkaline phenol, and hypochlorite are added. This reaction, the Berthelot reaction, produces the blue indophenol dye. The blue color is intensified by the addition of nitroferricyanide. The

resulting peak's absorbance is measured at 630 nm. The peak area is proportional to the concentration of ammonia in the original sample.

**1.2 Interferences:** Remove large or fibrous particulates by filtering sample through glass wool. Guard against contamination from reagents, water, glassware, and the sample preservation process. Some interferents are removed by distillation.

## 2 Apparatus

Flow injection analysis equipment consisting of:

**2.1** FIA injection valve with sample loop or equivalent.

**2.2** Multichannel proportioning pump.

**2.3** FIA manifold with tubing heater and flow cell. Tubing volumes may be scaled down proportionally to the relative flow. Use manifold tubing of an inert material such as TFE (Teflon or equivalent).

**2.4** Absorbance detector, 630 nm, 10-nm bandpass.

**2.5** Injection valve control and data acquisition system.

**3 Reagents:** Use reagent water (> 10 megohm) to prepare carrier and all solutions. To prevent bubble formation, degas carrier and buffer with helium. Use Heat 140 kPa (20 psi) through a helium degassing tube. Bubble He through 1 L solution for 1 min. As an alternative to preparing reagents by weight/ weight, use weight/ volume.

**3.1 Buffer:** To a 1L tared container add 50.0 gm disodium ethylenediamine tetraacetate and 5.5 gm sodium hydroxide, NaOH. Add 968 mL water. Mix with a magnetic stirrer until dissolved.

**3.2 Phenolate:** CAUTION: Wear gloves. Phenol causes severe burns and is rapidly absorbed into the body through the skin. To a tared 1-L container, add 888 gm water. Add 94.2 gm 88% liquefied phenol or 83 gm crystalline phenol, C<sub>6</sub>H<sub>5</sub>OH. While stirring, slowly add 32 gm NaOH. Cool and invert to mix thoroughly. Do not degas.

**3.3 Hypochlorite:** To a tared 500-mL container, add 250 gm 5.25% sodium

hypochlorite, NaOCl bleach solution and 250 gm water. Stir or shake to mix.

**3.4 Nitroprusside:** To a tared 1 L container add 3.50 gm sodium nitroprusside (sodium nitroferricyanide),  $\text{Na}_2\text{Fe}(\text{CN})_5\text{NO}\cdot 2\text{H}_2\text{O}$  and 1000gm water. Invert to mix.

**3.5 Stock ammonia standard, 1000 mg N/L :** In a 1 L volumetric flask dissolve 3.819 gm ammonium chloride,  $\text{NH}_4\text{Cl}$ , that has been dried for 2 h at  $110^\circ\text{C}$ , in about 800 mL water. Dilute to mark and invert to mix.

**3.6 Standard ammonia solutions:** Prepare ammonia standards in desired concentration range, using the stock standard 3.5 above), and diluting with water.

**4 Procedure:** Set up a manifold and follow method supplied by manufacturer or laboratory standard operating procedure for this method

**5 Calculations:** Prepare standard curves by plotting the absorbance of standards processed through the manifold versus ammonia concentration. The calibration curve is linear.

## 6 Reference:

**6.1.** IS: 3025 part 34 – 1988 (Reaffirmed 2003) - Methods of Sampling and Test (Physical and chemical) for water and Waste Water: Nitrogen

**6.2.** APHA 4500-NH<sub>3</sub>

## 2. ANIONIC SURFACE ACTIVE AGENTS

### Introduction:

Surfactants are a large group of surface active substances with a great number of (cleaning) applications. Most surfactants have degreasing or wash active abilities. They reduce the surface tension of the water so it can wet the fibres and surfaces, they loosen and encapsulate the dirt and in that way ensure that the soiling will not re-deposit on the surfaces. Surfactants have a hydrophobic (water repellent) part and a hydrophilic ('water loving') part. The hydrophobic part consists of an uncharged carbohydrate

group that can be straight, branched, cyclic or aromatic. Most surfactants are more or less toxic to aquatic organisms due to their surface activity which will react with the biological membranes of the organisms. The biological degradability varies according to the nature of the carbohydrate chain. Generally the linear chains are more readily degradable than branched chains. Also the toxic effects vary with the chain structure. Generally an increase of the chain length in the range of 10 to 16, leads to an increase in toxicity to aquatic organisms.

### **Methylene blue Method**

**1. Principle:** Methylene blue a cationic dye forms the salts with anionic surfactants in an alkaline medium. These salts are extracted with chloroform. Any interference present is eliminated by extraction of anionic surfactant-methylene blue complex from alkaline solutions and shaking with acidic methylene blue solution. The absorbance of the separated organic phase is measured at the maximum absorption wavelength of 650 nm. This method is applicable to limit of detection of about 0.05mg/L for solutions of standard surfactants in distilled water.

### **2. Apparatus**

**2.1** pH-Meter-With suitable electrodes made of glass.

**2.2** Spectrophotometer, capable of measurement at 650 nm, equipped with cells of optical path length of 10 mm & 50 mm

**2.3** Gas stripping Apparatus- One liter capacity.

**2.4** Separatory Funnels 500 mL capacity.

### **3. Reagents**

**3.1** Sodium Chloride

**3.2** Ethyl Acetate

**3.3** Chloroform

**3.4** Ethanol, 95%

**3.5** Methanol (Freshly Distilled)

**3.6** Sulphuric Acid Solution- 0.5 ml/L

**3.7** Ethanolic Sodium Hydroxide - 0.1 mol/L - Dissolve 4gm of sodium hydroxide pellets in ethanol and dilute to 1000 mL with the same ethanol.

**3.8 Methylene Blue, Neutral Solution** - Dissolve 0.350 gm of methylene blue in water and dilute to 1000 mL. Prepare the solution at least 24 hr before use.

**3.9 Methylene Blue, Acidic Solution--** Dissolve 0.350 gm of methylene blue in 500 mL of water and add 6.50 mL of sulphuric acid (Density -1.84 gm/mL). Dilute with water to 1000 mL after mixing. Prepare solution at least 24 hr before use. The absorbance of the chloroform phase of the blank test, measured against 0.02/10 mm of optical path length at 650 nm. In the case of higher blank absorbance, either wash the methylene blue solution twice with chloroform or use other batches of Methylene Blue.

**3.10 Buffer Solution, pH 10--** Dissolve 24 gm of sodium hydrogen carbonate ( $\text{NaHCO}_3$ ) and 27 gm of anhydrous sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) in water and dilute to 1000 mL.

**3.11 Phenolphthalein Indicator Solution--** Dissolve 1.0 gm of phenolphthalein in 50 mL of ethanol and add 50 mL of water, with stirring continuously. Filter off any precipitates.

**3.12 Dodecylbenzene Sulphonic Acid Methyl Ester (Tetra propylene Type), stock standard solution—**Weigh 400 mg to 450 mg of Dodecylbenzene Sulphonic acid methyl ester to the nearest 0.1 mg, into a round-bottom flask, and add 50 mL of ethanol sodium hydroxide solution and some anti-bumping granules. Attach the reflux condenser and boil for 1 hr. After cooling, rinse the condenser and the ground-glass joint with about 30 ml of ethanol and add the rinsing to the contents of the flask. Neutralize the solution with sulphuric acid against phenolphthalein until it becomes colorless. Transfer the solution to a 1000 mL volumetric flask, dilute to the mark with water and mix. This standard solution is stable for 6 months.

#### 4. Procedure

**4.1 Separation of the Surfactant** -- Non-surfactant methylene blue active substances can cause errors in the test of methylene blue index. Stripping is recommended for concentrating small amount of surfactants from water samples. Separate suspended matter by centrifugation, but note that adsorbed surfactants on

suspended matter will not be determined. Place a measured quantity of the test sample, up to 1000 mL in the gas-stripping apparatus. Install the stripping apparatus in well ventilated hood to carry off ethyl acetate vapour. Separation is improved by addition of sodium chloride. If sample volume exceeds 500 mL add 100 gm of sodium chloride dissolve by passing nitrogen gas or air through it. If a smaller test sample volume is used, dissolve 100 gm of sodium chloride in 400 mL of water and add this solution to test sample. If necessary, add water to bring the sample surface up to the level of the upper stopcock. Add 100 mL ethyl acetate. Fill the wash bottle in the gas line (nitrogen or air) two-third full with ethyl acetate. Pass a gas stream of 20 L/h to 50 L/h through the gas stripping apparatus. Adjust the gas flow in such a way that the phases remain separate and no turbulence is produced at the interface. The significant mixing of the phases and consequent solution of ethyl acetate in the water is avoided. Stop the gas flow after 5 min. If a loss of more than 20 percent ( $v/v$ ) of the organic phase has occurred due to solution in the water phase discard the test sample. Run off the organic phase completely into a separating funnel. Return any water in the separating funnel to the gas-stripping apparatus. Filter the ethyl acetate solution through a dry qualitative gas-filter paper into a 250 mL flask. Add a further 100 mL of ethyl acetate to the gas-stripping apparatus and again pass nitrogen or air through it for 5 min. separate the organic layer as described above, using the same separating funnel, filter, and add it to the first portion. Rinse the filter paper and funnel with 25 mL of ethyl acetate. Remove all the ethyl acetate solution on a water bath under a hood. To speed up the process direct a gentle air stream over the surface of the solution. Dissolve the residue in about 5 mL of methanol and 50 mL of water. Transfer the solution quantitatively to a 100 mL volumetric flask and dilute to the mark with water.

**4.2 Blank Test** -- Carry out a blank test at 650 nm and subtract the interpolated absorbance,  $A_0$  from the absorbance  $A_1$  of the test sample. Under the given conditions the absorbance  $A_0$  of the blank test shall not exceed 0.02 per 10 nm optical path length otherwise equipment and the reagents shall be checked carefully for any contamination.

### **4.3 Test with the sample**

4.3.1 Transfer a measured volume of the test sample into a separating funnel. This test portion should contain 20  $\mu\text{g}$  to 200 $\mu\text{g}$  of MBAS (methylene blue active

substances). In the lower MBAS range, a test portion up to 100 mL may be used. If the volume of the test portion is less than 100 mL, dilute with water to 100 mL.

4.3.2 Add 5.0 mL of neutral methylene blue solution, 10 mL of buffer solution and 15 mL chloroform.

4.3.3 Shake evenly and gently about twice a second for 1 min, preferably in a horizontal plane.

4.3.4 Allow the layers to separate as completely as possible and swirl the funnel to dislodge droplets from the sides of the funnel.

4.3.5 Allow to settle for 2 min, and then run as much as possible of the chloroform layer into a second separating funnel, containing 110 mL of water and 5.0 mL of acidic methylene blue solution.

4.3.6 Shake uniformly but not too vigorously for 1 min as previously described.

4.3.7 Filter the chloroform layer through a cotton or glass wool filter wetted with chloroform into a 50 mL volumetric flask.

4.3.8 Repeat the extraction of the alkaline and acidic solution using a 10 mL portion of chloroform for the extraction.

4.3.9 Separate the chloroform layer and filter it through the same filter, into the volumetric flask.

4.3.10 Repeat the extraction using a further 10 mL of portion of chloroform and filter that into a 50 mL of volumetric flask.

4.3.11 Dilute to the mark with chloroform and mix.

4.3.12 For each test sample carry out the complete extraction for a blank determination with 100 mL water.

4.3.13 Measure the absorbance for the test sample as well as for the blank test at 650 nm in cells of optical path length 10 mm to 50mm against chloroform. The absorbance of the test sample should not be more than that of the blank.

## 5 Reference:

5.1 IS: 13428 - 2005 (Reaffirmed- 2009) Packaged Natural Mineral water- Specifications. Annex: K (Method of test for Anionic Surface Active Agents)

### 3. BORON

#### Introduction:

Boron is an essential element for plant growth. However, in excess of 2mg/L in irrigation water, it is deleterious to certain plants and some plants may be affected adversely by concentrations as low as 1mg/L (or even less in commercial greenhouses). Drinking water rarely contain more than 1.0mg/L and generally less than 0.1mg/L concentrations considered innocuous for human consumption. Boron may occur naturally in some water or may find its way into a watercourse through cleaning compounds and industrial effluents. Seawater contains approximately 5mg/L Boron. The ingestion of large amounts of boron can affect the central nervous system. Protracted may result in a clinical syndrome known as "Borism".

Methods for determination of Boron in water are prescribed:-

- A. Azomethine Method
- B. Curcumin Method
- C. Carmine Method

#### A. Azomethine Method

##### 1. Principle

Reaction of azomethine-H, which is the condensation product of H-acid (8-amino-naphth-1-ol-3,6-disulfonic acid) and salicylaldehyde, with dissolved forms of borate at a pH of about 6, leads to the formation of a yellow complex that is measured spectrometrically at the absorption maximum in the range of 410 nm to 420 nm.

##### 2. Reagents

###### 2.1. Azomethine-H, Solution

Dissolve 1.0 gm of azomethine-H sodium salt [8-N-2-hydroxybenzylidene)-amino-naphth-1-ol-3,6 disulfonic acid] ( $C_{17}H_{12}NNaO_3S_2$ ) and 3.0 gm of  $\pm$  L ascorbic acid

(C<sub>6</sub>H<sub>8</sub>O<sub>6</sub>) in water and dilute to 100 mL in a volumetric flask. The solution is stable for up to one week when stored in a polyethylene bottle at a temperature of between 4-6°C.

## 2.2. Buffer Solution (pH 5.9)

Mix 250 gm of ammonium acetate (CH<sub>3</sub>COONH<sub>4</sub>) 250 mL of water, 80 mL of sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) (sp.gr-1.21 g/mL), 5 mL of phosphoric acid (H<sub>2</sub>PO<sub>4</sub>) (sp.gr -1.71 g/mL), 1.0gm of citric acid (C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>H<sub>2</sub>O) and add 1.0gm of disodiummethylenediamine-tetracetic acid dehydrate (C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>Na<sub>2</sub>O<sub>8</sub>H<sub>2</sub>O) with stirring and gentle heating.

## 2.3. Reagent Solution

Mix equal volumes of reagents prepared in 2.1 and 2.2. Prepare this solution on the day of use and store in a Polyethylene bottle.

## 2.4. Borate, stock solution corresponding to 1.0 of B per liter.

Dissolve 5.719 gm of boric acid (H<sub>3</sub>BO<sub>3</sub>) in 1000 mL of water. Store it in a polyethylene bottle. 1 mL of this stock solution contains 1.0 mg of borate, expressed as B.

## 2.5. Boron, standard solution-I corresponding to 10.0 mg of B per liter.

Dilute 10 mL of borate stock solution (see 2.4) to 1000 mL with water. 1 mL of this standard solution contains 10.0 µg of borate, expressed as B.

## 2.6. Boron, standard solution-II corresponding to 1.0 mg of B per liter.

Dilute 10 mL of borate solution (see 2.5) to 100 mL with water. 1mL of this standard solution contains 1.0 µg of borate, expressed as B.

## 2.7. Calcium hydroxide [Ca (OH)<sub>2</sub>]

## 3. Apparatus

3.1. Ordinary laboratory apparatus made of polypropylene, polyethylene or polytetrafluoroethylene, where applicable.

3.2. Spectrometer, for use in the wavelength range of 410 nm to 420 nm, with cells of an optical path length between 10 mm and 50 mm.

#### 4. Procedure

##### 4.1. Determination

Transfer 25.0 mL of the sample, or a smaller amount of the sample diluted to 25mL with distilled water, into a 100 mL polyethylene flask. Add 10mL of Azomethine-H. Mix and allow to stand in the dark for 2 hr at  $20 \pm 1^\circ\text{C}$ , then measure the absorbance at the absorption maximum in the range of 410 nm to 420 nm against distilled water in a cell of optical path length 10mm, using the spectrometer set up according to the manufacturer's instructions and after setting the zero with distilled water in the cell. Alternatively use a cell of 50 mm optical path length for low boron concentrations of up to about 0.2 mg of boron per litre. Check the wavelength of the absorption maximum whenever a new batch of this reagent is used.

##### 4.2. Blank Test

4.2.1. Carry out a blank test by treating 25 mL of water as described in 4.1. Ensure that the blank value is in the range of 0.1 absorption units to 0.17 absorption units per 10 mm. If the absorption is higher, then check the reagents and the distilled water for their borate content.

4.2.2. Measure it into three separate borate-free beakers (preferably polytetrafluorethylene), 25 mL, 100 mL and 250mL aliquots of the distilled water. Make slightly alkaline by the addition of the same small (for example 200 mg) amount of calcium hydroxide to each. Evaporate the 100 mL and 250 mL aliquots to a volume of just less than 25 mL and adjust their volumes to precisely 25 mL by the addition of a little extra distilled water, as necessary. Carry out the procedure given in 4.1 on these aliquots.

4.2.3. Carry out a blank determination with each of the aliquots. If borate is present in the distilled water, the borate found increases in proportion to the volume of the aliquot

taken. Erratic results indicate external borate contamination. Relatively high but constant results indicate impure reagents.

**4.3. Prevention of Contamination:** As borate is widespread in the environment, significant contamination may occur during trace determinations. The following sources of contamination, and remedies, should be considered.

4.3.1. Borosilicate glassware should be avoided to the extent possible as it may lead to positive contamination. Borosilicate glass, well rinsed in hydrochloric acid, may be used for acidic solutions, but should never be used for neutral or alkaline solutions, or for prolonged storage at any pH value. (Borosilicate glassware previously used with alkaline solutions shall not be used without very thorough acid rinsing.) Polyethylene flasks and plastic pipettes are preferable.

4.3.2. Detergents and soaps used for glassware and lab coats should be borate free, and the use of towels and tissues, for drying shall be avoided.

4.3.3. Toiletries, talcum powder and cosmetics used by technicians often contain borate and should be avoided or removed, especially prior to undertaking accurate low-level determinations.

4.3.4. Water and reagents may contain borate and blanks should be carried out at least in duplicate and should agree.

#### **4.4. Calibration**

##### **4.4.1. Zero mg/L to 0.20 mg/L of Boron Calibration Graph**

To a series of six 25 mL one mark plastic flasks add respectively 0 mL, 1 mL, 2 mL, 3 mL, 4 mL and 5 mL of boron standard solution-II (*see* 2.6), dilute to the mark with distilled water and mix. This gives concentrations of 0 mg; 0.04 mg; 0.08 mg; 0.12 mg; 0.16 mg and 0.20 mg of boron per liter respectively. Analyze each standard solution as described in 4.1, measuring the absorbance values in a 50 mm optical path length cell compared against distilled water. Prepare a calibration graph by plotting the absorbance values against the known concentrations in milligrams of boron per liter for each standard.

#### 4.4.2. Zero mg/L to 1.00 mg/L of Boron Calibration Graph

Repeat the above calibration, using 0 mL, 5 mL, 10mL, 15 mL, 20 mL and 25 mL of boron standard solution-II (see 2.6) respectively to give concentrations of 0mg; 0.2mg; 0.4mg; 0.6mg; 0.8mg and 1.0mg of boron per liter respectively. Analyze each standard Solution as described in 4.1, but this time measuring the absorbance values using a 10mm optical path length cell compared against distilled water. Prepare a separate Calibration graph.

#### 4.4.3. Calculation of Factor $f$

It is essential that a linear calibration graph be achieved in both cases; if not then check the solutions and repeat the calibration. Calculate the reciprocal value for the slope & factor  $f$ , for each graph.

**5. Calculations:** Calculate the borate content, in milligrams of boron per liter, from the formula

$$= \frac{(A_1 - A_0) f V_{1 \max}}{V_1}$$

Where

$A_1$  = absorbance of the sample

$A_0$  = absorbance of the blank

$V_1$  = volume, in milliliters, of the sample

$V_{1 \max}$  = maximum volume, in milliliters, of the sample

$f$  = calibration factor, determined from the appropriate calibration curve (reciprocal value of the slope, in milligrams of boron per liter).

## B. Curcumin Method

**1. Principle:** When a sample of water containing boron is acidified and evaporated in the presence of Curcumin, a red-colored product called rosocyanine is formed. The rosocyanine is taken a suitable solvent and the red color is compared with standards visually or photometrically.

**2. Interference:**  $\text{NO}_3\text{-N}$  concentrations above 20 mg/L interfere. Significantly high results are possible when the total of calcium and magnesium hardness exceeds 100 mg/L as calcium carbonate ( $\text{CaCO}_3$ ). Moderate hardness levels also can cause a

considerable percentage error in the low boron range. This interference springs from the insolubility of the hardness salts in 95% ethanol and consequent turbidity in the final solution. Filter the solution or pass the original sample through a column of strongly acidic cation-exchange resin in the hydrogen form to remove interfering cations. The latter procedure permits application of the method to samples of high hardness or solids content. Phosphate does not interfere.

**3. Minimum Detectable Quantity:** 0.2 µg/L

**4. Apparatus**

4.1 Colorimetric equipment: One of the following is required:

4.2 Spectrophotometer, for use at 540 nm, with a minimum light path of 1 cm.

4.3 Filter photometer, equipped with a green filter having a maximum transmittance near 540 nm, with a minimum light path of 1 cm.

4.4 Evaporating dishes, 100 to 150mL capacity, of high-silica glass, platinum, or other suitable material.

4.5 Water bath, set at 55± 2°C.

4.6 Glass-stoppered volumetric flasks, 25 and 50 mL capacity.

4.7 Ion-exchange *column*, 50 cm long by 1.3 cm in diameter.

**5. Reagents:** Store all reagents in polyethylene or boron-free containers.

**5.1 Stock boron solution:** Dissolve 571.6 mg anhydrous boric acid, H<sub>3</sub>B<sub>0</sub><sub>3</sub>, in distilled water and dilute to 1000 mL; 1.00 mL = 100µg Boron. Because H<sub>3</sub>B<sub>0</sub><sub>3</sub> loses weight on drying at 105°C, use a reagent meeting ACS specifications and keep the bottle tightly stoppered to prevent entrance of atmospheric moisture.

**5.2 Standard boron solution:** Dilute 10.00 ml stock boron solution to 1000 mL with distilled water; 1.00 mL = 1.00 µg Boron.

**5.3 Curcumin reagent:** Dissolve 40 mg finely ground Curcumin and: 5.0 gm oxalic acid

in 80 mL 95% ethyl alcohol. Add 4.2 mL conc. HCl, make up to 100 mL with ethyl alcohol in a 100 mL volumetric flask, and filter if reagent is turbid (isopropyl alcohol, 95% may be used in place of ethyl alcohol). This reagent is stable for several days if stored in a refrigerator.

#### 5.4 Ethyl or isopropyl alcohol 95%

#### 5.5 Reagent for removal of high hardness and cation interference:

##### 5.5.1 Strongly acidic cation- exchange resin

##### 5.5.2 Hydrochloric acid, HCl 1+5

## 6 Procedure

**6.1 Precautions:** Closely control such variables as volumes and concentrations of reagents, as well as time and temperature of drying. Use evaporating dishes identical in shape, size, and composition to insure equal evaporation time because increasing the time increases intensity of the resulting color.

**6.2 Preparation of calibration curve:** Pipet 0 (blank), 0.25, 0.50, 0.75, and 1.00  $\mu\text{g}$  boron into evaporating dishes of the same type, shape, and size. Add distilled water to each standard to bring total volume to 1.0 mL. Add 4.0 mL curcumin reagent to each and swirl gently to mix contents thoroughly. Float dishes on a water bath set at  $55 \pm 2^\circ\text{C}$  and let them remain for 80 min, which is usually sufficient for complete drying and removal of HCl. Keep drying time constant for standards and samples. After dishes cool to room temperature, add 10 mL 95% ethyl alcohol to each dish and stir gently with a polyethylene rod to insure complete dissolution of the red-colored product.

Wash contents of dish into a 25mL volumetric flask, using 95% ethyl alcohol. Make up to mark with 95% ethyl alcohol and mix thoroughly by inverting. Read absorbance of standards and samples at a wavelength of 540 nm after setting reagent blank at zero absorbance. The calibration curve is linear from 0 to 1.00  $\mu\text{g}$  boron. Make photometric readings within 1 h of drying samples.

**6.3 Sample treatment:** For waters containing 0.10 to 1.00 mg B/L, use 1.00 mL sample. For waters containing more than 1.00 mg B/L, make an appropriate dilution with boron-free distilled water, so that a 1.00 mL portion contains approximately 0.50 µg boron.

Pipet 1.00 mL sample or dilution into an evaporating dish. Unless the calibration curve is being determined at the same time, prepare a blank and a standard containing 0.50 µg boron and run in conjunction with the sample. Proceed as in 4.2 above, beginning with Add 4.0 mL Curcumin reagent. If the final solution is turbid, filter through filter paper before reading absorbance. Calculate boron content from calibration curve.

**6.4 Visual comparison:** The photometric method may be adapted to visual estimation of low boron concentrations, from 50 to 200 µg/L, as follows: Dilute the standard boron solution 1 + 3 with distilled water; 1 mL = 0.20 µg Boron. Pipette 0, 0.05, 0.10, 0.15, and 0.20 µg B into evaporating dish indicated in 6.3 above. At the same time add an appropriate volume of sample (1.00 mL or portion diluted to 1.00 mL) to an identical evaporating dish. The total boron should be between 0.05 and 0.20 µg. Proceed as in 4.2 above, beginning with "Add 4.0 mL curcumin reagent. ... "Compare color of samples with standards within 1 hr of drying samples.

**6.5 Removal of high hardness and cation interference:** Prepare an ion-exchange column of approximately 20 cm X 1.3 cm diameter. Charge column with a strongly acidic cation-exchange resin. Backwash column with distilled water to remove entrained air bubbles. Keep the resin covered with liquid at all times. Pass 50 mL 1 + 5 HCl through column at a rate of 0.2 mL acid/mL resin in column/min and wash column free of acid with distilled water.

Pipet 25 mL sample, or a smaller sample of known high boron content diluted to 25 mL, onto the resin column. Adjust rate flow to about 2 drops/s and collect effluent in a 50 mL volumetric flask. Wash column with small portions of distilled water until flask is filled to mark. Mix and transfer 2.00 mL in evaporating dish. Add 4.0 mL Curcumin reagent and complete the analysis as described above.

## 7 Calculation:

Use the following equation to calculate boron concentration from absorbance readings:

$$\text{Mg B/L} = \frac{A_2 \times C}{A_1 \times S}$$

Where:

A1 = absorbance of standard,

A2 = absorbance of sample,

C =  $\mu\text{g B}$  in standard taken, and

S = mL sample.

### C. Carmine Method

**1. Principle:** In the presence of boron, a solution of carmine or carminic acid in concentrated sulfuric acid changes from a bright red or bluish red, depending on the concentration of boron present.

**2. Interference:** The ions commonly found in water and waste water do not interfere.

**3. Minimum detectable quantity:** 2  $\mu\text{g/L}$ .

**4. Apparatus:**

**4.1** Colorimetric equipment: One of the following is required:

**4.2** Spectrophotometer, for use at 585 nm, with a minimum light path of 1 cm.

**4.3** Filter photometer, equipped with an orange filter having a maximum transmittance near 585 nm, with a minimum light path of 1 cm.

**5 Reagents:** Store all reagents in polyethylene or boron-free containers.

**5.1** Standard boron solution: Prepare as directed in 4500-B B.3.2

5.2 Hydrochloric acid, HCl, conc. and 1 + 11

5.3 Sulfuric acid, H<sub>2</sub>SO<sub>4</sub>, conc.

5.4 Carmine reagent: Dissolve 920 mg carmine N.F. 40, or carmine acid, in 1 L conc. H<sub>2</sub>SO<sub>4</sub>, (If unable to zero spectrophotometer, dilute carmine 1+1 with conc. H<sub>2</sub>SO<sub>4</sub> to replace above reagent)

## 6 Procedure

**6.1 Low-level sample concentration:** If sample contains less than 1mg Boron/L, pipet a portion containing 2 to 20 µgB into platinum dish, make alkaline with 1N NaOH plus a slight excess, and evaporate to dryness on a steam or hot water bath. If necessary destroy any organic material by ignition at 500 to 550°C. Acidify cooled residue (ignited or not) with 2.5 mL 1 + 11 HCl and triturate with a rubber policeman to dissolve. Centrifuge if necessary to obtain a clear solution. Pipet 2.00mL clear concentrate into a small flask or 30mL test tube. Treat reagent blank identically.

**6.2 Color development:** Prepare a series of boron standard solutions (100, 250, 500, 750, and 1000 µg) in 100 mL with distilled water. Pipet 2.00 mL of each standard solution into a small flask or 30 mL test tube. Treat blank and calibration standards exactly as the sample. Add 2 drops (0.1 mL) conc. HCl, carefully introduce 10.0 mL conc. H<sub>2</sub>SO<sub>4</sub>, mix, and let cool to room temperature. Add 10.0 mL carmine reagent, mix well, and after 45 to 60 min measure absorbance at 585 nm in a cell of 1cm or longer light path, using the blank as reference.

To avoid error, make sure that no bubbles are present in the optical cell while photometric readings are being made. Bubbles may appear as a result of incomplete mixing of reagents. Because carmine reagent deteriorates, check calibration curve daily.

## 7. Calculation

$$\text{mg Boron/L} = \frac{\mu\text{g B} \times D}{\text{mL sample}}$$

Where: D = dilution correction.

## 8. Reference:

8.1 IS: 13428 - 2005 (Reaffirmed- 2009) Packaged Natural Mineral water- Specifications. Annex: H (Determination of Borate).

8.2 APHA 4500-B

## 4. NITRATE

### Introduction:

Determination of nitrate ( $\text{NO}_3$ ) is difficult because of the relatively complex procedures required, the high probability that interfering constituents will be present and the limited concentration ranges of the various techniques. Nitrate is the most highly oxidized form of nitrogen compounds commonly present in natural waters. Significant sources of nitrate are chemical fertilizers, decayed vegetable and animal matter, domestic effluents sewage sludge disposal to land, industrial discharge, leachates from refuse dumps and atmospheric washout. Depending on the situation, these sources can contaminate streams, rivers, lakes and ground water. Unpolluted natural water contains minute amounts of nitrate. Excessive concentration in drinking water is considered hazardous for infants because of its nutrient taken up by plants and converted into cell protein. The growth stimulation of plants, especially of algae may cause objectionable eutrophication.

Methods for determination of Ammoniacal Nitrogen in water are prescribed:-

- A. Cadmium Reduction Method
- B. Chromotropic Acid Method
- C. Devarda's Alloy Reduction Method

## A. Cadmium Reduction Method

1. **Scope:** Prescribes cadmium reduction method for determination of Nitrate. This method is suitable for concentration below 0.1 mg per liter of nitrate nitrogen.

2. **Principle:** Nitrate is reduced to nitrite in presence of cadmium. The nitrite produced is determined by diazotizing with sulphanilamide and coupling with N-(1naphthyl) ethylenediamine to form a highly colored azo dye which is measured colorimetrically.

3. **Interference:** Higher concentrations of copper, iron etc lower the reduction efficiency. Add EDTA to remove this interference. Oil and grease & residual chlorine can interfere. Remove oil and grease by extraction with organic solvents and residual chlorine by adding sodium thiosulphate.

### 4. Apparatus

4.1. Reduction column – commercially available one or construct the column from a 100 mL volumetric pipette by removing the top portion. The column can also be constructed by two pieces of tubing joined end to end (join a 10 cm length of 3 cm internal diameter tubing to a 25 cm length of 3.5 cm Internal diameter tubing). A liquid leveling device is useful.

4.2. Colorimeter- One of the following

4.2.1. Spectrophotometer- for use near 543 nm with a light path of 1 cm or longer.

4.2.2. Filter photometer – provided with a yellow green filter having maximum transmittance near 540 nm and a light path of 1 cm or longer.

### 5. Reagents

5.1. **Nitrate free water-** the absorbance of a reagent blank prepared with this water should not exceed 0.01. Use for all solutions and dilution.

5.2. **Copper cadmium granules** – Wash 25 gm of 40-60 mesh cadmium granules with 6N hydrochloric acid and rinse with water. Swirl cadmium with 100 mL of 2 percent copper sulphate solution for 5 minutes or until blue color partially fades.

Decant, repeat with fresh copper sulphate until a brown colloidal precipitate develops. Wash copper cadmium copiously with water (at least 10 times) to remove all precipitated copper.

**5.3. Sulphanilamide reagent** – Dissolve 5 gm of sulphanilamide in a mixture of 50 mL concentrated hydrochloric acid and 300 mL of water. Dilute to 500 mL with water. The reagent is stable for months.

**5.4. N-(1-naphthyl)-ethylenediamine dihydrochloride (NED dihydrochloride) solution-** Dissolve 500 mg of NED dihydrochloride in 500mL of water. Store in dark colored bottle .Replace as soon as brown color develops.

**5.5. Ammonium chloride – EDTA solution-** Dissolve 13 gm ammonium chloride 1.7 gm of disodium ethylenediamine tetracetate in 900 mL of water. Adjust pH to 8.5 with liquid ammonia and dilute to 1 liter.

**5.6.** Dilute 300 mL of the above solution to 500 mL with water to get a dilute solution.

**5.7. Hydrochloric acid 6 N**

**5.8. Copper sulphate solution-** 2 percent (m/v).

**5.9. Stock nitrate solution** – Dissolve 0.7218 gm of dry potassium nitrate in water and dilute to 1000 mL. Preserve with 2 mL of chloroform per liter (1 mL = 100µg of nitrate nitrogen).

**5.10.** Dilute 50 mL of stock nitrate solution to 500 mL with water to get standard solution. 1.0 mL equal to 10.0 µg nitrate nitrogen.

**5.11. Stock nitrite solution-** Dissolve 0.6072 of dried potassium nitrite in nitrate free water and make up to 1000 mL. (1 mL = 100µg of nitrite nitrogen).Preserve with 2 mL of chloroform and keep in a refrigerator. The solution is stable for 3months.

**5.12.** Dilute 50.0 mL of above stock nitrite solution to 500 mL with nitrite free water (1 mL = 10µg of nitrite nitrogen).

## 6. Procedure

**6.1. Preparation of reduction column** – insert a glass wool plug into the bottom of the reduction column and fill with water. Add sufficient copper cadmium granules to produce a column 18.5 cm long. Maintain water level above Cu-Cd granules to prevent entrapment of air. Wash column with 200 mL dilute ammonium chloride EDTA solution. Activate column by passing, 100 mL of a solution comprising of 25 mL of 1.0 mg nitrogen (nitrate) per liter standard and 75 mL of ammonium chloride EDTA solution, through it, at 7 to 10 mL/ minute.

**6.2. Treatment of sample** – If turbidity or suspended solids are present, remove by filtering through a 0.45  $\mu\text{m}$  pore diameter membrane or glass fiber filter. Adjust pH to between 7 & 9 as necessary. To 25.0 mL sample or a portion diluted to 25.0 mL add 75 mL of ammonium chloride- EDTA solution and mix. Pour mixed sample into column and collect at the rate of 7 to 10 mL/minute. Discard first 25 mL. Collect the rest in original sample flask. There is no need to wash the column between samples but if columns are not to be reused for several hours or longer, pour 50 mL dilute ammonium chloride - EDTA solution on to the top and let it pass through the system. Store Cu-Cd column in this solution and never allow it to dry.

**6.3.** As soon as possible and not more than 15 min after reduction add 2.0 mL sulphanilamide reagent to 50 mL of sample. Let the reagent react for 2 to 8 min. add 2 mL of NED dihydrochloric acid solution and mix immediately. Measure absorbance between 10 min to 2 hr at 540 nm against a distilled water reagent blank. Using the standard nitrate nitrogen solution prepare standards in the range of 0.05 to 1.0 mg of nitrate nitrogen per liter by diluting the following volumes of standards to 100 mL in volumetric flasks: 0.5, 1.0, 2.0, 5.0 and 10.0 mL. Carry out reduction of standards exactly as described for samples. Compare at least one nitrite standard to a reduced nitrate standard at the same concentration to verify reduction column efficiency. Reactivate copper cadmium granules when reduction efficiency falls below 75 percent.

**7. Calculation:** Obtain a standard curve by plotting absorbance at standards against nitrate nitrogen concentration, compute sample concentration directly from standard curve report as milligrams of oxidized nitrogen per liter (sum of nitrate

nitrogen plus nitrite nitrogen) unless the concentration of nitrite nitrogen is separately determined and corrected for .

## **B. Chromotropic Acid Method**

**1. Principle-** Two moles of nitrate nitrogen react with one mole of chromotropic acid to form a yellow reaction product having maximum absorbance at 410 nm

**2. Interferences-** Residual chlorine, certain oxidants and nitrites yield yellow colour with chromotropic acid. Addition of sulphite removes interference from residual chlorine and oxidants. Urea converts nitrites to nitrogen gas. The minimum detectable quantity is 50 µg of nitrate nitrogen per litre.

### **3. Apparatus**

**3.1 Spectrophotometer-** for use of 410 nm and with a light path of 1 cm or longer.

**3.2 Photometer-** having maximum transmittance at 410 nm and having a light path of 1 cm or longer and equipped with violet filter.

## **4 Reagents**

**4.1 Nitrate free water-** The absorbance of a reagent blank prepared with this water should not exceed 0.01. Use for all solution and dilution.

**4.2 Stock nitrate solution –** Dissolve 0.7218 gm of dry potassium nitrate in water and dilute to 1000 mL preserve with 2 mL of chloroform per liter (1 mL = 100µg of nitrate nitrogen).

**4.3 Standard nitrate solution-** Dilute 50 mL of stock nitrate solution to 500 mL with water to get standard solution 100 mL equal to 10.0 µg nitrate nitrogen.

**4.4 Sulphite urea reagent-** Dissolve 5 gm of urea and 4 gm of anhydrous sodium sulphite in water and dilute to 1000 mL.

**4.5 Antimony reagent-** Dissolve 500 mg antimony metal by heating in 80 mL concentrated sulphuric acid. Cool and cautiously add to 20 mL of iced water. If crystals form upon standing overnight redissolve by heating

**4.6** Chromotropic acid reagent- Dissolve 100 mg of purified chromotropic acid crystals in 100 mL of concentrated sulphuric acid and store in a brown bottle. Prepare every 2 week. A colorless reagent solution signifies the absence of nitrate contamination from sulphuric acid.

**4.7** Sulphuric acid- concentrate nitrate free.

## 5 Procedure

Prepare nitrate standards in the range of 0.10 to 5.0 mg/L by diluting 0, 1.0, 5.0 10, 25, 25, 40 and 50 mL of standard nitrate solution to 100 mL with water. If appreciable amount of suspended matter is present, filter suitably. Pipette 2.0 mL portions of the standard nitrate solutions samples and a water blank into dry 10 mL volumetric flasks. To each flask, add 1 drop of sulphite urea reagent. Place flask in tray of cold water (10 to 20°C) and add 2 mL of antimony reagent. Swirl flasks during addition of each reagent. After about 4 minutes in the bath, add 1 mL of chromotropic acid reagent, swirl and let stand in cooling bath for 3 minutes. Add concentrated sulphuric acid to bring volume near the 10 mL mark. Stopper the flasks and mix by inverting each flask four times. Let it stand for 45 minutes at room temperature and adjust volume to 10 mL with concentrated sulphuric acid. Perform final mixing very carefully and gently to avoid introducing gas bubbles. Read absorbance at 410 nm between 15 minutes and 24 hours after last volume adjustment. Use nitrate free water in the reference cell of the spectrophotometer.

## 6 Calculation

$$\text{Nitrate nitrogen (as NO}_3\text{), mg/L} = \frac{\text{µg of nitrate nitrogen in 10 mL final volume}}{\text{Volume in ml of sample taken for test}}$$

### C. Devarda's Alloy Reduction Method

**1 Principle-** The nitrate and nitrite is reduced to ammonia under hot alkaline conditions in the presence of the reducing agent (Devarda's Alloy). The ammonia

formed distills and is trapped in a receiving flask containing boric acid. The ammonia can be determined either by direct nesslerization or acidimetrically. This method is recommended for nitrate nitrogen and nitrite nitrogen.

**2 Interference-** Ammonia is to be removed from sample by preliminary distillation. Nitrite also gets reduced to ammonia by this method. Therefore, a separate determination is made for nitrite and subtracts the result. This method is not recommended for levels of nitrate nitrogen below 2 mg/L.

### **3 Apparatus**

**3.1** Distillation assembly- Kjeldahl assembly is suitable

**3.2** Measuring scoop- to contain 1 gm of Devarda's alloy

**3.3** Spectrophotometer or photometer- suitable for use at 400-425 nm. The photometer should be equipped with a blue filter.

### **4 Reagents**

**4.1** Ammonia free water

**4.2** Borate buffer solution – add 88 mL of 0.1 N sodium hydroxide to 500 mL of 0.025 M sodium tetra borate (50 gm  $\text{Na}_2\text{B}_4\text{O}_7$  or 9.5 gm  $\text{Na}_2\text{B}_4\text{O}_7 \cdot \text{H}_2\text{O}$ ) and make up to 1 litre

**4.3** Sodium hydroxide- 6 N

**4.4** Devarda's alloy (An alloy of 50 percent Cu, 45 percent Al and 5 percent Zn)- 20 mesh or smaller containing less than 0.005 percent nitrogen

**4.5** Reagents for acidimetric titration

**4.5.1** Mixed indicator solution – Dissolve 200 mg of methyl red indicator in 100 mL 95%ethyl or isopropyl alcohol. Dissolve 100 mg of methylene blue in 50 mL of 95 % ethyl or isopropyl alcohol. Combine these two. Prepare monthly

**4.5.2** Indicating boric acid solution - dissolve 20 gm hydroboric acid in ammonia free water, add 10 mL of mixed indicator solution and dilute to 1 liter

**4.5.3** Standard sulphuric acid titrant – 0.02 N (1mL=280 µg of nitrogen)

## 4.6 Reagents for colorimetric estimation

4.6.1 Nessler's reagent- Dissolve 100 gm of mercuric iodide and 70 gm of potassium iodide in a small quantity of water and add this mixture slowly with stirring to a cool solution of 160 gm of sodium hydroxide dissolved in 500 mL of water. Dilute to 1 liter. Store in brown rubber stopper glass bottle. Reagent is stable up to one year. It is toxic and so avoid ingestion.

4.6.2 Stock ammonia solution- Dissolve 3.819 gm of anhydrous ammonium chloride in water and dilute to 1 liter (1.00 mL = 1.00 mg of nitrogen = 1.22 mg of ammonia).

4.6.3 Standard ammonia solution- Dilute 10.00 mL of stock solution to 1000 mL with water (1.00 mL = 12.2 µg of ammonia = 10.0 µg of N).

## 5 Procedure

If ammonia has not been determined by a method involving preliminary distillation dilute a portion of the sample to 500 mL with ammonia free water. Add 25 mL of borate buffer and adjust to pH 9.5 with 6 N sodium hydroxide using a pH meter or short range pH paper. Distil 250 to 300 mL into a dry receiving flask and discard. Make sure that the last part of the distillation is conducted with condenser tip out of the liquid in receiving flask. To the residue after removing ammonia, add 1 gm of Devarda's alloy and sufficient ammonia-free distilled water to bring total volume to 350 mL. Place in a receiving flask of 50 mL boric acid absorbent for each milligram of nitrate nitrogen in sample. Immerse the end of condenser in the absorbent. Heat distillation flask until boiling or vigorous bubbling occurs. Reduce heat and distil at the rate of 5 to 10 mL/min until at least 150 mL distillate have been collected. Lower receiver so that liquid is below the end of the condenser and continue distillation for 1 to 2 minutes to cleanse condenser. Determine ammoniacal nitrogen either by nesslerization or titration with standard strong acid as given in Nesslerization or Titrimetric Method.

## 6 Calculation

### 6.1 Nesslerization Method

$$\text{Ammoniacal Nitrogen (NH}_3\text{ - N), mg/L} = \frac{A \times B}{V \times C}$$

Where

A =  $\mu\text{g}$  of ammoniacal nitrogen (51 mL of final volume);

B = total volume of distillate collected, in mL, including acid absorbent;

C = volume distillate taken for nesslerization in mL, and

V = volume in mL of sample taken for test.

## 6.2 Titrimetric Method

$$\text{Ammoniacal nitrogen (NH}_3\text{ - N), mg/L} = \frac{(A - B) \times 280}{V}$$

Where

A = volume in mL of sulphuric acid titrated for sample

B = volume in mL of sulphuric acid titrated for blank, and

V = volume in mL of sample taken for test.

The above two represent the ammonia produced from reduction of nitrate and nitrite. To get nitrate nitrogen determine nitrite separately and subtract.

## 7. Reference:

7.1. IS 3025 (part 34) 1998: (Reaffirmed 2003) - Methods of Sampling and Test (Physical and chemical) for water and Waste Water: Nitrogen.

7.2. APHA 4500 NO<sub>3</sub>

## 5. CHLORIDE

### Introduction:

The presence of chloride in natural waters can be attributed to dissolution of salt deposits, discharges of effluents from chemical industries, oil well operations and seawater intrusion in coastal areas. Each of these sources may result in local contamination of both surface water and groundwater. The salty taste produced by chloride depends on the chemical compositions of the water. A concentration of 250 mg/L may be detected in some waters containing sodium ions. On the other hand, the

typical salty taste may be absent in water containing 1000mg/L chloride when calcium and magnesium ions are predominant. High chloride content may harm pipes and structures as well as agricultural plants.

Methods for determination of chloride in water are prescribed:-

- A. Argentometric method
- B. Potentiometric Method
- C. Automated Ferricyanide Method
- D. Mercuric Thiocyanate Flow Injection Analysis

#### A. Argentometric method

**1. Scope:** This method prescribes the determination of chloride. This method is suitable for use in relatively clear waters when 0.15 to 10mg of chloride is present in the portion titrated.

**2. Principle:** In a neutral or slightly alkaline solution, potassium chromate can indicate the end point of the silver nitrate titration of chloride. Silver chloride is precipitated before red silver chromate is formed.

**3. Interference:** Bromide, iodide and cyanide register equivalent chloride concentrations. Sulphite, thiosulphate and sulphide ions interfere but can be removed by treatment with hydrogen peroxide. Orthophosphates in excess of 25mg/L interfere. Iron in excess of 10mg/l interferes by masking the end point.

#### 4. Apparatus

4.1. Erlenmeyer flask — 250 mL.

4.2. Burette — 50 mL.

## 5. Reagents

**5.1 Potassium chromate indicator solution** — Dissolve 50 gm of potassium chromate in a little distilled water. Add silver nitrate solution until a definite red precipitate is formed. Let it stand for 12 hr, filter and dilute to 1 liter with distilled water.

**5.2 Standard silver nitrate titrant** —0.0141 N. Dissolve 2.395 gm of silver nitrate in distilled water and dilute to 1 liter. Standardize against 0.0141N sodium chloride solution. 1.00 mL = 500 µg of chloride. Store in a brown bottle.

**5.3 Standard sodium chloride solution** — 0.0141 N. Dissolve 824.0 mg of sodium chloride (dried at 140°C) in distilled water and dilute to 1 liter. 1 mL = 500 µg of chloride.

**5.4** Special reagents for removal of interferences:

**5.4.1 Aluminium hydroxide suspension** — Dissolve 1.25 gm of aluminum potassium sulphate or aluminium ammonium sulphate [ $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$  or  $\text{Al NH}_4(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ ] in 1 liter of distilled water. Warm to 60°C and add 55 mL of concentrated ammonium hydroxide slowly with stirring. Let it stand for 1 hr, transfer to a large bottle and wash precipitate by successive additions, with thorough mixing and decanting with distilled water, until free from chloride. When freshly prepared, the suspension occupies a volume of about 1 liter.

**5.4.2** Phenolphthalein indicator solution.

**5.4.3** Sodium hydroxide- 1 N.

**5.4.4** Sulphuric acid -1N.

**5.4.5** Hydrogen peroxide - 30 percent.

## 6 Procedure

**6.1** Use 100 mL sample or a suitable portion diluted to 100 mL. If the sample is highly colored, add 3 mL of aluminium hydroxide suspension, mix, let settle and filter.

**6.2** If sulphide, sulphite or thiosulphate is present, add 1 mL of hydrogen peroxide and stir for 1 minute. Directly titrate the samples in the pH range 7 to 10.

**6.3** Adjust sample pH to 7-10 with sulphuric acid or sodium hydroxide if it is not in the range.

**6.4** Add 1.0 mL of potassium chromate indicator solution.

**6.5** Titrate with standard silver nitrate solution to a pinkish yellow end point. Standardize silver nitrate solution and establish reagent blank value by titration method.

## 7 Calculation:

$$\text{Chloride, mg/L} = \frac{(v_1 - v_2) \times N \times 35450}{V_3}$$

Where

$V_1$  = Volume in mL of silver nitrate used by the sample

$V_2$  = Volume in mL of Silver nitrate used in the blank titration

$V_3$  = volume in mL of sample taken for titration

N = Normality of silver nitrate solution

## B. Potentiometric Method

**1. Principle:** Chloride is determined by potentiometric titration silver nitrate solution with a glass and silver-silver chloride electrode system. During titration an electronic voltmeter used to detect the change in potential between the two electrodes. The end point of the titration is that instrument reading at which the greatest change in

voltage has occurred for a small and constant increment of silver nitrate added.

**2. Interference:** Iodide and bromide also are titrated as chloride. Ferricyanide causes high results and must be removed. Chromate and dichromate interfere and should be reduced to the chromic state or removed. Ferric iron interferes if present in an amount substantially higher than the amount of chloride. Chromic ion, ferrous ion, and phosphate do not interfere. Grossly contaminated samples usually require pretreatment where contamination is minor, some contaminants can be destroyed simply by adding nitric acid.

### 3. Apparatus

**3.1** Glass and silver-silver chloride electrodes: Prepare in the laboratory or purchase a silver electrode coated with AgCl for use with specified instruments. Instructions on use and care of electrodes are supplied by the manufacturer.

**3.2** Electronic voltmeter, to measure potential difference between electrodes: A pH meter may be converted to this use by substituting the appropriate electrode.

**3.3** Mechanical stirrer, with plastic-coated or glass impeller.

### 4. Reagents

**4.1** Standard Sodium chloride solution, 0.0141M (0.0141N): Dissolve 824.0 mg NaCl (dried at 140°C) in distilled water and dilute to 100 mL: 1.00 mL = 500 µg Cl<sup>-</sup>

**4.2** Nitric acid, HNO<sub>3</sub>, conc.

**4.3** Standard silver nitrate titrant, 0.0141 (0.0141 N): Dissolve 2.395 gm AgNO<sub>3</sub> in distilled water and dilute to 1000 mL. Standardize against NaCl by the procedure; 1.00 mL = 500 µg Cl<sup>-</sup>

**4.4** Pretreatment reagents

4.4.1 Sulfuric acid H<sub>2</sub>SO<sub>4</sub>, 1+1

4.4.2 Hydrogen peroxide, H<sub>2</sub>O<sub>2</sub>, 30 %

4.4.3 Sodium hydroxide NaOH, 1N.

## 5 Procedure

**5.1 Standardization:** The various instruments that can be used in this determination differ in operating details; follow the manufacturer's instructions. Make necessary mechanical adjustments. Then, after allowing sufficient time for warm-up (10 min), balance internal electrical components to give an instrument setting of 0 mV or, if a pH meter is used, a pH reading of 7.0.

5.1.1 Place 10.0 mL standard NaCl solution in a 250mL beaker, dilutes to about 100 mL, and adds 2.0 mL conc HNO<sub>3</sub>. Immerse stirrer and electrodes.

5.1.2 Set instrument to desired range of mill volts or pH units Start stirrer

5.1.3 Add standard AgNO<sub>3</sub> titrant, recording scale reading after each addition. At the start, large increments of AgNO<sub>3</sub> may be added; then, as the end point is approached, add smaller and equal increments (0.1 or 0.2 mL) at longer intervals, so the exact end point can be determined. Determine volume of AgNO<sub>3</sub> used at the point at which there is the greatest change in instrument reading per unit addition of AgNO<sub>3</sub>.

5.1.4 Plot a differential titration curve if the exact endpoint cannot be determined by inspecting the data. Plot change in instrument reading for equal increments of AgNO<sub>3</sub> against volume of AgNO<sub>3</sub> added, using average of burette readings before and after each addition.

## 5.2 Sample analysis:

5.2.1 Pipet 100.0 mL sample or a portion containing not more than 10 mg Cl, into a 250mL beaker. In the absence of interfering substances, proceed with above.

5.2.2 In the presence of organic compounds, sulfite, or other interferences (such as large amounts of ferric iron, cyanide, or sulfide) acidify sample with H<sub>2</sub>SO<sub>4</sub>, using litmus paper. Boil for 5 min to remove volatile compounds. Add more H<sub>2</sub>SO<sub>4</sub>, if necessary, to keep solution acidic. Add 3 mL H<sub>2</sub>O<sub>2</sub> and boil for 15 min, adding chloride-free distilled water to keep the volume above 50 mL. Dilute to 100 mL, add NaOH solution dropwise until alkaline to litmus, then 10 drops in excess. Boil for 5 min, filter into a 250mL beaker, and wash precipitate and paper several times with hot distilled water.

5.2.3 Add conc.  $\text{HNO}_3$  drop wise until acidic to litmus paper, then 2.0 mL in excess. Cool and dilute to 100 mL if necessary. Immerse stirrer and electrodes and start stirrer. Make any necessary adjustments according to the manufacturer's instructions and set selector switch to appropriate setting for measuring the difference of potential between electrodes.

5.2.4 Complete determination by titrating according to 4.1above. If an end-point reading has been established from previous determination for similar samples and conditions, use this predetermined end point. For the most accurate work, make a blank titration by carrying chloride-free distilled water through the procedure.

## 6 Calculation

$$\text{mg Cl/L} = \frac{(A - B) \times N \times 35450}{\text{mL sample}}$$

where

A = mL  $\text{AgNO}_3$ ,

B = mL blank, and

N = normality of titrant

### C. Automated Ferricyanide Method

**1 Principle:** Thiocyanate ion is liberated from mercuric thiocyanate by the formation of soluble mercuric chloride. In the presence of ferric ion, free thiocyanate ion forms a highly colored ferric thiocyanate, of which the intensity is proportional to the chloride concentration.

**2 Interferences:** Remove particulate matter by filtration-or centrifugation before analysis. Guard against contamination from reagents, water, glassware, and sample preservation process. No chemical interferences are significant.

**3 Application:** The method is applicable to potable, surface, and saline waters, and domestic and industrial wastewaters. The concentration range is 1 to 200 mg Cl/L; it can be extended by dilution.

## 4 Apparatus

4.1 Automated analytical equipment: An example of the continuous-flow analytical instrument consists of the interchangeable components.

4.2 Filters, 480-nm.

## 5 Reagents

5.1 Stock mercuric thiocyanate solution: Dissolve 4.17 gm  $\text{Hg}(\text{SCN})_2$  in about 500 mL methanol, dilute to 1000 mL with methanol, mix, and filter through filter paper.

5.2 Stock ferric nitrate solution: Dissolve 202 gm  $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  in about 500mL distilled water, then carefully add 21 mL conc  $\text{HNO}_3$ . Dilute to 1000mL with distilled water and mix. Filter through paper and store in an amber bottle.

5.3 Color reagent: Add 150 mL stock  $\text{Hg}(\text{SCN})_2$  solution to 150 mL stock  $\text{Fe}(\text{NO}_3)_3$  solution. Mix and dilute to 1000 mL with distilled water. Add 0.5mL polyoxyethylene 23 lauryl ether.

5.4 Stock chloride solution: Dissolve 1.6482 gm NaCl, dried at  $140^\circ\text{C}$ , in distilled water and dilute to 1000 mL; 1.00 mL = 1.00 mg Cl.

5.5 Standard chloride solutions: Prepare chloride standards in the desired concentration range, such as 1 to 200 mg/L, using stock chloride solution.

6 **Procedure** Set up manifold and follow general procedure described by the manufacturer.

## 7 Calculation

Prepare standard curves by plotting response of standards processed through the manifold against chloride concentrations in standards. Compute sample chloride concentration by comparing sample response with standard curve.

### D. Mercuric Thiocyanate Flow Injection Analysis

1 **Principle:** A water sample containing chloride is injected into a carrier stream to

which mercuric thiocyanate and ferric nitrate are added. The chloride complexes with the Hg(II), displacing the thiocyanate anion, which forms the highly colored ferric thiocyanate complex anion. The resulting peak absorbance is measured at 480 nm. The peak area is proportional to the concentration of chloride in the original sample.

- 2 **Interferences:** Remove large or fibrous particulates by filtering sample through glass wool. Guard against contamination from reagents water, glassware, and the sample preservation process. Substances such as sulfite and thiosulfate, which reduce iron (III) to iron (II) and mercury (II) to mercury (I), can interfere. Halides, which also form strong complexes with mercuric ion (e.g., Br<sup>-</sup>, I<sup>-</sup>), give a positive interference.

### 3 Apparatus

3.1 Flow injection analysis equipment consisting of

3.1.1 FIA injection valve with sample loop.

3.1.2 Multichannel proportioning pump.

3.1.3 FIA manifold with flow cell.

3.1.4 Absorbance detector, 480 nm, 10nm band pass.

3.1.5 Valve control and data acquisition system.

### 4 Reagents

4.1 Use reagent water (> 10 mega ohm) to prepare carrier and all solutions.

4.2 Stock mercuric thiocyanate solution: In a 1L volumetric flask, dissolve 4.17gm mercuric thiocyanate, Hg(SCN)<sub>2</sub>, in about 500 mL methanol. Dilute to mark with methanol and mix.

**CAUTION: Mercuric thiocyanate is toxic. Wear gloves!**

4.3 Stock ferric nitrate reagent, 0.5M: In a 1L volumetric flask, dissolve 202 gm ferric nitrate, Fe(NO<sub>3</sub>)<sub>3</sub> · 9H<sub>2</sub>O, in approximately 800 mL water. Add 25 mL conc. HNO<sub>3</sub> and dilute to mark. Invert to mix.

4.4 Color reagent: In a 500mL volumetric flask, mix 75 mL stock mercuric thiocyanate solution with 75 mL stock ferric nitrate reagent and dilute to mark with water. Invert to mix. Vacuum filter through a 0.45 μm membrane filter. The color

reagent also is available as a commercially prepared solution that is stable for several months

**4.5** Stock chloride standard, 1000 mg Cl/L: In a 105°C oven, dry 3 gm primary standard grade sodium chloride, NaCl, overnight. In a 1L volumetric flask, dissolve 1.648 gm primary standard grade sodium chloride in about 500 mL water. Dilute to mark and invert to mix.

**4.6** Standard chloride solutions: Prepare chloride standards for the calibration curve in the desired concentration range, using the stock standard (as above), and diluting with water.

**5 Procedure:** Set up a manifold and follow method supplied by manufacturer, or laboratory standard operating procedure for this method.

**6 Calculations** Prepare standard curves by plotting absorbance of standards processed through the manifold versus chloride concentration. The calibration curve gives a good fit to a second-order polynomial.

## **7 Reference:**

**7.1** IS : 3025 (Part 32) – 1988 (Reaffirmed 2003)- Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : Chloride

**7.2** APHA 4500-Cl

## **6 MAGNESIUM**

### **Introduction:**

Magnesium occurs commonly in the minerals magnetite and dolomite. Magnesium is used in alloys, pyrotechnics, flash photography, drying agents, refractories, fertilizers, pharmaceuticals, and foods.

The common aqueous species is  $Mg^{2+}$ . The carbonate equilibrium reactions from magnesium are more complicated than for calcium and conditions for direct

precipitation of dolomite in natural waters are not common. Important contributors to the hardness of a water, magnesium salts break down when heated, forming scale in boilers. Chemical softening, reverse osmosis, ion exchange reduces magnesium and associated hardness to acceptable levels. Magnesium is an essential element in chlorophyll and in red blood cells. Some salts of magnesium are toxic by ingestion or inhalation. Concentrations greater than 125 mg/L also can have a cathartic and diuretic effect.

The methods for determination of Magnesium are prescribed as:

1. Calculation Method
2. Gravimetric Method as Magnesium Pyrophosphate

#### A. Calculation Method

**1. Principle:** Water sample containing Calcium as  $\text{CaCO}_3$  is estimated and Total Hardness is the sum of  $\text{CaCO}_3$  and  $\text{MgCO}_3$ . Magnesium hardness is determined by subtracting Calcium hardness from total hardness.

#### 2. Calculation

**2.1. Calculate the Total hardness as follows:** Analysis for Total Hardness is carried out at pH 10 using Erichrome Black T indicator.

$$\text{Total hardness (CaCO}_3\text{) mg/L} = [1000(V1 - V2)/V3] \times \text{CF}$$

Where

V1 = volume in mL of the EDTA standard solution used in the titration for the sample

V2 = volume in mL of the EDTA solution used in the titration for blank.

V3 = volume in mL of the sample taken for the test

CF =  $X1/X2$  correction factor for standardize ion of EDTA.

X1 = volume in mL of standard calcium solution taken for standardization

X2 = volume of mL of EDTA solution used in the titration

2.1.1. **Calculate the Calcium hardness as follows:** Analysis for Calcium is carried out at pH 12-14 using Patton & Reader Indicator.

$$\text{Calcium hardness (CaCO}_3\text{) mg/L} = [1000(V1 - V2)/V3] \times \text{CF}$$

Where

V1 = volume in mL of the EDTA standard solution used in the titration for the sample

V2 = volume in mL of the EDTA solution used in the titration for blank.

V3 = volume in mL of the sample taken for the test

CF = X1/X2 correction factor for standardize ion of EDTA.

X1 = volume in mL of standard calcium solution taken for standardization and

X2 = volume of mL of EDTA solution used in the titration

### **Calculate the Magnesium hardness as follows**

$$\text{Magnesium hardness} = \text{Total hardness} - \text{Calcium hardness (mg/L)}$$

$$\text{Magnesium (as Mg}^{+2}\text{)} = \text{Magnesium hardness} \times 0.2428 \text{ mg/L}$$

## **B. Gravimetric Method**

**1. Principle:** Diammonium hydrogen phosphate quantitatively precipitates magnesium in ammoniacal solution as magnesium ammonium phosphate. The precipitate is ignited and weighed as magnesium pyrophosphate. Below 1 mg/L atomic absorption Spectrophotometric method is desirable.

**2. Interferences:** The solution should be reasonably free from aluminum, calcium, iron, manganese, silica, strontium and suspended matter. It should not contain more than about 3.5 gm ammonium chloride.

### **3. Apparatus**

**3.1** Vacuum Pump or Other Source of vacuum

**3.2** Filter Flasks

3.3 Filter Crucibles – medium porosity; 30 MI

## 4 Reagents

4.1 Methyl Red Indicator Solution: Dissolve 100 mg of methyl red sodium salt in distilled water and dilute to 100 MI

4.2 Hydrochloric acid: 1:1, 1:9 and 1:99

4.3 Ammonium Oxalate Solution: Dissolve 10gm  $(\text{NH}_4)_2 \text{C}_2\text{O}_4 + \text{H}_2\text{O}$  in 250mL distilled water. Filter if necessary

4.4 Ammonium Hydroxide- Concentrated- 1:19

4.5 Nitric Acid- Concentrated

4.6 Diammonium hydrogen phosphate solution Dissolve 30gm of Diammonium pyrophosphate  $(\text{NH}_4)_2 \text{HPO}_4$  in distilled water and make up to 100 mL.

4.7 Urea

## 5 Procedure

5.1 **Pre treatment of Polluted Water and Wastewater Samples:** Mix the sample pretreated, if so required, and transfer a suitable volume (50 to 100 mL) to 250 mL conical flask or a beaker. Add 5 mL concentrated nitric acid and a few boiling chips or glass beads. Bring to a slow boil and evaporate on a hot plate to the lowest volume possible (about 10 to 20 mL) before precipitation or salting occurs. Add 5 mL concentrated nitric acid cover with a watch glass and heat to obtain a gentle refluxing action. Continue heating and adding concentrated nitric acid as necessary until digestion is complete as shown by a light coloured clear solution. Do not let sample dry during digestion. Add 1 to 2 mL concentrated nitric acid and warm slightly to dissolve any remaining residue. Wash down beaker walls and watch glass with water and then filter if necessary. Transfer filtrate to 100 mL volumetric flask with two 5 mL portions of water adding these risings to the volumetric flask. Cool dilute to mark and mix thoroughly. Take portions of this solution for the determination.

5.2 **Removal of calcium and other Metals as Oxalates:** To 200 mL of the sample pretreated if so required containing about 50 mg of calcium, add a few drops of methyl red indicator and 1:1 hydrochloric acid. Sufficient acid must be present in the solution

to prevent the precipitation of calcium oxalate when ammonium oxalate solution is added. Introduce 50 mL of ammonium oxalate solution and 15 gm of urea. Boil the solution gently until the methyl red changes its colour to yellow. Filter the precipitate and wash with small volume of cold water until free from chloride.

**5.3 Determination of Magnesium:** To the combined filtrate and washings from 5.2 containing not more than 60 mg magnesium add 50 mL of concentrated nitric acid and evaporate carefully to dryness on a hot plate. Do not let reaction become too violent during the later part of the evaporation stay in constant attendance to avoid losses through spattering. Moisten residues with 2 to 3 mL of concentrated hydrochloric acid, add, 20 mL of distilled water, warm, filter and wash. To the filtrate add 3 mL of concentrated hydrochloric acid 2 to 3 drops of methyl red solution, and 10 mL of  $(\text{NH}_4)_2\text{HPO}_4$  solution. Cool and add concentrated ammonium hydroxide drop by drop stirring constantly until color changes to yellow, stir for 5 minutes and add again 5 mL of concentrated ammonium hydroxide and stir vigorously for 10 min more. Let it stand overnight and filter through filter paper. Wash with 1:19 ammonium hydroxide. Transfer to an ignited, cooled and weighed crucible. Dry precipitate thoroughly and burn paper off slowly, allowing circulation of air. Heat at about  $500^\circ\text{C}$  until residue is white. Ignite for 30 minutes at  $1100^\circ\text{C}$  to constant mass.

#### 5.4 Calculation

$$\text{Magnesium, mg/L} = \frac{M \times 218.4 \times 103}{V}$$

Where

M= mass in mg of magnesium pyrophosphate, and

V= volume in mL of sample.

#### 6. Reference:

**6.1** IS : 3025 (Part 46) – 1994 Methods of Sampling and Test (Physical and chemical) for water and Waste Water

## 7 FLUORIDE

### Introduction:

Fluoride ions have dual significant in water supplies. High concentration of  $F^-$  causes dental fluorosis (disfigurement of the teeth). At the same time, a concentration less than 0.8 mg/L results in 'dental caries'. Hence it is essential to maintain the  $F^-$  concentration between 0.8 to 1.0 mg/L in drinking water. Among the many methods suggested for the determination fluoride ion in water, the Colorimetric method (SPANDS) and the ion selective electrode method are the most satisfactory and applicable to variety of samples. Because all of the Colorimetric methods are subject to errors due to presence of interfering ions, it may be necessary to distill the sample before making the fluoride estimation.

**1. Scope:** This standard method prescribes three methods of test for determination of fluoride content in water.

- A. Zirconium alizarin method without distillation
- B. Zirconium alizarin method with distillation
- C. Electrochemical probe method

The method without distillation and electrochemical probe method is reliable for samples of potable and lightly polluted water in which the interfering substances are not in excess of the limits given below:

**Table 1:** Maximum limits for interfering substances:

Chlorides (as Cl)	2000 mg/L
Sulphates (as $SO_4$ )	300 mg/L
Alkalinity (as $CaCO_3$ )	400 mg/L
Iron (as Fe)	2 mg/L
Aluminium (as Al)	0.5 mg/L
Phosphates (as $PO_4$ )	5 mg/L

Where the sample is highly coloured or turbid or has interfering substances in excess of the limits given above, the method with distillation shall be used or the sample shall be appropriately diluted before this test. With samples of unknown composition or where greater accuracy is needed, the method with distillation shall be employed.

## A. ZIRCONIUM ALIZARIN METHODS

**1.1 Principle:** The color (red to yellow with increasing concentration of fluoride) obtained with zirconium alizarin reagent is matched against that produced with a series of standard fluoride solutions.

**1.2 Range and Applicability:** This method is suitable for estimation of fluoride content up to 1.0 mg/L of fluoride as F. The minimum detection limit of this method is 0.05 mg/L fluoride as F.

**1.3 Interference:** Iron, alkalinity, phosphates interfere, if present above the values given in Table - 1. Interference of free residual chlorine can be removed by adding sodium arsenite. Aluminium gives negative error because of formation of Al-F complex which withdraws fluoride from the reaction of zirconium.

### 1.4 Apparatus

**1.4.1** Nessler Tubes, 100 mL capacity.

**1.4.2** Distillation Apparatus — The distillation apparatus shall consist of a Claisen flask of 100 mL capacity, a large flask for generating steam and an efficient condenser. The main neck of the Claisen flask shall be fitted with a two-holed rubber stopper through which shall pass a thermometer and a glass tube (for connecting with the steam supply), both the thermometer and the tube extending almost to the bottom of the flask. The side neck of the flask shall be closed with a rubber stopper and the side arm connected with the condenser. Steam shall be generated from water made alkaline with sodium hydroxide. Local overheating of the Claisen flask shall be avoided by use of an asbestos board with a hold which shall fit closely to the lower surface of the flask.

## 1.5 Reagents

**1.5.1 Sodium Thiosulphate Solution** — approximately 0.1 N.

**1.5.2 Standard Sodium Fluoride Solution** — Dissolve 0.221 gm of dry sodium fluoride in distilled water and make up to 1000 mL. Dilute 1000 mL of the solution to 1000 mL distilled water. One millilitre of this diluted solution contains 0.01 mg of fluoride (as F). The solution shall be kept in polyethylene or wax-lined glass bottles.

### 1.5.3 Zirconium Alizarin Reagent

1.5.3.1 Dissolve 0.3 gm of zirconium oxychloride ( $\text{ZrOCl}_2 \cdot 8\text{H}_2\text{O}$ ), or 0.25 gm of zirconium oxynitrate [ $\text{ZrO}(\text{NO}_3)_2 \cdot 2\text{H}_2\text{O}$ ] in 50 mL of distilled water. Dissolve 0.07 gm of alizarin sodium monosulphonate (alizarin S) in another 50 mL quantity of distilled water and add the latter solution slowly to the zirconium solution with continuous stirring. The resulting solution clears on standing for a few minutes.

1.5.3.2 Dilute 112 mL of concentrated hydrochloric acid to 500 mL with distilled water. Also add 37 mL of concentrated sulphuric acid to 400 mL of distilled water and then dilute to 500 mL. Mix the two diluted acids when cool.

1.5.3.3 Dilute the clear zirconium solution prepared in 1.5.3.1 to 1000 mL with the mixed acid solution prepared in 1.5.3.2. The reagent is at first red, but within an hour it changes to orange-yellow and is ready for use. The solution shall be stored in the dark, if kept in a refrigerator it is stable for 2 to 3 months. When 5 mL of this reagent is added to 100 mL of distilled water containing no fluorides, it soon turns pink. Fluorides discharge the pink colour of the lake so that the solution acquires a more yellow tint.

**1.5.4 Silver Sulphate**

**1.5.5 Perchloric Acid** — 60 percent.

**1.5.6 Phenolphthalein Indicator**

**1.5.7 Sodium Hydroxide Solution** — 10 percent w/v.

**1.5.8 Concentrated Sulphuric Acid**

## 1.6 Procedure

### 1.6.1 Method without Distillation

1.6.1.1 The sample shall not contain free chlorine; if necessary, it shall be dechlorinated with a slight excess of sodium thiosulphate solution before use.

1.6.1.2 Take 100 mL of the clear sample and a series of dilutions of standard sodium fluoride solution in 100 mL of distilled water in Nessler tubes and add 5.0 mL of the zirconium alizarin reagent to each. The sample and standards shall be at the same temperature to within 1°C to 2°C. Mix and compare the colours after standing for 1 hr exactly. Note the volume of standard sodium fluoride solution contained in the tube, with which a match with the sample under test is obtained.

### 1.6.2 Method with Distillation

1.6.2.1 Introduce into the Claisen flask a number of fragments of Pyrex glass or glass beads, 0.2 gm of silver sulphate, 7 mL of distilled water and 15 mL of Perchloric acid. Heat the flask until the temperature reaches 120°C to 125°C, connect to the steam supply and regulate the gas and steam so that the distillation proceeds at a temperature of 137°C to 140°C. Distil 150 mL in 25 to 35 min and steam out the condenser towards the end of the distillation. Discard the first distillate. Distil a further 150mL and determine the fluorides in it by the method given above. The figure for this blank shall not exceed 0.0015 mg and shall be approximately constant for any further 150 mL fraction.

1.6.2.2 Make 150 mL of the sample alkaline to phenolphthalein indicator with sodium hydroxide solution, add a few drops in excess and concentrate to 20 mL. When cool, transfer quantitatively to the distillation flask and carefully add 15 mL of concentrated sulphuric acid. If the amount of chloride in the aliquot exceeds 5 mg, add about 5 mg of silver sulphate for each milligram of chlorine. Connect up the apparatus and distil 150 mL as above. Determine the fluoride content of the total 150 mL of distillate as 1.6.1.2.

### 1.6.3 Calculation

#### 1.6.3.1 Method without Distillation

$$\text{Fluoride (as F), mg/L} = \frac{1000W}{V}$$

Where

W - Weight of fluorides (as F) in the standard solution matched by the sample in mg;

V - Volume of the sample taken in mL

#### 1.6.3.2 Method with Distillation

$$\text{Fluoride (as F), mg/L} = \frac{1000W}{V}$$

Where

W = weight of fluorides (as F) in the standard solution matched by 150 mL of the Distillate, in mg

V= volume of the sample taken in mL

## B. ELECTROCHEMICAL PROBE METHOD

**2.1 Range and Applicability:** The electrochemical technique method is directly suitable for measuring fluoride concentrations from 0.2 mg/L to 2.0 g/L. After the addition of a known amount of fluoride, concentrations as low as 0.02 mg/L can be detected.

**2.2 Interferences:** The electrode will respond directly to hydroxide ions. The formation of HF under acidic conditions will reduce the measured fluoride concentration. Therefore, buffer all test aliquots to a pH between 5 and 7 to prevent such interference. Cations such as calcium, magnesium, iron and aluminium form

complexes with fluoride or precipitates to which the electrode does not respond. Therefore the buffer solution also contains trans- 1, 2-diaminocyclohexane-N,N,N',N' - tetraacetic acid (CDTA) as a decomplexing agent to free bound fluoride. The boron tetrafluoride anion, is not decomplexed by the addition of buffer.

**2.3 Principle:** When a fluoride ion-selective electrode comes into contact with an aqueous solution containing fluoride ions, a potential difference develops between the measuring electrode and the reference electrode. The value of this potential difference is proportional to the logarithm of the value of the fluoride ion activity in accordance with the Nernst equation.

## 2.4 Apparatus

2.4.1 Millivolt Meter — a Millivolt meter with an impedance of not less than  $10^{12}\Omega$ , capable of resolving potential differences of 0.1 mV or better.

2.4.2 Fluoride Ion-selective Electrode — the e.m.f. response, using standard solutions, shall not be less than 55 mV per decade change in fluoride concentration at 25°C.

2.4.3 Reference Electrode -- Either a calomel electrode, filled with saturated potassium chloride (KCl) solution, or a silver/silver chloride electrode shall be used.

2.4.4 Measuring Cells — capacity 100 mL, made of polypropylene and fitted with a thermo stated jacket.

2.4.5 Water Bath — capable of supplying water to the jacket of the measuring cell at a temperature of  $25^{\circ}\text{C}\pm 0.2^{\circ}\text{C}$ .

2.4.6 Magnetic Stirrer, with a polytetrafluoroethylene (PTFE) -coated stirring bar.

2.4.7 Polyethylene Beaker, of capacity 100 mL.

**2.5 Reagents:** Purity of the Reagents —Unless specified otherwise, only pure chemicals and fluoride free distilled water shall be used in tests.

2.5.1 **Sodium Hydroxide (5M)** — Dissolve cautiously  $100 \pm 0.5$  gm of sodium hydroxide in water, cool and dilute to 500 mL.

2.5.2 **Total Ionic Strength Adjustment Buffer (TISAB)** — Add 58 gm of sodium chloride (NaCl) and 57 mL of glacial acetic acid [ $p(\text{CH}_3\text{COOH}) = 1.05 \text{ g/mL}$ ] to 500 mL of water in a 1 liter beaker. Stir until dissolved. Add 150 mL of the sodium hydroxide solution and 4 gm of CDTA (trans-1, 2- diaminocyclohexane-N,N,N,N'tetraacetic acid). Continue stirring until all the solids have dissolved and adjust the solution to pH 5.2 with sodium hydroxide solution using a pH meter. Transfer to a 1000 mL one mark volumetric flask, make up to the mark with water and mix. The solution is stable for about 6 months, but do not use if precipitation occurs in solution.

2.5.3 **Fluoride, Stock Solution, 1000 mg/L** - Dry a portion of sodium fluoride (NaF) at 150°C for 4 hr and cool in a desiccator. Dissolve  $2.210 \pm 0.001$  gm of the dried material in water contained in a 1000mL one-mark volumetric flask. Make up to the mark with water and mix. Store the solution in a screw-capped polyethylene container.

2.5.4 **Fluoride, working standard solution-I, 10 mg/L** - Pipette 10 mL of the fluoride stock solution into a 1000 mL one-mark volumetric flask. Make up to the mark with water and mix. Standard solutions should be stored in plastic bottles and are usable for one month.

2.5.5 **Fluoride, working standard solution-II, 5 mg/L** - Pipette 5 mL of the fluoride stock solution into a 1000 mL one-mark volumetric flask and make up to the mark with water.

2.5.6 **Fluoride, working standard solution-III, 1 mg/L** - Pipette 100 mL of the working standard solution I into a 1000 mL one-mark volumetric flask and make up to the mark with water.

2.5.7 **Fluoride, working standard solution-IV, 0.5 mg/L** - Pipette 100mL of the working standard solution-II into a 1000 mL one-mark volumetric flask and make up to the mark with water.

2.5.8 **Fluoride, working standard solution-V, 0.2 mg/L** - Pipette 20 mL of the working standard solution-I into a 1000 mL one-mark volumetric flask and make up to the mark with water.

## 2.6 Procedure

**2.6.1 Preparation for Measurement:** Since the electrode characteristics of a fluoride ion selective electrode generally vary with time, check the calibration curve on the day of use. To accelerate the establishment of the equilibrium potential, condition the electrode prior to measurement in the following way. Prior to measurement, immerse the electrode for 1 h in the cell which contains the reference solution-V (Refer Table-2). After rinsing with the first solution to be measured, the electrode is ready for use.

**Table-2: Preparation of Reference solution:**

S. No.	Reference Solution No. (mL)	Buffer Solution	Working Standard Solution		Fluoride Concentration mg/L
			No.	mL	
1.	1	25	I	25	10
2.	2	25	II	25	5
3.	3	25	III	25	1
4.	4	25	IV	25	0.5
5.	5	25	V	25	0.2

**2.6.2 Measurement:** Pipette 25 mL of the buffer solution, followed by 25mL of the water sample, into a measuring cell. Ensure that the pH is  $5.2 \pm 0.2$ ; if necessary, adjust the pH with hydrochloric acid or sodium hydroxide solution, using as little as possible.

For a series of determinations, start the measurement with the lowest concentration and finish with the highest following the anticipated concentration of the samples. After measuring the high concentrations, recondition the electrode before

measuring the low concentrations. Measure all the solutions according to the following procedure. Wait until constant temperature (for example  $25 \pm 0.5^\circ\text{C}$ ) is reached and carry out all the measurements at this temperature. Put a stirring bar into the measuring cell and place it on the magnetic stirrer. Insert the electrodes into the solution and fix them in place. Adjust the stirring rate to about 180 min/L to 200 min/L. When the potential does not change by more than 0.5 mV in 5 min, switch off the stirrer. After at least 15 sec, record the value obtained. Rinse the stirring bar and the electrodes with the next solution to be measured, before starting the next measurement.

**2.6.3 Measurement after Concentration Enhancement:** If a water sample contains less than 0.2 mg/L F, proceed as follows: Add 500mL of the fluoride standard solution-I to 25 mL of the sample using a piston pipette, and 25 mL of the buffer solution with a volumetric pipette; continue as described in 2.6.2. When calculating the result, subtract the amount of fluoride ions added from the total result.

**2.6.4 Calibration:** Establish a calibration function using the five reference solutions in the corresponding concentration range. For the range 0.2 mg/L to 10 mg/L, proceed as follows:

Pipette 25 mL of the buffer solution into each of five measuring cells. Pipette the respective volumes of the working standard fluoride solutions specified in Table 2 into the measuring flasks. For the establishment of the calibration function proceed step by step from the most dilute solution to the most concentrated solution, rinsing after each measurement with the solution of the next highest concentration. After the above measurements have been completed, recondition the electrode for 5 to 10 min, using the reference solution-V (see Table-2) in order to eliminate memory effects.

**2.6.5 Calculation and Expression of Result:** Plot the calibration values on semi logarithmic paper, with the fluoride concentrations, in milligrams per liter, on the abscissa and the cell potential, in millivolts, on the ordinate and establish the regression line. Read the value for the samples by using the regression line and express the mass concentration of fluoride in milligrams per liter.

**2.7 Reference :**

2.7.1 IS : 3025 (Part 60) – 2008 Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : Fluoride

2.7.2 APHA 4500 F<sup>-</sup>

**8. TOTAL HARDNESS****Introduction:**

Water hardness is a traditional measure of the capacity of water to precipitate soap. Hardness of water is not a specific constituent but is a variable and complex mixture of cations and anions. It is caused by dissolved polyvalent metallic ions. In fresh water, the principal hardness causing ions are calcium and magnesium which precipitate soap. Other polyvalent cations also may precipitate soap, but often are in complex form, frequently with organic constituents, and their role in water hardness may be minimal and difficult to define. Total hardness is defined as the sum of the calcium and magnesium concentration both expressed as CaCO<sub>3</sub> in mg/L. The degree of hardness of drinking water has been classified in terms of the equivalent CaCO<sub>3</sub> concentration as follows:

Soft	0-60 mg/L
Medium	60-120 mg/L
Hard	120-180 mg/L
Very Hard	>180 mg/L

Although hardness is caused by cation, it may also be discussed in terms of carbonate (temporary) and non-carbonate (permanent) hardness. Carbonate hardness refers to the amount of carbonates and bicarbonates in solution that can be removed or precipitated by boiling. This type of hardness is responsible for the deposition of scale in hot water pipes and kettles. When total hardness is numerically greater than that of total alkalinity expressed as CaCO<sub>3</sub> the amount of hardness is numerically equal to less than total alkalinity is called carbonate hardness. When the hardness is numerically equal to less than total alkalinity, all hardness is carbonate hardness. The amount of

hardness in excess of total alkalinity expressed as  $\text{CaCO}_3$  is non-carbonate hardness. Non-carbonate hardness is caused by the association of the hardness-causing cation with sulphate, chloride or nitrate and is referred to the “permanent hardness”. This type of hardness cannot be removed by boiling.

Public acceptability of the degree may vary considerably from community depending on local conditions, and the association. The taste threshold for magnesium is less than that for calcium.

Methods for determination of total hardness in water are prescribed:-

- A. Titrimetric Method
- B. Method based on Analytical Data

### A. Titrimetric Method

**1. Principle: (EDTA method for determination of total hardness)** depends on ability of ethylenediamine tetra acetic acid ( $\text{C}_{10}\text{H}_{16}\text{O}_8\text{N}_2$ ) or its disodium salt to form stable complexes with calcium and magnesium ions. When the dye eriochrome black T (EBT) ( $\text{C}_{20}\text{H}_{13}\text{N}_3\text{O}_7\text{S}$ ) is added to solution containing calcium and magnesium ions at pH 10.0, a Wine red complex is formed, this solution is titrated with standard solution of disodium salt of EDTA, which extracts calcium and magnesium from the dye complex and the dye is changed back to its original blue colour. Eriochrome black T is used to indicate the end point for the titration of calcium and magnesium together.

### 2. Interferences

**2.1.** The EDTA forms stable complexes with iron, manganese, copper, lead, cobalt, zinc and nickel. Heavy metal interferences can be eliminated by complexing the metals with cyanide. In the presence of cyanide the procedure may be used even when iron, copper, zinc or lead concentrations are as high as 10mg/L.

**2.2.** The higher oxidation states of manganese above  $\text{Mn}^{++}$  react rapidly with the indicator to form discolored oxidation products hydroxylamine hydrochloride reagent

may be used to reduce manganese to divalent state. The divalent manganese interference can be removed by adding of one or two crystal of potassium ferrocyanide.

**2.3.** In presence of high aluminum concentrations, the blue color near end point starts disappearing and reverts to red.

**2.4.** Phosphate and carbonate ion may precipitate calcium at the pH of titration.

**3. Reagents:** Purity of the reagents. Unless specified otherwise only pure chemicals and tannin free distilled water shall be used in tests.

**3.1. Buffer solution:** Dissolve 16.9 gm ammonium chloride in 143 mL concentrated ammonium hydroxide, add 1.25gm of magnesium salt of EDTA and dilute to 250 mL with distilled water. Store the solution in a polyethylene bottle tightly stopper to prevent loss of ammonia or pick up of carbon dioxide for no longer than 1 month. Dilute 10 mL of the solution to 100mL with distilled water and check that the pH value is  $10.0 \pm 0.1$ .

In the absence of magnesium salt of EDTA dissolve 1.179 gm disodium salt of EDTA and 780 mg magnesium sulphate or 644 mg magnesium chloride in 50mL of distilled water. Add this solution to 16.9 gm ammonium chloride and 143 mL concentrated ammonium hydroxide with mixing and dilute to 250mL with distilled water. To attain the highest accuracy adjust to exact equivalence through appropriate addition of a small amount of EDTA or magnesium sulphate or chloride the exact amount can be determined by taking an appropriate aliquot of buffer and titrate it with disodium salt of EDTA as above. Keep the solutions tightly Stoppard to prevent loss of ammonia or absorbance of carbon dioxide and do not store for more than a month. Dilute 10 mL of the solution to 100 mL with distilled water and check that the pH value is  $10.0 \pm 0.1$ .

**3.2. Standard calcium solution:** 1.0mL = 1.00mg calcium carbonate. Dry analytical grade calcium carbonate in a oven at  $180^{\circ}\text{C}$  for 1 hr. weigh 1.0gm, suspend it in distilled water and add 1:1 hydrochloric acid AR quality drop, wise slowly to dissolve the solid.

Use minimum amount of acid. Boil for a few minutes, cool add a few drop of methyl red indicator and adjust to orange color with 3N ammonium hydroxide or 1:1 hydrochloric acid. Dilute to 1000mL with distilled water.

**3.3. Eriochrome black T indicator solution:** Dissolve 0.40 gm Eriochrome black T and 4.5 gm hydroxylamine hydrochloride in 100mL 95% ethanol. This indicator is stable for more than 2 months. Alternatively dissolve 0.5 gm Eriochrome black T in 100mL triethanolamine or 2 methoxyethanol or mixed 0.5 gm EBT dye and 100gm sodium chloride in pestle and mortar. Store in tightly Stoppard bottle. All indicator formulation tends to deteriorate especially when exposed to moisture. If the end point color change is not sharp enough it is either due to the presence of some interfering ions or due to deterioration of the indicator. In the latter case, addition of inhibitor sodium cyanide or sodium sulphide does not sharpen the end point color change.

**3.4. Standard EDTA solution:** Dissolve 3.723gm EDTA which has been dried overnight in sulphuric acid desiccators, in demineralized water and dilute to 1000mL. The reagent is stable for several weeks and large volume is usually prepared. Check the reagent by titrating 25 mL of standard calcium solution as described above. Store in polyethylene bottles.

#### **4. Procedure**

**4.1. Standardization:** Pipette 25 mL of standard calcium solution in a porcelain basin and adjust the volume to 50 mL with distilled water. Add 1 mL buffer solution, add 1 to 2 drops of indicator, titrate slowly with continuous stirring until the reddish tinge disappears. adding last few drops at 3 to 5 second interval. At the end point the color is sky blue.

**4.2.** Pipette an aliquot of water sample maximum 50 mL in a porcelain dish or 150 mL beaker and adjust the volume to approximately 50 mL. Add 1 mL hydroxylamine hydrochloride solution.

**4.3.** Add 1 to 2 mL buffer solution so as to achieve pH of 10.0 to 10.1.

- 4.4. Add 2 mL Eriochrome black T indicator solution.
- 4.5. Titrate with standard EDTA solution stirring rapidly in the beginning and slowly towards the end till end point is reached when all the traces of red and purple color disappear and solution is clear sky blue in color. Blank titration carried out in a same way as that for sample may be used for comparison.

Note- Selection of sample volume may be made such that the result lies between 200 to 300 mg/L of hardness (as CaCO<sub>3</sub>)

## 5. Calculation

Calculate the hardness as follows

$$\text{Total hardness as (CaCO}_3\text{), mg/L} = [1000(V_1 - V_2)/V_3] \times CF$$

Where

V<sub>1</sub> = volume in mL of the EDTA standard solution used in the titration for the sample

V<sub>2</sub> = volume in mL of the EDTA solution used in the titration for blank.

V<sub>3</sub> = volume in mL of the sample taken for the test

CF = X<sub>1</sub>/X<sub>2</sub> correction factor for standardization of EDTA.

X<sub>1</sub> = volume in mL of standard calcium solution taken for standardization

X<sub>2</sub> = volume of mL of EDTA solution used in the titration

## 6. Report

Report hardness in mg/L as CaCO<sub>3</sub> rounded to the first decimal place when the value is less than 10 mg/L and to the nearest unit if the value is more than 10mg/L.

## B. Method based on Analytical Data

**1. Principle-** Total hardness computed from the concentration of the different metallic cation (other than alkali metals) in the sample but most often the cations taken into account are calcium, magnesium iron, aluminum zinc, strontium, barium and manganese.

## 2. Calculation

Total hardness (as CaCO<sub>3</sub>), mg/L= (2.497 x mg/L Ca) + (4.116 x mg/L Mg) + (2.69 x mg/L Fe) + (5.567 x mg/L Al) + (1.531 x mg/L Zn) + (1.822 x mg/L Mn) + (0.894 x mg/L Ba) + (1.319 x mg/L Sr).

## 3. Reference:

**3.1** IS: 3025 (Part 21) – 1983 (Reaffirmed 2002) - Methods of Sampling and Test (Physical and chemical) for water and Waste Water: Total Hardness

## 9. ALKALINITY

### Introduction:

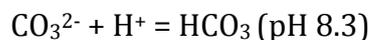
Alkalinity of sample can be estimated by titrating with standard sulphuric acid (0.02N) at room temperature using phenolphthalein and methyl orange indicator. Titration to decolourisation of phenolphthalein indicator will indicate complete neutralization of OH<sup>-</sup> and ½ of CO<sub>3</sub><sup>2-</sup> while sharp change from yellow to orange of methyl orange indicator total alkalinity (complete neutralization of OH<sup>-</sup>, CO<sub>3</sub><sup>2-</sup>, HCO<sub>3</sub><sup>-</sup>)

**1. Scope:** Prescribes the indicator methods for determination of alkalinity. This method is applicable to determine alkalinity in water in the range of 0.5 to 500mg/L alkalinity as CaCO<sub>3</sub>. The upper range may be extended by dilution of the original sample.

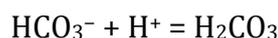
**2. Principle:** Alkalinity of water is the capacity of the water to accept protons. It may be defined as the quantitative capacity of an aqueous medium to react with

hydrogen ions to pH 8.3 (phenolphthalein alkalinity) and then to pH 3.7 (total alkalinity or methyl orange alkalinity).

The equation in its simplest form is as follows:



From pH 8.3 to 3.7 the following reaction may occur:



**3. Interferences:** Free available residual chlorine markedly affects the indicator color response. The addition of minimal volume of sodium thiosulphate eliminates this interference. Substances such as salt of weak organic or inorganic acids present in large amount may interfere.

**4. Sample Preparation:** The sample aliquot used for analysis should be either free from turbidity or should be allowed to settle prior to analysis.

## 5. Apparatus

5.1. pH meter

5.2. Burette- 50 mL capacity

5.3. Magnetic stirrer assembly

## 6. Reagents

**6.1. Distilled Water** – Distilled water used should have pH not less than 6.0. If the water has pH less than 6.0, it shall be freshly boiled for 15 minutes and cooled to room temperature. Deionized water may be used provided that it has a conductance of less than 2  $\mu\text{s}/\text{cm}$  and a pH more than 6.0.

**6.2. Sulphuric Acid** - Dilute 5.6 mL of concentrated sulphuric acid (relative density 1.84) to 1 liter with distilled water.

**6.3. Standard solution of sulphuric acid - 0.02N**

**6.4. Phenolphthalein indicator** - Dissolve 0.5 gm of phenolphthalein in 100mL, 1:1 (v/v) alcohol water mixture.

**6.5. Mixed indicator solution:** Dissolve 0.02gm methyl red and 0.01gm bromocresol green in 100mL, 95 percent, ethyl or isopropyl alcohol.

## 7. Procedure

**7.1.** Pipette 20 mL or a suitable aliquot of sample into 100 mL beaker.

**7.2.** If the pH of the sample is over 8.3 then add 2 to 3 phenolphthalein indicator and titrate with standard sulphuric acid solution till the pink color observed by indicator just disappears (equivalence of pH 8.3).

**7.3.** Record the volume of standard sulphuric acid solution used. Add 2 to 3 drops of mixed indicator to the solution in which the phenolphthalein alkalinity has been determined.

**7.4.** Titrate with the standard acid to light pink color (equivalence of pH 3.7). Record the volume of standard acid used after phenolphthalein alkalinity.

**8. Calculation :** Calculate alkalinity in the sample as follows

$$\text{Phenolphthalein alkalinity (as mg/L of CaCO}_3) = \frac{A \times N \times 50000}{V}$$

$$\text{Total alkalinity (as mg/L CaCO}_3) = \frac{(A + B) \times N \times 50000}{V}$$

Where

A= mL of standard sulphuric acid used to titrate to pH 8.3

B=mL of standard sulphuric acid used to titrate from pH 8.3 to pH 3.7

N= normality of acid used

V= Volume in mL of sample taken for test.

## 9. Reference:

9.1. IS : 3025 (Part 23) – 1986 (Reaffirmed 2003)- Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : Alkalinity

## 10. SULPHATES

### Introduction:

Sulphate ions usually occur in natural waters. Many sulphate compounds are readily soluble in water. Most of them originate from the oxidation of sulphate ores, the solution of gypsum and anhydrite, the presence of shales, particularly those rich in organic compounds, and the existence of industrial wastes. Atmospheric sulphur dioxide formed by the combustion of fossil fuels and emitted by the metallurgical roasting processes may also contribute to the sulphate compounds of water. Sulphur trioxide ( $\text{SO}_3$ ) produced by the photolytic oxidation of sulphur dioxide comes with water vapours to form sulphuric acid which is precipitated as acid rain or snow. Sulphur-bearing minerals are common in most sedimentary rocks. In the weathering process gypsum (calcium sulphate) is dissolved and sulphide minerals are partly oxidized, giving rise to a soluble form of sulphate that is carried away by water. In humid regions, sulphate is readily leached from the zone of weathering by infiltration waters and surface run off but in semiarid and arid regions the soluble salts may accumulate within a few tens of feet of land surface. Where this occurs, sulphate concentration in shallow ground water may exceed 5000mg/L and gradually decrease with depth.

Ingestion of water containing high concentration of sulphate can have a laxative effect, which is enhanced when sulphate is consumed in combination with magnesium. Water containing magnesium sulphate at levels about 1000 mg/L acts as a purgative in human adults. Taste threshold concentrations for the most prevalent sulphate salts are 200-500mg/L for sodium sulphate, 250-900 mg/L for calcium sulphate, and 400-600mg/L for magnesium sulphate. Essentially on the basis of above values which are also allied to the cathartic effect of sulphate, a guidelines value of 400 mg/L is proposed. Sulphates

cause scaling in water supplies, and problem of odour and corrosion in wastewater treatment due to its reduction to  $H_2S$ .

The methods for determination of Sulphates are prescribed:-

- A. Turbidity Method
- B. Gravimetric Method
- C. Thorin Method

### A. Turbidity Method

1. **Scope:** Turbidity method is applicable to surface and ground water in the range of 1 to 40 mg/L  $SO_4^{2-}$ . Samples having higher concentrations than this can be measured by appropriate dilution of sample.

2. **Principle** - Sulphate ion is precipitated in hydrochloric acid medium with barium chloride in such a manner as to form barium sulphate crystals of uniform size. The absorbance of barium sulphate suspension is measured by a nephelometer or transmission photometer (turbidity meter) and the sulphate ion concentration is determined by comparison of the reading with a standard curve.

### 3. Interferences

- 3.1. Color or suspended matter in large amounts will interfere.
- 3.2. In waters containing large quantities of organic material, it may not be possible to precipitate barium sulphate satisfactorily.

### 4. Apparatus & Reagents

- 4.1. Turbidity meter or spectrophotometer- for use at 420 nm
- 4.2. Usual laboratory glass apparatus
- 4.3. Barium chloride
- 4.4. Gelatin powder

4.5. Conditioning reagent (1) - Add 0.3 gm gelatin in 100mL distilled water and warm it on hot plate till it is dissolved. The gelatin solution is kept for about 12 hours, or overnight preferably, at 4°C after bringing the solution to room temperature, 3.0 gm barium chloride is added to gelatin solution and dissolved by mixing. The turbid solution is kept standing for 2 hours and mixed before use.

4.6. Conditioning reagent (2) - Mix 50 mL glycerol with a solution containing 30mL concentration hydrochloric acid, 300mL distilled water, 100 mL 95% ethyl or isopropyl alcohol and 75 gm sodium chloride.

4.7. Stock sulphate solution - Dissolve 0.1479 gm of anhydrous sodium sulphate ( $\text{Na}_2\text{SO}_4$ ) in distilled water and dilute to one liter.

4.8. Standard sulphate solution - prepare a series of standards by diluting stock solution of sulphate to cover the desired range in between 1 to 40 mg/L.

4.9. Hydrochloric acid (1+9) - dissolve one volume of concentrated hydrochloric acid with 9 volumes of distilled water.

**5. Sample preparation:** Filter the sample through 0.45  $\mu\text{m}$ , filter, if there is any turbidity.

## **6. Procedure**

6.1. Take 20 mL of clear aliquot of the water sample of suitable amount diluted to 20 mL in 100mL conical flask.

6.2. Add 1.0 mL hydrochloric acid solution and 1.0 mL conditioning reagent and mix well for 30 sec.

6.3. Read the absorbance on spectrophotometer after 10 min if glycerol conditioning reagent is used or 30 min, if gelatin is used, at 420 nm or read the turbidity occurred on turbidity meter following the manufacturer instruction to operate. If water sample is turbid take 20 mL sample or suitable amount dilute to 20 mL with distilled water. Do not add conditioning reagent. Read the absorbance of this sample and subtract this value from the above measured absorbance.

6.4. Calibration curve prepare a series of standards taking at least 4 standards and run a blank and follow the steps 6.2 and 6.3 and prepare a calibration curve of standards mg/L vs. absorbance

7. **Calculation:** Read the sulphate concentration of sample directly from the calibration curve.

## B. Gravimetric Method

1. **Scope-** This method is applicable for all the waters having sulphate concentrations above 10 mg/L; however, it is a time consuming method.

2. **Principle-** Sulphate is precipitated in hydrochloric acid medium as barium sulphate the addition of barium chloride solution. The precipitation is carried out near boiling temperature and after a period of digestion, the precipitate is filtered, washed with water until free of chlorides, ignited or dried and weighed as barium sulphate ( $\text{BaSO}_4$ ).

The reaction in its simplest form is:



3. **Interferences** - Suspended matter, silica, barium chloride precipitant, nitrate and sulphate are the principal factors in positive error. Alkali metal sulphates and heavy metals, such as chromium and iron cause low results. To minimize solubility of barium sulphite, the acid concentration while precipitating barium sulphate, should be minimized.

4. **Sampling and Storage-** Sampling and storage shall be done as prescribed in IS: 3025 (Part 1) 1986. Methods of sampling and test (physical and chemical) for water and wastewater: Part 1 Sampling (first revision). Highly polluted or contaminated samples should be stored at low temperature or treated with formaldehyde. Sulphite may be oxidized to sulphate by dissolved oxygen above pH 8.0; samples containing sulphite should have their

pH adjusted below this value.

## 5. Apparatus

5.1 Steam bath

5.2 Drying oven - equipped with thermostatic control.

5.3 Muffle furnace - with heat indicator.

5.4 Desiccator

5.5 Analytical balance - capable of weighing to 0.1 mg.

5.6 Filter paper - acid washed. Ashless hard finish filter paper sufficiently retentive for fine precipitates (preferably Whatman No. 42).

5.7 Crucible - Porous bottom silica or porcelain crucible with a maximum pore size of 5 microns.

5.8 Ion-exchange column -. The exchange column should be regenerated by passing hydrochloric acid (6.2) solution after five or six samples have passed through the column followed by washing with distilled water.

## 6. Reagents

6.1 Methyl red indicator - Dissolve 100 mg methyl red sodium salt in distilled water and dilute to 100 mL.

6.2 Hydrochloric acid (1: 4) - Dilute one volume of concentrated hydrochloric acid with four volumes of distilled water.

6.3 Barium chloride solution - Dissolve 100 gm of barium chloride ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in 1 litre distilled water. Filter through a membrane filter or hard finish filter paper (1 mL of this reagent is capable of precipitating approximately 40 mg  $\text{SO}_4$ ).

6.4 Silver nitrate-nitric acid reagent- Dissolve 8.5 gm of silver nitrate and 0.5 mL of nitric acid in 500 mL distilled water.

6.5 Ion exchange resin - Strong cation exchange resin, Amberlite IR-120 or

equivalent

## 7. Sample Preparation

7.1 The sample used for analysis should either be free from turbidity or filtered through 0.45  $\mu\text{m}$  filter.

7.2 If, the total cation concentration in the sample is more than 250mg/L or if the total heavy metal ion concentration is more than 10 mg/L, pass the sample through a cation removing ion exchange column.

7.3 If the silica concentration exceeds 25 mg/L, evaporate the sample nearly to dryness in a platinum dish on a steam bath. Add 2 mL hydrochloric acid (6.2) tilt the dish and rotate it until the acid comes in contact with the residue; continue the evaporation to dryness. Complete the drying in an oven at 180°C and if organic matter is present, char over the flame of a burner. Moisten the residue with 2 mL distilled water and 2 mL hydrochloric acid (6.2) and evaporate to dryness on steam bath. Add 5 mL hydrochloric acid (6.2), take up the soluble residue in hot water and filter. Wash the insoluble silica with several small portions of hot distilled water. Combine the filtrate and washings.

## 8. Procedure

8.1. Adjust the clarified sample, treated if, necessary to remove interfering agents, to contain approximately 100 mg of sulphate ion in 500 mL volume.

8.2. Add 2 to 3 drops of methyl red indicator solution (6.1). Add hydrochloric acid (6.2) drop till an orange red colour appears. Lower concentrations of sulphate ion may be tolerated if it is impracticable to concentrate the sample to the optimum level, but in such cases it is better to fix the total volume at 150 mL after concentration on hot plate.

8.3. Heat the solution to boiling, while stirring gently, add warm barium chloride solution (6.3) slowly until precipitation appears to be complete, then add about 2 mL in excess. Digest the precipitate at 80-90°C for at least 2 hours.

**8.4.** Filtration - Filter the precipitate through filter paper (5.6) and wash the precipitate with small portion of warm distilled water until the washings are free of chloride ions as indicated by testing with silver nitrate-nitric acid reagent (6.4).

**8.5.** Dry the precipitate in crucible and ignite at 800°C for 1 hour

*NOTE; Do not allow the filter paper to flame.*

**8.6.** Cool in a desiccator and weigh.

**8.7.** Calculation - Calculate the sulphate concentration in the sample from the equation:

$$\text{Sulphate concentration as mg/L BaSO}_4 = \frac{\text{mg BaSO}_4 \times 411.5}{\text{mL of sample}}$$

### C. Thorin Method

**1. Scope** - This method is applicable to surface and groundwater's with sulphate concentrations in the range 5 to 150 mg/L. Samples having higher concentrations can be measured by appropriate dilution of sample.

**2. Principle** - Sulphate ion is titrated in an alcoholic solution under controlled acid conditions with a standard barium chloride solution, using thorin as the indicator.

**3. Interferences** - There are no interferences in normal waters; however, chloride ions in concentrations greater than 1000 mg/L cause an indistinct end point when the sulphate present is low (less than 10 mg/L  $\text{SO}_4^{2-}$ ). To overcome this interference, a known amount of sulphate present is added to sample to increase the sulphate concentration

**4. Sampling and Storage** - Sampling and storage shall be done as

prescribed in IS: 3025 (Part 1) 1986. The sample container shall be tightly capped as soon as the sample has been collected

## 5. Apparatus

- 5.1 White porcelain basin-100 to 125mL capacity.
- 5.2 Burette - along with titration assembly.
- 5.3 Ion exchange column

## 6 Reagents

- 6.1 Ethyl alcohol- 95%.
- 6.2 Ammonium hydroxide solution (1 + 99) - Mix one volume of concentrated ammonia with 99 volumes of distilled water.
- 6.3 Hydrochloric acid solution (1 + 99) - Mix one volume of concentrated hydrochloric acid with 99 volumes of distilled water.
- 6.4 Hydrochloric acid solution (1 + 4) - Dilute one volume of concentrated hydrochloric acid with 4 volumes of distilled water.
- 6.5 Thorin solution - Dissolve 0.2 gm thorin (2, 2-Hydroxy-3, 6-disulpho-1-naphthylazo benzene arsenic acid) in 100 mL of distilled water.
- 6.6 Ion exchange resin - Strong cation-exchange resin. Aberlite IR-120 or equivalent.
- 6.7 Stock sulphate solution (100 mg/L  $\text{SO}_4^{2-}$ - Dissolve 1.479 gm anhydrous sodium sulphate ( $\text{Na}_2\text{SO}_4$ ) (dried at 110°C for 1 hour) in distilled water and make up to 1 litre in volumetric flask.
- 6.8 Standard sulphate solution - Prepare a series of standard solutions by diluting stock solution of sulphate with distilled water. The concentrations of standard solutions are 0 (blank), 10, 20, 30, 40, 50, 80, 100 and 150 mg/L  $\text{SO}_4^{2-}$ .
- 6.9 Standard barium chloride solution - Dissolve 0.4 gm barium chloride ( $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ) in 800 mL of distilled water and adjust the pH to 3.5 to 4.0 with dilute hydrochloric acid (6.3) or ammonia solution (6.2) and finally make up to one litre.

**7 Sample Preparation** - The sample should be free from turbidity or filtered through a 0.45 µm filter.

## **8 Procedure**

8.1 Pass the sample through ion exchange column (50 mL at a time). Discard the first 10 mL effluent and then collect in a 100mL beaker. Pipette 10 mL of this sample into a porcelain basin

8.2 Add 40 mL alcohol and 2 drops of thorin indicator. Adjust the pH to 3.8 to 4.0 by carefully adding drop by drop ammonia solution (6.2) until the solution just turns pink. Then add hydrochloric acid solution (6.3) drop by drop until the pink colour disappears; a drop is usually sufficient.

*Note -If the ammonia **is added** too fast. it is possible to overrun the colour change from yellow to pink and the sample continues to be yellow. It is then impossible to develop the pink colour by addition of ammonia solution.*

8.3 Titrate with standard barium chloride solution (6.9) until sample just turns pink.

**9 Calculation** - Prepare a calibration curve, mL of standard barium chloride solution needed to titrate standard sulphate solution (6.8) vs mg/L SO<sub>4</sub> and read the sulphate concentration of sample directly from the graph.

## **10 Reference:**

**10.1** IS: 3025 (Part 24) – 1986 (Reaffirmed 1992) - Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : Sulphate.

## 11. RESIDUAL FREE CHLORINE AND CHLORAMINES

### Introduction:

The chlorination of water supplies and polluted waters serves primarily to destroy or deactivate disease-producing micro-organisms. A secondary benefit, particularly in treating drinking water, is the overall improvement in water quality resulting from the reaction of chlorine with ammonia, iron, manganese, Sulphide and some organic substances.

Chlorination may produce adverse effects. Taste and odour characteristics of phenols and other organic compounds present in a water supply may be intensified. Potentially carcinogenic chloro-organic compounds such as chloroform may be formed. Combined chlorine formed on chlorination of ammonia-or amine-bearing waters adversely affects some aquatic life. To fulfill the primary purpose of chlorination and to minimize any adverse effects, it is essential that proper testing procedures be used with a foreknowledge of the limitations of the analytical determination.

Chlorine applied to water in its molecular or hypochloric form initially undergoes hydrolysis to form free chlorine consisting of aqueous molecular chlorine, hypochlorous acid and hypochlorite ion. The relative proportion of these free chlorine forms is pH and temperature-dependent. At the pH of most waters, hypochlorous acid and hypochlorite ion will predominate.

Free chlorine reacts readily with ammonia and certain nitrogenous compounds to form combined chlorine. With ammonia, chlorine reacts to form the chloramine, dichloramine and nitrogen trichloride. The presence and concentration of these combined forms depend chiefly on pH, temperature, initial chlorine-to-nitrogen ratio, absolute chlorine demand and reaction time. Both free and combined chlorine are formed in the treatment of raw waters containing ammonia or by the addition of ammonia or ammonium salts. Chlorinated wastewater effluents as well as certain chlorinated industrial effluents, normally contain only combined chlorine. Historically the principal analytical problem has been to distinguish between free and combined forms of chlorine.

Chloramines, the monochloramine form in particular, have also been used as a disinfectant since the 1930's. Chloramines are produced by combining chlorine and ammonia. While obviously toxic at high levels, neither pose health concerns to humans at the levels used for drinking water disinfection.

Chloramines are weaker disinfectants than chlorine, but are more stable, thus extending disinfectant benefits throughout a water utility's distribution system. They are not used as the primary disinfectant for water. Chloramines are used for maintaining a disinfectant residual in the distribution system so that disinfected drinking water is kept safe. Chloramines can also provide the Chloramines, like chlorine, are toxic to fish and amphibians at levels used for drinking water. Unlike chlorine, chloramines do not rapidly dissipate on standing. Neither do they dissipate by boiling. Fish owners must neutralize or remove chloramines from water used in aquariums or ponds.

#### **A. DPD Colorimetric Method**

**1. Principle:** DPD is used as indicator in this method to develop color. In absence of iodide ion, free chlorine reacts instantly with DPD indicator to produce a red color. Subsequent addition of small amount of iodide ions acts catalytically to cause monochloramines to produce color. Addition of iodide ion to excess evokes rapid response from dichloramine.

**2. Interference:** Compensate for color and turbidity by using sample to zero photometer. Organic contaminants may produce a false free chlorine reading in most colorimetric methods. Many strong oxidizing agents interfere in the measurement of the free chlorine in all methods. Such interferences include bromine, chlorine dioxide, iodine, permanganate, hydrogen peroxide, and ozone. However the reduced forms of these compounds –Bromide, Chloride, iodide, manganous ion and oxygen in absence of other oxidants, do not interfere. Reducing agents such as ferrous compounds, hydrogen sulfide, and oxidisable organic matter generally do not interfere. Minimize chromate interference by using the thioacetamide blank correction.

**3. Minimum detectable concentration:** Approximately 10 µg/L Cl as Cl<sub>2</sub>. This detection limit is achievable under ideal conditions; normal working detection limits typically are higher.

#### **4. Apparatus:**

**4.1.** Photometric equipment: One of the following is required:

**4.1.1** Spectrophotometer, for use at a wavelength of 515 nm and providing a light path of 1 cm or longer.

**4.1.2** Filter photometer, equipped with a filter having maximum transmission in the wavelength range of 490 to 530 nm and providing a light path of 1 cm or longer.

**4.2.** Glassware: Use separate glassware, including separate spectrophotometer cells, for free and combined (dichloramine) measurements to avoid iodide contamination in free chlorine measurement.

#### **5. Reagents:**

**5.1. Phosphate buffer Solution:** Dissolve 24 gm anhydrous Na<sub>2</sub>HPO<sub>4</sub> and 46 gm anhydrous KH<sub>2</sub>PO<sub>4</sub> in distilled water. Combine with 100 mL distilled water in which 800 mg disodium ethylenediamine tetracetate dihydrate (EDTA) have been dissolved. Dilute to 1L with distilled water and add optionally either 20mg HgCl<sub>2</sub> or two drops toluene to prevent mold growth. Interference from trace amounts of iodide in the reagents can be negated by optional addition of 20 mg HgCl<sub>2</sub> to the solution.

Caution: HgCl<sub>2</sub> is toxic-take care to avoid ingestion

**5.2. N,N Diethyl-p-phenylenediamine (DPD) indicator solution:** Dissolve 1 gm DPD Oxalate or 1.5 gm DPD Sulfate pentahydrate or 1.1 gm anhydrous DPD sulfate in chlorine free distilled water containing 8 mL 1+3 H<sub>2</sub>SO<sub>4</sub> and 200 mg disodium EDTA. Make up to 1L, store in brown glass stoppered bottle in the dark, and discard when discolored. Periodically check solution blank for absorbance and discard when absorbance at 515 nm exceeds 0.002/cm.

Caution: The oxalate is toxic-take care to avoid ingestion.

**5.3. Standard Sodium thiosulfate 0.1N:** Dissolve 25 gm sodium thiosulfate pentahydrate in 1 L freshly boiled distilled water and standardize against potassium dichromate after at least 2 weeks storage. This initial storage is necessary to allow oxidation of any bisulfite ion present. Use boiled distilled water and add few mL chloroform to minimize bacteria decomposition.

**5.4. Standardization of 0.1 N  $\text{Na}_2\text{S}_2\text{O}_3$  by Potassium Dichromate method:** Dissolve 4.904gm anhydrous potassium dichromate,  $\text{K}_2\text{Cr}_2\text{O}_7$ , of primary standard quality, in distilled water and dilute to 1000mL to yield a 0.1000N solution. Store in a glass-stoppered bottle. To 80 mL distilled water, add with constant stirring, 1 mL conc.  $\text{H}_2\text{SO}_4$ , 10 mL 0.1 N  $\text{K}_2\text{Cr}_2\text{O}_7$  and 1gm KI. Let the reaction mixture stand for 6 min in the dark before titrating. Titrate immediately with 0.1 N  $\text{Na}_2\text{S}_2\text{O}_3$  titrant until the yellow color of the liberated iodine is almost discharged. Add 1 mL starch indicator solution and continue titrating until the blue color disappears.

Normality of  $\text{Na}_2\text{S}_2\text{O}_3 = 1/\text{mL } \text{Na}_2\text{S}_2\text{O}_3 \text{ consumed}$

**5.5 Standard Sodium thiosulfate titrant (0.025 N):** Improve the stability of 0.025 N sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3$ ) by diluting an aged 0.1 N solution, made as directed above, with freshly boiled distilled water. Add 4 gm sodium borate and 10mg mercuric iodide per liter of the solution. For accurate work, standardize the solution daily in accordance with the directions given above, using  $\text{K}_2\text{Cr}_2\text{O}_7$ .

## 6. Procedure:

**6.1 Calibration of photometric equipment:** Calibrate instrument with chlorine or potassium permanganate solutions.

6.1.1. Chlorine solutions-Prepare chlorine standards in the range of 0.05 to 4 mg/L from about 100 mg/L chlorine water standardized as follows: Place 2 mL acetic acid and 10 to 25 mL chlorine-demand-free water in a flask. Add about 1gm KI. Measure into the flask a suitable volume of chlorine solution. In choosing a convenient volume, note that 1 mL 0.025N  $\text{Na}_2\text{S}_2\text{O}_3$  titrant is equivalent to about 0.9 mg chlorine. Titrate with

standardized 0.025N  $\text{Na}_2\text{S}_2\text{O}_3$  titrant until the yellow iodine color almost disappears. Add 1 to 2mL starch indicator solution and continue titrating to disappearance of blue color.

6.1.2. Determine the blank by adding identical quantities of acid, KI, and starch indicator to a volume of chlorine-demand-free water corresponding to the sample used for titration. Perform blank titration A or B, whichever applies.

$$\text{mg Cl as Cl}_2/\text{mL} = (A + B) \times N \times 35.45$$

Where:

N= Normality of  $\text{Na}_2\text{S}_2\text{O}_3$

A= mL titrant for sample.

B= mL titrant for blank (to be added or subtracted according to required blank titration)

6.1.3. Use chlorine-demand-free water and glassware to prepare these standards. Develop color by first placing 5 mL phosphate buffer solution and 5 mL DPD indicator reagent in flask and then adding 100 mL chlorine standard with thorough mixing as described below. Fill photometer or colorimeter cell from flask and read color at 515 nm. Return cell contents to flask and titrate with standard FAS titrant as a check on chlorine concentration.

6.1.4. Volume of sample: Use a sample volume appropriate to the photometer or colorimeter. The following procedure is based on using 10 mL volume; adjust reagent quantities proportionately for other sample volumes. Dilute sample with chlorine-demand free water when total chlorine exceeds 4 mg/L.

6.1.5. **Free chlorine:** Place 0.5 mL each of buffer reagent and DPD indicator reagent in a test tube or photometer cell. Add 10 ml sample and mix. Read color immediately(**A**).

6.1.6. **Monochloramines:** Continue by adding one very small crystal of KI (about 0.1 mg) and mix. If dichloramine concentration is expected to be high, instead of small crystal add 0.1 mL (2 drops) freshly prepared KI solution (0.1 gm/100 mL). Read color

immediately **(B)**.

6.1.7. **Dichloramines:** Continue by adding several crystals of KI (about 0.1 gm) and mix to dissolve. Let stand about 2 min and read color **(C)**.

## 7. Calculation

7.1. Free residual chlorine, mg/L = A\* Dilution factor Where A = Free Chlorine

7.2. Monochloramines mg/L = ( B - A)

7.3. Dichloramine mg/L =(C - B)

## 8. Reference

8.1. APHA 4500 Cl G

### B. Stabilized Neutral Ortho-toluidine Method

1. **Principle and Theory-** The stability of oxidized ortho-toluidine decreases as pH increases. Anionic surface active reagents stabilize the colour development by free chlorine and ortho-toluidine at pH 7.0. Sodium di (2-ethyl-hexyl) sulphasuccinate is the best stabilizing agent. The optimum concentration of stabilizer is 40 mg for each 100 mL of sample plus reagents. The final solution should have pH between 6.5 and 7.5

2. **Interferences** - Strong oxidizing agents, such as bromine, chlorine dioxide, iodine, manganic compounds and ozone interfere. However, the reduced forms of these compounds do not interfere. Reducing agents like nitrites, ferrous compounds, hydrogen sulphide and oxidizable organic matter do not interfere, but may interfere by reducing the chlorine residual by reaction with chlorine to produce chlorine ion. Turbidity and colour also interfere unless the background turbidity or colour is compensated for using a blank.

### 3. Apparatus

3.1 Spectrophotometer - Suitable for use at 625 nm providing a light path of 1 cm or longer for  $\leq 1\text{mg/L}$  free residual chlorine, or a light path from 1 to 10 mm for free

residual chlorine > 1.5 mg/L.

### 3.2 Magnetic stirrer assembly

## 4. Reagents

4.1 Distilled water - Chlorine demand free. Add sufficient chlorine to distilled water to destroy the ammonia and nitrate. The amount of chlorine required will be about 10 times the amount of ammoniacal nitrogen present; produce an initial residual of more than 1.0 mg/l free chlorine. Let the chlorinated distilled water stand overnight or longer; then expose to direct sunlight until all residual chlorine is discharged.

4.2 Neutral ortho-toluidine reagent - Add 5.0 mL of concentrated hydrochloric acid to 500 mL chlorine demand free distilled water. Add to 10 mL of this acid solution, 20 mg mercuric chloride ( $\text{Hg Cl}_2$ ), 30 mg of disodium salt of EDTA (dehydrated) and 1.5 gm of ortho-toluidine dihydrochloride to chlorine demand free distilled water and dilute to 1 litre. Store in a brown bottle or in the dark at room temperature. Protect at all times from direct sunlight. Use for not more than 6 months and avoid contact with rubber. Do not let the temperature fall below 0°C because the resulting crystallization of ortho-toluidine can lead to deficient subsequent colour development.

4.3 Buffer stabilizer reagent - Dissolve 34.4 gm dipotassium hydrogen phosphate ( $\text{K}_2\text{HPO}_4$ ) 12.6 gm potassium dihydrogen phosphate ( $\text{KH}_2\text{PO}_4$ ) and 8.0 gm 100 percent solid di (2-ethyl hexyl) sulphosuccinate in a solution of 500 mL chlorine demand free water and 200 mL diethylene glycol monobutyl ether. Dilute to 1 liter with chlorine demand free water.

4.4 Potassium iodide solution - Dissolve 0.4 gm potassium iodide in chlorine demand free distilled water and dilute to 100 mL, store in a brown glass stoppered bottle, preferably in a refrigerator. Discard when yellow colour is developed.

4.5 Sulphuric acid solution - Add cautiously 4.00 mL of concentrated sulphuric acid to chlorine demand free water and dilute to 100 mL

4.6 Sodium carbonate solution - Dissolve 5.0 gm of sodium carbonate in chlorine

demand free distilled water and dilute to 100 mL

4.7 Sodium arsenite solution - Dissolve 5.0 gm of sodium arsenite in distilled water and dilute to liter.

4.8 Standard chlorine solution - Obtain suitable solution from the chlorinator gas solution hose or by bubbling chlorine gas through distilled water. Improve the solution stability by storing in the dark or brown, glass-stoppered bottle. Standardize it each day of use. Alternatively, dilute household hypochlorite solution, which contains about 30 000 to 50 000 mg/L chlorine equivalent. This is more stable than chlorine solution, but do not use for more than 1 week without restandardization. Use the same chlorine concentration actually applied in plant treatment to determine chlorine demand. Depending on intended use, a suitable strength of chlorine solution usually will be between 100 and 1000 mg/L. Use a solution of sufficient concentration so that adding the chlorine solution will not increase the volume of treated portion by more than 5 percent. Standardize the solution using 0.025 N thiosulphate solution. 1 mL of 0.025N thiosulphate titrant is equivalent to 0.9 mg chlorine.

## 5 Procedure

5.1 Construct a calibration curve by making dilutions of standardized hypochlorite solution. Take care when diluting to low concentrations because of possible consumption of small amounts chlorine by trace impurities. Use chlorine demand free distilled water for dilution.

5.2 Use 5.0 mL neutral ortho-toluidine and 5.0 mL buffer stabilizer reagent with 100 mL sample. Place the neutral ortho-toluidine and buffer stabilizer mixture in a beaker on a magnetic stirrer. Mix and add sample to the reagents with gentle stirring. Measure the absorbance at 625 nm. The value obtained (**A**) from the calibration curve represents the free chlorine residual.

5.3 Monochloramine - Return any position used for measuring free chlorine in 4.5.1.1 to the sample. Add with stirring 0.5 mL potassium iodide solution to each 100 mL sample or similar ratio for other sample volumes. Again measure the

absorbance and obtain value **(B)** from calibration curve. This will give free residual chlorine plus monochloramine.

5.4 Dichloramine - Return any portion used for measuring the monochloramine in 4.5.1.2 to the sample. Add with stirring, 1 mL of sulphuric acid to each 100 mL of sample, or a similar ratio for other sample volumes. After 30 seconds for colour development add 1 mL of sodium carbonate solution, slowly with stirring or until a pure blue solution returns. Measure the absorbance of total residual chlorine, free chlorine, monochloramine and dichloramine and obtain the value **(C)** from the calibration curve.

5.5 Compensation for interferences - Compensate for the presence of natural colour or turbidity as well as manganic compounds by adding 5.0 mL arsenite solution to 100 mL of sample. Add this blank sample to the reagents as above. Use the colour of the blank to set zero absorbance on the spectrophotometer. Measure all samples in relation to this blank. Read from the calibration curve the concentrations of chlorine present in the sample.

## 6 Calculation

**Free residual chlorine, mg/L** = A, including 1/2 trichloramine, if present

**Monochloramine, mg/L** = (B - A) as mg/L of chlorine

**Dichloramine, mg/L** = 1.03 C - B as mg/L of chlorine

**Total chlorine, mg/L** = 1.03 C as mg/L chlorine

## 7 Reference:

7.1 IS 3025 (P-26)-1986

## 12. SULPHIDE

### Introduction:

Sulfide is often present in groundwater and sediment. It is produced by decomposition of organic matter and bacterial reduction of sulfate. It is sometimes found in industrial or municipal wastewater. Hydrogen sulfide escaping into the air from sulfide-containing wastewater causes odor nuisances. The threshold odor concentration of H<sub>2</sub>S in clean water is between 0.025 and 0.25 µg/L. Gaseous H<sub>2</sub>S is very toxic and has claimed the lives of numerous workers. At levels toxic to humans it interferes with the olfactory system, giving a false sense of the safe absence of H<sub>2</sub>S. It attacks metals directly and indirectly has caused serious corrosion of concrete sewers because it is oxidized biologically in the presence of oxygen to H<sub>2</sub>SO<sub>4</sub> on the pipe wall. Dissolved H<sub>2</sub>S is toxic to fish and other aquatic organisms.

The methods for the determination of Sulphide are prescribed as:-

- A. Methylene Blue Method
- B. Iodometric Method
- C. Ion Selective Method

### A. Methylene blue method

**1. Scope:** Prescribes method for the determination of dissolved sulphide in water by methylene blue method. The methylene blue method is applicable to sulphide concentration up to 20 mg/L.

**2. Principle-** The methylene blue method is based on the reaction of sulphide, ferric chloride and dimethyle-p-phenylenediamine to produce blue color. Ammonium phosphate is added after color development to remove ferric chloride color.

### 3. Apparatus

- 3.1. Matched Test tubes- Approximately 125 mm long and 15 mm OD.
- 3.2. Droppers- Capable of delivering 20 drops/mL of methylene blue solution.

**3.3.** Spectrophotometer- Suitable for use at 664 nm with cells providing light paths of 1 cm and 1 mm or filter photometer with filter providing maximum transmittance near 600 nm.

#### **4. Reagents**

**4.1. Amine sulphuric acid stock solutions-** Dissolve 27 gm N, N-dimethyl-p-phenylene diamine-oxalate in a cold mixture of 50 mL concentrated sulphuric acid and 20 mL of distilled water. Cool and dilute to 100 mL with distilled water. Use fresh oxalate as old stock may be oxidized and discolored to a degree that results interfering colors in the test. Store in a dark glass bottle. When this stock solution is diluted and used in the procedure with a sulphide free sample, it will first be pink but then should become colorless within 3 minutes.

**4.2. Amine-sulphuric acid reagent solution-** Dilute 25 mL of stock solution with 975 mL 1:1 sulphuric acid. Store in a dark glass bottle.

**4.3. Ferric Chloride solution-** Dissolve 100 gm of ferric chloride ( $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ ) in 40 mL water.

**4.4. Sulphuric acid solution – 1:1**

**4.5. Diammonium hydrogen phosphate solution-** Dissolve 400 gm of  $(\text{NH}_4)_2\text{HPO}_4$  in 800 mL of distilled water.

**4.6. Methylene blue solution I-** Dissolve 1.0 gm of dye (should be 84 percent or more) in distilled water and make up to 1 litre. Standardize this against sulphide solutions of known strength and adjust its concentration so that 0.05mL (1 drop) is equivalent to 1.0 mg of sulphide per litre.

**4.7. Methylene blue solution II-** Dilute 10 mL of adjusted methylene blue solution 1 to 100 mL.

## 5. Procedure

**5.1. Colour development-** Transfer 7.5 mL of sample to each two matched test tubes, using a special wide -tip pipette or filling to marks on test tubes. Add to tube A 0.5 mL of amine-sulphuric acid reagent and 0.15 mL of ferric chloride solution. Mix immediately by inverting slowly, only once (excessive mixing causes low results by loss of hydrogen sulphide as a gas before it has had time to react). To tube B add 0.5 mL of 1:1 sulphuric acid and 0.15 mL of ferric chloride solution and mix. The presence of sulphide will be indicated by the appearance of blue color in tube A. Colour development is generally complete in about 1 minute, but a longer time often is required for fading out of the initial pink color. Wait 3 to 5 minutes and make color comparisons. If zinc acetate was used, wait at least 10 minutes before making visual color comparison.

### 5.2. Colour determination

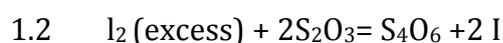
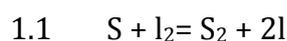
5.2.1. Visual color estimation- Add methylene blue solution I or II, depending on sulphide concentration and desired accuracy, dropwise, to the second tube, until color matches that developed in the first tube, If the concentration exceeds 20 mg/L, repeat test with a portion of sample diluted to one tenth. With methylene blue solution I adjusted so that 0.05 mL (1 drop) is equivalent to 1.0 mg of sulphide per liter when 7.5 mL of sample are used:

$$\text{mg of sulphide/liter} = \text{No. of drops of solution I} + 0.1 (\text{No. of drops of Solution II})$$

5.2.2. Photometric method- A cell with a light path of 1 cm is suitable for measuring sulphide concentration from 0.1 to 2.0 mg/L. Use shorter or longer light paths for higher or lower concentrations. The upper limit of the method is 20 mg/L. Zero instrument with a portion of treated sample from tube B. Prepare calibration curves on the basis of colorimetric tests made on sodium sulphide solutions simultaneously analyzed by the Iodometric method, plotting concentration as absorbance. A straight line relationship between concentration and absorbance can be assumed from 0 to 1.0 mg/L. Read sulphide concentration from calibration curve.

## B. Iodometric Method

**1. Principle** – Sulphides are stripped from the acidified sample with an inert gas and collected in zinc acetate solution. Excess iodine solution added to the zinc sulphide suspension reacts with the sulphide under acidic condition. Thiosulphate is used to measure unreacted iodine to indicate the quantity of iodine consumed by sulphide. The reaction may be given as follows:



## 2. Interferences

2.1 Reduced sulphur compounds such as sulphite thiosulphate and hydrosulphite which decompose in acid may yield erratic results.

2.2 Volatile iodine consuming substances will give high results.

2.3 Eliminate interferences due to sulphite, thiosulphate, iodide and many other soluble substances but not ferro-cyanide, by first precipitating zinc sulphide removing the supernatant, and replacing it with distilled water. Use the same procedure even when not needed for removal of interferences, to concentrate sulphide.

## 3. Procedure

Put required quantity of 2 N zinc acetate solution into 500 mL glass bottle, fill with sample and add required quantity of 6 N sodium hydroxide solution. Stopper with no air bubbles under stopper and mix by rotating back and forth vigorously about a transverse axis. Addition of reagents may be varied in volume so that the resulting precipitate is not excessively bulky and settles rapidly. Add enough sodium hydroxide to produce a pH above 9. Let the precipitate settle for 30 minutes. Filter the precipitate through glass fiber filter paper and carry out titration immediately.

## 4. Sampling and Storage

4.1 Sampling and storage shall be done as prescribed in IS: 3025 (Part I) - 1986 'Methods of sampling and test (physical and chemical) for water and waste water : Part 1 sampling (first revision). Samples must be taken with a minimum of aeration and preserved at low temperature (Sulphide may be volatilized by aeration and any oxygen is advertently added to the sample may be convert the sulphide to an unmeasurable form).

4.2 Preserve the sample with addition of 2 ml/L of zinc acetate. Samples not preserved must be analyzed immediately.

## 5. Apparatus

5.1 Reaction Flask- Wide mouth bottle of 1 litre capacity with a 2 hole stopper, fitted with a fritted gas-diffusion tube (plastic, ceramic or glass and a gas outlet tube).

5.2 Absorption flasks- Two 250 mL capacity long necked flask with 2 hole stoppers fitted with glass tubes and suitable connections to pass gas through in series.

## 6. Reagents

6.1 Zinc acetate solution (2 N) – Dissolve 110 gm Zn (C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub>.2H<sub>2</sub>O on 400 mL distilled water and finally make up to 1 litre

6.2 Inert gas- A cylinder of nitrogen [pure grade, see IS: 1747- 1972 Specification for nitrogen (first revision)] or CO<sub>2</sub> or a CO<sub>2</sub> gas generator [Grade 1 see IS: 307- 1966 specification for carbon dioxide (second revision)]

6.3 Sulphuric Acid concentrated

6.4 Standard iodine solution (0.025 N) - Dissolve 20-25 gm potassium iodide (KI) in a little water and add 3.175 gm iodine. After iodine has dissolved, dilute to 1 litre with distilled water, standardize this solution against 0.025 N sodium thiosulphate using starch indicator.

6.5 Hydrochloric acid concentrated

6.6 Standard thiosulphate solution (0.025 N) - Dissolve 6.205 gm  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  in 800 mL boiled and cooled distilled water. Add 0.4 gm NaOH or 5 mL chloroform as a preservative and finally make up to 1 litre.

6.7 Starch indicator solution- Add 5.0 gm starch to 800 mL boiling distilled water & stir. Dilute to one litre and boil for few minutes and let settle over night. Use the clear supernatant. (This solution may be preserve by adding 1.25 gm of salicylic acid/litre or by adding a few drops of toluene)

6.8 Aluminum Chloride solution (6 N) - Take the 100 gm  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  from a previously unopened reagent bottle and dissolve in 144mL distilled water.

*Note- because of the hygroscopic and caking tendencies of this chemical it will be convenient to purchase in small packing*

6.9 Sodium hydroxide (6 N)- Dissolve 240 gm NaOH in distilled water and dilute to 1 litre

## 7. Procedure

### 7.1 Total sulphide

7.1.1 Take 5 mL zinc acetate solution and 95 mL distilled water into each of the two absorption flasks

7.1.2 Connect the reaction flask and two absorption flasks in series and purge the system with  $\text{CO}_2$  or  $\text{N}_2$  for 2 minutes. Measure 500 mL well mixed with sample into the reaction flask

7.1.3 Acidify the sample with 10 mL concentrated  $\text{H}_2\text{SO}_4$  and replace the prepared 2 holes stopper tightly pass  $\text{N}_2$  or  $\text{CO}_2$  (Not air or oxygen) through the sample for 1 hour or until the experiments show no more sulphide coming over

7.1.4 To each of the absorption flasks, then add iodine solution well in excess of the amount necessary to react with collected sulphide

7.1.5 Add 2.5 mL concentrated HCl acid to each flask, stopper and shake to mix thoroughly

7.1.6 Transfer contents of bath flasks and back titrate with 0.025 N sodium thiosulphate solution using starch solution as indicator. Run a blank parallel for accurate results.

## 7.2 Dissolved Sulphide

7.2.1 Remove suspended solids in the sample by flocculation and settling.

7.2.2 Fill 1 litre bottle with flowing sample in such a way that the sample, which has had the least possible contact with air. Add 2 mL aluminium chloride solution and 2 mL NaOH solution and stopper with no air bubbles under the stopper. Rotate back and forth about a transverse axis as vigorously as possible for at least 1 minute in order to flocculate the contents thoroughly.

*Note- The volume of these chemicals may be varied according to experience, the idea being to get good clarification without using excessively large amounts.*

7.2.3 Allow to settle for 15 minutes, or until supernatant liquid is reasonably clear. Alternatively remove suspended matter by centrifugation.

7.2.4 Proceed as for total sulphide after taking 500 mL sample into the reaction flask.

## 8 Calculation

$$\text{Mg/L sulphide} = \frac{(V_1 - V_2) \times 400}{V}$$

Where

V<sub>1</sub> = volume in mL of standard iodine solution added

V<sub>2</sub> = Volume in mL of standard thiosulphate solution used, and

V = Volume in mL of sample taken.

### C. Ion Selective Electrode

- Principle:** The potential of a silver/sulfide ion-selective electrode (ISE) is related to the sulfide ion activity. An alkaline antioxidant reagent (AAR) is added to samples and standards to inhibit oxidation of sulfide by oxygen and to provide a constant ionic strength and pH. Use of the AAR allows calibration in terms of total dissolved sulfide concentration. All samples and standards must be at the same temperature. Sulfide concentrations between 0.032 mg/L ( $1 \times 10^{-6}$ M) and 100 mg/L can be measured without preconcentration. For lower concentrations, preconcentration is necessary.

**2. Interferences:** Humic substances may interfere with Ag/S-ISE measurements. For highly colored water (high concentration of humic substances), use the method of standard additions to check results. Sulfide is oxidized by dissolved oxygen. Sulfide oxidation may cause potential readings to drift in the direction of decreasing concentration, i.e., to more positive values. Flush surface of samples and standards with nitrogen to minimize contact with atmospheric oxygen for low-level measurements. Temperature changes may cause potentials to drift either upward or downward. Therefore, let standards and samples come to the same temperature. If samples cannot be analyzed immediately, preserve dissolved sulfide by precipitating with zinc acetate (4500-S<sup>2-</sup>.C).

### 3. Apparatus

3.1 Silver/sulfide electrode. \*

3.2 Double-junction reference electrode.

3.3 Electrode polishing strips.

3.4 pH meter with mill volt scale, capable of 0.1-mV resolution. Meters that can be calibrated in concentration and that perform standard-additions calculations are available.

3.5 Electrochemical cell: Make suitable cell from a 150mL beaker and a sheet of rigid plastic (PVC or acrylic) with holes drilled to allow insertion of the electrodes and a tube for flushing the headspace with nitrogen. Alternatively, purchase a polarographic cell with gas transfer tube.

3.6 Gas dispersion tube: Use to deaerate water for preparing reagents and standards.

3.7 Magnetic stirrer and stirring bar: Use a piece of Styrofoam or cardboard to insulate the *cell* from the magnetic stirrer.

### 4. Reagents

4.1 Alkaline antioxidant reagent (AAR): To approximately 600 mL deaerated reagent water (DRW) in a 1L volumetric flask, add 80 gm NaOH, 35 gm ascorbic acid, and 67 gm Na<sub>2</sub>H<sub>2</sub>EDTA. Swirl to dissolve and dilute to 1L. The color of freshly prepared AAR will range from colorless to yellow. Store in a tightly capped brown glass bottle. Discard

when solution becomes brown.

4.2 *Lead perchlorate, 0.1 M*: Dissolve 4.60 gm  $\text{Pb}(\text{ClO}_4)_2 \cdot 3\text{H}_2\text{O}$  in 100 mL reagent water. Standardize by titrating with  $\text{Na}_2\text{H}_2\text{EDT}$ . Alternatively, use commercially available 0.1 M  $\text{Pb}(\text{ClO}_4)_2$  solutions.

4.3 *Sulfide stock solution*: Dissolve 3.75 gm of  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  and diluted to 500 mL will give a stock solution of which 1.00 mL = 1 mg  $\text{S}^{2-}$ . Dilute 13.0 mL of 1.00 mg  $\text{S}^{2-}$  /mL stock to 100.0 mL with AAR. Alternatively, add 500 mL AAR and 1 g  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  to a 1L volumetric flask; dissolve. Dilute to 1L with DRW. Use deaerated artificial seawater (DASW) or 0.7M NaCl if sulfide concentrations are to be determined in seawater. Standardize stock solution by titrating with 0.1M  $\text{Pb}(\text{ClO}_4)_2$ . Pipet 50 mL sulfide stock solution into the electrochemical cell. (Use 10 mL with a small-volume polarographic cell.) Insert Ag/S electrode and reference electrode and read initial potential. Titrate with 0.1 M  $\text{Pb}(\text{ClO}_4)_2$ . Let electrode potential stabilize and record potential after each addition. Locate equivalence point as in Section 4500-Cl-D 4a. Alternatively, linearize the titration curve<sup>1</sup> Calculate the function  $F_1$  for points before the equivalence point.

$$F_1 = (V_0 + V) 10^{E/m}$$

where:

$V_0$  = volume of stock solution, mL,

$V$  = titrant volume, mL,

$E$  = potential, mV, and

$m$  = slope of calibration curve, mV/log unit.

Plot  $F_1$  as a function of titrant volume. Extrapolate to find the intersection with the x-axis; that is, the equivalence point. Calculate sulfide concentration in the stock solution from:

$$C = \frac{V_{\text{eq}} [\text{Pb}]}{V_0}$$

where:

$C$  = sulfide concentration, mg/L,

$V_{\text{eq}}$  = equivalence volume, mL,

$[\text{Pb}]$  = concentration of Pb in titrant, mg/L, and

$V_o$  = volume of stock solution, mL.

Store stock solution in a tightly capped bottle for 1 week or less. The stock solution also can be standardized iodometrically. CAUTION: Store in a fume hood.

4.4 *Sulfide standards:* Prepare sulfide standards daily by serial dilution of stock. Add AAR and Zn ( $C_2H_3O_2$ )<sub>2</sub> solutions to 100 mL volumetric flasks. Add sulfide solutions and dilute to volume with DRW (or DASW). Prepare at least one standard with a concentration less than the lowest sample concentration.

**5 Procedure:** Check electrode performance and calibrate daily. Check electrode potential in a sulfide standard every 2 hr. The procedure depends on the sulfide concentration and the time between sample collection and sulfide determination. If the total sulfide concentration is greater than 0.03 mg/L ( $1 \times 10^{-6}M$ ) and the time delay is only a few minutes, sulfide can be determined directly. Otherwise, precipitate ZnS and filter as described in 4500-S<sup>2-</sup>.C.

5.1 *Check electrode performance:* Pipet 50 mL AAR, 50 mL DWR, and 1 mL sulfide stock solution into the measurement cell. Place Ag/S and reference electrodes in the solution and read potential. Add 10 mL stock solution and read potential. The change in potential should be  $-28 \pm 2$  mV. If it is not, follow the troubleshooting procedure in the electrode manual.

5.2 *Calibration:* Place electrodes in the most dilute standard but use calibration standards that bracket the sulfide concentrations in the samples. Record potential when the rate of change is less than 0.3 mV/min (This may take up to 30 min for very low sulfide concentrations, i.e., less than 0.03 mg/L.) Rinse electrodes, blot dry with a tissue, and read potential of the next highest standard. For a meter that can be calibrated directly in concentration, follow manufacturer's directions. For other meters plot potential as a function of the logarithm (base 10) of the sulfide concentration. For potentials in the linear range, calculate the slope and intercept of the linear portion of the calibration plot.

5.3 *Sulfide determination by comparison with calibration curve, no ZnS precipitation:* Add 40 mL AAR, 0.15 mL (3 drops) zinc acetate, and 50 mL sample to a

100mL volumetric flask. Dilute to 100 mL with AAR. Pour into the electrochemical cell and insert the electrodes. Record potential when the rate of change is less than 0.3 mV/min. Read sulfide concentration from the calibration curve. Alternatively, for potentials in the linear range, calculate the sulfide concentration from:

$$S_{\text{Tot}} = 10^{(E-b)/m}$$

Where:

$E$  = electrode potential and

$B$  and  $m$  are the intercept and slope of the calibration curve.

For a meter that can be calibrated directly in concentration, follow the manufacturer's directions.

5.4 *Sulfide determination by comparison with calibration curve, with ZnS precipitation:* Place filter with ZnS precipitate in a 150mL beaker containing a stir bar. Wash sample bottle with 50 mL AAR and 20 mL DRW and pour the washings into the beaker. Stir to dissolve precipitate. Remove filter with forceps while rinsing it into the beaker with a minimum amount of DRW. Quantitatively transfer to a 100mL volumetric flask and dilute to mark with DRW. Pour into the electrochemical cell and place the electrodes in the solution. Measure potential as in 4.3 above. Calculate sulfide concentration (4.3 above).

5.5 *Sulfide determination by standard addition with or without ZnS precipitation:* Measure the Ag/S-ISE electrode potential as in 4.3 or 4.4 above. Add sulfide stock solution and measure potential again. Calculate sulfide concentration as follows:

$$C_o = \frac{fC_s}{(1+f)10^{(E_s-E_o)/m} - 1}$$

where:

$C_o$  and  $C_s$  = sulfide concentrations in sample and known addition,

$E_o$  and  $E_s$  = potentials measured for sample and known addition,

$m$  = slope of calibration curve (approximately 28 mV/log  $S^{2-}$  and

$f$  = ratio of known-addition volume to sample volume.

## 6 References

- 6.1 IS 3025 (PART 29) 1986  
6.2 APHA 4500S-2

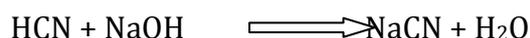
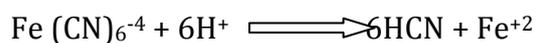
## 13. CYANIDE

### Introduction:

Cyanide refers to all of the CN groups in cyanide compounds that can be determined as the cyanide ion, CN<sup>-</sup>; by the methods used. The cyanide compounds in which cyanide can be obtained as CN<sup>-</sup> are classed as simple and complex cyanide. Two methods for determination of total cyanides in water have been given.

### A. Colorimetric Method

**1. Principle** - Distillation of sample in the presence of sulphuric acid converts simple and complex cyanides into hydrocyanic acid. The hydrogen cyanide gas is absorbed in a solution of sodium hydroxide and the cyanide is determined colorimetrically.



In the colorimetric measurement the cyanide in the sodium hydroxide solution after distillation is converted to cyanogen chloride by reaction with chloramine-T. The cyanogen chloride then forms a blue dye on the addition of pyridine-pyrazolone reagent and the absorbance is measured at 620 nm or pyridine-barbituric acid reagent and the absorbance is measured at 575 and 582 nm.

### 2. Sampling Procedure and storage

2.1 The sample should be collected in 2- litre polyethylene bottle and analyzed as soon as possible after collection

2.2 Samples should be preserved by addition of sufficient hydroxide to raise the pH to 11.0 or above and be stored in a cool place.

### 3 Apparatus

3.1 Boiling flask- 1 litre with inlet tube and provision for water cooled condensers

3.2 Heating mantle

3.3 Gas absorber- with gas dispersion tube equipped with medium- porosity fritted outlet

3.4 Ground glass ST joints- TFE sleeved or with an appropriate lubricant for the boiling flask and condenser. Neoprene stopper and plastic threaded joints may also be used.

3.5 Spectrophotometer- for use at 620 nm, providing a light path of 1 cm used in

#### **pyridine-Pyrazolone reagent Method**

3.6 One of the following is required in case of **Pyridine-barbituric acid reagent**:

i) Spectrophotometer, for use at 578 nm, providing a light path of 10 mm or longer

ii) Filter photometer, providing a light path of at least 10 mm and equipped with a red filter having maximum transmittance at 570 to 580 nm.

### 4 Reagents

4.1 Sodium hydroxide solution- Dissolve 50 gm sodium hydroxide in 1 litre distilled water.

4.2 Lead carbonate- Powdered.

4.3 Sulphamic acid (NH<sub>2</sub>SO<sub>3</sub>H).

4.4 Magnesium Chloride solution- Dissolve 51 gm magnesium chloride (MgCl<sub>2</sub>.6H<sub>2</sub>O) in 100 mL distilled water.

4.5 Sulphuric acid concentrated.

4.6 Sodium Hydroxide solution (0.2 N) - Dissolve 8.0 gm sodium hydroxide in 1 litre distilled water.

4.7 Acetic Acid- Make by diluting 1 part of glacial acid with 4 parts of water

#### **Pyridine-Pyrazolone reagent Method:**

4.8.1 Stock cyanide solution- Dissolve 2.51 gm potassium cyanide, in 1 litre water, standardise this solution with 0.019 2 N silver nitrate solution. This solution loses strength gradually and must be rechecked every week.

(1 mL of this solution = 1 mg CN)

Caution: Potassium cyanide is highly toxic, take care to avoid ingestion; use gloves while preparing solution.

4.8.2 Standard cyanide solution- Dilute 10 mL stock solution (2.6.8) to 1 litre with distilled water, mix and make a second dilution of 10mL to 100 mL.

One mL = 1 µg CN

Note = this solution must be prepared daily

(Caution: Toxic, take care to avoid ingestion)

4.8.3 Chloramine- T-Dissolve 1 gm of chloramines- T in 100 mL distilled water. Prepare daily.

4.8.4 Pyridine

4.8.5 1-phenyl-3-methyl-5 pyrazolone solution- Prepare a saturated aqueous solution (approximately 0.5 g/100 mL) by adding the pyrazolone to water at 75°C. Agitate occasionally as the solution cools to room temperature. If necessary, the pyrazolone (melting point 127° to 128°C) can be purified by recrystallisation from ethyl alcohol. Usually this is not required.

4.8.6 Bis-pyrazolone (3,3'-dimethyl-1-diphenyl) (4,4'-bis-2pyrazolone)- (5,5' dione)

4.8.7 Mixed pyridine-Pyrazolone reagent- Mix 125 mL of the filtered saturated aqueous solution of pyrazolone with a filtered solution containing 0.025 gm bis-pyrazolone dissolved in 25 mL pyridine. Several minutes of mixing is usually necessary to dissolve the bis-pyrazolone in pyridine.

4.8.8 Note- Prepare the reagent daily. This reagent develops a pink colour on standing

4.8.9 Standard silver nitrate solution- Dissolve 3.27 gm of silver nitrate in 1 litre of distilled water. Store in dark bottle.

1 mL of this solution= 1 mg CN

4.9 **Pyridine-barbituric acid reagent Method:**

4.9.1 **ChloramineT- Solution:** Dissolved 1.0 gm white, water soluble powder in 100 mL water. Prepare a weekly and store in refrigerator.

4.9.2 **Stock cyanide solution:** Dissolve approximately 1.6 gm NaOH and 2.51 gm KCN in 1 L distilled water. (**CAUTION- KCN is highly toxic; avoid contact or inhalation**)

standardized against standard silver nitrate ( $\text{AgNO}_3$ ) titrant using 25 mL KCN solution. Check titer weekly because the solution gradually loses strength; 1 mL = 1 mg  $\text{CN}^-$

**4.9.3 Standard cyanide solution:** Based on the concentration determined for the KCN stock solution (as above) calculate volume required (approximately 10 mL) to prepare 1 liter of 10  $\mu\text{g CN}^-$  /mL. Dilute with NaOH dilution solution. Dilute 10 mL of the 10  $\mu\text{g CN}^-$  /mL solution to 100 mL with the NaOH dilution solution; 1.0 mL = 1.0  $\mu\text{g CN}^-$ . Prepare fresh daily and keep in a glass-stoppered bottle. (**CAUTION- toxic; take care to avoid ingestion**)

**4.9.4 Pyridine-barbituric acid reagent:** Place 15 gm barbituric acid in a 250 mL volumetric flask and add just enough water to wash sides of flask and wet barbituric acid. Add 75 mL pyridine and mix. Add 15 mL conc. hydrochloric acid (HCl), mix, and cool to room temperature. Dilute to volume and mix until barbituric acid is dissolved. The solution is stable for approximately 6 months if stored in an amber bottle under refrigeration; discard if precipitate develops

**4.9.5 Acetate buffer:** Dissolve 410 gm sodium acetate trihydrate.  $\text{NaC}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$  in 500 mL of water. Add glacial acetic acid to adjust to pH 4.5, approximately 500 mL.

**4.9.6 Sodium Hydroxide dilution solution:** Dissolve 1.6 gm NaOH in 1 L distilled water.

## 5 Procedure

### 5.1 Distillation

**5.1.1** Add 500 mL sample to the boiling flask. Add 10 mL of sodium hydroxide solution to gas scrubber and dilute, if necessary, with distilled water to obtain an adequate liquid depth in the absorber. Do not use more than 225 mL total volume of absorber solution. When sulphide generation from the distilling flask is anticipated, add 50 or more mg powdered lead carbonate to the absorber solution to precipitate sulphide. Connect the train, consisting of boiling flask air inlet, flask condenser, gas washer, suction flask trap and aspirator. Adjust suction so that approximately 1 air bubble per second enters the boiling flask. The air rate will carry hydrogen cyanide gas from flask to absorber and usually will prevent a reverse flow of hydrogen cyanide gas through the air inlet. If this air rate does not prevent sample backup in the delivery tube, increase air flow rate to 2 air bubbles per second. Observe air purge rate in the absorber where the liquid level should be raised not more than 6.5 to 10 mm. Maintain airflow through the reaction.

5.1.2 Add 2 gm of Sulphamic acid through the air inlet tube and wash down with distilled water

5.1.3 Add 50 mL of concentrated sulphuric acid through the air inlet tube with distilled water and let air mix flask contents for 3 minutes. Add 20 mL of magnesium chloride reagent through air inlet and wash down with stream of water. A precipitate that may form re-dissolves on heating

5.1.4 Heat with rapid boiling, but do not flood condenser inlet or permit vapors to rise more than halfway into condenser. Adequate refluxing is indicated by reflux rate of 40 to 50 drops/min from the condenser lip. Reflux for at least 1 hour. Discontinue heating but continue air flow. Cool for 15 minutes and drain gas washer contents into separate container. Rinse connecting tube between condenser and gas washer with distilled water, and rinse water to drained liquid, and make upto 250 mL in a volumetric flask.

## 5.2 For colorimetric measurement (By pyridine-Pyrazolone reagent)

5.2.1 Transfer 15 mL of distillate to a 50 mL beaker.

5.2.2 To prepare standard solutions for the calibration curve, use cyanide standard 1 mL = 1 mg CN. Pipette 0 (blank), 0.2, 0.5, 0.8 & 1.0 mL into 50 mL beaker and make up to 15 mL with 0.2 N sodium hydroxide solution proceed with 4.8.3 to 4.8.7, treating samples and standards in the same manner.

5.2.3 Adjust pH at 6-7 with acetic acid (4.7); transfer to 25 mL volumetric flask

5.2.4 Add 0.2 mL chloramines- T solution and mix. Allow 2 minutes for the reaction.

5.2.5 Add 5.0 mL mixed pyridine- pyrazolone reagent (4.8.7) and make up to the mark, mix allow 20 minutes for colour development

5.2.6 Read absorbance at 620 nm in a 1 cm cell

5.2.7 As a check on the distillation step, periodically process cyanide standard solutions through the complete procedure

## 5.3 For colorimetric measurement (By Pyridine-barbituric acid reagent)

5.3.1 **Preparation of standard curve:** Pipette a series of standards containing 1 to 10  $\mu\text{g CN}^-$  into 50mL volumetric flasks (0.02 to 0.2  $\mu\text{g CN}^-/\text{mL}$ ). Dilute to 40 mL with NaOH dilution solution. Use 40 mL of NaOH dilution solution as blank. Develop and measure absorbance in 10mm cells as described in 5.3.2 below for both standards and blank. For concentrations lower than 0.02  $\mu\text{g CN}^-/\text{ml}$  use 100 mm cells

Recheck calibration curve periodically and each time a new reagent is prepared

**5.3.2 Color Development:** Pipette a portion of absorption solution into a 50mL volumetric flask and dilute to 40 mL with NaOH dilution solution. Add 1 mL acetate buffer and 2 mL chloramines-T solution, stopper, and mix by inversion twice. Let stand exactly 2 min.

**5.3.3** Add 5 mL pyridine-barbituric acid reagent, dilute to volume with distilled water, mix thoroughly and let stand exactly 8min. Measure absorbance against distilled water at 578 nm. Measure absorbance of blank (0.0 mg CN<sup>-</sup>/L) using 40 mL NaOH dilution solution and procedures for color development.

## 6 Calculations

### 6.1 For colorimetric measurement (By pyridine-Pyrazolone reagent)

6.1.1 Prepare a calibration curve derived by plotting concentrations versus absorbances

6.1.2 Determine the micrograms of cyanide in the samples by comparing on calibration curve.

6.1.3 Calculate the cyanide concentration as follows:

$$\text{mg/L, CN} = \frac{AXB}{CXD}$$

Where

A= cyanide determined in mg by calibration graph

B= diluted absorbing solution in mL

C= original sample in mL, and

D= sample taken for colorimetric measurement in mL

### 6.2 For colorimetric measurement (By Pyridine-barbituric acid reagent)

Use the linear regression feature available on most scientific calculators, or compute slope and intercept of standard curve as follows:

$$M = \frac{n \sum ca - \sum c \sum a}{n \sum a^2 - (\sum a)^2}$$

$$b = \frac{\sum a^2 \sum c - \sum a \sum a c}{2n \sum a^2 - (\sum a)^2 a}$$

Where:

a= absorbance of standard solution,

c= concentration of CN<sup>-</sup> in standard, mg/L

n= number of standard solutions,

m= slope of standard curve, and

b= intercept on c axis

Include the blank concentration, 0.0 mg CN<sup>-</sup>/L and blank absorbance in the calculations above.

$$\text{CN}^-, \text{ mg/L} = (\text{ma}_1 + \text{b}) \times \frac{50}{X} \times \frac{250}{Y}$$

Where:

X= absorption solution, mL,

Y= original sample mL and

a<sub>1</sub>= absorbance of sample solution.

## 7 Reference:

7.1 IS 3025 (PART 27)-1986

7.2 APHA 4500 CN

## B. Selective Electrode Method

**1. Principle-** Cyanide in the alkaline distillate from the preliminary treatment, as given in 5.1 can be determined potentiometrically by using a cyanide in selective electrode in combination with a double junction reference electrode and a pH meter having an expanded millivolt scale, or specific ion meter.

## 2. Apparatus

- 2.1. Expanded scale pH meter or specific ion meter
- 2.2. Cyanided-ion-selective electrode
- 2.3. Reference electrode, double junction
- 2.4. Magnetic mixer with TFE coated stirring bar

## 3. Reagents

- 3.1. Stock standard cyanide solution
- 3.2. Sodium hydroxide diluent- Dissolve 1.6 gm sodium hydroxide in water and dilute to 1 litre
- 3.3. Intermediate standard cyanide solution – Dilute a calculated volume (approx. 100 mL) of stock potassium cyanide solution, based on the determined concentration, to 1000 ml with sodium hydroxide diluent. Mix thoroughly; 1 mL = 100  $\mu\text{g CN}^-$
- 3.4. Dilute standard cyanide solution- Dilute 100 mL intermediate cyanide standard solution to 1000 mL with sodium hydroxide diluents; 1.00 mL = 10.0  $\mu\text{g CN}^-$ . Prepare daily and keep in a dark, glass Stoppard bottle
- 3.5. Potassium nitrate solution- Dissolve 100 gm potassium nitrate in water and dilute to 1 liter. Adjust to pH 12 with potassium hydroxide. This is the outer filling solution for the double-junction reference electrode.

## 4. Procedure

4.1 **Calibration-** Use the dilute and intermediate standard cyanide solutions and sodium hydroxide diluent to prepare a series of three standards, 0.1, 1.0 and 10.0 mg  $\text{CN}^-/1$ . Transfer approximately 100 mL of each of these standard solutions into a 250 mL beaker pre-rinsed with a small portion of standard being tested. Immerse  $\text{CN}^-$  and double-junction reference electrodes. Mix well on a magnetic stirrer at 27°C maintaining as closely as possible the same stirring rate for all solutions.

Always progress from the lowest to the highest concentration of standard otherwise equilibrium is reached only slowly. The electrode membrane dissolves in solutions of high cyanide concentration; do not use with a concentration above 10 mg/L. After making measurements remove electrode and soak in water.

After equilibrium is reached (at least 5 min and not more than 10 min) record potential (millivolt) readings and plot  $\text{CN}^-$  concentrations versus readings on semi-logarithmic graph paper. A straight line with a slope approximately 59 mV per decade indicates that the instrument and electrodes are operating properly. Record slope of line obtained (millivolts/decade of concentration). The slope may vary somewhat from the theoretical value of 59.2 mV per decade because of manufacturing variation and reference electrode (liquid junction) potentials. The slope should be a straight line and is the basis for calculating sample concentration.

**4.2 Measurement of sample-** Place 100 mL of absorption liquid obtained into a 250 mL beaker. When measuring low cyanide concentrations, first rinse beaker and electrodes with a small volume of sample. Immerse cyanide and double-junction reference electrodes and mix on a magnetic stirrer at the same stirring rate used for calibration. After equilibrium is reached (at least 5 min and not more than 10 min) record values indicated on ion meter or found from graph prepared above. Calculate concentration as given below.

## 5. Calculation

$$\text{Cyanide, mg/L} = \frac{A \times B}{C}$$

Where

A= mg cyanide per liter found from meter reading or graph

B= total volume of absorption solution after dilution, mL; and

C= volume of original sample used in the distillation, mL

## 14. CALCIUM

### Introduction:

The average abundance of Ca in the earth's crust is 4.9%; in soils it is 0.07 to 1.7 % in streams it is about 15 mg/L; and in groundwater it is from 1 to >500 mg/L. The most common forms of calcium are calcium carbonate (calcite) and calcium-magnesium carbonate (dolomite). Calcium compounds are widely used in pharmaceuticals photography, lime, de-icing salts, pigments, fertilizers, and plasters. Calcium carbonate

solubility is controlled by pH and dissolved CO<sub>2</sub>. The CO<sub>2</sub>, HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> equilibrium is the major buffering mechanism in fresh waters. Hardness is based on the concentration of calcium and magnesium salts, and often is used as a measure of potable water quality.

The methods for the determination of Sulphide are prescribed as:-

- A. EDTA Titrimetric Method
- B. Permanganate Titrimetric Method

#### A. EDTA Titrimetric Method

**1. Scope:** This standard prescribes EDTA Titrimetric Method for determination of calcium.

**2. Principle:** In a solution containing both calcium and magnesium, calcium can be determined directly with EDTA (ethylenediamine tetra-acetic acid or its salts) when the pH is made sufficiently high (12 to 13) so that the magnesium is largely precipitated as the hydroxide and an indicator is used which combines, only with calcium.

**3. Interferences:** Under conditions of this test, the following concentrations of ions cause no interference with the calcium determination: Copper, 2 mg/L; ferrous iron 20 mg/L; ferric iron, 20 mg/L; manganese 20 mg/L; zinc 5 mg/L. Orthophosphate precipitates calcium at the pH of the test. Strontium and barium give a positive interference and alkalinity in excess of 300 mg/L may cause an indistinct end point in hard waters.

#### 4. Apparatus

4.1. Hot plate- One 30 x 50 cm heating surface is adequate

**5. Reagents:** Quality of Reagents. Unless specified otherwise, pure chemicals and distilled water shall be used in tests

5.1. Sodium Hydroxide Solution- 1N

5.2. Hydrochloric Acid- 0.1 N

5.3. Indicator solution: Any of the following indicates shall be used

5.3.1. **Murexide (ammonium purpurate) indicator solution:** This indicator changes from pink to purple at the end point. An indicator solution can be prepared by dissolving 150 mg of the dye in 100 gm of absolute ethylene glycol. Water solutions of the dye are not stable for longer than a day. A ground mixture of the dye powder and sodium chloride provides a stable form of the indicator. It is prepared by mixing 200 mg of murexide with 100 gm of solid sodium chloride and grinding the mixture to 300 to 425 microns. The titration should be performed immediately after the addition of the indicator because it is unstable under alkaline conditions. End point recognition is facilitated by the preparation of color comparison blank containing 2.0 ml of sodium hydroxide solution, 0.2 gm of solid indicator mixture (or 1 to 2 drops if a solution is used), and sufficient standard EDTA titrant (0.05 to 0.10 mL) to produce an unchanging color.

5.3.2. **Patton and Reeder's indicator solution:** This indicator solution permits the direct titration of calcium in the presence of magnesium. It produces a sharp color change from wine red to pure blue at the end point. It is prepared by mixing 1 gm of Patton and Reeder's (Eriochrome blue Black R) reagent with 100 gm of sodium sulphate or potassium sulphate.

#### 5.4. **Standard EDTA Solution- 0.01 M:**

Dissolve 3.75 gm of disodium ethylenediamine tetra-acetate, dihydrate in water and make up to 1000 mL in a volumetric flask. Standardize this with standard zinc solution. Pipette out 25 mL of standard zinc solution in a 250 mL conical flask. Adjust the pH to approximately 10 with buffer solution. Dilute to about 100 mL and add 3 to 4 drops of Eriochrome Black T indicator solution. This will give red color. Titrate with 0.01 M EDTA solution to a clear blue end point free from violet tinge. This solution will be slightly stronger than 0.01 M, dilute the solution to exactly 0.01 M by adding calculated amount of water and recheck the strength by titrating 25 mL of standard zinc solution by exactly the same manner as mentioned above. This should consume exactly 25.0 mL of standard EDTA solution.

Alternatively, calcium solution may be used for standardization of EDTA subject to the availability of certified  $\text{CaCO}_3$  according to the method given below:

Weigh 3.723 gm of dry analytical reagent grade disodium ethylene diamine tetra acetate, dihydrate, dissolve in distilled water and dilute to 1000 mL. Check the strength by standardizing against standard calcium solution. An exactly 0.01 M solution is equivalent to 0.4008 mg of calcium per milliliter

#### 5.5. **Stock Calcium Solution:**

Dry calcium carbonate ( $\text{Ca CO}_3$ ) at  $180^\circ\text{C}$  for one hour and allow it to cool in a desiccator. Suspend  $2.50 \pm 0.01$  gm of the dried material in 100 mL of water. Add slowly the minimum amount of 0.1N hydrochloric acid to dissolve the calcium carbonate (approximately 500 mL). Boil briefly to expel dissolved carbon dioxide, cool and transfer the solution quantitatively to a 1000 mL volumetric flask and dilute to mark with 0.1N hydrochloric Acid.

#### 5.6. **Standard Calcium Solution:**

Dilute 100 mL of the stock solution (5.5) to 250 mL using 0.1N hydrochloric acid. This solution is equivalent to 1.00mg of calcium carbonate or 0.400 8 gm of calcium per milliliter. Store the solution in a polyethylene bottle.

### 6. **Procedure**

#### 6.1. **Pretreatment**

Mix the sample pretreated, if so required and transfer a suitable volume (50 to 100 mL) to 250 mL conical flask or a beaker. Add 5 mL of concentrated nitric acid and evaporate on a hotplate at a slow boil to the lowest volume possible (about 15 to 20 mL) before precipitation or salting occurs. Add 5 mL of concentrated nitric acid, cover with a watch glass and heat to obtain a gentle refluxing action. Continue heating and adding concentrated nitric acid as necessary until digestion is complete as shown by a light colored clear solution. Do not let sample dry during digestion. Add 1 to 2 mL of concentrated nitric acid and warm slightly to dissolve any remaining residue. Wash down beaker walls and watch glass with water and then filter, if necessary. Transfer the filtrate to a 100 mL volumetric flask. Cool, dilute to mark and mix thoroughly. Take a portion of this solution for the determination of calcium.

## 6.2. Sample preparation

Because of the high pH used in this procedure, the titration should be performed immediately after the addition of the alkali and indicator. Use 50mL of sample or a smaller portion diluted to 50 mL so that the calcium content is about 5 to 10 mg. Analyze hard waters with alkalinity higher than 300 mg/LCaCO<sub>3</sub> by taking a smaller aliquot and diluting to 50 mL or by neutralization of the alkalinity with acid, boiling for one minute and cooling before beginning the titration.

6.2.1. Add 2.0 mL of sodium hydroxide solution or a volume sufficient to produce pH of 12 to 13. Stir. Add 0.1 to 0.2 gm of the indicator murexide-sodium chloride mixture selected (or 1 to 2 drops if a solution is used). Alternatively, approximately 1 gm of the mixture of Patton and Reeder's reagent and sodium sulphate or potassium sulphate may be used. Add EDTA titrant slowly with continuous stirring to the proper end point. Check the end point by adding 1 to 2 drop of titrant in excess to make certain that no further color change occurs.

## 7. Calculation

$$\text{Calcium (CaCO}_3\text{) mg/L} = \frac{A \times CF \times 1000}{V}$$

$$\text{Calcium (Ca}^{2+}\text{) mg/L} = \frac{A \times CF \times 1000 \times 0.4004}{V}$$

Where

A= volume in mL of EDTA solution used for titration.

CF= mass in mg of calcium equivalent to 1 mL of EDTA solution,

(X<sub>1</sub>/X<sub>2</sub> correction factor for standardize ion of EDTA)

X<sub>1</sub> = volume in mL of standard calcium solution taken for standardization

X<sub>2</sub> = volume of mL of EDTA solution used in the titration

V= volume in mL of the sample taken for the test.

## 8. Precision and accuracy

A synthetic unknown sample containing 108 mg/L of calcium, 82 mg/L of magnesium, 3.1 mg/L of potassium, 19.9 mg/L of sodium, 241 mg/L of chloride, 1.1 mg/L of nitrate, 0.25 mg/L of nitrite, 259 mg/L of sulphate and 42.5 mg/L of total alkalinity (contributed by Na HCO<sub>3</sub>) in distilled water was analyzed in 44 laboratories by the EDTA titrimetric method, with a relative standard deviation of 9.2 percent and a relative error of 1.9 percent.

### B. Permanganate Titration Method

#### 1. Principle:

The calcium present in the solution is precipitated as oxalate filtered off and washed. The washed precipitate is dissolved in dilute sulphuric acid and the oxalic acid liberated is titrated against standard potassium permanganate solution. The homogeneous precipitation approach using the urea hydrolysis method is best suited for the precipitation of calcium oxalate. Initially the pH of the solution is adjusted to approximately 1.0 by adding sufficient amount of acid. This is followed by ammonium oxalate and urea. Upon boiling the solution, the urea gradually undergoes hydrolysis and the pH rises to the point of calcium oxalate precipitation. The precipitate is filtered off immediately after formation. This eliminates the digestion period which is otherwise required. The solution must remain clear until boiling is commenced to hydrolyse the urea.

**2. Interference:** The sample should be free of interfering elements of strontium, silica, aluminium, iron, manganese, phosphate and suspended matter. Strontium may precipitate as oxalate and cause high results. In such cases, determine strontium by flame photometry. Interference of silica may be eliminated by classical dehydration procedure. Precipitate aluminum, iron, and manganese by ammonium hydroxide after treatment with persulphate. Precipitate phosphate as the ferric salt. Remove suspended matter by centrifuging or by filtration through sintered glass crucible or a cellulose acetate membrane.

### 3. Apparatus

- 3.1 Beakers with Glass Rod - 400mL capacity and cover glass.
- 3.2 Filtration Set Up - A coarse filter paper or a small filter paper supported in a Gooch crucible with suction.

### 4. Reagents

- 4.1 Quality of Reagents Unless specified otherwise pure chemicals and distilled water shall be used in the tests. NOTE - Pure Chemicals shall mean chemicals that do not contain impurities which affect the results of analysis.
- 4.2 Hydrochloric Acid - 1 N.
- 4.3 Methyl Red Indicator Solution Dissolve 100 mg of methyl red sodium salt in 100 mL of hot water or dissolve in 60 mL of ethanol dilute with 40 mL of water.
- 4.4 Ammonium Oxalate Solution- Saturated solution in water.
- 4.5 Urea
- 4.6 Dilute Sulphuric Acid - 1 N
- 4.7 Sodium Oxalate
- 4.8 Standardization of Potassium Permanganate Solution

Weigh about 1.6 gm of AR grade potassium permanganate on a watch glass, transfer it to a 1500mL beaker, add 1 litre of water, cover the beaker with a watch glass, heat the solution to boiling; boil gently for 15-30 minutes and allow the solution to cool to the laboratory temperature. Filter the solution through a funnel, containing a plug of purified glass wool, or through a Gooch crucible provided with a pad of purified asbestos, or most simply, through a sintered glass or porcelain filtering crucible. Collect the filtrate in a vessel which has previously been cleaned with chromic acid mixture and then thoroughly washed with distilled water. Store the filtered solution in a clean, glass stoppered bottle. Keep it in the dark or in an amber coloured bottle or in diffused light except while in use.

Weigh out accurately about 1.7 gm of dry sodium oxalate into a 250mL volumetric flask, dissolve it in water and make up to the mark. Pipette out 25mL of this solution into a 400mL beaker and add 150mL of 1 N sulphuric acid. Titrate this solution rapidly at room temperature with potassium permanganate solution to

be standardized while stirring, to a slight pink end point that persists 'for at least 1 minute. Do not let the temperature fall below 85°C. If necessary, warm beaker contents during titration. Repeat the titration with two more aliquots of the oxalate solution.

Calculate the normality of the permanganate solution using the following relationship:

$$\text{Normality of potassium Permanganate solution} = \frac{100 \times m_1}{67 \times V_1}$$

Where

$m_1$  = mass in gm of sodium oxalate taken, and

$V_1$  = volume in mL of the potassium permanganate solution consumed by 25mL of the oxalate solution.

## 5. Procedure

Pipette out 50 mL of the sample (containing about 10 mg of calcium) into a 250mL beaker. Add dilute hydrochloric acid drop by drop to a pH of approximately 1.0. Add a few drops of methyl red indicator solution (sufficient acid must be present in the solution to prevent the precipitation of calcium oxalate when ammonium oxalate solution is added). Add about 10 mL of saturated ammonium oxalate solution gently until the methyl red changes colour to yellow (pH 5). Filter through a coarse filter paper or with suction on a small filter paper supported in a Gooch crucible. Wash the precipitate with cold water till the filtrate is free from chloride. Transfer the filter paper and the precipitate (or the Gooch crucible and precipitate) to the original beaker, dissolve the precipitate in hot dilute sulphuric acid and titrate immediately with standard 0.05N potassium permanganate solutions as described in 4.8.

## 6. Calculation

$$\text{Calcium (as Ca) mg/L} = \frac{A \times B \times 100}{V}$$

where

$A$  = volume in mL of permanganate solution used for the titration,

$B$  = mass in mg of calcium equivalent to 1 mL of potassium permanganate solution, and

$V$  = volume of the sample taken for the test.

7. **Reference:** IS 3025 (PART 40)

## 15. PHENOLS

### 1. Introduction:

The presence of organic compounds in water and wastewater is a matter of increasing concern to the water industry, environmentalists and general public. The impact of such compounds may differ. For example, biologically oxidizable compounds will deplete oxygen in the water body and petroleum products. Oil and grease will adversely affect biological activity. Phenols detergents and other organic materials may be toxic to phyto and zoo-plankton beyond certain levels. Organic compounds may enter water environment through human waste disposal and industrial discharges. A variety of sensitive instruments have been developed to quantify the organic pollutants. BOD, COD, TOC, oil and grease, phenols, detergents-surfactants, and TFP's are certain demand parameters considered to evaluate levels of contamination.

Phenols are defined as hydroxyl derivatives of benzene, and its condensed nuclei occur in domestic and industrial wastewaters, natural wastes and potable water supplies. Odoriferous and objectionable tasting chlorophenols are formed as a result of chlorination of water containing phenol. Phenols may be present in raw water owing to the discharge of wastewaters from coke distillation plants, the petrochemical industry and numerous other industries where phenols serve as intermediates. They are also present in municipal wastewaters.

Presence of phenols may lead to objectionable taste in chlorinated drinking water and hence its monitoring is essential. Phenols can be removed from drinking water by

super-chlorination (chlorine-dioxide or chloramines treatment) ozonation and activated carbon absorption.

**2. Principle:** Most phenols react with 4-aminoantipyrine at  $\text{pH} 7.9 \pm 0.1$  in the presence of potassium ferricyanide to form a coloured antipyrine dye. This dye is extracted from water with chloroform and the absorbance is measured at 460nm. The minimum detectable quantity is 1  $\mu\text{g}$  of phenol in 460 mL distillate. This method is more sensitive and is adoptable for use in water sample containing less than 0.1 mg of phenol/litre.

**3. Interferences:** Interference such as phenol decomposition bacteria, oxidizing and reducing substances, and alkaline pH values are dealt with by acidification. If the sample has been preserved as mentioned in 2.2, further acidification is not required. The interferences may be eliminated as follows:

**3.1.1. Oxidizing agents, such as chlorine:** Remove immediately after sampling by adding excess ferrous sulphate.

**3.1.2. Sulphur compounds:** Remove by acidifying to pH 4.0 with phosphoric acid and aerating briefly by stirring. This eliminates interference of gases like hydrogen sulphite and sulphur dioxide.

#### **4. Sample Preservation and Storage**

**4.1.** Preserve and store samples at  $4^{\circ}\text{C}$  or lower but do not allow freezing unless analyzed within 4 hours after collection.

**4.2.** After acidification with phosphoric acid to pH 4 or slightly below the sample can be stored up to 4 weeks at  $4^{\circ}\text{C}$

#### **5. Procedure**

##### **5.1. Preliminary Step of Steam Distillation**

**5.1.1.** Measure 500 mL of sample into a beaker. Lower the pH to approximately 4.0 with 8.5 percent phosphoric acid. If the sample was already preserved using

phosphoric acid, omit the addition of phosphoric acid again. Transfer to the distillation apparatus made up of glass, consisting of a 1 litre borosilicate glass distilling apparatus with Graham condenser. Distil 450 mL of sample and stop the distillation. When boiling ceases, add 50 mL of warm distilled water to the distilling flask and resume distillation until 500 mL have been collected.

5.1.2. If the distillate is turbid, filter through a pre washed membrane filter.

## 6. Apparatus:

**6.1 Spectrophotometer:** For the use at 460 nm and equipped with 1 to 10 cm cells.

**6.2** Filter Funnels

**6.3** Buchner type with fritted disc

**6.4** Filter Paper

**6.5** Alternative to Buchner type funnel, use Whatman No. 40 filter paper and anhydrous sodium sulphate for filtration of chloroform phase.

**6.6** pH Meter.

**6.7** Separating Funnel -1000 mL capacity with ground glass stoppered and TFE stop cock.

**7. Reagents:** All reagents should be prepared with distilled water free from phenols and chlorine.

**7.1.1. Phenol Stock Solution.** Dissolve 1.0 gm phenol in freshly boiled and cooled distilled water and dilute to 1000 mL.

*CAUTION - 'TOXIC, HANDLE WITH CARE',*

**7.1.2. Intermediate Phenol Solution:** Dilute 10.0 mL of stock phenol solution in freshly boiled and cooled distilled water to 1000 mL. 1 mL = 10.0 µg of phenol. Prepare daily.

7.1.3. **Standard Phenol Solution:** Dilute 50.0 mL of intermediate phenol solution to 500 mL with freshly boiled and cooled distilled water. 1 mL of this solution is equivalent to 1.0 µg of phenol. Prepare this solution within 2 hours of use.

7.1.4. **Ammonium Hydroxide-** 0.5 N Dilute 35 mL of fresh concentrated ammonium hydroxide to 1 litre with distilled water.

7.1.5. **Phosphate Buffer Solution:** Dissolve 104.5 gm of potassium hydrogen phosphate ( $K_2HPO_4$ ) and 72.3 gm of potassium dihydrogen phosphate ( $KH_2PO_4$ ) in distilled water and dilute to 1 liter. The pH of the resulting solution should be 6.8.

7.1.6. **4-Aminoantipyrine Solution:** Dissolve 2.0 gm of 4-aminoantipyrine in distilled water and dilute to 100 mL. Prepare daily.

7.1.7. **Potassium Ferricyanide Solution:** Dissolve 8.0 gm of material in water and dilute to 100 mL. Filter, if necessary and store in brown glass bottle. Prepare fresh weekly.

7.1.8. Chloroform

7.1.9. Sodium Sulphate – Anhydrous

## 8. Procedure

Place 500 mL of distillate or a suitable portion containing not more than 50 µg phenol, diluted to 500 mL in 1 litre beaker. Prepare a 500 mL distilled water blank and a series of 500 mL phenol standards containing 5,10,20,30,40 and 50 µg phenol. Treat sample, blank, and standards as follows:

Add 12.0 mL of 0.5 N ammonium hydroxide and adjust pH to  $7.9 \pm 0.1$  with phosphate buffer (10 mL may be sufficient). Transfer to a 1 litre separating funnel, add 3.0 mL aminoantipyrine solution, mix well and add 3.0 mL of potassium ferricyanide and let color develop for 3 minutes. The solution should be clear and light yellow. Extract immediately with chloroform using 25 mL for 1 to 5 cm cells and 50 mL for 10 cm cell. Let chloroform settle, shake again for 10 minutes add let the chloroform settle again. Filter each chloroform extract through filter paper or fritted glass funnels containing a 5 gm layer of anhydrous sodium sulphate. Make up the volume

to 25 mL or 50 mL as the case may be. Read absorbance of sample and standards against the blank at 460 nm.

**Calibration Curve:** Prepare a standard curve by plotting the absorbance values of standards versus corresponding phenol concentrations.

For infrequent analysis, prepare only one standard phenol solution. Prepare 500 mL standard phenol solution of strength approximately equal to the phenolic content of that portion of original sample used for final analysis. Also prepare a 500mL distilled water blank. Measure absorbance of sample and standard phenol solution against the blank at 460 nm

**9. Calculation:** After obtaining the absorbance values, depending upon the volume of sample chosen for test, calculate the amount of phenol present in 1000 mL as given below:

**Using calibration curve:**

$$C \text{ phenol, } \mu\text{g/L} = \frac{C * 1000}{V}$$

Where,

C = concentration of phenol in  $\mu\text{g}$  in sample from the calibration curve.

V = volume in mL of original sample.

**10. Reference:** IS 3025(PART 43): 1992)

## 16. SODIUM

### Introduction

Sodium ranks sixth among the elements in order of abundance and is present in most natural water. The levels may vary from less than 1 mg Na/L to more than 500 mg Na/L. Relatively high concentrations may be found in brines and hard water softened by the sodium exchange process. The ratio of sodium of total cations is important in

agriculture and human pathology. Soil permeability can be harmed by a high sodium ratio. Persons afflicted with certain diseases require water with low sodium concentration. A limiting concentration of 2 to 3 mg/L is recommended in feed water destined for high pressure boilers. Ehen necessary, sodium can be removed by the hydrogen-exchange process or by distillation. Sodium compounds are used in many applications, including caustic soda, sat fertilizers and water treatment chemicals.

### Flame Photometric Method

- 1. Scope:** This standard prescribes method for determination of sodium by flame emission photometric method using flame photometry method.
- 2. Sampling & Storage:** Acidify the sample to pH 2 with concentrated nitric acid. Store all samples/stock solutions in polyethylene bottles.
- 3. Principle:** A flame photometer measures photo electrically the intensity of color imparted to the flame of a Meker- type burner where the sample is introduced into the flame under carefully standardized conditions. The intensity of color is proportional to the sodium content in the sample. Sodium is determined at a wavelength of 589 nm.

### 4. Interferences

4.1 **Radiation** interference caused by elements other than those being determined is the chief contributing factor for error in flame photometry. Of the elements encountered in these analyses, the major effect is due to interference of one alkali-metal on another. Some effects are positive and some are negative. Among the other common ions capable of causing interference are  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$  and  $\text{HCO}_3^-$  in relatively higher concentration. The foreign element effects cannot be entirely compensated without employing calibration standards closely duplicating the composition of the samples or by applying an experimentally determined correction in those instances where the sample contains a single important interference. However, the effects may be minimized by operating at the lowest practical sodium concentration range or by removal of the interfering elements. For example, aluminum has a depressing effect on alkali-metal emission, which may be of serious consequence.

4.2 Suspended matter which may interfere mechanically by clogging the burner shall be removed by filtration prior to the analysis. Organic colouring matter does not cause interference and need not be removed.

4.3 Flame photometers operating on the internal standard principle may require adding a standard lithium solution to each working standard and sample. Follow the manufacturer's instructions for the optimum lithium concentration.

4.4 Incorporate a non-ionic detergent in the standard lithium solution to assure proper aspirator function when using the internal standard type flame photometer.

## 5.0 Apparatus

5.1 **Flame photometer:** Either direct-reading or internal standard type or an atomic absorption spectrophotometer in the flame emission mode.

5.2 **Glassware:** Rinse all glasswares with dilute nitric acid (1:15) followed by several portions of deionized distilled water.

6.0 **Reagents:** Use deionized distilled water to prepare all reagents, calibrations, standards and dilution water.

6.1 **Stock sodium solution:** Dissolved in deionized distilled water, 2.542 gm of sodium chloride dried to constant mass at 140°C and make up to 1000 mL with water, 1 mL = 1mg of sodium

6.2 **Standard lithium Solution:** Weigh rapidly 6.109 gm of lithium chloride (LiCl) or 9.93 gm of lithium nitrate (LiNO<sub>3</sub>) dried overnight in an oven at 105°C. Dissolve in water and make up to 1000 mL with water, 1 mL = 1 mg of lithium.

**NOTE-** prepare a new calibration curve whenever the standard lithium solution is changed.

## 7 Procedure

**7.1 Direct Intensity Measurement:** Prepare a blank and sodium standards in stepped amounts by diluting the stock solutions described 4.4.1 and for any of the following applicable ranges: 0 to 1.0 mg/L, 0 to 10 mg/L or 0 to 100 mg/L so that within each range there are equally spaced standards in tenths of the maximum. Starting with the highest calibration standard and working towards the most dilute standard, measure emission at 589 nm for sodium. Repeat the operation with both calibration standards and samples enough number of times to secure a reliable average reading for each solution. Construct a calibration curve, by plotting emission intensity (scale reading) versus concentration of each calibration standard on a linear graph paper. Determine the Sodium concentration of the sample solution from the respective calibration curve.

**7.2 Internal Standard Measurement:** Add an appropriate volume of standard lithium solution to carefully measured volume of sample (or diluted portion), each sodium calibration standard and the blank and then follow all the steps described above.

## 8. Calculation

For direct intensity measurement and internal standard measurements

$$\text{Sodium} = \text{Sodium in mg/L in portion} \times D.$$

Where

$$D = \text{dilution Ratio.}$$

## 9. Reference: IS 3025(Part 45)

## 17. HEXAVALENT CHROMIUM

### Introduction:

The average abundance of Cr in the earth's crust is 122 ppm; in soils Cr ranges from 11 to 22 ppm; in streams it averages about 1 µg/L. Chromium is found chiefly in chrome-iron ore (FeO.Cr<sub>2</sub>O<sub>3</sub>). Chromium is used in alloys, in electroplating, and in pigments.

Chromate compounds frequently are added to cooling water for corrosion control. In natural waters trivalent chromium exists as  $\text{Cr}^{3+}$ ,  $\text{Cr}(\text{OH})^{2+}$ ,  $\text{Cr}(\text{OH})_4^-$ ; in the hexavalent form chromium exists as  $\text{CrO}_4^{2-}$  and  $\text{Cr}_2\text{O}_7^{2-}$ .  $\text{Cr}^{3+}$  would be expected to form strong complexes with amines and would be absorbed by clay minerals.

Chromium is considered nonessential for plants, but an essential trace element for animals. Hexavalent compounds have been shown to be carcinogenic by inhalation and are corrosive to tissue. The chromium guidelines for natural water are linked to the hardness or alkalinity of the water (i.e. the softer the water, the lower the permitted level for chromium). The United Nations Food and Agriculture organization recommended maximum level for irrigation water is 100  $\mu\text{g}/\text{L}$ . The U.S. EPA primary drinking water standard MCL is 100  $\mu\text{g}/\text{L}$  for total chromium.

### **1. Scope:**

This standard prescribes Diphenylcarbazide method for the determination of hexavalent chromium.

### **2. Sampling & Storage:**

The sampling bottles shall be cleaned thoroughly with dilute nitric acid (6N), prior to the final rinsing with water. The water samples should be collected and stored preferably in polypropylene bottles or chemically resistant glass containers. For the determination of dissolved chromium content filtration through 0.45  $\mu\text{m}$  membrane filter, at the time of sampling, is required. The analysis of such samples is to be carried out within 24 hours of sampling. For preservation, the samples should be acidified with concentrated nitric acid (2 mL of conc. nitric acid in 1 liter sample, just to bring down the pH below 2). The acidified samples can be stored for a few days (up to 5 days) in a refrigerator. In case, when dissolved hexavalent chromium is to be estimated adjust the pH of the samples to 8 or above with 1N NaOH and refrigerate.

**2.1. Principle:** This procedure measures only hexavalent chromium ( $\text{Cr}^{6+}$ ). The hexavalent chromium is determined spectrophotometrically by reaction with Diphenylcarbazide in acid solution. A red violet color of unknown composition is produced. The colored complex obeys Beer's law and is suitable for spectrophotometric

measurements at 540 nm. This method is applicable in range of 30 to 20000 $\mu\text{g/l}$  of chromium.

**2.2. Interferences:** The reaction with Diphenylcarbazide is nearly specific for chromium. Hexavalent molybdenum and mercury salts will react to form color with the reagent, but the intensities are much lower than that for chromium at the specified pH. Concentrations as high as 200 mg/L of Mo or Hg can be tolerated. Pentavalent vanadium interferes, strongly but concentrations up to 10 times that of chromium will not cause trouble. Potential interference from permanganate is eliminated by prior reduction with sodium azide. Iron in concentrations greater than 1 mg/L may produce a yellow color, but the color is not strong and no difficulty is encountered normally if the absorbance is measured spectrophotometrically at 540 nm.

### 2.3. Apparatus

2.3.1. Spectrophotometer, for use at 540 nm, with a light path of 1 cm.

2.3.2. pH meter

2.3.3. Standard volumetric glassware

*NOTE: Thoroughly cleaned glassware with nitric acid or hydrochloric acid to remove chromium traces. Do not use glassware previously treated with chromic acid. New and unscratched glassware will minimize chromium absorption on glassware during oxidation procedure.*

### 2.4. Reagents

2.4.1. **Stock Chromium Solution:** Dissolve 141.4 mg of  $\text{K}_2\text{Cr}_2\text{O}_7$  in water and dilute to 100 mL (1.0 mL = 500  $\mu\text{g}$  of Cr).

2.4.2. **Standard Chromium Solution:** dilute 1 mL of stock Chromium Solution to 100mL; (1 mL = 5  $\mu\text{g}$  of Cr).

2.4.3. **Nitric acid-** Concentrated (16N).

2.4.4. **Sulphuric acid** - Concentrated 36 N; 1:1;6 N and 0.2 N.

2.4.5. **Phosphoric acid-** concentrated (41N).

2.4.6. **Methyl orange indicator solution** – Dissolve 50 mg of methyl orange in 100mL of Distilled water.

2.4.7. **Ammonium hydroxide-** concentrated (14N)

**2.4.8. Potassium permanganate solution-** Dissolve 4 gm of  $\text{KMnO}_4$  in 100mL Distilled water.

**2.4.9. Sodium Azide solution-**Dissolve 0.5gm of sodium Azide ( $\text{NaN}_3$ ) in 100mL distilled water.

**2.4.10. Diphenylcarbazide Solution-** Dissolve 250 mg of 1, 5-diphenylcarbazide in 50 mL acetone. Store in an amber colored bottle. Discard when the solution becomes discolored.

**2.4.11. Acetone**

## **2.5. Procedure**

**2.5.1. Preparation of calibration Curve:** Pipette out measured volumes of standard chromium solution ranging from 2 to 20 mL (to give standards for 10-100  $\mu\text{g}$  of Cr), into 100 mL beakers. Make up the volume to about 50 mL with water. Use 0.2 N  $\text{H}_2\text{SO}_4$  and a pH meter to adjust the pH of each solution to  $1.0 \pm 0.3$ . Transfer quantitatively each of these solutions into 100 mL volumetric flasks and add 2.0 mL of diphenylcarbazide solution. Dilute to 100 mL with water, mix and let these stand for 5 to 10 min for full color development. Meanwhile, prepare a reagent blank in an identical manner using 10mL of water. Measure the absorbance of the standard solutions at 540 nm, using reagent blank as reference solution. Construct a calibration curve by plotting absorbance values against micrograms ( $\mu\text{g}$ ) Cr in 100 mL of the final volume.

**2.5.2. Determination of Hexavalent Chromium ( $\text{Cr}^{6+}$ ):** Pipette out a portion of filtered sample (filtered through 0.45  $\mu\text{m}$  membrane filter), containing 10 to 100  $\mu\text{g}$  of Cr into a 100 mL beaker. Make up the volume to about 50 mL with water. Adjust pH of this solution to  $1.0 \pm 0.3$  using 0.2 N  $\text{H}_2\text{SO}_4$ , and a pH meter. Transfer quantitatively into a 100 mL volumetric flask, add 2.0 mL of diphenylcarbazide solution. Dilute to 100 mL water, mix well and allow to stand for 5 to 10 min. Measure absorbance at 540 nm, using reagent blank as reference solution. From the absorbance data, determine the micrograms of chromium present in 100 mL of the final solution using the calibration curve.

**2.6. Calculation:**

$$\text{Soluble Hexavalent Chromium (Cr}^{6+}\text{) mg /L} = \frac{\mu\text{g of Cr (in 100 mL of the final solution )}}{V}$$

Where

V = volume in mL, of the sample used.

**2.7. References**

2.7.1. IS: 3025 part 52 -2003- Methods of Sampling and Test (Physical and chemical) for water and Waste Water: Chromium & Hexavalent chromium.

**18. TOTAL SOLIDS****Introduction:**

The term 'Solid' refers to the matter either filterable or non- filterable that remains as residue upon evaporation and subsequent drying at a defined temperature. Further categorization depends upon the temperature employed for drying and ignition. Different forms of solids are defined on the basis of method applied for their determination. Solids may affect water or effluent quality adversely in number of ways. Water with high dissolved solids may include an unfavorable physiological reaction in the transient consumer and generally are of inferior palatability. Highly mineralized waters are unsuitable for many industrial applications. High suspended solids in waters may be aesthetically unsatisfactory for such purposes as bathing. Analysis of total solids are important to decide upon the various unit operations and processes in physical and biological wastewater treatment and to asses its performance evaluation. For assessing compliance with regulatory agency, wastewater effluent limitations for various form of solids act as indicating parameters.

**1. Principle-** The sample is evaporated in a weighed dish on a stream-bath and is dried to a constant mass in an oven either at 103-105°C or 179-181°C. Total residue is calculated from increase in mass.

**NOTE-** In general by evaporating and drying water samples at 103-105°C or 179-181°C values are obtained which conform more closely to those obtained by summation of individually determined mineral salts.

## 2. Interferences

**2.1** Highly mineralized waters containing significant concentration of calcium, magnesium, chloride and/or sulphate may be hygroscopic. These may require prolonged drying, desiccation and rapid weighing. However, prolonged drying may also cause loss of constituents, particularly nitrates and chlorides.

**2.2** A large amount of residue in the evaporating basin may crust over and entrap water preventing its evaporation during drying. For this reason, the volume of the sample should be adjusted so that the residue left after drying should be about 100-200 mg.

## 3. Apparatus

**3.1 Evaporating Dish-** of 90 mm diameter, 100 mL capacity made of platinum, nickel, porcelain, silica or borosilicate glass. Platinum is suitable for all tests. Nickel is satisfactory if residue is not to be ignited. Porcelain, silica and glass may be used for samples with a pH value less than 9.0.

**3.2 Steam-Bath.**

**3.3 Drying Oven-** Drying oven with thermostatic control for maintaining temperature up to  $180 \pm 2^\circ\text{C}$ .

**3.4 Desiccator-** Provided with a color indicating desiccants.

**3.5 Analytical Balance-** 200gm capacity and capable of weighing to nearest 0.1 mg.

**4. Sample handling and Preservation-** Preservation of the samples is not practical. Analysis should begin as soon as possible. Refrigeration or chilling to  $4^\circ\text{C}$ , to minimize microbiological decomposition of solids is recommended.

## 5. Procedure

5.1 Heat the clean evaporating dish to 180°C for 1 hour. Cool desiccate, weigh and store in desiccator until ready for use.

5.2 Select volume of the sample which has residue between 25 and 250 mg, preferably between 100 and 200 mg. This volume may be estimated from values of specific conductance. To obtain a measurable residue; successive aliquots of sample may be added to the sample dish.

5.3 Pipette this volume to a weighed evaporating dish placed on a steam-bath. Evaporation may also be performed in a drying oven. The temperature should be lowered to approximately 98°C to prevent boiling and splattering of the sample. After complete evaporation of water from the residue, transfer the dish to an oven at 103-105°C, or 179-181°C and dry to constant mass, that is, till the difference in the successive weighing is less than 0.5 mg. Drying for a long duration (usually 1 to 2 hours) is done to eliminate necessity of checking for constant mass. The time for drying to constant mass with a given type of sample when a number of samples of nearly same type are to be analyzed should be determined by trial.

5.4 Weigh the dish as soon as it has cooled avoiding residue to stay for long time as some residues are hygroscopic and may absorb water from desiccant which may not be absolutely dry.

6. **Calculation-** Calculate the total residue using following equation:

$$\text{Total residue, mg/L} = \frac{1000 M}{V}$$

Where, M= Mass in mg of total residue, and

V= volume in mL of the sample.

7. **Report-**Report in whole numbers for less than 100 mg/L and above 100 mg/L to three significant figures. Report the temperature of determination also.

## 8. References

8.1. IS:3025 part 15 – 1984 (Reaffirmed 2003)- Methods of Sampling and Test (Physical and chemical ) for water and Waste Water : (Total Solids)

## 19. NITRITE

Nitrite in water is either due to oxidation of ammonium compounds or due to reduction of nitrate. As an intermediate stage in the nitrogen cycle it is unstable. A usual concentration in natural water is in the range of some tenths of mg/L. Higher concentrations are present in industrial wastes. Sewage and in biologically purified effluents and in polluted streams. In chlorinated supplies, levels of nitrite are often less than the limit of detection, i.e. 0.005mg/L  $\text{NO}_2^-$  N but high levels may occur in unchlorinated water. Very high nitrite levels are usually associated with water of unsatisfactory microbiological activity.

**1. Principle-** Nitrite is determined through formation of a reddish purple azo dye produced at pH 2.0 to 2.5 by coupling diazotized sulphanalic acid with N- (1 naphthyl)-ethylene diamine dihydrochloride (NED dihydrochloride). The color obeys Beer's law up to 180  $\mu\text{g/L}$  with 1 cm path length at 543 nm.

**2. Interference-** Nitrogen trichloride ( $\text{NCl}_3$ ) imparts a false red color when normal order of reagents addition is followed. It can be minimized by adding NED dihydrochloride first and then sulphanalic acid. Ions like  $\text{Sb}^{3+}$ ,  $\text{Au}^{3+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Bi}^{3+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{PtCl}_6^{2-}$  interfere. Cupric ions cause low results.

### 3. Apparatus

**3.1 Spectrophotometer or photometer-** for use at 543 nm in case of spectrophotometer or photometer having a green filter and having maximum absorbance near 540 nm.

**3.2 Nessler tubes-**matched, 50 mL capacity.

### 4 Reagents

**4.1 Nitrite free water-** If the distilled water is not nitrite free, prepare as follows:

**4.1.1** Add to 1 liter of distilled water, a small crystal each of potassium permanganate and barium hydroxide or calcium hydroxide. Redistill in a borosilicate glass bottle.

**4.1.2** Add 1 mL of concentrated sulphuric acid and 0.2 mL of manganese sulphate (36.48 gm  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ /100 mL) solution to each 1 liter of distilled water and make pink with 1 to 3 mL of potassium permanganate solution (400 mg/L). Redistill as in 4.1.1 above. Use this water in making all reagents and dilutions.

**4.2 Sulphanilamide reagent-** Dissolve 5 gm of the material in a mixture of 50 mL of concentrated hydrochloric acid and 300 mL of water. Dilute to 500 mL with water. The reagent is stable for several months.

**4.3 NED dihydrochloride-** Dissolve 500 mg of the material in 500 mL of water. Store in colored bottle in dark. Replace monthly or when it turns dark brown in color.

**4.4 Hydrochloric acid-** 1:3.

**4.5 Sodium oxalate-** 0.05 N. Dissolve 3.350 gm of sodium oxalate (primary standard grade) in 1000 mL of water.

**4.6 Ferrous ammonium sulphate-** 0.05 N. Dissolve 19.607 gm of ferrous ammonium sulphate in 20 mL of concentrated sulphuric acid and water and dilute to 1 litre. Standardize with standard dichromate.

**4.7 Stock nitrite solution-** Dissolve 1.232 gm of sodium nitrite in water and dilute to 1000 mL (1 mL = 250  $\mu\text{g}$  of N). Preserve with 1 mL of chloroform. Standardize using sodium oxalate (4.5) and standard potassium permanganate solution.

**4.8 Intermediate nitrite solution** – Calculate the volume, G, of stock nitrite solution required for intermediate nitrite solution from  $G = 12.5/A$ , where A is the stock solution in mg/L. Dilute the volume G to 250 mL with water (1.00 mL = 50.0  $\mu\text{g}$  N).

**4.9** Standard nitrite solution- Dilute 10.00 mL of intermediate nitrite solution to 1000 mL with water (1.00 mL = 0.500 µg N).

## 5 Procedure

**5.1** If the sample is turbid, filter through a 0.45 µm membrane filter. To 50.0 mL of clear sample neutralized to pH 7 or to a portion diluted to 50mL add 1 mL of sulphanimide solution. Let the reagent react for 2 to 8 minutes. Add 1.0 mL of NED dihydrochloride solution and mix immediately. Let stand for at least 10 minutes but not more than 2 hours. Measure absorbance at 543 nm. As a guide, use the following light paths for the indicated nitrite nitrogen concentrations:

Light Path Length, cm	Nitrite Nitrogen, µg/L
1	2 - 25
5	2 - 6
10	2

Run parallel checks frequently against nitrite standards.

**5.2 Color standards for visual comparison** – Prepare a suitable series of visual color standards in Nessler tubes by adding the following volumes of standard nitrite solutions and diluting to 50 mL with water: 0, 0.1, 0.2, 0.4, 0.7, 1.0, 1.4 1.7, 2.0 and 2.5 mL, corresponding, respectively to 0, 1.0, 2.0, 4.0, 7.0, 10, 14, 17, 20 and 25 µg of nitrite per liter. Develop color as described above. Compare samples to visual standards in matched Nessler tubes between 10 and 120 minutes after adding NED dihydrochloride reagent. Select the concentration where the sample tube color matches the standard tube color.

## 5.3 Calculation

**5.3.1** Calculate nitrite nitrogen from the following:

$$\text{Nitrite Nitrogen (as NO}_2\text{-N) per liter} = \frac{\text{mg NO}_2\text{-N (in 52 mL final volume)}}{\text{mL of sample}}$$

## 6 Reference

6.1 IS:3025 part 34 – 1988 (Reaffirmed 2003)- Methods of Sampling and Test (Physical and chemical ) for water and Waste Water: Nitrogen

## 20. MINERAL OIL

**1.0 Principle:** The sample of water is extracted with tetrachloroethylene (TCE) as extraction solvent followed by analysis by infra-red (IR) spectrometry using peak heights at  $2930 \pm 5 \text{ cm}^{-1}$ .

**2.0 Interference:** Thoroughly rinse cuvette with distilled TCA while reading blank(s) and sample(s).

### 3.0 Reagents and Material:

#### 3.1 Reagents:

3.1.1 TCE

3.1.2 Anhydrous Sodium Sulphate dried at 200 to 250°C.

3.1.3 HCl- 35% GR grade

#### 3.2 Glassware:

3.2.1 Separating Funnel of 2L

3.2.2 Volumetric Flask

3.2.3 Micro Pipettes

3.2.4 Measuring cylinder

#### 3.3 Apparatus/Instruments:

3.3.1 Filter paper – Whatman No. 40 or equivalent

3.3.2 Cells – Infra- red, silica/quartz (1 or 5cm path length; for low range, 5cm path length will be appropriate).

3.3.3 FTIR- Fourier Transformer Infrared Spectrometer

#### 4.0 Preparation of reagents:

**4.1 Preparation of Stock solution**-Prepare a reference mixture by volume of 37.5 percent isooctane, 37.5 percent hexadecane and 25 percent benzene. Store in a stoppered 100mL volumetric flask to prevent loss of evaporation.

**4.2 Preparation of Calibration Solutions**- Take 20mL of TCE in 100mL volumetric flask, stopper it and weigh it. Add 1mL standard to it and obtain its exact weight by difference. Make up the volume with solvent and calculate the exact concentration in *mg/L*. Prepare the calibration standards in the range of 0-50 *mg/L*.

#### 5.0 Procedure:

**5.1** Acidify sample using HCl to pH ~ 2.

**5.2** Transfer 1L sample to 2L separating funnel.

**5.3** Add 20mL TCE and shake vigorously for about 2 min. and leave the funnel undisturbed till layers separate. If emulsion will form, shake gently for 5 to 10 min or centrifuge.

**5.4** Collect lower organic layer in glass vials after passing through anhydrous sodium sulphate and repeat the extraction step four times. Collect and combine the layers.

**5.5** Prepare the method blank with reagent grade water adopting same extraction process (without reference oil).

**5.6** Scan the standards and samples from 3200 to 2700 $\text{cm}^{-1}$ . Measure absorbance of standards at peak height at  $2930 \pm 5 \text{ cm}^{-1}$  on solvent TCE background. Similarly, measure the absorbance of samples at same peak height on blank background.

**5.7** Prepare a calibration curve of absorbance against the concentration of standards. If the absorbance exceeds more than that of highest standard sample, dilute the sample as required.

#### 6.0 Calculations:

Mineral oil = Mass of oil in the extract as determined from calibration curve  
(mg) x 1000

**7.0 Quality control- Spiked** Sample must be added with batch of 10 samples for quality check.

## **8.0 References -**

**8.1** Clause (6) of IS 3025 (part 39), Amendment No. 2

## **21A. ESTIMATION OF ANIONS BY ION CHROMATOGRAPHY**

**1. Scope** – This procedure is used for the determination of Anions ( $F^-$ ,  $Cl^-$ ,  $NO_2^-$ ,  $Br^-$ ,  $NO_3^-$ ,  $PO_4^-$ ,  $SO_4^-$ ) in Drinking Water

**2. Principle-** A water sample is injected into a stream of effluent and passed through a series of ion exchangers. The anions of interest are separated on the basis of their relative affinities for a low capacity, strongly basic anion exchanger (guard & analytical columns). The separated anions are directed through a suppressor device that provides continuous suppression of effluent conductivity and enhance analyte response. In the suppressor the separated anions are converted to their highly conductive acid forms while the conductivity of the effluent is greatly decreased. The separated anions in their acid forms are measured by conductivity. They are identified on the basis of retention time as compared to standards. Quantitation is by measurement of peak area or peak height.

**3. Interference-** Sample with higher concentration may interfere which may lead to tailing and heading of the Peaks. Flush column before next analysis.

## **4. Materials and Reagents -**

**4.1** Reagent water ASTM type 1 water

**4.2** Sulphuric Acid

**4.3** Eluent Solution: Appropriate to column used to resolve target anions. Prepare 1.7 mM sodium bicarbonate and 1.8 mM sodium carbonate as eluent. Dissolve 0.5712 gm sodium bicarbonate 0.7632 gm sodium carbonate in water and make up 4 L. Degas eluent before use either by vacuum filtration to simultaneously remove particle greater than 0.45 micron or by purging with helium for 10 mins.

**4.4** Regeneration solution: Required with some types of suppressors. See manufacturer's recommendations.

**4.5** Standard Anion solutions:- Stock standards solutions traceable to NIST are available from a number of commercial suppliers (Merck / sigma) or alternatively prepare from salt.

## **5. Apparatus :**

**5.1** Ion Chromatograph, including an injection valve, a sample loop, Guard column, analytical column, suppressor device, a temperature - compensated small volume conductivity cell and detector and an electronic peak integrator or chromatography data acquisition system. Use an ion chromatography capable of delivering 2 to 5 mL eluent per minute at a pressure of 5600 to 28000 KPa (800 Psi).

**5.2** Analytical Column: Any commercially available anion-exchange column capable of resolving Fluoride, Bromide, Chloride, Nitrate, Nitrite, Phosphate and Sulphate is acceptable.

**5.3** Guard Column: Identical to separator column to protect analytical column from fouling by particulates or organics.

**5.4** Suppressor device: Place this ion- exchange based device between column and detector to reduce background conductivity of the eluent and enhance conductivity of the target analytes. Several such devices with different operational principles are available commercially; any that provides the required sensitivity and baseline stability may be used.

## **6. Procedure :**

**6.1** *System Equilibration*: Turn on ion chromatograph and adjust eluent flow rate to manufacturer's recommendations for the column/ eluent combination being used. Adjust detector to desired setting (10 $\mu$ s to 30  $\mu$ s) and let system come to equilibrium (15-20 min). A stable base line indicates equilibrium conditions. Adjust detector offset to zero out eluent conductivity. If regenerant is used with the suppressor, adjust flow rate to manufacturer's specifications.

## **7. Calibration:**

**7.1** Inject standards containing a single anion or a mixture and determine approximate retention times. Observed times vary with conditions. Retention time always is in order  $F^-$ ,  $Cl^-$ ,  $NO_2^-$ ,  $Br^-$ ,  $NO_3^-$ ,  $HPO_4^-$  and  $SO_4^-$ . Inject at least three different concentrations for each anion to be measured. Construct a calibration by plotting peak height or area versus concentration using appropriate software. Verify calibration curve with a mid range check standard from a source independent of that of the calibration standards. Check validity of existing calibration curves daily with a mid range calibration standard. Result should be within 10% of original curve at mid range. Recalibrate whenever the detector setting, eluent or regenerant is changed. To minimize the effect of the water dip on  $F^-$  analysis. Eliminate water dip by diluting sample with eluent or by adding concentrated eluent to the sample to give the same concentration as in eluent. If sample adjustments are made, adjust standards and blanks identically.

**7.2** If linearity is established ( $r \geq 0.99$ ) over the calibration range the average response factor is acceptable. Record peak height or area for calculations of the response factor, RF.  $HPO_4^{2-}$  is non linear below 1 mg/L.

**7.3** *Sample analysis:* If sample is collected with an auto sampler that does not automatically filter samples, remove particulates by filtering through a prewashed 0.45  $\mu m$  pore membrane. With either manual or automated injection, flush loop with several volumes of sample. Take care to prevent carryover of analytes from samples of high concentration. After last peak has appeared and detector signal has returned to base line, another sample can be injected.

## **8. Calculation :**

Determine concentration of each anion, in milligrams per liter, by referring to the appropriate calibration curve. Alternatively, when the response is shown to be linear, use the following equation:

$$C = H \times RF \times D$$

Where

C = mg anion/L

H = Peak height or area

RF = response factor = concentration of standard/ height (or area) of standard

D = dilution factor

## 9. Special Precautions:

**9.1** Do not inject any high concentration analyte samples or standards into the column. This may overload the column thereby leading to fronting or tailing of peaks. Only diluted samples and flow concentration standards are preferred.

**9.2** Even if high concentration sample were injected, they have to be completely flushed out of the column before next analysis.

**9.3** After the final analysis the column has to be flushed for 15-20 minutes with mobile phase to remove any ion present in the column. The column should not be stored with any ion.

## 10. References

**10.1** APHA 4110

## 21 B. Estimation of Bromate using Ion Chromatography

### Introduction

Bromate in drinking water is the most commonly formed during ozonation of bromide containing waters for disinfection purposes. Bromate can also be introduced into drinking water as a contaminant in the sodium hypochlorite used for disinfection. The determination of the concentration of bromate is correspondingly a prerequisite measure for the elimination of risks for consumers' health.

### 1. Principle:

**1.1** Sample pretreatment is carried out in order to remove ozone and solids, and to reduce chloride, sulfate, carbonate, hydrogen carbonate and metals present by use of cation exchangers.

**1.2** Measurement of bromate is made in the range 0.5 µg/L to 1000 µg/L, with or without preconcentration.

**1.3** Liquid chromatographic separation of bromate is carried out either by means of a separator column or after elution of bromate from a concentrator column, if used. An anion exchange resin is used as the stationary phase, and usually, aqueous solutions of salts of weak mono- and dibasic acids as eluent (Annex A)

**1.4** A conductivity detector (CD) with chemical suppression is used. A UV detector (wavelength = 190 nm to 205 nm) is suitable to confirm the CD results only.

**NOTE** When using conductivity detectors it is essential that the eluents have a sufficiently low conductivity. For this reason, conductivity detectors are combined with a suppressor device (cation exchanger) which reduces the conductivity of the eluent and transforms the sample species into their respective acids. UV detection measures absorbance directly.

**1.5** Strongly retained ions (e.g. nitrate, phosphate, sulphate) are removed from the separator column, e.g. by flushing the separator column with a more concentrated eluent.

## **2. Reagents**

Use only reagents of recognized analytical grade. Carry out weighing of the reagents with an accuracy of  $\pm 1$  % of the nominal mass, unless stated otherwise.

**2.1 Water**, complying with ASTM type-1.

**2.2 Sodium hydrogen carbonate**, NaHCO<sub>3</sub>.

**2.3 Sodium carbonate**, Na<sub>2</sub>CO<sub>3</sub>.

**2.4 Disodium tetraborate decahydrate**, Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub> · 10 H<sub>2</sub>O

**2.5 Boric acid**, H<sub>3</sub>BO<sub>3</sub>.

**2.6 Potassium bromate**, KBrO<sub>3</sub>.

**2.7 Nitric acid**,  $c(\text{HNO}_3) = 0,1\text{mol/L}$ .

**2.8 Sulfuric acid**,  $\rho(\text{H}_2\text{SO}_4) = 1,84\text{ g/mL}$ .

**2.9 Ethylenediamine**, C<sub>2</sub>H<sub>8</sub>N<sub>2</sub>

## 2.10 Eluents

Degas all water used for eluent preparation. Take steps to avoid any renewed air pick-up during operation (e.g. by helium sparging). In order to minimize the growth of bacteria or algae, store the eluents in the dark and renew every 3 day.

See annex A for examples of eluents.

Two different types of eluent are used.

**2.10.1 Eluent of Type 1**, of a lower concentration level (for example see clause A.1) to be applicable for the separation of bromate.

**2.10.2 Eluent of Type 2**, of a higher concentration level (for example see clause A.2) to be applicable to remove strongly retained ions (e.g. nitrate, phosphate) from the concentrator and separator column.

The choice of eluent is dependent on the choice of column and detector.

A selection of reagents for common eluents is presented in 2.2 to 2.5.

### **2.11 Bromate stock standard solution**, $\rho(\text{BrO}_3^-) = 1\,000\text{ mg/L}$

Dry approximately 1.5 gm of potassium bromate for at least 1 h at  $105\text{ °C} \pm 5\text{ °C}$ . Store the dried solid in a desiccator.

Dissolve  $1.306\text{ gm} \pm 0.001\text{ gm}$  of the dried potassium bromate in approximately 800 mL of water in a 1000mL volumetric flask, and dilute to volume with water (2.1). Store the solution at  $2\text{ °C}$  to  $6\text{ °C}$  in polyethylene or glass bottles and renew it every 12 months.

Alternatively, use commercially available stock solutions of the required concentration

### **2.12 Bromate standard solutions.**

### 2.12.1 General

Depending upon the concentrations expected, prepare the following standard solutions of different bromate concentrations from the stock standard solution (2.11). Note the possible risk of changes in concentration caused by interaction with the vessel material increases with decreasing bromate concentration. Store the standard solutions in polyethylene or glass bottles.

### 2.12.2 Bromate Standard Solution I

The mass concentration of this solution is  $\rho(\text{BrO}_3^-) = 100\text{mg/L}$ .

Pipette 10.0 mL of stock standard solution (2.11) into a 100 mL volumetric flask, and dilute to volume with water.

Store the solution at 2°C to 6°C in polyethylene or glass bottles and renew every 6 months.

### 2.12.3 Bromate Standard Solution II

The mass concentration of this solution is  $\rho(\text{BrO}_3^-) = 1\text{mg/L}$ .

Pipette 1.0 mL of Standard Solution I (2.12.2) into a 100 mL volumetric flask, dilute to volume with water.

Store the solution at 2°C to 6°C in polyethylene or glass bottles and renew every 3 months.

### 2.13 Bromate calibration solutions.

Depending on the bromate concentration expected in the sample, use the Bromate Standard Solution I or II to prepare five to ten calibration solutions distributed over the expected working range as evenly as possible.

For example, proceed as follows for the range 0.5  $\mu\text{g/L}$  to 5.0  $\mu\text{g/L}$   $\text{BrO}_3^-$ :

Pipette, into a series of 100 mL volumetric flasks, the following volumes: 50  $\mu\text{L}$ , 100  $\mu\text{L}$ , 150  $\mu\text{L}$ , 200  $\mu\text{L}$ , 250  $\mu\text{L}$ , 300  $\mu\text{L}$ , 350  $\mu\text{L}$ , 400  $\mu\text{L}$ , 450  $\mu\text{L}$  or 500  $\mu\text{L}$  of Bromate Standard Solution II and dilute to volume with water.

The concentrations of  $\text{BrO}_3^-$  in these calibration solutions are: 0.5  $\mu\text{g/L}$ , 1.0  $\mu\text{g/L}$ , 1.5  $\mu\text{g/L}$ , 2.0  $\mu\text{g/L}$ , 2.5  $\mu\text{g/L}$ , 3.0  $\mu\text{g/L}$ , 3.5  $\mu\text{g/L}$ , 4.0  $\mu\text{g/L}$ , 4.5  $\mu\text{g/L}$  and 5.0  $\mu\text{g/L}$  respectively.

Prepare the calibration solutions on the day of use.

### 2.14 Regeneration solutions

The choice is dependent on the type of metal clean-up columns or suppressor devices. Therefore, follow the column manufacturer's instructions for the exact composition of the regeneration solutions (for examples of compositions see annex B)

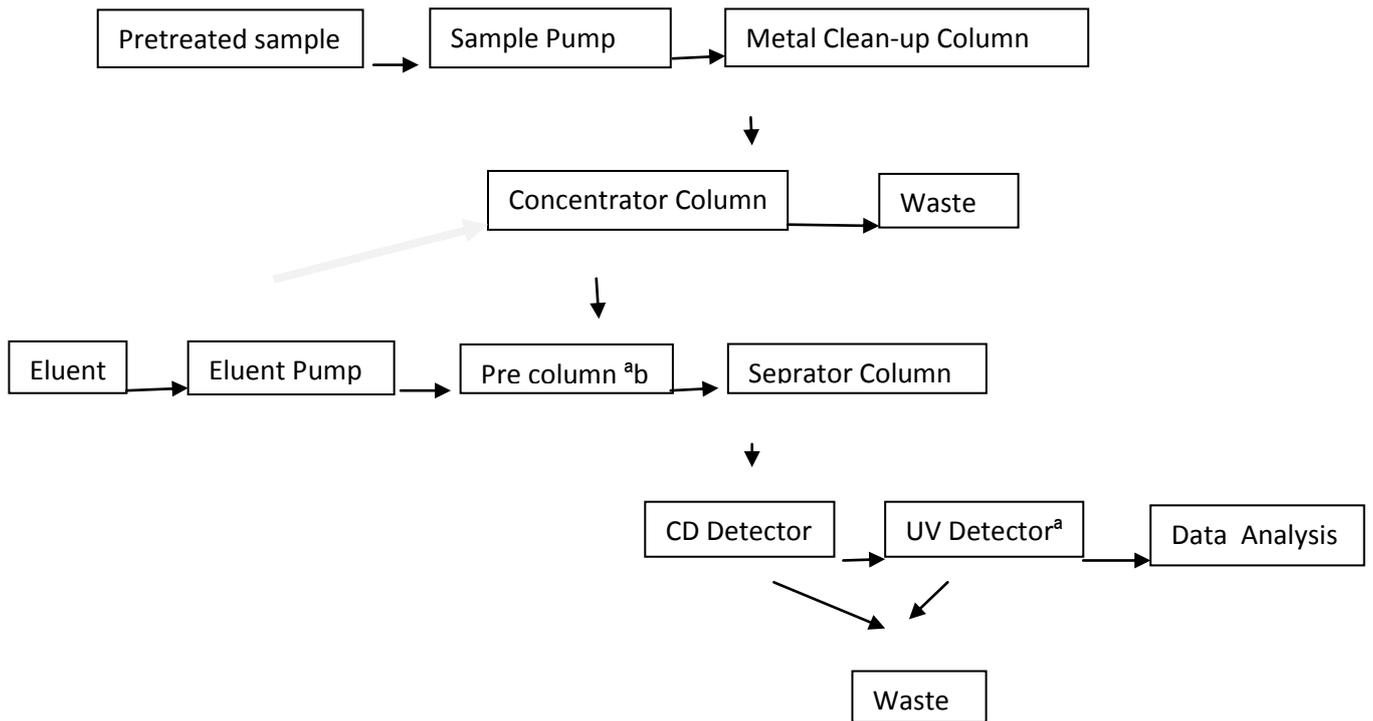
### 2.15 Blank solution

1. Fill a 100 ml volumetric flask with water.

## 3. Apparatus

**3.1 Ion chromatographic system**, in general, it shall consist of the following components :

- a) eluent reservoirs, and a degassing unit for two eluents;
- b) pump, suitable for step gradient technique;
- c) sample delivery device (e.g. sample pump) including a sample injection system incorporating a sample loop of appropriate volume (e.g. 0.05 mL to 2 mL) or autosampler device;
- d) column-switching valves (e.g. 6-port-valve) including a device for timing and controlling valves and pump;
- e) concentrator column (may be required for low concentrations);
- f) separator column with the specified separating performance;
- g) conductivity detector with an anion suppressor device assembly;
- h) UV detector (e.g. spectrophotometer: 190 nm to 400 nm);
- i) recording device (e.g. recorder, integrator with printer, PC with software for data acquisition and evaluation).



**Fig;4.1 Schematic representation of an ion chromatography system, including on-line preconcentration system**

### 3.2 Cartridges

- \_ cation exchanger in the Ag-form (cartridge);
- \_ cation exchanger in the Ba-form (cartridge);
- \_ cation exchanger in the H-form (cartridge);
- \_ optional: metal clean-up column for on-line use;
- \_ cartridges with non-polar phases to be used for sample preparation (e.g. polyvinylpyrrolidone).

## 4. Sample pretreatment

### 4.1 General requirements

**4.1.1** Treat the calibration solutions (2.13) and the blank solution (2.15) in the same manner as the sample solution (see Figure 5.1, steps 1 to 5 and 5.1.2 to 5.2.6).

**4.1.2** Avoid any further formation of bromate after sampling by immediately removing any ozone present. For example, add 50 mg of ethylenediamine to 1L of sample immediately after sampling (see Figure 5.1, step 1).

**4.1.3** Store the sample in a polyethene vessel at 2°C to 6°C until analysis is carried out.

## **4.2 Elimination of dissolved sulfate, chloride, carbonate, hydrogen carbonate and metals**

**4.2.1** If considered necessary, remove chloride, sulfate, carbonate and hydrogen carbonate with the aid of the ion-exchange cartridges described, by carrying out the following elution steps with a constant flow rate of between 1 mL/min and 1.5 mL/min (see Figure 5.1 , steps 2 to 4). Rinse ion-exchange cartridges with water before use according to the manufacturer's instructions. In addition, purge the sample with an inert gas (e.g. N<sub>2</sub> or He) to eliminate carbon dioxide (formed from carbonate and hydrogen carbonate salts).

The presence of nitrate, chloride, carbonate and sulfate may affect the capacity of the concentrator column and may lead to poor recovery of bromate. This effect should be checked for every matrix by standard addition, and the recovery of bromate should be in the range 80 % to 120 %.

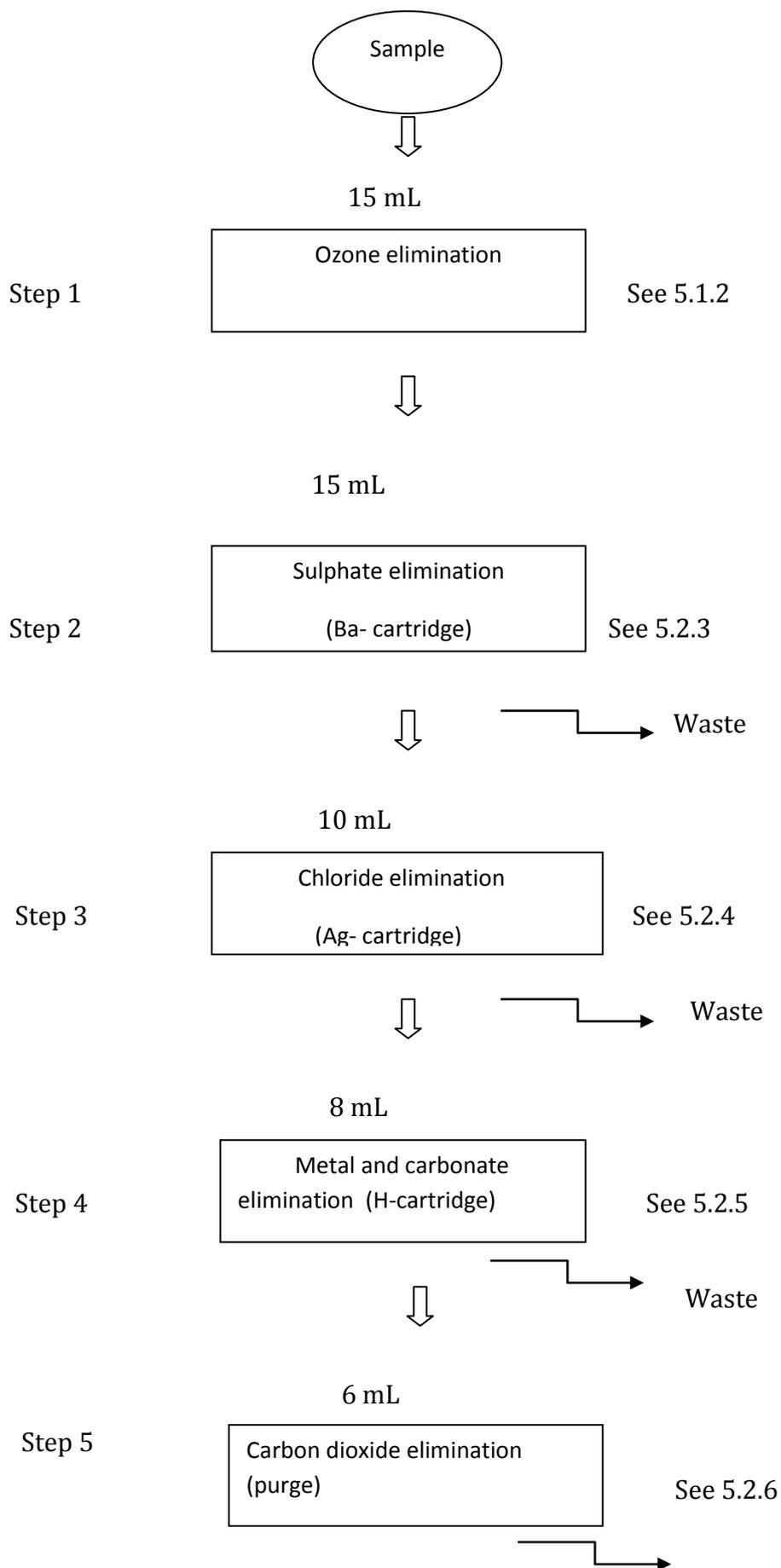




Figure 5.1 — Pretreatment steps for samples, calibration and blank solution

**4.2.2** Prepare the samples as described in 4.1

**4.2.3** Pass approximately 15 mL of the sample through a cation exchanger in the Ba-form (cartridge, 3.2) to remove dissolved sulfate ions from the sample (see Figure 5.1, step 2). Discard the first portion of 2 mL.

**4.2.4** Pass approximately 10 mL of the remaining sample through a strongly acid cation exchanger in the Ag-form (cartridge, 3.2) to remove dissolved halides from the sample (see Figure 5.1, step 3). Discard the first portion of 2 mL.

**4.2.5** Pass approximately 8 mL of the remaining sample through a cation exchanger in the H-form (cartridge, 3.2) to remove dissolved metal ions, carbonate and hydrogen carbonate from the sample (see Figure 5.1, step 4). Discard the first portion of 2 mL.

**NOTE** Alternatively, connect all the clean-up columns/cartridges (see Figure, steps 2 to 4). In this case, the first 3 mL of eluate of the sample leaving the last cartridge should be discarded (see Figure 5.1, step 4).

## 5. Procedure

### 5.1 General

Set up the ion chromatographic system (3.1) according to the instrument manufacturer's instructions.

Run the starting eluent; once the baseline is stable analysis can begin.

If metal clean-up, concentrator columns and suppressor devices are being used, regenerate according to the instrument manufacturer's instructions before use. Perform the calibration as described in 5.2. Measure the samples and blank solution as described in 5.5.

## 5.2 Calibration

Inject the pretreated bromate calibration solutions. In calculating concentrations, use the characteristic that the area (or height) of the peak (signal) is proportional to the concentration of the bromate ion.

When the analytical system is first evaluated, and at intervals afterwards, establish a calibration function for the measurement as follows.

- a) Prepare the bromate calibration solutions.
- b) Analyse the calibration solutions chromatographically.
- c) Use the data obtained to calculate the regression line.
- d) Subsequently, verify the continuing validity of the established calibration function (5.5).

## 5.3 Measurement of bromate

After establishing the calibration function, inject the pretreated sample into the chromatograph and measure the peaks. Identify the bromate peak by comparing the retention time with that of bromate in the standard solutions.

Take into account the fact that the retention times can be dependent on concentration and matrix. If a concentrator column is not used, the use of a precolumn is recommended, especially for the injection of waters strongly contaminated with organics. It serves to protect the analytical separator column.

### NOTE 1

In general, two different types of precolumns can be used: those containing the same resin material as the analytical separator column and those packed with a macroporous polymer.

If the bromate concentration of the sample exceeds the calibration range, dilute the sample and re-analyse it.

If the bromate concentration of the sample falls short of the calibration range, establish a separate calibration function for the lower working range, preconcentrate the bromate solution, if necessary, and analyse it.

#### **NOTE 2**

There are a number of available systems which can carry out a preconcentration step. The manufacturer's instructions for each system should be followed.

If matrix interferences are expected, use the method of standard addition to confirm the results (verify the peaks by comparing the retention time of the spiked sample with those of the original sample).

Measure the blank solution in the same manner

.

#### **5.4 Confirmation of bromate results**

If required, confirm bromate concentrations greater than 2 µg/L by UV detection (wavelength= 200 nm) as follows.

a) Calculate the bromate slopes of the CD ( $b_1$ ) and the UV detector ( $b_2$ ) from calibration experiments according to 5.2, and calculate factor  $B$  using equation (1).

$$B = b_1/b_2 \quad (1)$$

Where

$b_1$  is the slope of the calibration function for the CD detector, e.g. mm. l/mg;  $\mu\text{V} \cdot \text{s} \cdot \text{l}/\text{mg}$ ;

$b_2$  is the slope of the calibration function for the UV detector, e.g. mm .l/mg;  $\mu\text{V} \cdot \text{s} \cdot \text{l}/\text{mg}$ .

- b) Analyse a bromate calibration solution, e.g.  $\rho(\text{BrO}_3) = 10 \mu\text{g}/\text{L}$ .
- c) Record the measured CD value ( $Y_1$ ) and the measured UV value ( $Y_2$ ) for bromate.
- d) Calculate the ordinate intercept for CD ( $a_1$ ) and UV ( $a_2$ ) according to 5.2.
- e) Calculate the response ratio  $r$  [equation (2)]:

$$r = [Y_1 - a_1 / Y_2 - a_2] \quad (2)$$

where

$r$  is the response ratio;

$Y_1$  is the measured value (size of signal) for the CD detector, in terms of peak height or peak area, respectively in millimetres or microvolt seconds;

$Y_2$  is the measured value (size of signal) for the UV detector, in terms of peak height or peak area, respectively in millimetres or microvolt seconds;

$a_1$  is the ordinate intercept of the calibration function (calculated blank) for the CD detector, e.g. mm,  $\mu\text{V} \cdot \text{s}$  (5.2);

$a_2$  is the ordinate intercept of the calibration function (calculated blank) for the UV detector, e.g. mm,  $\mu\text{V} \cdot \text{s}$  (5.2).

$r$  shall be in the range of  $B \pm 10 \%$ . If  $r$  exceeds the range of 10 %:

- use the method of standard addition;
- calculate  $r$  again; if  $r$  still exceeds the range of  $B \pm 10 \%$  then mark the result as "bromate not confirmed".

## 5.5 Validity check of the calibration function

In order to verify the continuing validity of the calibration function, measure standard solutions of different bromate concentrations in the lower and upper thirds of the working range. Carry this out after the set-up procedure and after each sample series at least, but in any case after 20 measurements. Recalibrate, if necessary.

## 6. Calculation

Calculate the mass concentration,  $\rho$ , in micrograms per litre, of bromate in the solution using the peak areas or peak heights (5.3).

Take into account all of the dilution steps.

## 7. Interference

**7.1** The presence of nitrate, chloride, carbonate and sulfate may affect the capacity of the concentrator column and lead to poor recovery of bromated.

**7.2** The presence of chloride, sulfate, carbonate and hydrogen carbonate can cause interference with the determination of bromate. Depending on the column utilized, other ions may interfere; this should be checked.

**7.3** Metals present (e.g. barium and silver ions released from sample pretreatment steps) will bind to the resin material of concentrator and separator columns, resulting in a loss of performance. Metal ions may be eliminated with the aid of a metal clean-up column or special exchangers

**7.4** The interference of some organic acids with the determination of bromate was checked and found not to be significant to the concentrations tested.

**7.5** Solid particles and organic compounds such as mineral oils, detergents and humic acids shorten the life-time of the concentrator and separator column.

## **8. References**

**8.1** ISO 15061, First Edition, 2001-07-01: Water quality— Determination of dissolved Bromate — Method by liquid chromatography of ions

**Annex A**  
**(Informative)**  
**Eluents**

**A.1 Examples of eluents of Type 1 to be used for bromate separation**

**A.1.1 General**

Solutions of sodium hydroxide and salt solutions of weakly dissociated acids, such as sodium carbonate/sodium hydrogen carbonate, sodium hydrogen carbonate and sodium tetraborate, can be used.

**A.1.2 Sodium hydrogen carbonate concentrate I**

The addition of the following eluent concentrate is appropriate for the eluent preparation

**(A.1.3):**

Place 58.8 gm of sodium hydrogen carbonate (see 2.2) into a 1000 mL volumetric flask, dissolve in water (2.1) and dilute to volume with water (2.1).

The solution contains 0.7 mol/L of sodium hydrogen carbonate. This solution is stable for several months if stored at 2°C to 6°C.

**A.1.3 Sodium hydrogen carbonate eluent I**

The following eluent is applicable for the determination of bromate:

Pipette 5 mL of the concentrate (A.1.2) into a 5000 mL volumetric flask and dilute to volume with water (2.1).

The solution contains 0,000 7 mol/L of sodium hydrogen carbonate. The solution should be renewed every 3 d.

### **A.1.4 Borate eluent I**

The following eluent is applicable for the determination of bromate:

Place 76.3 gm of disodium tetraborate decahydrate (2.4) into a 5000 mL volumetric flask, dissolve in approximately 4000 mL of water (2.1), and dilute to volume with water (2.1).

The solution contains 0.04 mol/L of disodium tetraborate. The solution should be renewed every 3 d.

## **A.2 Examples of eluent of Type 2 to be used to remove strongly retained ions**

### **A.2.1 General**

Solutions of sodium hydroxide and salt solutions of weakly dissociated acids, such as sodium carbonate/sodium hydrogen carbonate, sodium hydrogen carbonate and sodium tetraborate, can be used.

### **A.2.2 Sodium carbonate/sodium hydrogen carbonate concentrate II**

The addition of the following eluent concentrate is appropriate for the eluent preparation (A.2.3).

Place 10.6 gm of sodium carbonate (2.3) and 8.4 gm of sodium hydrogen carbonate (2.2) into a 1000 mL volumetric flask, dissolve in water (2.1) and dilute to volume with water (2.1).

The solution contains 0.1 mol/L of sodium carbonate and 0.1 mol/L of sodium hydrogen carbonate. The solution is stable for several months if stored at 2°C to 6°C.

### **A.2.3 Sodium carbonate/sodium hydrogen carbonate eluent II**

The following eluent is applicable for the removal of strongly retained ions from the separator column.

Place 50 mL of the concentrate (A.2.2) into a 500 mL volumetric flask and dilute to volume with water (2.1).

The solution contains 0.01 mol/L of sodium carbonate and 0.01 mol/L of sodium hydrogen carbonate. The solution should be renewed every 3 d.

#### **A.2.4 Borate eluent II**

The following eluent is applicable for the removal of strongly retained ions from the separator column.

Place 477 gm of disodium tetra borate decahydrate (2.4) into a 5000 mL volumetric flask, dissolve in approximately 4000 mL of water (2.1), and dilute to volume with water (2.1).

The solution contains 0.25 mol/L of disodium tetra borate. The solution should be renewed every 3 d.

**Annex B**  
**(Informative)**  
**Regeneration solutions**

**B.1 General**

If metal clean-up columns and/or suppressor devices are used, these should be regenerated on a regular basis. The timing of the regeneration shall be determined for each system, and the following regenerant solutions may be used.

**B.2 Example of a regenerant solution for metal clean-up columns**

The use of nitric acid (2.7) is applicable for the regeneration of metal clean-up columns.

**B.3 Example of a regenerant solution for suppressor devices**

The use of sulfuric acid is applicable for the regeneration of suppressor devices.

Pipette 3.5 mL of sulfuric acid (2.8) into approximately 4000 mL of water (2.1) in a 5000 mL volumetric flask and dilute to volume with water (2.1).

The solution contains 0.012 6 mol/L of H<sub>2</sub>SO<sub>4</sub> and is stable for several months if stored at ≤ 30°C.

## CHAPTER III: METALS

### 1. By Atomic Absorption Spectrophotometer (AAS)

**Introduction:** Atomic Absorption Spectrophotometer (AAS) is a technique used for determining the concentration of a particular metal element within a sample. In this, light of a specific wavelength is passed through the atomic vapor of an element of interest, and measurement is made of the attenuation of the intensity of the light as a result of absorption.

Quantitative analysis by AAS depends on: accurate measurement of the intensity of the light and the assumption that the radiation absorbed is proportional to atomic concentration

AAS can be used to analyze the concentration of over 62 different metals in water. There are three techniques of AAS to determine metals in water:

- i) **Flame AAS:** Flame Atomic Absorption Spectroscopy is a fast and easy technique with an extremely high sensitivity. In this, the sample is atomized in the flame, through which radiation of a chosen wavelength (using a hollow cathode lamp) is sent. The amount of absorbed radiation is a quantitative measure for the concentration of the element to be analyzed. This technique is used for the determination of metals in water where the requirements are at ppm levels.
  
- ii) **Electrothermal AAS - Graphite Furnace:** Graphite furnace atomic absorption spectrometry is a highly sensitive spectroscopic technique that provides excellent detection limits for measuring concentrations of metals in water. This technique is used for the determination of metals in water where the requirements are at very low levels (ppb).

iii) **Cold Vapour AAS - FIAS Flame AAS:** Since atoms for most AA elements cannot exist in the free, ground state at room temperature, heat must be applied to the sample to break the bonds combining atoms into molecules. The only notable exception to this is mercury. Free mercury atoms can exist at room temperature and, therefore, mercury can be measured by Cold Vapour atomic absorption without a heated sample cell.

## A. By Flame AAS

1. **Scope** – This procedure is used for the determination of Zn, Mg, Ca, Na, in all type of water up to parts per million (ppm) levels.

2. **Principle-** Sample is aspirated into flame and atomized. A light beam is directed through the flame, into a monochromator and onto a detector that measures the amount of light absorbed by the atomized element in the flame. The amount of energy at the characteristic wavelength absorbed in the flame is proportional to the concentration of element in the sample over a limited concentration range.

3. **Interference-** Molecular absorption and light scattering caused by solid particles in the flame can erroneously cause high absorption values resulting in positive errors. When such phenomena occurs use background correction to obtain accurate values.

### 4. Materials & Reagents

4.1. Nitric Acid (Suprapure 70%)

4.2. Reagent water (ASTM type-1 Complying the parameters given in Annexure))

4.3. Standard of metal - Stock standard solutions traceable to NIST are available from a number of commercial suppliers (Merck / Sigma) or alternatively prepare from NIST traceable salt.

4.4. Air

**4.5.** Acetylene- Standard commercial grade acetylene. Acetone, which is always present in acetylene cylinders can be prevented from entering and damaging the burner head by replacing a cylinder when its pressure has fallen to 689 kPa (100psi) acetylene.

**5. Apparatus :**

**5.1.** Atomic Absorption Spectrometer (AAS) with flame technique.

**6. Procedure :**

**6.1. Sample Preparation:**

Colorless & transparent water samples with turbidity of <1.0 can be directly analyzed by AAS for total metals after acidifying with conc. HNO<sub>3</sub> (1.5 mL HNO<sub>3</sub> /L of water). Sample digestion is not required.

**6.2. Standard Preparation:**

Prepare a series of standard Zinc solutions in the optimum concentration range by appropriate dilution from its stock solution with ASTM type 1 water containing 1.5 mL conc. HNO<sub>3</sub>/L, using the following formula.

$$N_1V_1 = N_2V_2$$

**6.3. Sample Analysis:**

Prepare standard solutions of at least three different concentrations, measure their absorbance and prepare a calibration curve. Then measure the absorbance of the test solution adjusted in concentration to a measurable range and determines the concentration of the element from the calibration curve. Before sample analysis, rinse nebulizer by aspirating ASTM type-1 containing 1.5 mL conc. HNO<sub>3</sub>/L.

**6.4. Calculation :**

Read concentration directly from the instrument readout and multiply by appropriate dilution factor if sample has been diluted. Report the result in mg/L.

***Metal conc. in sample (mg/L) = Sample conc. from instrument (mg/L) X Dilution factor (if any)***

**7. References :**

- 7.1. APHA 3111 B
- 7.2. IS 3025 Part 49

**B. By Electrothermal AAS - Graphite Furnace**

**1. Scope** – This procedure is used for the determination of micro quantities of metals like aluminum, antimony, arsenic, barium, beryllium, cadmium, chromium, cobalt, copper, iron, lead, manganese, molybdenum, nickel, selenium, silver and tin in water samples up to parts per billion (ppb) level.

**2. Principle-** Electrothermal atomic absorption spectroscopy is based on the same principle as direct flame atomization, but, an electrically heated atomizer or graphite furnace replaces the standard burner head. A discrete sample volume is dispensed into the graphite sample tube. Typically, determinations are made by heating the sample in three or more stages. First, a low current heats the tube to dry the sample. The second or charring stage destroys organic matter and volatilizes other matrix components at an intermediate temperature. Finally, the current heats the tube to incandescence and in an inert atmosphere, atomizes the element being determined. Additional stages frequently are added to aid in drying and charring, and to clean and cool the tube between samples. The resultant ground-state atomic vapour absorbs monochromatic radiation from the source. A photoelectric detector measures the intensity of transmitted radiation. The inverse of transmittance is related logarithmically to the absorbance, which is directly proportional to the number density of vaporized ground-state atom over a limited concentration range.

**3. Interference**

**3.1** Electro thermal atomization determinations may be subjected to significant interferences from molecular absorption as well as chemical and matrix effect. Molecular absorption may occur when components of sample matrix volatilize during

atomization, resulting in broadband absorption. When such phenomena occurs use background correction to compensate for this interference.

**3.2** Matrix modification can be useful in minimizing interference and increasing analytical sensitivity. Chemical modifier generally modifies relative volatilities of matrix and metal. Some modifiers inhibit metal volatilization, allowing use of higher ashing/charring temperatures and increasing efficiency of matrix removal

#### **4. Reagents**

**4.1** Reagent water (ASTM type-1)

**4.2** Nitric acid (Suprapure 70%)

**4.3** Standard of metals - stock standard solutions traceable to NIST are available from a number of commercial suppliers (Merck & Sigma) or alternatively prepare from reagent as mentioned in APHA 3111B

**4.4** Air- Air is cleaned & dried through a suitable filter to remove oil, water and other foreign substances. The source may be a compressor or commercially bottled gas.

**4.5** Argon Gas- Minimum purity 99.99%

**4.6** Matrix modifier

**4.6.1** Magnesium nitrate- (10000 mg/L): Dissolve 10.5 gm  $Mg(NO_3)_2 \cdot 6H_2O$  in water. Dilute to 100 mL.

**4.6.2** Palladium nitrate- (4000 mg/L): Dissolve 8.89 gm  $Pd(NO_3)_2 \cdot H_2O$  in water. Dilute to 1000 mL.

**4.6.3** Phosphoric acid- (10% v/v): Add 10 mL conc.  $H_3PO_4$  to water. Dilute to 100 mL.

**4.6.4** Nickel nitrate - (10000 mg/L): Dissolve 4.96 gm  $Ni(NO_3)_2 \cdot 6H_2O$  in water. Dilute to 100 mL.

**4.6.5** Citric acid - (4%): Dissolve 40 gm citric acid in water. Dilute to 1L.

#### **5. Apparatus :**

**5.1** Atomic Absorption Spectrometer with Graphite furnace technique.

#### **6. Procedure**

**6.1 Sample Preparation :-** Same as given under method by Flame AAS

**6.2 Standard Preparation:** - Prepare a series of standard metal solution in the optimum concentration range by appropriate dilution from their stock solution with ASTM type 1 water containing 1.5 mL conc.  $\text{HNO}_3/\text{L}$ , using the following formula.

$$N_1V_1 = N_2V_2$$

**6.3 Determination by instrument:** - Inject a measured portion of pretreated sample into the graphite furnace .Use same volume as was used to prepare the calibration curve. Add modifier immediately after adding the sample, preferably using an automatic sampler or a micropipette. Use the same volume and concentration of modifier for all standards and samples as given in the table. Dry, char, and atomize according to the preset program in the method. Repeat until reproducible results are obtained.

Compare the average absorbance value or peak area to the calibration curve to determine concentration of the element of interest .Alternatively, read results directly if the instrument is equipped with this capability. If absorbance (or concentration) or peak area of the sample is greater than absorbance (concentration) or peak area of the most concentrated standard solution, dilute sample and reanalyze.

**Table – Potential Matrix Modifiers for Graphite furnace AAS**

<b>Modifier</b>	<b>Analyses for which modifier May be Useful</b>
1500 mg Pd/L + 100mg $\text{Mg}(\text{NO}_3)_2$	Ag, As, Cu, Mn, Hg, Sb, Se, Tl
00-2000 mg Pd/L + Reducing agent (Citric acid 1-2% preferred)	As, Cd, Cr, Cu, Fe, Mn, Hg, Ni, Pb, Sb
5000 mg $\text{Mg}(\text{NO}_3)_2/\text{L}$	Co, Cr, Fe, Mn,
100-500 mg Pd/L	As ,
50 mg Ni/L	As , Se , Sb
2% $\text{PO}_4$ + 1000mg $\text{Mg}(\text{NO}_3)_2$	Cd , Pb
<b><i>Use 10<math>\mu\text{L}</math> modifier/ 10 <math>\mu\text{L}</math> sample</i></b>	

## 7. Calculation:

Read concentration directly from the instrument readout and multiply by appropriate dilution factor if sample has been diluted. Report the result in mg/L

Metal conc. in sample (mg/L) = Sample conc. from instrument (mg/L) X Dilution factor (if any)

## 8. References : APHA 3113 B

### C. By Cold Vapour AAS - FIAS Flame AAS

**1. Scope** – This procedure is used for the determination of mercury in Packaged Drinking water, drinking water and waste water. Lower detection limit of 0.2 ppb can be achieved using this technique.

**2. Principle-** The flameless atomic absorption procedure is a physical method based on the absorption of radiation at 253.7 nm by mercury vapour. The mercury is reduced to the elemental state and aerated from solution in closed system .The mercury vapour passes through a cell positioned in the light path of Mercury lamp of an atomic absorption spectrophotometer. Absorbance (peak height) is measured as a function of mercury concentration and recorded.

### 3. Interference

**3.1** Interferences of sulfide, chloride, copper and tellurium have been reported for waters containing Organic compounds which have broad band UV absorbance are confirmed interferences. The concentration levels for interferants are difficult to define. This suggests that quality control procedures must be strictly followed.

**3.2** Volatile materials (e.g., chlorine) which absorb at 253.7 nm will cause a positive Interference. In order to remove any interfering volatile materials, the dead air space in the digestion vessel (BOD bottle) should be purged before addition of stannous chloride solution.

**3.3** Low level mercury sample preparation, digestion, and analysis may be subject to environmental contamination if performed in areas with high ambient backgrounds where mercury was previously employed as an analytical reagent in analyses such as total Kjeldahl nitrogen (TKN) or chemical oxygen demand (COD).

#### **4. Reagents**

**4.1** Reagent water (ASTM type-1)

**4.2** Nitric acid (Suprapure 70%)

**4.3** Sulphuric acid (Suprapure 98%)

**4.4** Hydrochloric acid (Suprapure 30%)

**4.5** Stannous chloride solution: Dissolve 25 gm of stannous chloride ( $\text{SnCl}_2$ ) in water containing 50 mL of Conc. Hydrochloric acid and dilute to 250 mL. If a suspension forms, stir the reagent continuously during use.

**4.6** Sodium chloride & Hydroxylamine sulphate solution: Dissolve 12 gm of Sodium chloride and 12 gm of Hydroxylamine sulphate ( $(\text{NH}_2\text{OH})_2 \cdot \text{H}_2\text{SO}_4$ ) in water and dilute to 100mL.

**4.7** Potassium permanganate solution: Dissolve 5 gm of Potassium permanganate in water and dilute to 100mL.

**4.8** Potassium per sulphate solution: Dissolve 5 gm of Potassium per sulphate in water and dilute to 100mL

**4.9** Standard Solution of Mercury: Stock standard solutions traceable to NIST are available from a number of commercial suppliers (Merck /Sigma) or alternatively prepared by dissolving 1.354 gm Mercuric chloride in about 700 mL of water. Add 10mL of conc. Nitric acid and make up the volume to 1000 mL (1 mL = 1mg Hg).

**4.10** Air- Air is cleaned & dried through a suitable filter to remove oil, water and other foreign substances. The source may be a compressor or commercially bottled gas.

**4.11** Argon Gas- Minimum purity 99.99%

## 5. Apparatus :

5.1 Atomic Absorption Spectrometer (AAS) with Mercury vapour generation assembly (FIAS).

## 6. Procedure

### 6.1 Sample Preparation

Transfer 100mL of sample or portion diluted to 100 mL containing not than 5.0 µg/L and a blank of 100 mL water to a 300mL BOD bottles. Add 5 mL Sulphuric acid (98%) and 2.5 mL of Nitric acid (70%) to each bottle .Add 15 mL Potassium permanganate solution to each bottle and let it stand for at least 15 minutes. Add 8 mL Potassium persulphate solution to each bottle and heat for 2 h in a water bath at 95°C. Cool and add 6 mL of Sodium chloride-hydroxylamine sulphate to reduce the excess permanganate.

### 6.2 Standard Preparation :-

Prepare a series of standard metal solution in the optimum concentration range (1µg/L to 5µg/L) by appropriate dilution from their stock solution with ASTM type 1 water using the following formula.

$$N_1V_1 = N_2V_2$$

### 6.3 Analysis of sample

Prepare calibration curve by transfer 100mL of each of the 1.0, 2.0 and 5.0µg/L standard of mercury solution and a blank of 100 mL water to a 300mL BOD bottles. Add 5 mL Sulphuric acid and 2.5 mL Nitric acid to each bottle. Add 15 mL Potassium permanganate solution to each bottle and let it stand for at least 15 min. Add 8 mL Potassium persulphate solution to each bottle and heat for 2 h in a water bath at 95°C. Cool and add 6 mL of Sodium chloride-hydroxylamine sulphate to reduce the excess permanganate. After decolourization add 5 mL stannous chloride solution and attached the to the aeration apparatus forming a closed system. As mercury is volatilized and carried into the absorption cell, absorbance will increase to a maximum within a few seconds. As soon as recorder returns approximately to the base line, remove stopper holding the aeration frit from the reaction bottle and replace with a bottle containing

distilled water .Flush the system for few seconds and run the next standard in the same manner. Construct a standard calibration curve by plotting absorbance (peak height) versus concentration in  $\mu\text{g}$ .

After calibration digested sample is analyzed in the same manner as standard after addition of stannous chloride solution.

#### **6.4 Calculation :**

Mercury concentration in sample ( $\mu\text{g/L}$ ) =  $\mu\text{g Hg in aliquot} \times 1000/\text{mL of aliquot}$

### **7. Special Precautions**

**7.1** Place a vent about 15 to 30 cm above the flame/ furnace to remove fumes and vapours to protect the laboratory personnel from toxic vapours, corrosion of instrument and flame stability from room drafts.

**7.2** Acetylene gas represents an explosive hazard in the laboratory. Follow instrument manufacturer's directions in plumbing and using this gas. Do not allow contact of gas with copper, brass with >65% copper, silver or liquid mercury. For this it is not advisable not to use copper or brass regulators, or fittings

**7.3** Furnace technique is highly sensitive and extremely susceptible to contamination; extra care in sample handling and analysis may be required.

**7.4** Mercury compounds are highly toxic if swallowed, inhaled, or absorbed through the skin. Analyses should be conducted in a laboratory exhaust hood. The analyst should use chemical resistant gloves when handling concentrated mercury standards.

**7.5** The acidification of samples containing reactive materials may result in the release of toxic gases, such as cyanides or sulfides. Acidification of samples should be done in a fume hood.

### **8. References :**

**8.1** APHA

**8.2** USEPA 245.1

## 2. By ICP-MS

The Inductively Coupled Plasma coupled with a mass spectrometer give very high sensitivity for the determination of multi elements and even isotopes. This technique has the ability to detect very low levels (parts per billion) of most elements in a sample. Rapid data acquisition and data reduction enable the measurement of large numbers of samples in a short period of time. ICP-MS is the technique of choice for trace element analysis of waters. High precision is achieved by using multiple internal standards.

**1. Scope** – This procedure is used for the determination of metals like aluminium, antimony, arsenic, barium, cadmium, chromium, copper, iron, lead, manganese, molybdenum, nickel, selenium and silver in water up to ppb level.

**2. Principle-** Sample material introduced into an argon –based, high-temperature radio-frequency plasma, usually by pneumatic nebulization. Energy transfer from plasma to the sample stream causes desolvation, atomization, and ionization of target elements. Ions generated by the energy –transfer processes are extracted from the plasma through a differential vacuum interface, and separated on the basis of their mass –to-charge ratio by a mass spectrometer. The mass spectrometer usually is of the quadrupole or magnetic sector type. The ions passing through the mass spectrometer are counted, usually by an electron multiplier detector, and the resulting information processed by a computer based data handling system.

**3. Interference:** Several interference sources may cause inaccuracies in the determination of trace elements by ICP-MS. These are:

**3.1 Isobaric elemental interferences** - Are caused by isotopes of different elements which form singly or doubly charged ions of the same nominal mass-to-charge ratio and which cannot be resolved by the mass spectrometer in use. All elements determined by this method have, at a minimum, one isotope free of isobaric elemental interference. of the analytical isotopes only molybdenum-98 (ruthenium) and selenium-82 (krypton) have isobaric elemental interferences. If alternative analytical isotopes

having higher natural abundance are selected in order to achieve greater sensitivity, an isobaric interference may occur. All data obtained under such conditions must be corrected by measuring the signal from another isotope of the interfering element and subtracting the appropriate signal ratio from the isotope of interest. A record of this correction process should be included with the report of the data. It should be noted that such corrections will only be as accurate as the accuracy of the isotope ratio used in the elemental equation for data calculations. Relevant isotope ratios should be established prior to the application of any corrections.

**3.2 Abundance sensitivity** - Is a property defining the degree to which the wings of a mass peak contribute to adjacent masses. The abundance sensitivity is affected by ion energy and quadrupole operating pressure. Wing overlap interferences may result when a small ion peak is being measured adjacent to a large one. The potential for these interferences should be recognized and the spectrometer resolution adjusted to minimize them.

**3.3 Isobaric polyatomic ion interferences** - Are caused by ions consisting of more than one atom which have the same nominal mass-to-charge ratio as the isotope of interest, and which cannot be resolved by the mass spectrometer in use. These ions are commonly formed in the plasma or interface system from support gases or sample components. Most of the common interferences have been identified<sup>3</sup>, and these are listed in Table 2 together with the method elements affected. Such interferences must be recognized, and when they cannot be avoided by the selection of alternative analytical isotopes, appropriate corrections must be made to the data. Equations for the correction of data should be established at the time of the analytical run sequence as the polyatomic ion interferences will be highly dependent on the sample matrix and chosen instrument conditions. In particular, the common <sup>82</sup>Kr interference that affects the determination of both arsenic and selenium, can be greatly reduced with the use of high purity krypton free argon.

**3.4 Physical interferences** - Are associated with the physical processes which govern the transport of sample into the plasma, sample conversion processes in the

plasma, and the transmission of ions through the plasma mass spectrometer interface. These interferences may result in differences between instrument responses for the sample and the calibration standards. Physical interferences may occur in the transfer of solution to the nebulizer (e.g., viscosity effects), at the point of aerosol formation and transport to the plasma (e.g., surface tension), or during excitation and ionization processes within the plasma itself. High levels of dissolved solids in the sample may contribute deposits of material on the extraction and/or skimmer cones reducing the effective diameter of the orifices and therefore ion transmission. Dissolved solids levels not exceeding 0.2% (w/v) have been recommended<sup>3</sup> to reduce such effects. Internal standardization may be effectively used to compensate for many physical interference effects. Four Internal standards ideally should have similar analytical behavior to the elements being determined.

**3.5 Memory interferences** - Result when isotopes of elements in a previous sample contribute to the signals measured in a new sample. Memory effects can result from sample deposition on the sampler and skimmer cones, and from the buildup of sample material in the plasma torch and spray chamber. The site where these effects occur is dependent on the element and can be minimized by flushing the system with a rinse blank between samples. The possibility of memory interferences should be recognized within an analytical run and suitable rinse times should be used to reduce them. The rinse times necessary for a particular element should be estimated prior to analysis. This may be achieved by aspirating a standard containing elements corresponding to 10 times the upper end of the linear range for a normal sample analysis period, followed by analysis of the rinse blank at designated intervals. The length of time required to reduce analyte signals to within a factor of 10 of the method detection limit, should be noted. Memory interferences may also be assessed within an analytical run by using a minimum of three replicate integrations for data acquisition. If the integrated signal values drop consecutively, the analyst should be alerted to the possibility of a memory effect, and should examine the analyte concentration in the previous sample to identify if this was high. If a memory interference is suspected, the sample should be reanalyzed after a long rinse period. In the determination of mercury, which suffers from severe memory effects, the addition of 100 µg/L gold will effectively rinse 5 µg/L mercury in approximately two minutes. Higher concentrations will require a longer rinse time.

#### 4. Materials and Reagents

Reagents may contain elemental impurities that might affect the integrity of analytical data. Owing to the high sensitivity of ICP-MS, high-purity reagents should be used whenever possible. All acids used for this method must be of ultra high-purity grade. Suitable acids are available from a number of manufacturers or may be prepared by sub-boiling distillation. Nitric acid is preferred for ICP-MS in order to minimize polyatomic ion interferences. Several polyatomic ion interferences result when hydrochloric acid is used, however, it should be noted that hydrochloric acid is required to maintain stability in solutions containing antimony and silver. When hydrochloric acid is used, corrections for the chloride polyatomic ion interferences must be applied to all data.

4.1 Nitric acid (specific gravity 1.41).

4.2 Hydrochloric acid (specific gravity 1.19).

4.3 Ammonium hydroxide (specific gravity 0.902).

4.4 Reagent water - All references to reagent grade water in this method refer to ASTM Type I water (Complying the parameter given in Annexure)

4.5 Standard Stock Solutions - Stock standards and tuning solution may be purchased from a reputable commercial source or prepared from ultra high-purity grade chemicals or metals (99.99 - 99.999% pure). All salts should be dried for one hour at 105°C, unless otherwise specified. Stock solutions should be stored in FEP bottles.

5. **Apparatus** : Inductively coupled plasma/mass spectrometer (ICP-MS)

#### 6. Procedure

6.1 **Sample Preparation**: - Colorless & transparent, water samples with turbidity of <1.0 can be directly analyzed by ICP-MS for total metals after acidifying with HNO<sub>3</sub> (1.5 mL HNO<sub>3</sub> /L of water). Sample digestion is not required.

**6.2 Standard Preparation:** - Prepare a series of standard metal solution in the optimum concentration range (1µg/L to 5µg/L) by appropriate dilution from their stock solution with ASTM type 1 water using the following formula.

$$N_1V_1 = N_2V_2$$

**6.3 Analysis of sample:** Follow manufacturer's standard operating procedure for initialization, mass calibration, gas flow optimization, and other instrument operating conditions. Maintain complete and detailed information on the operational status of the instrument whenever it is used.

A suggested analytical run sequence, including instrument tuning/optimization, checking of reagent blanks, instrument calibration and calibration verification, analysis of samples, and analysis of quality control samples and blanks.

Follow manufacture's instruction for optimizing instrument performance. The most important optimization criteria include nebulizer gas flows, detector and lens voltages, radio-frequency forward power, and mass calibration. Periodically check mass calibration and instrument resolution. Ideally, optimize the instrument to minimize oxide formation and doubly-charged species formation. Measure the CeO/Ce ratio to monitor oxide formation, and measure doubly-charged species by determination of the Ba<sup>2+</sup>/Ba<sup>+</sup> ratio. Both these ratios should meet the manufacture's criteria.

After optimization and tuning, calibrate instrument using an appropriate range of calibration standards. Use appropriate regression techniques to determine calibration lines or curves for each analyte. For acceptable calibrations. Correlation coefficients for regression curves are ideally 0.995 or greater. Immediately after calibration, run initial calibration verification standard; acceptance criteria are +10% of known analyte concentration. Next run initial calibration verification blank; acceptance criteria are ideally ± the absolute value of the instrument detection limit for each analyte, but in practise ± the absolute value of laboratory reporting limit or the laboratory method detection limit for each analyte is acceptable. Verify low-level calibration by running 0.3- and/or 1.0 µg/L standards if analyte concentration are less than 5 µg/L.

Ensure that all vessels and reagents are free from contamination. During analytical run include quality control analyses. Internal standard recoveries must be between 70% and 125% of internal standard response in the laboratory-fortified blank; otherwise, dilute sample, add internal standard mix, and reanalyze. Make known-addition analyses for each case separate matrix in a digestion or filtration batch.

For every new or unusual matrix, it is highly recommended that a semi-quantitative analysis be carried out to screen the sample for elements at high concentration. Information gained from this may be used to prevent potential damage to the detector during sample analysis and to identify elements which may be higher than the linear range should be diluted into range and reanalyzed. Select abundant masses for the metals as given below in the table

**Table**

<b>Element of Interest</b>	<b>Masses</b>
Aluminum	27
Antimony	123
Arsenic	75
Barium	137
Beryllium	9
Cadmium	111
Chromium	52
Cobalt	59
Copper	63
Lead	206, 207, and 208
Manganese	55
Mercury	202
Molybdenum	98
Nickel	60
Selenium	82

Silver	107
Thallium	205
Thorium	232
Uranium	238
Vanadium	51
Zinc	66

## 7. Calculation :

$$\text{Metal conc. in sample (mg/L)} = \frac{\text{Sample conc. from instrument (mg/L)}}{\text{X Dilution factor (if any)}}$$

## 8. Special precautions

**8.1** Concentrated nitric and hydrochloric acids present various hazards and are moderately toxic and extremely irritating to skin and mucus membranes. Use these reagents in a fume hood whenever possible and if eye or skin contact occurs, flush with large volumes of water. Always wear safety glasses or a shield for eye protection, protective clothing and observe proper mixing when working with these reagents.

**8.2** The acidification of samples containing reactive materials may result in the release of toxic gases, such as cyanides or sulfides. Acidification of samples should be done in a fume hood.

**8.3** Analytical plasma sources emit radiofrequency radiation in addition to intense UV radiation. Suitable precautions should be taken to protect personnel from such hazards. The inductively coupled plasma should only be viewed with proper eye protection from UV emissions

**9. References**

**9.1** APHA 3125 B

**9.2** USEPA METHOD 200.8

**APPENDIX-I****ASTM Type -1 Water**

ASTM Type -1 is type of reagent water meeting the following requirement.

<b>S. No.</b>	<b>Parameter</b>	<b>Requirement</b>
<b>1.</b>	<b>Electrical conductivity,max, <math>\mu\text{S}/\text{cm}</math> at 298 K (25°C)</b>	<b>0.056</b>
<b>2.</b>	<b>Electrical resistivity, min, <math>\text{M}\Omega\text{-cm}</math> at 298 K (25°C)</b>	<b>18</b>
<b>3.</b>	<b>Total organic carbon (TOC), max, <math>\mu\text{g}/\text{L}</math></b>	<b>50</b>
<b>4.</b>	<b>Sodium, max, <math>\mu\text{g}/\text{L}</math></b>	<b>1</b>
<b>5.</b>	<b>Chlorides, max, <math>\mu\text{g}/\text{L}</math></b>	<b>1</b>
<b>6.</b>	<b>Total silica, max, <math>\mu\text{g}/\text{L}</math></b>	<b>3</b>

**CHAPTER IV: OTHER TOXIC SUBSTANCES (PESTICIDE RESIDUES,  
POLYCHLORINATED BIPHENYLS, POLYAROMATIC HYDROCARBON,  
TRIHALOMETHANE)**

**1. Pesticide residues, PCBs and PAHs**

**A. By GC/GCMS/GCMSMS**

**1.1 Scope:**

This procedure is applicable for the determination of following in water samples:

<b><u>Pesticide Residues</u></b>	<b><u>Polychlorinated Biphenyls</u></b>	<b><u>Polyaromatic Hydrocarbons</u></b>
1.p,p' DDD	1.1. Heptachloro Biphenyl	1. Acenaphthene
2.o,p' DDD	1.2. Octachloro Biphenyl	2. Acenaphthylene
3.o,p' DDE	1.3. Pentachloro Biphenyl	3. Anthracene
4.p,p' DDE	1.4. Hexachloro Biphenyl	4. Benzo(a)anthracene
5.o,p' DDT	1.5. Tetrachloro Biphenyl	5. Benzo(a)anthracene
6.p,p' DDT	1.6. Dichloro Biphenyl	6. Benzo(a)pyrene
7.Alpha BHC	1.7. Trichloro Biphenyl	7. Benzo(b)fluoranthene
8.Beta BHC	1.8. Chloro Biphenyl	8. Benzo(g,h,i)perylene
9.Gamma BHC		9. Benzo(k)fluoranthene
10. Delta BHC		10. Chrysene
11. Alfa Endosulfan		11. Dibenzo(a,h)anthracene
12. Beta Endosulfan		12. Fluoranthene
13. Endosulfan sulfate		13. Fluorene
14. Monocrotoph os		14. Indeno(1,2,3- c,d)perene
15. Ethion		15. Napthalene
16. Chlorpyrifos		16. Phenanthrene
17. Phorate		17. Pyrene

18. Phorate sulfone		
19. Phorate sulfoxide		
20. 2,4-D Acid		
21. Butachlor		
22. Isoproturon		
23. Alachlor		
24. Atrazine		
25. Parathion methyl		
26. Paraoxon methyl		
27. Malathion		
28. Malaixon		
29. Aldrin		
30. Dieldrin		
31. Heptachlor		
32. Heptachlor		
33. epoxide		

**1.2 Principle** – The pesticide residues, PCB & PAH are extracted by liquid - liquid partitioning with Dichloromethane in separatory funnels and estimated by Gas Chromatographically in an instrument equipped with Electron Capture Detector (ECD), Nitrogen Phosphorus Detector (NPD) or Mass Spectrometer (MS). The contents of the residue are determined by comparing the instrument response with those of the standards of similar concentrations.

### 1.3 Reagents & Materials

#### 1.3.1 Reagents

1.3.1.1 Methanol

1.3.1.2 Dichloromethane (DCM)

- 1.3.1.3 N- Hexane
- 1.3.1.4 Acetone
- 1.3.1.5 1% Formic Acid
- 1.3.1.6 Sodium Sulphate
- 1.3.1.7 Sodium Chloride
- 1.3.1.8 Pesticide Standards of known Purity.

### 1.3.2 Material

- 1.3.2.1 2.5 L Separating Funnel
- 1.3.2.2 Separating Funnel Stands
- 1.3.2.3 250 mL Rotary evaporator flask Funnels
- 1.3.2.4 Glass test tubes
- 1.3.2.5 What man Filter paper (42 no.)
- 1.3.2.6 C18 bond elute cartridges
- 1.3.2.7 Glass Test tubes
- 1.3.2.8 Injection Vials

### 1.3.3 Apparatus /Instruments

- 1.3.3.1 Weighing Balance
- 1.3.3.2 Vortex Mixer
- 1.3.3.3 Separatory Funnel Shaker
- 1.3.3.4 Solid Phase Extractor (SPE)
- 1.3.3.5 Rota vapor
- 1.3.3.6 Nitrogen Concentrator
- 1.3.3.7 Micro pipettes
- 1.3.3.8 Instruments:
  - 1.3.3.8.1 Gas Chromatograph with Electron Capture Detector & Nitrogen Phosphorus Detector, or
  - 1.3.3.8.2 GCMS or GCMSMS

## 1.4 Preparation of reagents

**1.4.1 Preparation of Stock Solution:** Into a 100 mL volumetric flask, weigh approximately 10 mg Reference Standard and dissolve it in Acetone/Hexane and make up to mark with.

$$\text{Concentration of Stock Solution} = \frac{\text{Weight of Standard (mg)} \times 1000 \times \text{Purity}}{100 \times 100}$$

(µg/mL)

**1.4.2 Preparation of Calibration Solutions:** Prepare calibration standards from stock solutions for calibration curve by diluting stock standard solution with Acetone/Hexane to get calibration solutions of 0.02 ppm, 0.04 ppm, 0.06 ppm, 0.08 ppm, 0.1 ppm, 0.4 ppm, 0.6 ppm, 0.8 ppm and 1.0 ppm.

**1.4.3 Preparation of Matrix Match:** Spike the sample with known amount of the standard and process it in the same manner like sample processing.

## 1.5 Procedure

### 1.5.1 Sample preparation

#### 1.5.1.1 Liquid- Liquid Partitioning

**1.5.1.1.1** Take 750 mL water into 1L separating funnels and check pH of the sample (adjust to ~7 pH by adding H<sub>2</sub>SO<sub>4</sub> or NaOH if necessary).

**1.5.1.1.2** Add 10gm NaCl to the water sample, and shake vigorously.

**1.5.1.1.3** Add 80mL dichloromethane. Shake vigorously for 2 min.

**1.5.1.1.4** Shake the funnel manually or on separatory funnel shaker at 250 rpm for 5 minutes and place the funnel on stand for about 5 minutes to separate organic and aqueous layer.

**1.5.1.1.5** If the emulsion interface, employ mechanical techniques like stirring, filtration through glass wool, centrifugation etc.

**1.5.1.1.6** Collect the lower organic layer (DCM) in a conical flask, and repeat partitioning twice with 60mL and 40mL of DCM.

- 1.5.1.1.7 Collect and combine DCM layer through pre-wet sodium sulfate in a 250mL round bottom flask.
- 1.5.1.1.8 Concentrate the filtrate on rotary evaporator up to 5mL at 40°C.
- 1.5.1.1.9 Transfer the extract to glass test tubes with 3 washing of 2mL dichloromethane/n-hexane.
- 1.5.1.1.10 Load the test tube in Nitrogen Concentrator and evaporate the extract to dryness at 40°C bath temperature, 10-15psi nitrogen pressure.
- 1.5.1.1.11 Equilibrate the tube to room temperature.
- 1.5.1.1.12 Reconstitute the residues with 1mL n-hexane/Acetone.
- 1.5.1.1.13 Shake tubes on vortex and transfer the extract into GC vial for GC, GCMS, or GCMSMS analysis.

#### 1.5.1.2 Solid Phase Extraction

**Principle** – The pesticides residues are extracted by Solid phase extractor with Methanol and Acetone by using C18 bond elute cartridges and finally eluted by acetone and analyzed by GCFID/GCECD/GCMS/GCMSMS. The contents of the residue are determined by comparing the instrument response with those of the standards of similar concentrations.

##### 1.5.1.2.1 Sample extraction

- 1.5.1.2.1.1 Take 1500mL water into 2L reagent bottles and check pH of the sample (~7, add H<sub>2</sub>SO<sub>4</sub> or NaOH if necessary).
- 1.5.1.2.1.2 Wash syringe with 5mL of Methanol.
- 1.5.1.2.1.3 Rinse cartridges with 5mL of Methanol followed by 5mL of acetone.
- 1.5.1.2.1.4 Finally rinse cartridges with 5mL of 1% of formic acid into aqueous waste.
- 1.5.1.2.1.5 Load 1025 mL of sample into the cartridges (this is the above level of 300-400mL filled beaker, since tubing filters should be properly dipped even after 1025ml of sample is loaded).
- 1.5.1.2.1.6 Dry cartridges with nitrogen gas for 10 min.
- 1.5.1.2.1.7 Soak and collect 5mL fraction using acetone.
- 1.5.1.2.1.8 Soak and collect 5mL fraction using acetone.

**1.5.1.2.1.9** Concentrate the extract in Nitrogen concentrator.

**1.5.1.2.1.10** Reconstitute the residues with 1mL Hexane: Acetone (80:20) and inject in GC, GCMS or GCMSMS.

## 1.5.2 Instrumental Analysis

**1.5.2.1 Column-** 30m x 0.25mm I.D x 0.25µm TR-1 or DB-5 bonded fused silica column or equivalent.

**1.5.2.2 Integration-** Observe the retention time (RT) of the analytes with the respective standards and integrate the peak to record the area and calculate the concentration with the following formula:

$$\text{Residue } (\mu\text{g/mL}) = \frac{\text{Peak area of sample} \times \text{Std. Conc.} \times \text{Vol. of std injected} \times \text{Final vol. of sample made}}{\text{Peak area of Std.} \times \text{Wt. of sample} \times \text{Vol. of sample injected}}$$

**Note:** In case of trace analysis, some compounds are highly sensitive and are not easily detected at GC level, for such analysis, GCMS or GCMSMS required.

## 1.6 Special Precautions:

**1.6.1** All glass ware should be properly washed to avoid the cross contamination.

**1.6.2** Always wear gloves and masks to avoid direct exposure to solvents and standards.

**1.6.3** MSDS should be available to the concerned analyst for awareness so that hazards will be reduced to its lowest level.

## 1.7 Interference:

**1.7.1** Method interferences may be caused by contaminants in solvents, reagents, glassware and other sample processing apparatus that lead to discrete artifacts or elevated base line in gas chromatograms. All reagents and apparatus must be routinely demonstrated to be free from interferences under the conditions of the analysis by running reagent blank.

**1.7.2** Clean all glass wares as soon as possible after use by thoroughly rinsing with acetone. Follow by washing with hot water and detergent and through rinsing with tap

and reagent water. Finally rinse the apparatus with acetone. Heat in an oven at 200°C for ca. 1 hour. After drying and cooling, seal and store glassware in a clean environment to prevent any accumulation of dust or other contaminants.

**1.7.3** The use of high purity reagents and solvents helps to minimize interference problems. Purify solvents by distillation in all glass systems.

**1.7.4** Interfering contamination may occur when a sample containing low concentration of analytes is immediately analyzed following a sample containing high concentration of analytes. After analysis of the sample containing high concentrations of analytes, one or more injections of pure solvents should be made to ensure that accurate peak area is obtained for the next sample.

**1.7.5** Matrix interferences may be caused by contaminants that are co-extracted from the sample. Clean up the sample extract if required. Analyte identification should be confirmed.

**1.7.6** It is important that samples and working standards be contained in the same solvent. The solvent for working standard must be same as the final solvent used in sample preparation. If this is not the case, chromatographic comparability of standard to sample may be affected.

**1.7.7** Sample transfers and glass surface contacts should be minimized, and that adequate rinsing of glass surface be performed.

## **1.8 References**

**1.8.1** USEPA, Method No. 508 (Revision 3.1).

**1.8.2** AOAC Method 990.06

**1.8.3** Pesticide Residue Analysis Manual, 2<sup>nd</sup> edition- By Dr. K.K. Sharma.

**1.8.4** Official Journal of the European Communities-implementing council decision 2002/65/EC

**1.8.5** Concerning the performance of analytical methods and the interpretation of results

**1.8.6** SANCO/12495/2011 – Method Validation and Quality control procedures for pesticide residue analysis in food and feed.

**1.8.7** USFDA Laboratory information Bulletin No. 4303, vol. 19 No.4, April 2003

## **2. Trihalomethanes**

The use of chlorine to disinfect water produces various disinfection by products, which have been classified mainly as halogenated and non-halogenated byproducts. These primary byproducts are trihalomethanes (THMs) and haloacetic acids. THMs are the byproducts of chlorination of water that contains natural organic matter. A U.S. Environmental Protection Agency (EPA) survey shows that THMs are present in most chlorinated water supplies. Even though they pose a less acute health risk than do waterborne diseases, THMs are still among the important water quality issues. The most common THM compounds are dibromochloromethane ( $\text{CHClBr}_2$ ), bromoform ( $\text{CHBr}_3$ ), chloroform ( $\text{CHCl}_3$ ), and dichlorobromomethane ( $\text{CHCl}_2\text{Br}$ ). The sum of these four compounds is referred to as Total Trihalomethanes (TTHMs). THMs, chloroform and dibromochloromethane, are carcinogens; and another THM, bromodichloromethane, has been identified as a mutagen, which alters DNA. Mutagens are considered to affect the genetics of future generations in addition to being carcinogenic.

### **A. By Gas Chromatography with ECD detector method**

**1. Scope:** This method is applicable to the determination of four Trihalomethanes (THMs), i.e., Chloroform, Bromodichloromethane, Dibromochloromethane, and Bromoform, and the selected chlorinated solvents in finished drinking water, drinking water during intermediate stages of treatment, and in both surface and ground water.

**2. Principle:** Sample is extracted once with pentane and the extract is injected into

a gas chromatograph equipped with a linearized electron capture detector (ECD) for separation and analysis. Extraction and analysis time is 10 to 30 min per sample, depending on analytical conditions. Confirmatory evidence, where necessary is obtained by using dissimilar columns, other selective detectors, or mass spectrometry. Component concentrations must be sufficiently high (i.e., >50 µg/L) for confirmatory analyses using a mass spectrometer. Standards added to organic-free water and samples are extracted and analyzed in the same manner, under identical conditions. This step is essential to adjust for the less-than 100% extraction efficiency of the simplified extraction technique. Extreme differences in ionic strength or organic content between standards and samples can result in different equilibria of sample constituents with the extracting solvent and a method bias may result.

### 3. Reagents:

**3.1 Extraction solvent:** For capillary column split injection technique, preferably use only pentane. For other techniques, recommended solvent is pentane; alternatively, use hexane, methylcyclohexane, methyl-tert-butyl ether (MtBE) or 2, 2, 4-trimethylpentane.

**3.2 Methyl alcohol,** to be free from interferences

**3.3 Neat standard materials:** Use materials of 96% purity or greater for:

1) **Calibration standards:** Bromoform (CHBr<sub>3</sub>), Bromodichloromethane (BDCM), Dibromochloromethane (DBCM), Chloroform (CHCl<sub>3</sub>), 1, 1, 1-Trichloroethane (TCA), Tetrachloroethene (PCE), Trichloroethene (TCE), and Carbon tetrachloride (CCl<sub>4</sub>).

2) **Internal standard:** 1, 2 dibromopropane. A compound selected as an internal standard should have baseline resolution to separate it from constituents of interest and any interference. Because this requirement is highly dependent on the samples and the analytical conditions and equipment used, no single internal standard is universally applicable. 1, 2-dibromopropane has been found to be generally useful.

**3.4 Reagent water:** Generate VOC-free water, defined as water free of interference by passing tap water through a carbon filter. Alternatively, prepare VOC-free water as follows:

Boil water for 15 min, then maintain at 90°C while bubbling a contaminant-free inert gas through water at 100 mL/min for 1 h. While water is still hot, transfer to a narrow-mouth screw-cap bottle with a TFE seal. Test VOC-free water each day before use by analyzing a method blank for constituents of interest. If any chlorine residual remains after such treatment, destroy it.

**3.5. Stock standard solutions:** Prepare from pure standard materials or purchase as certified solutions. Prepare stock standard solutions in methanol using assayed liquids or gases as appropriate.

**CAUTION: Toxic substances.** The toxicity or carcinogenicity of each analyte has not been precisely defined. Benzene, Carbon tetrachloride, Bis (1-chloroisopropyl) ether, 1,4-dichlorobenzene, 1,2-dichloroethane, hexachlorobutadiene, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, chloroform, 1,2-dibromoethane, tetrachloroethene, trichloroethene, and vinyl chloride have been classified tentatively as known or suspect carcinogens. Handle pure standard materials and stock standard solutions of these compounds in a hood and wear a NIOSH/MESA-approved toxic gas respirator when handling high concentrations.

Place about 9.8 mL methanol in a 10 mL ground-glass-stoppered volumetric flask. Let stand unstoppered for about 10 min or until all alcohol-wetted surfaces have dried. Weigh flask to nearest 0.1 mg. Add assayed reference materials as follows: For liquids, using a 100- $\mu$ L syringe or disposable capillary-tip glass pipette, immediately add two or more drops of assayed reference material to flask, then reweigh. Ensure that the drops fall directly into the alcohol without contacting flask neck. For halocarbon gases that boil below 30°C (bromomethane, chloroethane, chloromethane, dichlorofluoromethane, trichlorofluoromethane, vinyl chloride), attach a vinyl plastic tube to port of gas bottle containing reference material, with open end bubbling into a beaker of methanol showing flow through the tubing; insert needle of 5 mL valved gastight syringe into tube and pull gas into syringe slowly to 5.0 mL mark. Lower syringe needle to within 5 mm of methanol surface and slowly force gas onto surface. The gas will dissolve into the methanol and will be seen as a vortex as it dissolves into the solvent. Reweigh flask (difference is amount of gas dissolved into methanol), dilute to volume, stopper, and mix by inverting several times. Calculate concentration in

micrograms per microliter from net gain in weight. When compound purity is assayed to be 96% or greater, calculate concentration of stock standard from uncorrected weight. Preferably use commercially prepared stock standards at any concentration if they are certified by the manufacturer or an independent source. Transfer stock standard solution into a TFE-sealed screw-cap bottle. Store with minimum headspace at -10 to -20°C away from light

Alternatively, purchase prepared standard solutions in methanol.

**CAUTION:** Trihalomethanes and chlorinated solvents are toxic and may be carcinogenic: prepare primary stock solutions in a hood and wear appropriate personal protective equipment.

**3.6. Secondary dilution standards:** From standard stock solutions, prepare multi-component secondary standards in methyl alcohol so that standards over the working range of instrument can be prepared using no more than 20 $\mu$ L methanolic standard solution /100 mL reagent water.

Using stock standard solutions, prepare in methanol secondary dilution standards that contain the compounds of interest, either singly or mixed together. Prepare secondary dilution standards at concentrations that will permit aqueous calibration standards (as mentioned below) (to bracket working range of the analytical system. Store secondary dilution standards with minimal headspace in a freezer and check frequently for signs of evaporation (which would indicate need for regeneration). Always bring to room temperature before preparing calibration standards. Prepare standards fresh weekly for gases. Replace all other standards monthly, or sooner if comparison with check standards indicates a problem.

**3.7. Calibration standards:** Prepare at least five concentration levels for each compound by adding appropriate amounts of secondary standard solution to reagent water and inverting water sample twice. Prepare one standard at a concentration near, but above, the MDL (i.e., 4 X MDL for potable-water-type samples) or a level that defines the low end of the working range and the others to correspond to the expected range of sample concentrations or to define the detector working range. Aqueous calibration standards can be stored up to 24 h if

held in sealed vials with zero headspace. Otherwise, discard within 1 h. Alternatively, prepare calibration standards by injecting, with a solvent flush, an appropriate amount of a standard mix dilution and internal standard surrogate mix, directly into a 25mL syringe filled with reagent water; immediately inject water standard into purge vessel.

**3.8. Internal standard solution:** Prepare stock solution from neat material in hexane. Make secondary dilution directly into storage container of pentane extracting solvent to produce a concentration of 30 µg/L internal standard in pentane.

**3.9. Aqueous calibration standards:** Construct a calibration curve for each constituent using a minimum of three different concentrations, but preferably use five to seven concentrations. Bracket each sample with two of the concentrations. Use one concentration near, but above, the laboratory-determined detection limit. Where a sample component exceeds the range bracketed by standards, dilute a fresh volume of sample and re-extract, or prepare new standards in reagent water to bracket the concentration and dilute sample and standard extracts to bring them into the linear range of the detector. To prepare calibration standards, rapidly inject the required volume of alcoholic standard into the expanded area of a reagent-water-filled volumetric flask. Using an extended barrel syringe, inject the methanol well below the reagent water surface. Preferably incline the volumetric flask at an approximately 45 degree angle while injecting the standard. Remove syringe and stopper flask. Mix aqueous standards by gently inverting flask three times only. Discard to waste the contents in the neck of flask before transferring standards to sample vials. Add any preservatives to both samples and standards before extraction. Process standards through extraction in conjunction with sample sets. Aqueous standards, when stored with a headspace, are not stable; discard after 1 h. When stored in headspace-free sample storage vials, aqueous standards may be used for 24 h. Avoid standard preparation procedures that require delivery of less than 10 µL of methanolic standards into volumetric flasks. Instead, use a larger volumetric flask and a larger volume of methanolic standard.

#### **4. Apparatus:**

**4.1 Sample storage vials:** Clean, baked 40mL glass open screw-top TFE-faced septum VOA vials or equivalent.

**4.2 Microsyringes, 10, 25, and 100  $\mu$ L:** Microsyringes with extended barrels are suggested for proper injection of methanolic standards when preparing aqueous standards in volumetric flasks.

**4.3 Volumetric flasks, glass-stoppered, 10, 25, 50, 100, 250, 500, and 1000 mL** Class A. Choose size according to final volume of aqueous standard required and concentration of methanolic standards.

**4.4 Extraction vessels:** Use sample storage vials. Optionally, where samples are transferred to smaller extraction vessels select an extraction container on the basis of specific requirements for final extract volume, solvent-to-water volume ratio and availability. If a separate vessel is used for extraction, place standards along with samples in sample storage vials and follow identical procedures for preservation and transfer to the extraction vessel. Use the same lots of vessels for sample and standard extraction. Use of clean, oven-baked glass vessels and TFE faced septa is critical.

**4.5 Extract storage vials:** 1.8mL autosampler vials with open screw-top caps and TFE septa, or equivalent.

**4.6 Gas Chromatograph,** preferably temperature-programmable with linearized electron-capture detector.

**4.7 Chromatographic columns:**

**4.7.1** 0.32mm ID X 30 m fused silica capillary, 1  $\mu$ m DB-5, or equivalent, at linear velocity of 20 cm/s. Temperature program: 35°C for 5 min. ramp 10°C/min to 70°C, then 20°C/min to 200°C.

**4.7.2** 0.53-mm ID X 30 m, 1.5  $\mu$ m DB-5 or equivalent, at 25 cm/s. Starting at 30°C for 1 min, ramp 6°C/min to 150°C.

**4.7.3** 2-mm ID X 2 m long glass packed with 1% SP-1000 on Carbopack B (60/80) operated at 50°C with 60 mL/min flow, or, if temperature-programmable GC is available, 45°C for 1 min, ramp 8°C/min to 240°C.

**4.7.4** 2-mm ID X 2 m long glass packed with 10% squalene on Chromosorb WA W (80/100 mesh) operated at 67°C with 25 mL/min flow.

**4.7.5** 2-mm ID X 3 m long glass packed with 6% OV-11/4% SP-2100 on Supelcoport (100/120 mesh); temperature program 45°C for 12 min, then 1 to 70°C with 25-mL/min flow rate.

**4.8 Mechanical shaker:** Optionally, a rotary platform shaker.

**4.9 Solvent pipettor:** 2mL transfer pipette, pipettor, or all-glass and TFE repipettor

that attaches to the pentane storage bottle.

**4.10 Transfer pipettes** 5 mL. Preferably use a pipettor with disposable tips cleaned and dried as recommended for TFE septa.

**4.11 Analytical balance**, capable of measuring to  $\pm 0.01$  gm.

## 5. Procedure :

### 5.1 Extraction:

**5.1.1** Let samples and standards come to room temperature. Open each sample vial and remove 5 mL of sample and discard to waste, preferably using a transfer pipettor with disposable tips. Replace cap, weigh vial to nearest 0.1 gm, and record weight. Alternatively, use a 25mL gastight syringe to measure sample volume (method described below) and perform subsequent extraction.

**5.1.2 Sample analysis:** Bring sample to ambient temperature. Remove plunger from 25mL syringe and close attached valve. Open sample bottle and carefully pour sample into syringe barrel to just short of overflowing. Replace syringe plunger, invert syringe, and open valve. Vent any air and adjust sample volume to 25.0 mL, in duplicate if sufficient sample is available (once sample cap has been removed, sample cannot be stored, because of headspace). Add an appropriate amount of surrogate/internal standard through valve bore, and close valve. Attach to purge device, open valves, and inject sample into purge vessel. Close valves and purge sample for 11.0 min at ambient temperature at a flow rate of 40 mL/min (helium or nitrogen). If water vapor causes problems in the mass spectrometer, use a 3-min dry purge and/or a moisture control module. Desorbs trapped materials onto head of chromatographic column at 180°C while back-flushing trap for 4 min with inert gas at a flow rate compatible with the column of choice, and begin GC temperature program.

**5.1.3** Set system auto-drain to empty purge chamber while trap is being desorbed into GC, or alternatively, use sample syringe to empty vessel. Washing chamber with two 25mL flushes of reagent water is useful if highly contaminated samples are being analyzed. Be sure all areas wetted during purging are also wetted during rinsing to maximize flushing.

**5.1.4** Recondition trap by baking at conditioning temperature for 5 to 7 min. Let trap cool to ambient before introduction of next sample into purge vessel. When all sample

compounds have been eluted from chromatographic column, end data acquisition and store data files. Use data system software to display full range mass spectra and appropriate extracted ion current profiles (EICP). If any ion abundances exceed system working range, dilute sample in second syringe with reagent water and analyze.

*(NOTE: Take care with sample because compounds can be very volatile and can be lost if sample is reopened.)*

**5.1.5** Estimate amount of dilution needed and expel excess sample from second syringe, inject that portion into purge vessel, and with a second syringe, add necessary reagent water to a total of 25.0 mL in purge vessel.

**5.1.6** Using a clean, dedicated volumetric measurement device (2mL transfer pipette, pipettor, or all-glass and TFE repipettor attached to the pentane storage bottle.) carefully measure 2.00 mL pentane and add to sample vial. Vigorously shake by hand for 1 min or use a rotary platform shaker set at 60 to 100 rpm.

**5.1.7** Let phases separate for at least 2 min. Where emulsions do not separate on standing, centrifuge or transfer entire emulsion to a separate vial and cool extract below 4°C to promote separation.

**5.1.8** Using a disposable glass pipette, transfer at least 1 mL of upper pentane extract to extract storage vials. Optionally, transfer half of the pentane extract to each of two vials to provide for reanalysis where necessary. Protect pentane extracts from warm temperatures and minimize extract holding time at room temperature.

**5.1.9** Store extracts at 4°C.

**5.1.10** Empty sample/extraction vial to waste, rinse, and shake dry.

**5.1.11** Reweigh empty container with original cap to nearest 0.1 gm and record weight.

**5.1.12** Calculate weight of sample extracted to the nearest 0.1 gm by subtraction of vial-only weight from sample-plus- vial weight. For an assumed density of 1 g/mL, weight of sample extracted is equal to volume of sample extracted, in milliliters. Convert volume in milliliters to liters and record.

## **5.2 Sample and standard analysis:**

Before extraction of samples or standards, prepare and analyze a method blank to verify freedom from interferences. Once extracts have been prepared, analyze standards and

calculate a calibration curve or calibration factor.

Inject 1 to 5  $\mu\text{L}$  of standard extract depending on the configuration of the instrument and the required sensitivity. Inject exactly the same volume of extract each time, preferably using an autosampler. To test that injection volumes are repeatable, inject replicates of a single standard extract, and determine the standard deviation. Percent relative standard deviation (%RSD) should not be more than 5%. If this precision is not routinely achievable, use the internal standard calibration procedure. After calibration, analyze the method blank, samples, and quality control samples. Extract and analyze a quality-control check standard each twentieth analysis (5%) and at the end of the analytical sequence. The percent recovery for the QC check standard should be between 80 and 120%. Develop historical mean control charts of QC check standard recovery for each compound and use the 99% confidence about historical data as the control criteria for rejection of QC check standards validity. Where criteria are failed, repeat analysis of any samples analyzed since the last QC check standard was in control.

### **5.3 Internal standard analysis procedure:**

Add the internal standard to the pentane solvent in the storage container at the concentration specified, and proceed with extraction and analysis of samples and standards as outlined above.

**5.4 Compound identification:** Identification of compounds in samples is based on comparison of retention times (RT) of suspect peaks to the confidence limits for RT of the authentic compounds in standards.

Using the retention times of the standards analyzed, determine the average retention time for each compound and the standard deviation of the retention time. Tentatively identify peaks in sample chromatograms as compounds on basis of the 99% confidence interval around the calculated mean value using the calculated standard deviation. Nominally, the retention time window would be expected to be no wider than 0.25 min (packed column) and 0.05 min (capillary) before and after the average retention time calculated for the standards. When the 99% confidence limits for the data set are wider than the nominal value, institute corrective action.

Additional evidence of compound identity may be obtained by adding standard material to the suspect extract (standard addition) and reanalyzing. Presence of separate peaks in the extract with the known addition confirms that the suspect peak is not the compound of interest.

If chromatographic data systems are used to identify compounds, follow manufacturer's specifications. If the RT windows calculated by a computerized system are wider than the nominal values, investigate sources of retention time variability and take corrective action.

## 5.5 Calculation

**5.5.1 External standard Procedure:** Use this procedure only if the volume of the injection can be held constant. Calculate individual Response Factor (RFs) for each standard analysed as follows:

$$\text{RF} = \frac{\text{Nominal amount compound extracted, } \mu\text{g}}{\text{Response (peak area or peak height)}}$$

Calculate the amount of compound for each standard as:

$$W_s = V_s \times C_s$$

Where:

$W_s$  = amount of compound,  $\mu\text{g}$ ,

$V_s$  = volume of standard extracted, L, and

$C_s$  = concentration of prepared standard,  $\mu\text{g/L}$ .

For each compound determine average RF and standard deviation of the RFs using all calibration standards analyzed.

If the percent relative standard deviation [%RSD = (SD/mean RF) X 100] is less than 10%, use average RF to calculate sample concentration.

If the %RSD is greater than 10%, plot a calibration curve of amount injected versus response. Use the graph to determine the amount of compound present in each sample.

Then determine the concentration by dividing amount,  $\mu\text{g}$ , by the volume, L, of sample extracted.

Optionally use a data system to prepare a linear regression and use the linear regression equation to calculate compound amounts in samples from response values.

Where average RF is used, determine sample concentration as follows:

$$C_x = RF \times R_x / V_x$$

Where:

$C_x$  = compound concentration,  $\mu\text{g/L}$

$R_x$  = sample response (mm, area etc.)

$V_x$  = volume of sample extracted, L

Round all final sample results to two significant figures.

**5.5.2 Internal standard procedure:** For all analyses made in a given analytical sequence, determine average internal standard response and standard deviation of the internal standard response. Calculate percent relative standard deviation. If the %RSD is greater than 25%, take corrective action to improve method precision. Establish the 99% confidence interval for the internal standard response using the calculated mean and standard deviation for the sample set. Reject analyses where the internal standard response is outside these confidence limits, and reanalyze. After analysis of calibration standards, calculate individual relative response factors (RRF) for each compound in standard as follows:

$$RRF = \frac{R_s \times C_i}{R_i \times C_s}$$

where:

$R_s, R_i$  = responses for calibration standard and internal standards, respectively, and

$C_s, C_i$  = compound concentrations in calibration and internal standards, respectively.

Calculate average RRF for each compound, standard deviation of the RRFs, and %RSD. If %RSD is less than 10%, use the average RRF; if it is greater, develop a calibration curve or a linear regression equation as outlined in the external standard procedure.

When using the average internal standard RRF, calculate concentration in samples as follows:

$$C_x = \frac{R_x \times C_i}{R_i \times \text{RRF}}$$

Where:

$C_x$  = compound concentration in sample,  $\mu\text{g/L}$ , and

$R_x$  = sample response.

**5.5.3 Total Trihalomethane concentration:** Calculate total trihalomethane concentration by summing the concentration of the four individual trihalomethanes in each sample. This is required for USEPA reporting purposes but it is preferable to report only individual THMs.

## 6.0 Interference

Impurities contained in the extracting solvent account for many analytical problems. Maintain records of the reagent's manufacturer, lot number, purity, date bottle were opened, and expiration date.

Analyze solvent blanks before using a new bottle of solvent.

Make indirect daily checks on the extracting solvent by monitoring the method blanks. Whenever an interference is noted in the method blank, analyze a solvent blank..

Discard (or use for another purpose) extraction solvent if a high level of interference is traced to it. Low-level interferences can be removed by distillation or column chromatography however, it usually is more economical to obtain new solvent or select an approved alternative solvent.

Interference-free solvent is defined as a solvent containing less than the laboratory

determined detection limit of interference for each constituent. Protect interference-free solvents by storing in an area known to be free of organohalogen solvents.

*Note: Do not subtract blank values from sample analysis results as a correction for contamination.*

Sample contamination has been attributed to diffusion of volatile organics through the septum seal on a sample bottle during shipment and storage. Use the trip blank to monitor for this problem.

Contamination also may occur whenever equipment and materials used to store, extract, or analyze samples are inadequately cleaned, prepared, tested, or stored. There are many sources of contamination including contamination of reagents during storage and contamination of equipment reused in the sequential extraction of samples and standards. Maintain records of dates of preparation and cleaning and inclusive dates of use of reagents, standards, bottles, and equipment. Test all reagents and standards before initial use. To reduce possibility of carryover contamination, always clean equipment thoroughly after each use. Where equipment contamination is a concern, processing and analysis of additional method blanks beyond the minimum requirements of this method may be useful. Start by placing reagent water in a sample vial of the same lot that was used for samples and add preservative as was done for samples. Process this method blank in conjunction with samples using the same reagents, materials, and equipment. Where analysis of a method blank indicates contamination, investigate possible sources and isolate the cause. Take and document corrective action. Following analysis of a sample containing late-eluting interferences, or containing over-range concentrations of constituents of interest, analyze a solvent blank or method blank to demonstrate freedom from carry-over.

This liquid-liquid extraction technique efficiently extracts a wide boiling range of nonpolar organic compounds and also extracts polar organic components with varying efficiencies. To analyze rapidly for trihalomethanes and chlorinated solvents with sensitivities in the low microgram-per-liter range in the presence of these other organic compounds, use the semi-specific electron capture detector. Trihalomethanes are primarily products of the chlorination process and seldom appear in raw unchlorinated

source water. The absence of peaks with retention times similar to the trihalomethanes in raw source water analysis is supporting evidence of an interference-free finished drinking water analysis. Because of possible interferences, analysis of representative raw source water when analyzing finished drinking water provides evidence of freedom from this interference source. When potential interferences are noted in the raw source water, use the alternate chromatographic columns to reanalyze the sample set. If interferences still are noted, make confirmatory qualitative identifications as directed above. If the peaks are determined to be other than the constituents of interest and they add significantly to the constituents' value in the finished drinking water, analyze sample set by the purge and trap method.

Where chlorinated solvents are present in finished drinking water the most likely source is the raw water. Analyze individual raw water samples to isolate the source of contamination. Always consider the possibility of co-eluting interferences. Analysis using capillary chromatography minimizes this possibility. Analysis using dissimilar columns may confirm the presence of interferences through differences in retention time between the constituent of interest and the unknown compound in the sample; however, the most definitive confirmation routinely available is GC/MS.

because the simplified extraction technique depends on equilibria between solvent and water, because extraction is not 100% efficient, and because efficiency is dependent on concentration, it is important to: extract samples and standards in the same manner; monitor matrix recoveries to assess differences in equilibria and, where the concentration is found to be above the linear range of the method, to either dilute samples carefully before extraction, or prepare standards in water at the estimated sample concentration and carefully dilute both sample and standard extracts. Always process standards and samples together and hold constant such variables as water temperature, solvent temperature, room temperature, extraction time, and separation times.

Some interferences in GC analyses occur as a result of sample, solvent, or carrier gas contamination, or because large amounts of a compound may be injected into the GC and linger in the detector. Methylene chloride, chloroform, and other halocarbon and hydrocarbon solvents are ubiquitous contaminants in environmental laboratories. Make

strenuous efforts to isolate the analytical system from laboratory areas where these or other solvents are in use. An important sample contaminant is sulfur, which is encountered generally only in base/neutral extracts of water, although anaerobic groundwaters and certain wastewaters and sediment/sludge extracts may contain reduced sulfur compounds, elemental sulfur, or polymeric sulfur. Eliminate this interference by adding a small amount of mercury or copper filings to precipitate the sulfur as metallic sulfide. Sources of interference originating in the chromatograph, and countermeasures, are as follows:

**6.1 Septum bleed**-This occurs when compounds used to make the septum on the injection port of the GC bleed from the heated septum. These high-molecular-weight silicon compounds can be distinguished readily from compounds normally encountered in environmental samples by their peak shape or baseline rise. Nevertheless, minimize septum bleed by using septum sweep, in which clean carrier gas passes over the septum to flush out the "bleed" compounds.

**6.2 Column bleed**-This term refers to loss of column coating or breakdown products when the column is heated. This interference is more prevalent in packed columns, but also occurs to a much lesser extent in capillary columns. It occurs when the column temperature is high or when water or oxygen is introduced into the system. Solvent injection can damage the stationary phase by displacing it. Certain organic compounds acting as powerful solvents, acids, or bases can degrade the column coating. Injection of large amounts of certain Surface-active agents may destroy GC columns. Signs of column bleed are a rising baseline or a large peak at end of run.

**6.3 Ghost peaks**-These peaks occur when an injected sample contains either a large amount of a given compound, or a compound that adsorbs to the column coating or injector parts (e.g., septum). When a subsequent sample is injected, peaks can appear as a result of the previous injection. Eliminate ghost peak by injecting a more dilute sample, by producing less reactive derivatives of a compound that may interact strongly with the column material, by selecting a column coating that precludes these interactions, by injecting solvent blanks between samples, or by heating the column for a longer time or to a higher temperature at the end of the run.

## 7.0 Detection levels:

The method is useful for trihalomethane and selected chlorinated solvents at concentrations from approximately 0.1 to 200 µg/L. Actual detection levels are highly dependent on the characteristics of the gas chromatographic system used, the ratio of solvent to water, and interferences present in the solvent.

## 8.0 Quality Control:

For THMs, the ISTD (internal standard) is optional.

A minimum program of quality control consists of an initial demonstration of proficiency for each analyst and each instrument system and an ongoing program of quality control analysis. Record initial quality by documenting initial performance relative to published performance criteria. Maintain records of performance by comparing ongoing quality control checks to performance criteria and objectives for data quality.

**8.1 Analyst proficiency:** The analyst should be experienced in the operation of a GC/ECD and produce an initial demonstration of proficiency.

**8.2 Method blanks:** Prepare and analyze method blanks on each shift after calibration and before first sample analysis. Concentrations of compounds in the method blank should not exceed the experimentally determined method detection level. If the method blank is out of control isolate the source of contamination, apply corrective action, and process a new method blank. Under no circumstances subtract method blank values from the sample result.

**8.3 Quality control (QC) check standards:** Preferably obtain QC standards from a separate source and prepare independently from calibration standards. Analyze QC check standards as though they were samples at a frequency of 5% (every 20 samples) and at the end of the analytical sequence. Compare results to known concentration of the check standard and calculate percent recovery. Percent recovery nominally should be between 80 and 120%. Develop mean recovery control charts of QC check standards results and use historical 99% confidence limits to accept or reject the ongoing calibration. Where historical confidence limits are wider than the nominal limits, investigate standard materials, preparation and storage procedures, and other potential

sources of error. Take and document corrective actions.

**8.4 Detector sensitivity:** Maintain a log of detector response, in area counts or peak height, using one standard that is analyzed each day, to monitor changes in detector sensitivity. Optionally, plot these data to observe trends in detector sensitivity. Note the sensitivity at which method detection level studies were performed and replace or repair detectors where minimum detectable quantities are significantly affected by declining detector sensitivity.

**8.5 Laboratory-fortified samples with known additions:** In a laboratory analyzing more than 10 samples daily, extract and analyze a known addition on each tenth sample. Be sure this is representative of different sample types because there is some evidence of matrix effects with liquid-liquid extraction methods. In a laboratory analyzing fewer than 10 samples daily, each time sample extractions are performed, extract and analyze at least one laboratory -generated known addition sample.

**8.6 Duplicate analysis:** Randomly select, then extract and analyze in duplicate, 10% of all samples. Maintain an up-to-date log on bias and precision data collected on known-addition samples, duplicate samples and evaluate results

**8.7 Laboratory control standards (performance evaluation standards):** Quarterly, add an external reference laboratory evaluation standard to organic-free water, extract, and analyze. Obtain this standard from an authorized proficiency-testing provider. The results from this sample should agree within 20% of the true value for each compound. If not, check each step in preparation and analysis to isolate the problem. Document external reference standard results and any corrective action taken.

## 9.0 References:

9.1 American Public Health Association. 22<sup>nd</sup> edition. 2012. Method No. 6232B.

## B. By Gas Chromatography Mass Spectrometer (GC-MS) method

### 1. Scope:

This method is applicable to the determination of a wide range of purge able organic compounds (Table 1) .The method can be extended to include other volatile organic compounds, provided that all performance criteria are met. It should be used only by

analysts experienced in the operation of GC/MS systems and in evaluation and interpretation of mass spectra.

Table 1: Compounds Determinable By Gas Chromatographic Methods for Purgeable Organic Compounds

<b>Analyte</b>	<b>Chemical Abstract Services Registry Number</b>
Benzene	71-43-2
Bromobenzene	108-86-1
Bromochloromethane	74-97-5
Bromodichloromethane	75-27-4
Bromoform	75-25-2
Bromomethane	74-83-9
n-Butylbenzene	104-51-8
sec-Butylbenzene	135-98-8
tert-Butylbenzene	98-06-6
Carbon tetrachloride	56-23-5
Chlorobenzene	108-90-7
Chloroethane	75-00-3
Chloroform	67-66-3
Chloromethane	74-87-3
2-Chlorotoluene	95-49-8
4-Chlorotoluene	106-43-4
Dibromochloromethane	124-48-1
1,2-Dibromo-3- chloropropane	96-12-8
1,2-Dibromoethane	106-93-4
Dibromomethane	74-95-3
1,2-Dichlorobenzene	95-50-1
1,3-Dichlorobenzene	541-73-1
1,4-Dichlorobenzene	106-46-7
Dichlorodifluoromethane	75-71-8

1,1-Dichloroethane	75-34-3
1,2-Dichloroethane	107-06-2
1,1-Dichloroethene	75-35-4
cis-1,2-Dichloroethene	156-59-4
trans-1,2-Dichloroethene	156-60-5
1,3-Dichloropropane	78-87-5
1,3-Dichloropropane	142-28-9
2,2-Dichloropropane	590-20-7
1,1-Dichloropropene	563-58-6
cis-1,3-Dichloropropene	10061-01-5
trans-1,3-Dichloropropene	10061-02-6
Ethylbenzene	100-41-4
Hexachlorobutadiene	87-68-3
Isopropylbenzene	98-82-8
p-Isopropyltoluene	99-87-6
Methyl t-butyl ether	1634-04-4
Methylene chloride	75-09-2
Naphthalene	91-20-3
n-Propylbenzene	103-65-1
Styrene	100-42-5
1,1,1,2-Tetrachloroethane	630-20-6
1,1,1,2,2-Tetrachloroethane	79-34-5
Tetrachloroethene	127-18-4
Toluene	108-88-3
1,2,3-Trichlorobenzene	87-61-6
1,2,4-Trichlorobenzene	120-82-1
1,1,1-Trichloroethane	71-55-6

Analyte	Chemical Abstract Services Registry Number
1,1,2- Trichloroethane	79-00-5
Trichloroethene	79-01-6
Trichlorofluoromethane	75-69-4
1,2,3- Trichloropropane	96-18-4
1,2,4- Trimethylbenzene	95-63-6
1,3,5- Trimethylbenzene	108-67-8
Vinyl chloride	75-01-4
o-Xylene	95-47-6
m-Xylene	108-38-3
p-Xylene	106-42-3

## 2. Principle:

Volatile organic compounds are transferred efficiently from the aqueous to the gaseous phase by bubbling an inert gas (e.g., helium) through a water sample contained in a specially designed purging chamber at ambient temperature. The vapor is swept through a sorbent trap that adsorbs the analytes of interest. After purging is complete, the trap is heated and back-flushed with the same inert gas to desorb the compounds onto a gas chromatographic column. The gas chromatograph is temperature-programmed to separate the compounds. The detector is a mass spectrometer.

## 3.0 Reagents:

**3.1 Reagent water**, in which no interferent is observed at or above the MDL of the constituents of interest. Prepare by passing tap water through a carbon filter bed containing about 0.5 kg activated carbon, by distillation, or by using a water purification system.

### 3.2 Trap packing materials:

**3.2.1** 2, 6 -Diphenylene oxide polymer, 60/80 mesh: chromatographic grade.

**3.2.2** Methyl silicone packing, 3 OV-1.

**3.2.3** Silica gel, 35/60 mesh.

**3.2.4** Methanol, purge-and-trap grade.

**3.2.5** Hydrochloric acid: HCl, 1 + 1.

**3.2.6** Vinyl chloride, 99.9% pure.

**3.2.7** Ascorbic acid.

**3.3 Stock standard solutions:** Prepare from pure standard materials or purchase as certified solutions. Prepare stock standard solutions in methanol using assayed liquids or gases as describe below:

**3.3.1** Place about 9.8 mL methanol in a 10mL ground-glassstoppered volumetric flask. Let stand unstoppered for about 10 min or until all alcohol-wetted surfaces have dried. Weigh flask to nearest 0.1 mg.

**3.3.2** Add assayed reference materials as follows: For liquids, using a 100 $\mu$ L syringe or disposable capillary-tip glass pipet, immediately add two or more drops of assayed reference material to flask, then reweigh. Ensure that the drops fall directly into the alcohol without contacting flask neck.

**3.3.3** For halocarbon gases that boil below 30°C (bromomethane, chloroethane, chloromethane, dichlorofluoromethane, trichlorofluoromethane, vinyl chloride), attach a vinyl plastic tube to port of gas bottle containing reference material, with open end bubbling into a beaker of methanol showing flow through the tubing; insert needle of 5mL valved gastight syringe into tube and pull gas into syringe slowly to 5.0mL mark.

**3.3.4** Lower syringe needle to within 5 mm of methanol surface and slowly force gas onto surface. The gas will dissolve into the methanol and will be seen as a vortex as it dissolves into the solvent.

**3.3.5** Reweigh flask (difference is amount of gas dissolved into methanol), dilute to volume, stopper, and mix by inverting several times.

**3.3.6** Calculate concentration in micrograms per microliter from net gain in weight. When compound purity is assayed to be 96% or greater, calculate concentration of stock standard from uncorrected weight.

**3.3.7** Transfer stock standard solution into a TFE-sealed screw-cap bottle. Store with minimum heads pace at -10 to -20°C away from light.

**CAUTION: Toxic substances**

**Safety:**

The toxicity or carcinogenicity of each analyte has not been precisely defined. Benzene,

Carbon tetrachloride, Bis(1-chloroisopropyl)ether, chloroform, 1,4-dichlorobenzene, 1,2-dichloroethane, hexachlorobutadiene, trichloroethene, vinyl chloride, 1,1,2,2-tetrachloroethane, 1,1,2-trichloroethane, 1,2-dibromoethane, and tetrachloroethene have been classified tentatively as known or suspect carcinogens. Handle pure standard materials and stock standard solutions of these compounds in a hood and wear a NIOSH/MESA-approved toxic gas respirator when handling high concentrations.

**3.4 Secondary dilution standards:** Using stock standard solutions, prepare in methanol secondary dilution standards that contain the compounds of interest, either singly or mixed together. Prepare secondary dilution standards at concentrations that will permit aqueous calibration standards (3.10) to bracket working range of the analytical system. Store secondary dilution standards with minimal headspace in a freezer and check frequently for signs of evaporation (which would indicate need for regeneration). Always bring to room temperature before preparing calibration standards. Prepare standards fresh weekly for gases. Replace all other standards monthly or sooner if comparison with check standards indicates a problem.

**3.5 Internal standard/surrogate standard known addition:** Prepare a solution containing Fluorobenzene (internal standard) and 1,2-dichlorobenzene- $d_4$  (surrogate) in methanol. Alternate internal standard and surrogate compounds may be used, provided that they meet method criteria and do not interfere with any method analyte(s). Prepare secondary dilution standard at a concentration of  $5\mu\text{g}/\text{mL}$  of each compound. Adding  $5.0\mu\text{L}$  standard to  $25.0\text{mL}$  sample or calibration standard yields a concentration equivalent to  $1.0\mu\text{g}/\text{L}$ . Alternate secondary standard concentrations can be used if addition volume is adjusted accordingly and all internal standard criteria are met. Add this mixture to each sample, standard, and blank.

**3.6 Calibration standards:** Prepare at least five concentration levels for each compound by adding appropriate amounts of secondary standard solution to reagent water and inverting water sample twice. Prepare one standard at a concentration near, but above, the MDL (i.e., 4 X MDL for potable-water-type samples) or a level that defines the low end of the working range and the others to

correspond to the expected range of sample concentrations or to define the detector working range. Aqueous calibration standards can be stored up to 24 h if held in sealed vials with zero headspace. Otherwise, discard within 1 h. Alternatively, prepare calibration standards by injecting, with a solvent flush, an appropriate amount of a standard mix dilution and internal standard surrogate mix, directly into a 25-mL syringe filled with reagent water; immediately inject water standard into purge vessel.

## 4.0 Apparatus

**4.1 Purge and trap system:** The purge and trap system consists of purging device, trap, and desorber. Several complete systems are available commercially.

**4.1.1** Purging device, designed to accept 25 mL samples with a water column at least 5 cm deep. A smaller 5 mL purging device is acceptable if required method detection levels and performance criteria are met. Keep gaseous headspace between water column and trap to a total volume of less than 15 mL. Pass purge gas through water column as finely divided bubbles with a diameter of less than 3 mm at the origin. Introduce purge gas no more than 5 mm from base of water column. Needle spargers may be used instead of the glass frit. However, in either case, introduce purge gas at a point <5 mm from base of water column.

**4.1.2** Trap, at least 25 cm long and with an inside diameter of at least 3 mm, packed with the following minimum lengths of adsorbents: 1.0 cm methyl silicone coated packing, 7.7 cm 2, 6-diphenylene oxide polymer, 7.7 cm silica gel, and 7.7 cm coconut charcoal. If analysis is not to be made for dichlorodifluoromethane, the charcoal may be eliminated and the polymer section lengthened to 15 cm.

**4.1.3** Alternative sorbents may be used provided that all quality control criteria are met. Various sorbent traps are available commercially; ensure that trap keeps total purge gas volume and purge time constant (i.e., 40 mL/min for 11 min) and that performance will meet all quality control criteria. Methyl silicone coated packing is recommended, but not mandatory. The packing protects the diphenylene oxide polymer adsorbent from aerosols, re-coating any active site that may develop during the heating process, and ensures that the polymer is fully enclosed within the heated zone of the trap, thus eliminating potential cold spots. Alternatively, silanized glass wool may be

used as a spacer in the trap inlet. Before initial use, condition trap overnight following manufacturer's instructions. Vent trap effluent to the room, not to analytical column. Before daily use, condition trap for 10 min with back-flushing. Optimally, vent trap to analytical column during daily conditioning; however, run column through temperature program before sample analysis.

**4.2 Gas chromatograph (GC):** Use a temperature-programmable GC, suitable for on-column injection. Deactivate all glass components (e.g., injector liners) with a silanizing agent.

**4.3 Capillary GC columns:** Use any capillary GC column that meets all performance criteria. Ensure that desorb flow rate is compatible with the column of choice. Four examples of acceptable columns are listed below.

**4.3.1** 60-m-long X 0.75-mm-ID VOCOL wide-bore capillary column with 1.5 $\mu$ m film thickness.

**4.3.2** 30-m-long X 0.53-mm-ID DB-624 mega-bore capillary column with 3 $\mu$ m film thickness.

**4.3.3** 30-m-long X 0.32-mm-ID DB-5 capillary column with 1 $\mu$ m film thickness.

**4.3.4** 30-m-long X 0.25-mm-ID DB-624 capillary column with 1.4 $\mu$ m film thickness.

**4.4 Mass spectrometer,** capable of scanning from 35 to 300 amu every 2s or less, utilizing 70 eV (nominal) electron energy in the electron impact ionization mode, and producing a mass spectrum that meets all criteria of table given below when 25 ng or less of 4-bromofluorobenzene is introduced into GC.

**Table 2: BFB Key m/z Abundance Criteria**

Mass	m/z Abundance Criteria
50	15 to 40% of mass 95
75	30 to 60% of mass 95
95	Base Peak, 100% relative Abundance
96	5 to 9% of mass 95

173	<2% of mass 174
174	>50% of mass 95
175	5 to 9% of mass 174
176	95 to 101 % of mass 174
177	5 to 9% of mass 176

To ensure sufficient precision, the desired scan rate permits acquisition of at least five spectra while a sample component elutes from the GC

**4.5 Purge and trap-GCIMS interface:** Use an open-split or direct-split interface, depending on which column is used. Alternatively, if the narrow-bore column (column mentioned in 4.3.4) is used, a capillary concentrator preceding the GC may be necessary. This interface condenses desorbed materials onto an uncoated fused silica pre-column and when flash-heated transfers compounds onto the capillary column. The uncoated section of column is cooled to -150°C during desorption and heated to 250°C to transfer condensed materials.

**4.6 Data System:** To the mass spectrometer attach a computer that allows continuous acquisition and storage of all mass spectra obtained throughout the chromatographic program. Computer software should allow for a search of all acquired spectra for specific m/z (masses) and the plot of such m/z abundances versus time or scan number. This type of plot is an extracted ion current profile (EICP). Software also should allow the integration of abundances in any EICP over a specified time or scan limit.

**4.7 Syringes,** 0.5, 1.0, 5, and 25 mL glass hypodermic with detachable tip.

**4.8 Syringe valves,** two-way, with detachable tip.

**4.9 Microsyringes,** 10,25, and 100 µL with a 5 cm X 0.15mm-ID and 220 bevel needle.

**4.10 Bottles,** 40mL with TFE-lined screw cap.

## 5.0 Procedure:

**5.1 Operating conditions:** Table below provides recommended operating

conditions for the gas chromatograph and gives estimated retention times and MDLs that can be achieved under these conditions. Other chromatographic columns or conditions can be used if the quality control criteria are met.

**Table 3: Primary Quantitation Ion, Retention Times and Method Detection Levels**

Analyte	Retention Time (min)	MDL (µg/L)	Primary(m/z)
Dichlorodifluoromethane	1.49	0.19	85
Chloromethane	1.71	0.15	50
Vinyl chloride	1.79	0.12	62
Bromomethane	2.16	0.22	94
Chloroethane	2.28	0.23	64
Trichlorofluoromethane	2.57	0.059	101
1,1- Dichloroethene	3.22	0.13	96
1,1,2-Trichloro-1,2,2-triflloerhane	3.25	0.065	101
Methylene chloride	3.96	0.099	49
trans-1,2-Dichloroethene	4.4	0.2	96
Methyl t-butyl ether	4.45	0.45	73
1,1 - Dichloroethane	5.14	0.047	63
cis-1,2- Dichloroethene	6.3	0.13	96
2,2-Dichloropropane	6.24	0.041	77
Bromochloromethane	6.77	0.032	128
Chloroform	7	0.126	83
1,1 ,1- Trichloroethane	7.24	0.043	97
1,1 - Dichloropropene	7.67	0.04	75
Carbon tetrachloride	7.65	0.042	]17
Benzene	8.07	0.036	78
1,2-Dichloroethane	8.14	0.055	62
Trichlorethene	9.44	0.045	95
1,2-Dichloropropane	9.85	0.053	63

Dibromomethane	10.07	0.035	93
<b>Analyte</b>	<b>Retention Time (min)</b>	<b>MDL (µg/L)</b>	<b>Primary(m/z)</b>
Bromodichloromethane	10.47	0.112	83
cis-1,3-Dichloropropene	11.29	0.048	75
Toluene	11.81	0.047	91
trans-1,3-Dichloropropene	12.27	0.051	75
1,1,2- Trichloroethane	12.56	0.043	83
1,3-Dichloropropane	12.83	0.09	76
Dibromochloromethane	13.24	0.133	129
1,2-Dibromoethane	13.35	0.133	107
Chlorobenzene	14.21	0.052	112
1,1,1 ,2- Tetrachloroethane	14.37	0.048	131
Ethylbenzene	14.42	0.032	91
m,p-Xylene	14.63	0.038	91
o-Xylene	15.27	0.038	91
Styrene	15.3	0.031	104
Bromoform	15.6	0.131	173
Isopropylbenzene	15.9	0.074	105
Bromobenzene	16.34	0.14	156
1,1,2,2- Tetrachloroethane	16.41	0.066	83
1,2,3- Trichloropropane	16.44	0.072	75
n-Propylbenzene	16.57	0.26	91
2-Chlorotoluene	16.68	0.042	126
4-Chlorotoluene	16.86	0.04	126
1,3,5- Trimethylbenzene	16.88	0.035	105
tert-Butylbenzene	17.38	0.1	119
sec-Butylbenzene	17.46	0.025	105
1,2,4-Trimethylbenzene	17.74	0.046	105
4- Isopropyltoluene	17.99	0.037	119
1,3-Dichlorobenzene	17.89	0.045	146

1,4-Dichlorobenzene	18.04	0.033	146
1,2-Dichlorobenzene	18.64	0.031	146
n-Butylbenzene	18.65	0.028	91
Hexachlorobutadiene	21.69	0.033	225
1,2,4- Trichlorobenzene	21.34	0.043	180
Naphthalene	21.8	0.049	128
1,2,3- Trichlorobenzene	22.32	0.047	180

**5.2 GC/MS performance tests:** At the beginning of each 12h period during which analyses are to be performed, check GCIMS system by a performance test with BFB before any samples, blanks, or standards are analyzed. Performance tests require the following instrument parameters:

Electron energy: 70 eV (nominal)

Mass range: 35 to 300 amu

Scan time: at least 5 scans/peak but not more than 2 s/scan

Inject 25 ng BFB directly on GC column. If direct injection is not easily performed, add 1µL 25µg/mL BFB solution to 25 mL reagent water in syringe used for sample transfer to purge device and analyze as a sample. Obtain a background-corrected mass spectrum of BFB and confirm that all key m/z criteria in table 2 are achieved. If all criteria are not achieved, re-tune mass spectrometer and repeat test until all criteria are met.

**5.3 Calibration:** Calibrate system as follows:

**5.3.1 System setup** - Condition trap initially overnight at 180°C by back-flushing with inert gas at 20 mL/min. Condition trap daily for 10 min at manufacturer's suggested temperature. Connect purge and trap system to GC using recommended temperature program and flow-rate conditions. Calibrate system by either the internal or the external standard technique.

**5.3.2 Internal standard calibration technique** - Select one or more internal standards similar in analytical behavior to the compounds of interest. Fluorobenzene is

a recommended internal standard compound. Demonstrate that measurement of internal standard is not affected by method or matrix interference. Because of such limitations, no one internal standard may be applicable to all samples. The compounds used as surrogates (e.g., 1,2-dichlorobenzene-d<sub>4</sub>) for quality control also can be used successfully as internal standards. Prepare calibration standards at a minimum of five concentration levels for each compound (described in 3.10).

Prepare a secondary dilution standard containing each of the internal standards (3.9). Analyze each calibration standard according to procedure for samples, adding internal standard solution directly to syringe. Tabulate peak height or area responses against concentration for each compound and internal standard, and calculate response factors (RF) for each compound in each calibration standard as follows:

$$RF = \frac{(A_s)(C_{is})}{(A_{is})(C_s)}$$

Where,

$A_s$  = response for compound to be measured,

$A_{is}$  = response for internal standard

$C_{is}$  = concentration for internal standard, and

$C_s$  = concentration of compound to be measured.

Calculate % RSD for the average RFs for each compound. Average RF can be used if RSD is less than 20%.

**5.3.3. External standard calibration technique** - Prepare standards (as directed in 3.10). Analyze each calibration standard and tabulate peak area responses versus concentration. Prepare calibration curve for each compound. Alternatively, if ratio of response to concentration (calibration factor) is a constant over the working range (<20% RSD), assume linearity through the origin and use average calibration factor in place of a calibration curve.

**5.3.4. Calibration check**, please refer (5.7.2.2) for calibration check.

**5.4 Sample analysis:** Bring sample to ambient temperature. Remove plunger from 25mL syringe and close attached valve. Open sample bottle and carefully pour sample into syringe barrel to just short of overflowing. Replace syringe plunger, invert syringe, and open valve. Vent any air and adjust sample volume to 25.0 mL, in duplicate if sufficient sample is available (once sample cap has been removed, sample cannot be stored, because of headspace). Add an appropriate amount of surrogate/internal standard through valve bore, and close valve. Attach to purge device, open valves, and inject sample into purge vessel. Close valves and purge sample for 11.0 min at ambient temperature at a flow rate of 40 mL/min (helium or nitrogen). If water vapor causes problems in the mass spectrometer, use a 3-min dry purge and/or a moisture control module. Desorb trapped materials onto head of chromatographic column at 180°C while back-flushing trap for 4 min with inert gas at a flow rate compatible with the column of choice, and begin GC temperature program. Set system auto-drain to empty purge chamber while trap is being desorbed into GC, or alternatively, use sample syringe to empty vessel. Washing chamber with two 25mL flushes of reagent water is useful if highly contaminated samples are being analyzed. Be sure all areas wetted during purging are also wetted during rinsing to maximize flushing. Recondition trap by baking at conditioning temperature for 5 to 7 min. Let trap cool to ambient before introduction of next sample into purge vessel. When all sample compounds have been eluted from chromatographic column, end data acquisition and store data files. Use data system software to display full range mass spectra and appropriate extracted ion current profiles (EICP). If any ion abundances exceed system working range, dilute sample in second syringe with reagent water and analyze.

***(NOTE: Take care with sample because compounds can be very volatile and can be lost if sample is reopened.)***

Estimate amount of dilution needed and expel excess sample from second syringe, inject that portion into purge vessel, and with a second syringe, add necessary reagent water to a total of 25.0 mL in purge vessel.

**5.5 Calculation:**

When compounds have been identified, base quantitation on integrated area abundance from the EICP of the primary characteristic  $m/z$  given in Table 3. If sample produces an interference for the primary  $m/z$ , calculate a response factor or calibration curve using a secondary characteristic  $m/z$ , and use secondary  $m/z$  to quantitate. Report results in micrograms per liter. Report all quality control data with sample results.

### 5.6 Interferences:

Impurities in the purge gas and organic compounds outgassing from the plumbing upstream of the trap account for most contamination problems. Demonstrate that the system is free from contamination under operational conditions by analyzing laboratory reagent blanks daily.

***(NOTE: Use blanks for monitoring only; corrections for blank values are unacceptable.)***

Avoid using non- TFE plastic tubing, non- TFE thread sealants, or flow controllers with rubber components in the purge and trap system. Ensure that the analytical area is not subject to contamination from laboratory solvents particularly methylene chloride and methyl tert-butyl ether (MtBE).

Samples can be contaminated by diffusion of volatile organics (particularly fluorocarbons and methylene chloride) through the septum seal during shipment and storage. Use a field reagent blank prepared from reagent water and carried through the sampling, handling, and shipping procedures as a check on such contamination. Contamination by carryover can occur whenever high-level and low-level concentration samples are analyzed sequentially. To reduce carryover, rinse purging device and sample syringe with reagent water between samples. Follow analysis of an unusually high concentration sample with a LRB to check for carryover contamination. For samples containing large amounts of water-soluble materials, suspended solids, high boiling compounds, or high levels of volatile compounds, wash purging device with a detergent solution, rinse it with distilled water, and dry it in an oven at 105°C between analyses. The trap and other parts of the system also are subject to contamination; therefore, frequently bake and purge entire system.

## 5.7 Quality control:

### 5.7.1 Initial quality control:

**5.7.1.1 Initial demonstration of capability**-Conduct initial demonstration of capability study at least once, before analysis of any sample, by each analyst, to demonstrate proficiency with the method of choice. Include at least analysis of a reagent blank and four reagent blank samples fortified at a concentration between 10 times the minimum reporting level and the midpoint of the libration curve. The blank should not contain any compound of interest at a concentration greater than minimum reporting level. Mean percent recovery for each compound calculated from the four fortified samples should be 80 to 120%, and the relative standard deviation (RSD) should be <20%.

**5.7.1.2 Method detection level (MDL)** - The MDL is a statistical determination of the minimum concentration that can be measured by the method with a confidence level of 99% that the analyte concentration is greater than zero. Determine MDL before any samples are analyzed. For MDL calculation, start with a concentration about five times the estimated instrument detection level. Perform MDL determination as an iterative process. (The values listed in Table 3 were generated using a concentration of 0.5 µg/L) Conduct MDL determination at least annually. Analyze samples for MDL determination over a 3- to 5-days period to generate a more realistic value.

**5.7.1.3 Quality-control sample**-Analyze an externally generated quality-control sample as a laboratory fortified blank at least quarterly or whenever new stock solutions are generated. Obtain this sample from sources external to the immediate laboratory, and use it to validate the laboratory's standards both qualitatively and quantitatively. Acceptance criteria are supplied by the manufacturer. If all criteria are not met, determine cause of error, and correct it before continuing.

**5.7.1.4 Minimum quantitation level (MQL)**-The MQL is the lowest level that can be quantified accurately. The MQL is defined as four times the MDL.

### 5.7.2 Calibration:

**5.7.2.1 Initial calibration-**Perform initial calibration with "a minimum of five concentrations of analytical calibration standards (CALs) for the compound(s) of interest. The lowest concentration should be at the working reporting level; the highest concentration should be at the upper end of the calibration range. Do not report values that are outside of the defined calibration range. For the calibration concentrations, there should be no more than one order of magnitude between concentrations.

Use any of the following calibration functions, as appropriate: response factor for internal standard calibration, calibration factor for external standard calibration, or calibration curve. Calibration curves may be linear through the origin, linear not through the origin, or quadratic through or not through the origin. Use the following recommended acceptance criteria for the various calibration functions.

If using response factors or calibration factors, relative standard deviation (RSD) for each compound of interest should be less than 20%. If the RSD is not less than 20% for any compound of interest, then identify and correct source of lack of linearity before sample quantitation. When using response factors (i.e., for GC/MS analysis), check performance or sensitivity of the instrument for the compound of interest against minimum acceptance values for the response factors. See specific analytical method for the acceptance criteria for the response factors for each compound.

For a linear regression, the correlation coefficient should be  $>0.994$ . Recalculate each calibration point compared to curve. Resulting values should be within  $\pm 20\%$ . If any of the recalculated values are not within  $\pm 20\%$ , identify and correct source of outlier(s) before sample quantitation.

Use initial calibration, with any of the above functions (response factor, calibration factor, or calibration curve) for quantitation of the analytes of interest in samples. Use continuing calibration, only for checks on initial calibration and not for sample quantitation. Perform initial calibration when instrument is set up and whenever continuing calibration criteria are not met.

**5.7.2.2 Continuing calibration-Continuing calibration (CCAL)** is the periodic analysis of a calibration standard used to verify that the instrument response has not changed significantly from the initial calibration. Perform continuing calibration every

10 samples for GC analysis, every 20 samples for GC/MS analysis, or every 12 h, whichever is more frequent.

Perform continuing calibration with one or more of the concentrations of analytical standards in the initial calibration. Vary actual concentration of continuing calibration standard over calibration range, with a minimum concentration greater than two times the reporting limit. The acceptance criterion for continuing calibration is 70 to 130% recovery compared to the known or expected value of the calibration standard (at the analyst's discretion, the acceptance criterion for the gases may be extended to 60 to 140% recovery).

If the acceptance criteria are not met, re-analyze continuing calibration standard or repeat initial calibration. When using response factors, check performance or sensitivity of instrument for analytes of interest against minimum acceptance values for response factors.

**5.7.2.3 Closing standard**-Finish all sample sets with a closing standard to demonstrate that performance was still acceptable for the last sample analyzed. Use acceptance criteria as for the CCAL.

### **5.7.3 Batch quality control:**

**5.7.3.1 Analytical day**-An analytical day is defined as a 12h analytical period.

**5.7.3.2 Sample set (batch)** - A sample set (batch) is defined as those samples extracted in an analytical day, not to exceed 20 samples.

**5.7.3.3 Laboratory reagent blank (LRB)**-A LRB is a blank sample consisting of all reagents that normally contact a sample when carried through the entire analytical procedure. Use reagent blank to determine contribution of reagents and preparative analytical steps to observed value. No compound of interest should be present in reagent blank at a level greater than the MQL. Include a minimum of one reagent blank with each sample set (batch).

**5.7.3.4 Laboratory-fortified blank (LFB)** (as per 5.7.2.2)

For this method, the LFB and CCAL are the same.

**5.7.3.5 Internal standard (IS)**-An internal standard is a compound of known

concentration added to each standard and sample just before sample analysis. Because of the nature of purge and trap analysis, the IS is taken through the entire analytical process, just as is the surrogate standard (as per 5.7.3.6). However, the IS is used for quantitation, whereas the surrogate standard is used to monitor ongoing purge recovery. Use IS to monitor retention time, relative response, and concentration of analytes in each sample. When quantifying by the internal standard method, measure all compound responses relative to this standard. Internal standard area response should be in the range of  $\pm 30\%$  compared to the mean calibration curve area response.

The IS compound should mimic the chromatographic conditions of the analytes of interest. The retention time of this compound should separate from all analytes of interest and elute in a representative area of the chromatogram. If a specific compound cannot be found to meet these criteria, use additional compounds to satisfy analytical needs.

**5.3.7.6 Surrogate standard (SS)**-A surrogate standard is a compound added to each standard and sample at a known concentration before extraction. Choose a compound(s) that is chemically similar to the analytes and that is unlikely to be found in environmental samples. Carry surrogate standard through entire sample extraction and analytical process to monitor extraction recovery for each sample. Surrogate recovery should remain reasonably constant over time. Recovery should not vary more than 30% from the known value. Refer to method of choice for specific surrogates.

**5.3.7.7 Laboratory-fortified sample (LFS)**-A LFS is an additional portion of a sample to which the analytes of interest have been added at a concentration at least two times the MRL or around the middle of the calibration range. Include a minimum of one LFS with each sample set (batch). Make LFSs at sufficient concentrations that sample background levels do not adversely affect recovery calculations. (If this is a known sample, adjust addition concentrations to be about five times background level). Base sample batch acceptance on results of CCALs and LFBs rather than on LFSs, because the matrix of the sample may interfere with method performance. Prepare fortifying solution for blanks and samples from a different primary mix than that used to develop working standard mix.

**5.3.7.8 LFS duplicates**-A LFS duplicate is a second LFS used to evaluate the precision of the method in a matrix sample. If sufficient sample volume is collected,

fortify a large enough volume to yield two sample portions for analysis. If sufficient sample volume is not collected, use a second bottle of the same sample fortified to the same concentration as the first. Include a minimum of one LFS duplicate with each sample set (batch). Compare precision and bias to those listed in the method. Base sample batch acceptance on results of CCAL and LFB additions rather than LFS duplicates.

## **6      References:**

- 6.1    American Public Health Association. 22<sup>nd</sup> edition. 2012. Method No. 6200B.
- 6.2    American Public Health Association. 22<sup>nd</sup> edition. 2012. Method No. 6200A.5

**SECTION B: MICROBIOLOGICAL PARAMETERS**

## CHAPTER I: TEST METHODS FOR DRINKING WATER

### 1. Aerobic Microbial count/Standard plate count

#### 1.1 Principle:

Plate count agar (PCA) is a general purpose growth medium commonly used to assess "total" or "viable" bacterial growth of a water sample. The number of microorganisms per milliliter of sample is calculated from the number of colonies obtained on PCA plate from selected dilution. Poured plates are prepared using a specified culture medium and a specified quantity of the sample. The plates are aerobically incubated at two different temperatures i.e. 37°C for 24 hr and 20 – 22°C for 72 hr

#### 1.2 Culture Media:

##### 1.2.1 Plate count Agar (PCA)

##### 1.2.2 Buffered Peptone water (BPW)

##### 1.2.3 Overlay Medium (Agar Medium)

#### 1.3 Procedure:

**1.3.1** Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution.

**1.3.2** Aseptically inoculate 1ml of the water sample using sterile pipette into sterile petri plates in duplicate in two sets. The petri plates should be labeled with the sample number, date and any other desired information.

**1.3.3** Pour into each plate 15–18 mL of the molten sterilized PCA media (cooled to 44°C–47°C). Avoid pouring of molten medium directly onto the inoculum. Mix the media and inoculum by swirling gently clockwise and anti-clockwise so as to obtain homogenous distribution of inoculum in the medium.

**1.3.4** Allow to cool and solidify. In case, where in sample microorganism having spreading colonies is expected, add 4ml of overlay medium onto the surface of solidified plates.

**1.3.5** After complete solidification, invert the prepared plates and incubate one set at 37°C for 24 hr and other set at 20 – 22°C for 72 hr.

**1.3.6** After specified incubation period count all colonies including pinpoint colonies. Spreading colonies shall be considered as single colony. If less than one quarter of dish is overgrown by spreading, count the colonies on the unaffected part of the dish and calculate corresponding number in the entire dish. If more than one quarter is overgrown by spreading colonies discard the plate.

#### **1.4 Calculation & Expression of results:**

**Case 1:** Plates having microbial count between 10 and 300cfu

$$N = \frac{\text{ColoniesPlate1} + \text{ColoniesPlate2}}{2}$$

**Case 2:** Plates having microbial count less than 10cfu but at least 4.

**Calculate the results as given in Case 1.**

**Case 3:** If microbial load is from 3 to 1 then reporting of results shall be:

**“Microorganisms are present, but, less than 4 per mL”**

**Case 4:** When the test sample/plates contains no colonies then reporting of results shall be:

**“Less than 1 cfu/ml”.**

#### **1.5 References:**

**1.5.1** IS 5402:2012

**1.5.2** ISO 7218:1996

**1.5.3** IS 14543:2004

## 2. Detection of *E.coli* and Coliform

Coliform bacteria are members of Enterobacteriaceae family that express  $\beta$ -D-galactosidase activity and *E.coli* are members of coliform group that express  $\beta$ -D-galactosidase and  $\beta$ -D-glucuronidase activity

### 2.1 Principle:

A test portion of water sample is passed through a membrane filter, which is then placed for incubation on Chromogenic coliform agar plate. All presumptive coliform bacteria grow as pink to red colonies which are then confirmed by negative oxidase test. Due to  $\beta$ -D-galactosidase and  $\beta$ -D-glucuronidase activity *E.coli* bacteria appear as Dark blue to violet in color. This method is not applicable to  $\beta$ -D-glucuronidase negative *E.coli* strains like *E.coli* O157.

### 2.2 Culture Media:

2.2.1 Chromogenic coliform Agar (CCA)

2.2.2 Tryptone Soya agar (Soyabean Casein digest agar)

### 2.3 Procedure:

2.3.1 Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution

2.3.2 Filter the sample (required volume) through a sterile membrane filter (0.45 $\mu$ m pore size) and place the filter in CCA plate and Incubate overnight at 36 $\pm$ 2 $^{\circ}$ C for 18-24hr.

### 2.4 Observation:

2.4.1 Count all pink to red colonies as presumptive coliform. Confirm all presumptive colonies by negative oxidase test.

2.4.2 Count all dark blue to violet colonies as *E.coli*.

2.4.3 **Oxidase test:** Add 2-3 drops of Oxidase reagent (1% alpha naphthol in 95% ethanol and equal amount of para-amino diamethylaniline hydrochloride in water) on

to a filter paper in a petridish. Take a colony to be confirmed by inoculating loop on pretreated filter paper. A positive reaction is indicated by the appearance of blue color within 30 s. commercially available oxidase disc can be used as an alternative

**2.4.4** Further subculture the presumptive colonies on non-selective agar (Tryptone Soy Agar) and incubate at  $36\pm 2^{\circ}\text{C}$  for  $21\pm 2$  hr to carry out oxidase test.

**2.4.5** Consider all colonies giving negative oxidase reaction as Coliform.

## **2.5 Expression of results:**

Presence/Absence of *E.coli* and Coliform given after confirmation of presumptive colonies in the sample examined.

Test for *E.coli* and Coliform= Present/absent per X mL of sample

## **2.6 References:**

**2.6.1** ISO 9308-1:2014

**2.6.2** IS 15185:2002

**2.6.3** IS 14543:2004 IS15188:2012

## **3. Detection of Coliform (Alternative method)**

Coliform bacteria are members of Enterobacteriaceae family that are gram negative, non sporeforming rods, capable of fermenting lactose and forming acid & gas within 48 hr at  $30-37^{\circ}\text{C}$ .

### **3.1 Principle:**

A test portion of water sample is passed through a membrane filter, which is then placed for incubation on Violet red Bile lactose Agar plate. The medium is selective due to the presence of the inhibitors - bile salts and crystal violet. Crystal violet inhibits gram-positive microorganisms especially Staphylococci. Organisms which rapidly ferment lactose produce red colonies surrounded by redpurple halo. Lactose non-fermenters and late lactose fermenters produce pale colonies.

### 3.2 Culture Media:

#### 3.2.1 Violet Red Bile Lactose Agar (VRBL)

### 3.3 Procedure:

**3.3.1** Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution

**3.3.2** Filter the sample (required volume) through a sterile membrane filter (0.45µm pore size) and place the filter in VRBL agar plate and Incubate overnight at 36±1°C for 24±2hr.

### 3.4 Observation:

**3.4.1** Coliform bacteria grow as purplish red colonies surrounded by a reddish zone of precipitated bile.

**3.4.2** Count all purplish red colonies as coliform.

### 3.5 Expression of results:

Presence/Absence of Coliform given in per unit of the sample examined.

Test for Coliform= Present/absent per X mL of sample

### 3.6 References:

**3.6.1** IS 5401(Part 1): 2002

**3.6.2** IS 14543:2004

**3.6.3** IS15188:2012

## 4. Detection of *Enterococci* (Faecal *Streptococci*)

*Enterococci* are Gram-positive cocci that often occur in pairs or short chains. *Enterococci* are facultative anaerobic organisms capable of forming spores, catalase negative and coccoid to egg shaped.

#### 4.1 Principle:

Slantez and Bartley medium contains sodium azide (to suppress the growth of gram negative bacteria) and 2,3,5-triphenyltetrazolium chloride, that is reduced to red formazon by *Enterococci*. Confirmation is done by transfer of membrane with all the colonies on Bile Aesculin Azide agar where aesculin is hydrolyzed within 2 hr to form 6, 7-dihydroxycoumarin, and combines with iron (III) to give tan colored to black compound which diffuses into the medium.

#### 4.2 Culture Media:

4.2.1 Slantz and Bartley medium

4.2.2 Bile-aesculin-azide agar

#### 4.3 Procedure:

4.3.1 Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution

4.3.2 Filter the sample (requisite volume) through a sterile membrane filter (0.45 $\mu$ m pore size) and place the filter on Slantez and Bartley medium plates.

4.3.3 Incubate the plates at 36 $\pm$ 2 $^{\circ}$ C for 44 $\pm$ 4hr. After incubation observe the plates showing red, maroon or pink colonies as presumptive *Enterococci*.

4.3.4 If there are presumptive colonies, confirmation can be done by transferring the membrane with sterile forceps without inverting it on plate of bile-aesculin-azide agar which has been preheated to 44 $^{\circ}$ C.

4.3.5 Incubate at 44  $\pm$  0.5 $^{\circ}$ C for 2hr and observe the plates immediately.

4.3.6 Consider all colonies showing tan to black color as *Enterococci*.

4.4 **Expression of results:** Presence/Absence of *Enterococci* is given after confirmation on Bile -aesculin-azide agar and given as.

*Enterococci*: Present/Absent per X mL of sample.

#### 4.5 Special Precautions:

4.5.1 Sodium Azide is highly toxic and mutagenic; precautions shall be taken to avoid contact with it, especially through the inhalation of fine dust during the preparation of media.

4.5.2 Azide containing media should not be mixed with strong inorganic acids, as toxic hydrogen azide (HN<sub>3</sub>) may be produced.

4.5.3 Solution containing azide can also form explosive compounds when in contact with metal pipework e.g. from sinks.

#### 4.6 References:

4.6.1 IS 15186:2002

4.6.2 IS 14543:2004

IS15188:2012

### Alternative Method: Detection of *Enterococci* (Faecal *Streptococci*)

#### 4.7 Principle:

Ethyl violet azide dextrose Agar contain sodium azide and ethyl violet that inhibit gram-positive bacilli and gram-positive cocci other than *Enterococci*. Confirmation is done by Gram stain reaction and colony characteristics on MacConkey Agar at 44°C.

#### 4.8 Culture Media:

4.8.1 Ethyl Violet Azide Dextrose Agar

4.8.2 MacConkey Agar

4.8.3 Nutrient Agar

#### 4.9 Procedure:

**4.9.1** Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution

**4.9.2** Filter the sample (requisite volume) through a sterile membrane filter (0.45µm pore size) and place the filter on Ethyl Violet Azide Dextrose Agar plates.

**4.9.3** Incubate the plates at 37±1°C for 48hr. After incubation observe the plates showing dark red colonies or colonies having red or pink centres as presumptive *Enterococci*.

**4.9.4** If there are presumptive colonies, confirmation can be done by Gram stain reaction and colony characteristics on MacConkey Agar at 44°C.

**4.9.5** On MacConkey agar *Enterococci* appear as small pink colonies. Streak suspected or typical colonies on nutrient agar prior to Gram staining.

**4.9.6** *Enterococci* usually appear as Gram positive cocci in pairs or short chains.

**4.10 Expression of results:** Presence/Absence of *Enterococci* is given after confirmation as.

*Enterococci*: Present/Absent per X mL of sample.

#### **4.11 Special Precautions:**

**4.11.1** Sodium Azide is highly toxic and mutagenic; precautions shall be taken to avoid contact with it, especially through the inhalation of fine dust during the preparation of media.

**4.11.2** Azide containing media should not be mixed with strong inorganic acids, as toxic hydrogen azide (HN<sub>3</sub>) may be produced.

**4.11.3** Solution containing azide can also form explosive compounds when in contact with metal pipework e.g. from sinks.

#### **4.12 References:**

4.12.1 IS 5887 (Part II):1976

4.12.2 IS 14543:2004

4.12.3 IS15188:2012

## 5. Detection of Salmonella

*Salmonella* are Gram negative, oxidase negative, non-spore forming, rod shaped bacteria which is able to ferment glucose and capable of facultative anaerobic growth.

### 5.1 Principle:

Detection of *Salmonella* is based on pre-enrichment, selective enrichment followed by isolation on selective media. Presumptive *Salmonella* colonies are confirmed by biochemical and serological tests. Pre-enrichment broth is necessary to enable injured cells to grow. Further selective enrichment is done to increase the proportion of *Salmonella* in relation to background flora. Selective media are used for further isolation and preliminary confirmation.

### 5.2 Culture Media:

5.2.1 Buffered peptone water

5.2.2 Rappaport-Vassiliadis (RVS) broth

5.2.3 Brilliant green/phenol red lactose agar (BGA)

5.2.4 Xylose lysine deoxycholate agar (XLD)

5.2.5 Bismuth sulphite agar (optional)

5.2.6 Nutrient agar

### 5.3 Procedure:

5.3.1 Pre-enrichment: Aseptically clean the surface of bottle/pouch/cups containing sample with 70% ethanol and filter 250ml (or as specified) of water sample through a

membrane filter of 0.45µm pore size using sterile membrane filtration assembly. Place the filter disk in 50 mL buffered peptone water and incubate at 36±2°C for 16-20 hrs.

**5.3.2 Selective enrichment:** Transfer 0.1ml of the pre-enrichment culture to 10mL or 1mL to 100 mL of malachite green/magnesium chloride (Modified RVS broth) and incubate at 42±0.5°C for 18-24h.

**5.3.3 Confirmation on Selective agar media:** After incubation streak a loopful from RVS broth on selective medium i.e. BGA, XLD and BSA (Optional). Incubate the plates at 36±2°C for 24 hr (48 hr for BSA).

**5.3.4** In order to detect slow growing *Salmonella* reinoculate BGA, XLD and BSA (optional) after continued incubation of RVS broth for further 24 hr.

#### **5.4 Observation:**

**5.4.1** Colonies on BGA are red or slightly pink-white and opaque with red surrounding. Colonies on XLD agar are colorless but appear red usually with a black centre. On BSA medium black colonies appear surrounded by a metallic sheen. *Salmonella* H<sub>2</sub>S negative strains appear on XLD agar as pink with a darker pink center. Lactose positive *Salmonella* strains grow on XLD agar as yellow with or without blackening

**5.5 Confirmation:** If there are typical/suspected colonies, plate at least 1 selected colony from each positive agar medium and further four colonies if the first colony tests negative on nutrient agar plates and incubate at 36°C±2°C for 18-24 hr and thereafter proceed for further biochemical & serological confirmation.

#### **5.5.1 Biochemical confirmation:**

**5.5.1.1 Lactose/Glucose fermentation & Hydrogen Sulphide formation:** Streak a colony on Iron/two-sugar agar (Kligler Iron agar slant) and stab the butt. Incubate at 36±2°C for 24 hr. Typical *Salmonella* show red slant with gas formation and yellow butts with blackening of agar.

**5.5.1.2 Urea degradation:** Incubate a colony on slant of urea agar and incubate at 36±2°C for 24hr. Typical *Salmonella* culture show a negative reaction i.e. no rose pink color followed by deep cerise.

**5.5.1.3 Lysine decarboxylase medium:** Inoculate a colony just below the surface of the liquid Lysine decarboxylase medium. Overlay the medium with sterile liquid paraffin or oil. Incubate at  $36\pm 2^{\circ}\text{C}$  for 24hr. Typical *Salmonella* show a purple color.

**Table1 show the Biochemical characterization of *Salmonella***

Sr. No.	Biochemical test	Reaction	Observation
1.	Lactose	-	Red slant with gas formation and yellow butts with blackening of the agar.
2.	Glucose	+	
3.	Hydrogen sulfide	+	
4.	Urea	-	No rose-pink color followed by deep cerise.
5.	Lysine decarboxylase	+	Development of purple color.

Note: (+) means positive reaction, (-) means negative reaction

**5.5.2 Serological confirmation:** Carry out slide agglutination test to check the presence of *Salmonella* O-, Vi- and H-antigens with the appropriate antisera from pure colonies on Nutrient agar as per manufacturer's instructions after elimination of auto-agglutinable strain.

**5.6 Expression of results:** Presence/Absence of *Salmonella* is given after confirmation of presumptive colonies.

Test for *Salmonella* = Present/absent per X mL of sample.

## 5.7 References:

5.7.1 IS 15187:2002

5.7.2 IS 14543:2004

### 5.7.3 IS15188:2012

## 6. Detection of *Shigella sp.*

*Shigella* is a Gram-negative, aerobic, nonspore forming, non-motile, rod-shaped bacteria.

### 6.1 Principle:

Detection of *Shigella* is based on pre enrichment, selective enrichment followed by isolation on selective media. Presumptive *Shigella* colonies are identified by biochemical and serological test. Pre enrichment broth enables injured cells to grow. A selective enrichment is necessary to increase the proportion of *Shigella sp.* in relation to background flora.

### 6.2 Culture Media:

6.2.1 Nutrient broth

6.2.2 Kauffmann-Muller's Tetrathionate broth

6.2.3 Selenite F broth

6.2.4 Deoxycholate citrate agar

6.2.5 MacConkey agar

6.2.6 Nutrient agar No. 2

### 6.3 Procedure:

6.3.1 Pre-enrichment: Aseptically clean the surface of bottle/pouch/cups containing sample with 70% ethanol and filter 250mL (or as specified) of water sample through a membrane filter of 0.45µm pore size using sterile membrane filtration assembly and place the filter in 50mL of Nutrient broth. Incubate at 37°C for 18-24 hr.

6.3.2 Selective enrichment: Transfer 1ml from pre-enriched nutrient broth to each of 100mL Selenite-F broth and tetrathionate broth. Incubate at 37°C for 24hr.

6.3.3 After incubation streak out a loopful from Selenite F broth and tetrathionate broth deoxycholate citrate agar (DCA). Incubate the plates at 37°C for 24 hr. If there is no typical/suspected growth on DCA plate, reincubate further for 24 hr.

**6.4 Observations:** *Shigella* colonies on deoxycholate citrate agar (DCA) appear opaque with a ground-glass appearance and with even margins.

**6.5 Confirmation:** If there are typical/suspected colonies, pick at least 5 colonies from each plate and streak on MacConkey agar. Incubate the plate(s) overnight at 37°C to isolate pure colonies. Pure colonies are transferred on to Nutrient agar No. 2 prior to biochemical and serological confirmation.

#### **6.5.1 Biochemical Confirmation:**

**6.5.1.1 Gram staining:** Typical colonies of *Shigella* are gram -ve rods.

**6.5.1.2 Motility:** Nutrient agar tubes are inoculated by stabbing with a straight wire to a depth of 5 mm and incubated at 37°C for 18-24 hr to check the motility. Out growth in whole tube indicates motility. If no motility is observed within 24 hr. the tubes are incubated at room temperature for further 4-6 days to check the Motility. *Shigella* is non-motile.

**6.5.1.3 Catalase Test:** Streak a colony on nutrient agar slant and incubate at 37°C for 24 hr. Add 1 ml of hydrogen peroxide over the growth. *Shigella* is Catalase positive and show release of oxygen, as bubbles of hydrogen peroxide indicating the presence of catalase.

**6.5.1.4 Oxidase Test:** Streak a colony on nutrient agar slant and incubate at 37°C for 24 hr. To nutrient agar slant containing the culture, add a few drops of mixed test reagents (1% alpha naphthol in 95% ethanol and equal amount of para-amino diamethylaniline hydrochloride in water). *Shigella* being an Oxidase negative does not develop blue color within two minutes of addition of reagent.

**6.5.1.5 Hugh-Leifson's test:** Stab a fresh culture from nutrient agar slant into two tubes of Hugh Leifson medium. One tube is over layered with a small amount of sterile paraffin liquid to create anaerobic conditions, whereas, other is incubated as such. Both are incubated at 37°C and examined daily upto 4 days. In case of *Shigella* Acid formation in both the tubes indicates fermentative reaction.

**6.5.1.6 Test for H<sub>2</sub>S Production:** Inoculate Triple sugar iron agar (TSI) by stabbing the butt and streaking the slope. Incubate at 37°C and examine daily upto 7 days. In case of H<sub>2</sub>S production blackening of butt is observed.

**6.5.1.7 Test for Urease:** Inoculate culture in Nutrient broth and incubate for 37°C for 24hr. Add inoculated culture from Nutrient broth to urea agar slant and incubate at 37°C for 18-24 hr. A positive urease is shown by the medium becoming pink or red on incubation. If no color formed, continue incubation for 4 days and record for color development.

**6.5.1.8 Phenyl pyruvic acid production:** Suspend overnight grown culture in 0.5 mL of normal saline and transfer to a test tube (dia 1.5 cm). Add 0.5 mL of 0.2% di-phenylalanine and mix. Keep it in horizontal position for at least 3 hr at room temperature. Add few drops of half saturated ferric chloride solution. A positive reaction is indicated by the formation of immediate deep color which fades on keeping.

**6.5.1.9 Test for Citrate utilization:** Inoculate the culture on Simmons citrate agar slant using a straight wire. Incubate at 37°C and examine daily upto 4 days. No change in color of media indicates negative reaction.

**6.5.1.10 Test for Indole:** Inoculate Peptone water medium with a loopful of 24hr grown culture in Nutrient broth and incubate at 37°C for 48 hr. Add 0.5mL of KOVAC'S reagent and shake the tube gently. Observe for the appearance of red color which indicates the presence of Indole.

**6.5.1.11 Test for Fermentation of carbohydrates:** Inoculate each of Andrade peptone water medium tubes for carbohydrates i.e. Glucose, Lactose, Sucrose, Salicin, Dulcitol, Mannitol (@ 1% concentration) with freshly grown culture from Nutrient broth/agar and incubate at 37°C for 18-48 hr. Record the presence of acid from pink color and that of gas in Durham tube.

**6.5.1.12 Test for Dihydrolase & decarboxylase activity:** Inoculate each of the tubes of Dihydrolase and Decarboxylase medium through liquid paraffin with freshly grown culture from Nutrient agar. Incubate at 37°C and examine upto 4 days. Medium first become yellow due to acid production from the glucose. Later if dehydrolation or decarboxylation of respective amino acid occur, the medium become violet in color.

**6.5.1.13 Test for utilization of Malonate:** Inoculate the medium with freshly grown culture from Nutrient broth/agar for Malonate test and incubate at 37°C for 24hr. Positive Malonate function is indicated by deep blue color and negative reaction by unchanged greenish or yellow color of medium.

**6.5.1.14 Gelatin liquification test:** Inoculate the culture into gelatin liquification test medium and as a stab culture. Incubate at 22°C for 4 days in an upright position. Before taking reading keep the tube at refrigerated temperature to check liquification of the media.

**Table 2 Show the biochemical characteristics of *Shigella sp***

S. No	Tests	Positive Test observations	<i>Sh. dysenteriae</i>	<i>Sh. Flexneri, Sh. boydii</i>	<i>Sh. sonnei</i>
1.	Gram reaction	Gram negative, Rods	Gram negative, rods	Gram negative, rods	Gram negative, rods
2.	Motility	Growth present outside of inner glass tube	-	-	-
3.	Catalase	Oxygen released as bubble	+	+	+
4.	Oxidase	Blue color within 2 min	-	-	-
5.	Hugh-Leifson's test	Fermentative (Acid & yellow color formation in both tube)	F	F	F
6.	TSI for H <sub>2</sub> S	Blackening of Butt	-	-	-
7.	Urease	Medium color change to pink or red	-	-	-
8.	Phenyl pyruvic acid	Deep green color formed	-	-	-
9.	Citrate	Medium	-	-	-

		color change to Blue			
10.	Glucose fermentation	Acid formation (Pink color), gas +	+	+	+
11.	Lactose(1%) fermentation		-	-	(++)
12.	Sucrose fermentation		-	-	(++)
13.	Salicin fermentation		-	-	-
14.	Mannitol fermentation		-	+	+
15.	Lysine	First yellow color(Acid formation) then purple color due to decarboxylation of amino acid	-	-	-
16.	Ornithine		-	-/+	+
17.	Malonate	Medium color change to deep Blue	-	-	-
18.	Gelatin	Gelatin is liquified	-	-	-

Note: (+) means positive reaction, (-) means negative reaction, (++) means late positive, (F) means fermentative reaction

**6.5.2 Serotyping:** Use slide agglutination with the appropriate antisera from discrete single colonies on Nutrient agar as per manufacturer's instructions after elimination of auto-agglutinable strain. *Shigella* strain is confirmed by Polyvalent *Shigella* sera, on the basis of serotyping.

## 6.6 Expression of results:

Presence/Absence of *Shigella* species given after Biochemical & Serological confirmation of presumptive colonies in the sample examined.

Test for *Shigella* = Present/absent per X mL of sample

## 6.7 References:

6.7.1 IS 5887 (Part-7):1999 (Reaffirmed 2005)

6.7.2 IS 14543:2004

IS15188:2012

## 7. Detection of *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* a Gram negative, non-sporing rod is oxidase and catalase positive. When grown on asparagine and ethanol, it produces water soluble fluorescent pigment.

### 7.1 Principle:

Detection of *Pseudomonas aeruginosa* is based on selective enrichment followed by isolation on confirmation media. Asparagine proline broth is used as selective medium for cultivation of *P. aeruginosa*. Milk Agar is used for selective isolation of *Pseudomonas aeruginosa*. Strains of *Pseudomonas aeruginosa* are identified by their pigment i.e. pyocyanin production. *Pseudomonas aeruginosa* hydrolyzes casein and produces a yellow to green diffusible pigment on Milk agar.

### 7.2 Culture Media:

7.2.1 Pseudomonas asparagine proline broth

7.2.2 Milk agar with cetrimide

### 7.3 Procedure:

**7.3.1** Aseptically clean the surface of bottle/pouch/cups containing sample with 70% ethanol and filter 250mL (or as specified) of water sample through a membrane filter of 0.45µm pore size using sterile membrane filtration assembly and place the filter in 50mL of concentrated Asparagine proline broth. Incubate at 37±1°C for 48 hr.

**7.3.2** After incubation examine the medium showing either growth or fluorescence under U.V. light (360±20 nm).

**7.3.3** Subculture a loopful on Milk agar plate and incubate for 24h at 42±0.5°C.

#### **7.4 Observation:**

Observe the plates for culture growth, pigment production and casein hydrolysis (clearing of medium around the colonies).

**Table 3 Show the Characteristics of *Pseudomonas aeruginosa* on Milk agar**

S. No.	Reaction	<i>Pseudomonas aeruginosa</i> characteristics	
		Typical	Atypical
1.	Casein hydrolysis	+	+
2.	Growth at 42°C	+	+
3.	Fluorescence under UV light	+	-
4.	Pyocyanine (Blue/green) pigment production	+	-

Note: (+) means positive reaction, (-) means negative reaction

Culture showing either growth or fluorescence in Asparagine proline broth, which further produce colonies on Milk agar plates and show pigment production and casein hydrolysis are regarded positive for presence of *Pseudomonas aeruginosa*.

The colonies (atypical) showing casein hydrolysis, but no fluorescence or pigment productions are further confirmed by biochemical tests.

#### **7.5 Confirmation of Atypical colonies:**

If there are Atypical/suspected colonies, streak out atleast 5 selected colonies further on Milk agar medium and incubate at  $37^{\circ}\text{C}\pm 1^{\circ}\text{C}$  for 24 hr. Proceed for further biochemical confirmation.

**7.5.1 Catalase Test:** Streak a colony on Nutrient agar slant and allow to grow at  $37^{\circ}\text{C}$  for 24 hr. Add 1 mL of hydrogen peroxide over the growth in slanting position. Release of oxygen, as bubbles, from hydrogen peroxide indicates presence of catalase.

**7.5.2 Oxidase Test:** Streak a colony on Nutrient agar slant and allow to grow at  $37^{\circ}\text{C}$  for 24 hr. Add a few drops of mixed test reagents (1% alpha naphthol in 95% ethanol and equal amount of para-amino diamethylaniline hydrochloride in water) to Nutrient agar slant of culture. A positive reaction is indicated by the appearance of blue color within two minutes of addition of reagent.

**7.5.3 Hugh-Leifson's test:** Stab a fresh culture from Nutrient agar slant into two tubes of Hugh Leifson medium, one of which is then layered over with a small amount of sterile liquid paraffin. Incubate at  $37^{\circ}\text{C}$  and examine daily upto 4 days. Acid formation, in both the tubes indicates fermentative reaction.

**7.5.4 Nitrate reduction:** Add Reagent A (Sulfanilic acid) and Reagent B (N,N-dimethyl-1-naphthylamine) to 18hr old culture inoculated in Nitrate broth. Formation of red color indicates nitrate reduction. If no change in color is observed, add Zinc granules to tube, and observe color change. If the broth turns to red, test is negative but if no red color is developed after addition of zinc granules, then test is positive.

**7.5.5 Gelatin liquification test:** Inoculate the strain into Gelatin liquification test medium as a stab culture. Incubate at  $22^{\circ}\text{C}$  for 4 days in an upright position. Before taking reading keep the tube at refrigerated temperature to check liquification of the media.

**Table 4 Show the Biochemical characteristics *Pseudomonas aeruginosa***

S. No.	Tests	Observation
1.	Catalase test	+
2.	Oxidase test	+

3.	Growth in Hugh and Liefson medium	Oxidative reaction
4.	Nitrate reduction to ammonia	+
5.	Gelatine Liquefaction	+

## 7.6 Expression of results:

Presence/Absence of *Pseudomonas* sp. is given after Biochemical & Serological confirmation of presumptive colonies in the sample examined.

Test for *Pseudomonas aeruginosa* = Present/absent per X mL of sample

## 7.7 References:

7.7.1 IS 13428(Annex D):2005

7.7.2 IS 14543:2004

IS15188:2012

## 8. Method for detection of Yeast & Moulds

Microorganism which are capable of forming colonies in a selective medium (Chloroamphenicol Glucose Yeast Extract Agar medium) at 25°C

**8.1 Principle:** Chloramphenicol Yeast Glucose Agar is a selective medium recommended for isolation and enumeration of Yeast & Moulds. The medium contains yeast extract, which provides nitrogenous nutrient and vitamin B complex. Dextrose is the energy source. Chloramphenicol a thermostable antibiotic, suppresses the bacterial flora. Aerobic incubation of plates is done at 25°C±1°C and count is taken on 3<sup>rd</sup>, 4<sup>th</sup> or 5<sup>th</sup> day.

### 8.2 Culture media:

8.2.1 Chloroamphenicol Glucose Yeast Extract Agar (CGYEA)

### 8.3 Procedure:

**8.3.1** Aseptically clean the surface of bottle/pouch/cups containing sample with 70% ethanol and filter 250mL (or as specified) of water sample through a membrane filter of 0.45µm pore size using sterile membrane filtration assembly.

**8.3.2** Place the filter on CGYEA media and incubate at 25±1°C

#### **8.4 Observation:**

**8.4.1** Observe the plates for colonies of Yeast & Moulds on 3<sup>rd</sup>, 4<sup>th</sup> and 5<sup>th</sup> days of incubation.

It is advisable to examine the plates at the end of three days for yeast colonies, which are smooth, moist, elevated or surface colonies. Mould colonies are easily recognized by the profuse growth of hyphae.

#### **8.5 Expression of results:**

Presence/Absence of yeast & Moulds given after examining the plates of sample.

Test for Yeast and Moulds = Present/absent per X mL of sample

#### **8.6 References:**

**8.6.1** IS 5403:1999 (Reaffirmed 2005)

**8.6.2** IS 14543:2004

IS15188:2012

### **9. Detection of Sulphite-Reducing anaerobes (*Clostridia*)**

Sulphite-Reducing anaerobes (*Clostridia*) are relatively large, gram-positive, rod-shaped, anaerobic bacteria.

#### **9.1 Principle:**

Detection of sulphite reducing anaerobes by inoculating 50 mL of sample into equal volume of double strength of Differential Reinforced Clostridial Medium (DRCM), followed by anaerobic incubation at 37°C±1°C for 44±4 hr. As a result of reduction of

sulphite and the precipitation of iron (II) sulphide in medium developing, Black color in medium, sample is considered as positive.

**9.2 Culture Media:** Differential Reinforced Clostridial Medium (DRCM)

**9.3 Procedure:**

**9.3.1** Aseptically clean the surface of the bottle (1lt./5lt./20lt.) or water pouch/cups containing sample with 70% ethanol. Aseptically withdraw 100 mL sample to sterile bottle and heat at  $75\pm 5^{\circ}\text{C}$  for 15 min.

**9.3.2** Add 50mL of sample after heat shock to 100ml bottle containing 50mL of the double strength DRCM. Cap the bottles tightly and incubate under anaerobic conditions at  $37\pm 1^{\circ}\text{C}$  for  $44\pm 4$ hr. Iron wire, heated to redness can be placed in the medium before inoculation to enhance anaerobic conditions.

**9.4 Observation:**

Observe the bottle for blackening.

**9.5 Expression of results:**

Test for Sulphite-Reducing anaerobes (*Clostridia*): = Present/absent per X mL of sample.

**9.6 References:**

**9.6.1** IS 13428(Annex C):2005

**9.6.2** IS 14543:2004

## **10. Detection of *Vibrio cholerae***

*Vibrio cholera*- a Gram negative bacterium is a curve shaped rod. It is catalase and oxidase positive and ferment glucose without gas production. *V. cholera* occurs naturally in the plankton of fresh, brackish and salt water

**10.1 Principle:**

Bile Salt agar and Thiosulphate Citrate Bile salt sucrose agar (TCBS) is used selectively for the isolation, identification and enumeration of *Vibrio cholerae*. *Vibrio* species grow in the presence of relatively high levels of bile salts which inhibit the growth of gram-positive microorganisms. TCBS has a very high pH (8.5-9.5) which suppresses growth of intestinal flora other than *Vibrio* sp. *V. cholerae* ferment sucrose, which results in a pH shift and production of yellow colonies.

## **10.2 Culture Media:**

**10.2.1** Alkaline peptone water

**10.2.2** Thiosulphate-Citrate-Bile salts-Sucrose agar (TCBS)

**10.2.3** Bile salt agar (BSA)

**10.2.4** Nutrient agar No. 2

## **10.3 Procedure:**

**10.3.1** Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution.

**10.3.2** Filter the sample (requisite volume) through a sterile membrane filter (0.45µm pore size) and place the filter in 50 mL of alkaline peptone water. Incubate at 37°C overnight.

**10.3.3** After incubation streak a loopful on TCBS and BSA medium plates and incubation at 37°C overnight.

## **10.4 Observation:**

**10.4.1** Suspicious colonies of *V. cholera* on TCBS appear as opaque yellow colored with entire round margins.

**10.4.2** *V. cholera* colonies show a distinctive appearance on BSA medium.

## 10.5 Confirmation:

**10.5.1 Biochemical Confirmation:** If there are typical/suspected colonies, plate out at least 5 selected colonies from each positive agar medium on nutrient agar No. 2 plates and incubate at  $36^{\circ}\text{C}\pm 2^{\circ}\text{C}$  for 18-24 hr. Proceed for further biochemical & serological confirmation

**10.5.1.1 Gram Staining:** Gram stain the isolated colony. Typical *Vibrio cholerae* are gram -ve curved shaped rods when observed under microscope.

**10.5.1.2 Test for Motility:** Nutrient agar for motility is used to check the motility of *V. cholera*. Inoculate by stabbing with a straight wire into glass tube to a depth of 5 mm and incubate at  $37^{\circ}\text{C}$  for 18-24 hr. Motile strain shall be found to show growth on the surface of the medium, outside the inner glass tube; travelled through entire medium. If no motility observed keep at room temperature for 4-6 days to see if any evidence of motility is present.

**10.5.1.3 Test for Catalase:** Streak a colony on Nutrient agar slant and allow to grow for 24 hr at  $37^{\circ}\text{C}$ . Add 1 ml of hydrogen peroxide over the growth in slanting position. Release of oxygen, shown as bubbles, from hydrogen peroxide indicates presence of catalase.

**10.5.1.4 Test for Oxidase:** Streak a colony on Nutrient agar slant and allow to grow at  $37^{\circ}\text{C}$  for 24 hr. To the slant culture, add a few drops of mixed test reagents (1% alpha naphthol in 95% ethanol and equal amount of para-amino diamethylaniline hydrochloride in water). A positive reaction is indicated by the appearance of blue color within two (2) minutes of addition of reagent.

**10.5.1.5 Hugh-Leifson's test:** Stab a fresh culture from Nutrient agar slant into two tubes of Hugh Leifson medium, one of which is then layered over with a small amount of sterile paraffin liquid. Incubate at  $37^{\circ}\text{C}$  and examine daily upto 4 days. Acid formation, shown by yellow color in the tube without paraffin indicates oxidative utilization of glucose. Acid in both tubes indicates fermentative reaction.

**10.5.1.6 Test for Fermentation of carbohydrates:** Inoculate each of Andrade Peptone water medium tubes for carbohydrates i.e. Glucose, inositol, Mannitol (@ 1% concentration) and incubate at 37°C for 18-48 hr. observe color change for carbohydrate fermentation. Ferment glucose without gas production and in case of mannitol ferment with acid production

**10.5.1.7 TSI for H<sub>2</sub>S production:** Inoculate Triple Sugar Iron agar (TSI) medium by stabbing into the butt and streaking the slope. Incubate at 37°C and examine daily upto 7 days. In case of H<sub>2</sub>S production blackening of butt is observed.

**10.5.1.8 Test for the growth in 1% Tryptone Broth:** Inoculate the culture in Tryptone broth (without sodium chloride) and incubate at 37°C for 18hr. *V. cholera* grows in 1% tryptone broth.

**10.5.1.9 Test for Dihydrolase & Decarboxylase activity:** Inoculate each of the tubes of Dihydrolase & Decarboxylase medium through liquid paraffin from freshly grown culture from Nutrient agar. Incubate at 37°C and examine up to 4 days. Medium first become yellow due to acid production from the glucose. Later if dehydrolation or decarboxylation of respective amino acid occur, the medium become violet in color.

**Table 5** Show the Biochemical characteristics of *Vibrio cholerae*

S. No.	Tests	Reaction
1.	Gram Reaction	Gram Negative , Rods
2.	Motility test	+
3.	Catalase test	+
4.	Oxidase test	+
5.	Hugh-Leifson's test	F
6.	H <sub>2</sub> S production	-
7.	Glucose fermentation	+ (without gas)
8.	Mannitol fermentation	+
9.	Inositol fermentation	-
10.	Growth in 1% tryptone broth	+

11.	Lysine decarboxylase	+
12	Arginine dihydrolase	-
13	Ornithine decarboxylase	+

Note: (+) means positive reaction, (-) means negative reaction, (F) means fermentative reaction

**10.5.2 Serological Confirmation:** Suspicious growth of *V. cholerae* is tested by slide agglutination using polyvalent cholera typing serum (high titre serum of combined Ogawa & Inaba Serotypes). The growth is emulsified in a drop of normal saline and smooth suspensions mixed with a drop of Cholera serum. Positive reaction is shown by the appearance of clumps within 30 seconds.

### 10.6 Expression of results:

Presence/Absence of *V. cholera* species given after confirmation of presumptive colonies in the sample examined.

Test for *V. cholera* = Present/absent per X mL of sample filtered

### 10.7 References:

10.7.1 IS 5887 (Part-V) -1976 (Reaffirmed 2005)

10.7.2 IS 14543:2004

IS15188:2012

## 11. Detection of *Vibrio parahaemolyticus*

*Vibrio parahaemolyticus*: Gram negative, oxidase negative, ferment glucose without gas production and do not produce H<sub>2</sub>S in TSI (Triple sugar iron agar) medium. It is a motile bacterium that causes enteritis in humans.

### 11.1 Principle:

Glucose salt teepol broth is used to enrich *Vibrio parahaemolyticus*. Glucose is utilized by the organism while teepol inhibits the migration of halophilic organisms and the growth of the gram-positive organisms. After overnight incubation at  $35\pm 2^{\circ}\text{C}$ , inoculation on TCBS agar plates gives round green or bluish colonies.

### 11.2 Culture Media:

#### 11.2.1 Glucose-salt-teepol broth

#### 11.2.2 Thiosulphate-Citrate-Bile salts-Sucrose (TCBS)

### 11.3 Procedure:

**11.3.1** Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution

**11.3.2** Filter the sample (required volume) through a sterile membrane filter (0.45 $\mu\text{m}$  pore size) and place the filter in 50ml of Glucose-salt-Teepol broth. Incubate overnight at  $37^{\circ}\text{C}$ .

**11.3.3** After incubation streak a loopful from pre-enrichment culture on TCBS prepared plates and incubate for 18hr at  $37^{\circ}\text{C}$ .

### 11.4 Observation:

**11.4.1** Suspicious colonies of *V. parahaemolyticus* on TCBS medium are 2-3 mm and round with green or blue centres.

**11.5 Identification:** Suspicious growth of *V. parahaemolyticus* is confirmed through biochemical confirmation.

**11.5.1 Biochemical tests:** If there are typical/suspected colonies, plate out at least 5 selected colonies from each positive agar medium on nutrient agar plates and incubate at  $36^{\circ}\text{C}\pm 2^{\circ}\text{C}$  for 18-24 hr. Proceed for further biochemical & serological confirmation

**11.5.1.1 Gram Staining:** Gram stain the isolated colony. Typical *Vibrio parahaemolyticus* are gram -ve rods.

**11.5.1.2 Test for Oxidase:** Streak a colony on Nutrient agar slant (with 2-3% added NaCl) and allow to grow for 24 hr. To Nutrient agar slant of culture, add a few drops of mixed test reagents (1% alpha naphthol in 95% ethanol and equal amount of para-amino diamethylaniline hydrochloride in water). A positive reaction is indicated by the appearance of blue color within two minutes of addition of reagent.

**11.5.1.3 Hugh-Leifson's test:** Stab a fresh culture from Nutrient agar slant into two tubes of Hugh Leifson medium (with 2-3% added NaCl), one of which is then overlaid with a small amount of sterile liquid paraffin. Incubate at 37°C and examine daily upto 4 days. Acid formation, shown by yellow color in the tube without paraffin indicates oxidative utilization of glucose. Acid in both tubes indicates fermentative reaction.

**11.5.1.4 Test for Fermentation of carbohydrates:** Inoculate each of Andrade Peptone water medium tubes for carbohydrates i.e. Sucrose, Mannitol (@ 1% concentration) and incubate at 37°C for 18-48 hr. observe color change for carbohydrate fermentation

**11.5.1.5 TSI for H<sub>2</sub>S production:** Inoculate Triple Sugar Iron Agar (TSI) medium (with 2-3% added NaCl) by stabbing into the butt and streaking the slope. Incubate at 37°C and examine daily upto 7 days. In case of H<sub>2</sub>S production blackening of butt is observed.

**11.5.1.6 Test for Dihydrolase & Decarboxylase activity:** Inoculate each of the tubes of Dihydrolase & Decarboxylase medium (with 2-3% added NaCl) through liquid paraffin from freshly grown culture on Nutrient agar medium. Incubate at 37°C and examine up to 4 days. Medium first turns yellow due to acid production from glucose and later if dehydration or decarboxylation of respective amino acids occur, the medium changes to violet in color.

**11.5.1.7 Test for Voges-Proskauer reaction:** Inoculate the medium with added NaCl(2-3%) and incubate for 48hr at 37°C. To 1 mL of growth, add 0.6ml of 5% alpha

nephthol. Shake and add 0.2 ml of KOH solution 40%. Shake and slope the tube for upto 4 hr for color change. Pink color indicate positive reaction.

**11.5.1.8 Test for the growth in Tryptone broth:** Inoculate the culture in Tryptone broth with different concentration (0%, 1%, 8% & 10%) of Sodium chloride.

**11.5.1.8.1** Growth in Tryptone broth with added NaCl incubated at 42°C for 24 hr shows positive growth.

**11.5.1.8.2** Growth in 1% Tryptone broth, with added 8% NaCl and incubated at 37°C is positive

**11.5.1.8.3** Growth in 1% Tryptone broth, with added 10% NaCl and incubated at 37°C is negative

**11.5.1.8.4** Growth in 1% Tryptone broth, without NaCl and incubated at 37°C is negative

**11.5.1.9 Kanagawa test:** Grow the culture in Trypticase-Soy-Sodium Chloride broth by incubating for 18hr at 37°C. Further streak on blood agar and incubate at 37°C for not more than 24hr. A positive result in case of *V. parahaemolyticus* consists of a zone of transparent clearing of the red blood cells around the colony. It is important that the reading is not taken beyond 24hr of incubation as any haemolysis seen beyond this time is not to be recorded as Kanagawa positive.

**Table 6 Show the biochemical characteristics of *Vibrio parahaemolyticus***

S. No.	Tests	Reaction
1.	Gram Reaction	Gram Negative , Rods
2.	Oxidase test	+
3.	Hugh-Leifson's test	F
4.	H <sub>2</sub> S production	-
5.	Glucose fermentation	+ (without gas)

6.	Mannitol fermentation	+
7.	Sucrose fermentation	-
8.	Voges –Proskauer test	-
9.	Lysine decarboxylase	+
10	Arginine dihydrolase	-
11	Ornithine decarboxylase	+
12	Growth in 1% tryptone brothwith added NaCl at 42°C	+
13	Growth in 1% tryptone broth + 8% NaCl	+
14	Growth in 1% tryptone broth + 10% NaCl	-
15	Growth in 1% tryptone broth without naCl	-

Note: (+) means positive reaction, (-) means negative reaction, (F) means fermentative reaction

### 11.6 Expression of results:

Presence/Absence of *V. parahaemolyticus* given after confirmation of presumptive colonies in the sample examined.

Test for *V. parahaemolyticus* = Present/absent per X mL of sample

### 11.7 References:

11.7.1 IS 5887 (Part-V) -1976 (Reaffirmed 2005)

11.7.2 IS 14543:2004

IS15188:2012

## 12. Detection of *Staphylococcus aureus*

***Staphylococcus aureus***: Aerobic, gram positive cocci in cluster show coagulase activity. Produce golden yellow colored colonies on Nutrient agar and Blood agar and shiny black colonies on Baird-Parker medium.

### 12.1 Principle:

Baird Parker agar is used for the isolation and differentiation of coagulase-positive *Staphylococci*. *Staphylococci* can reduce tellurite to telluride, which results in grey to black coloration of the colonies. With the addition of egg yolk, the medium becomes yellow, slightly opaque. A clear halo develops around colonies from coagulase positive *Staphylococcus aureus*. Grey-black colonies and a halo on this medium are presumed to be indicative of coagulase positive staphylococci.

### 12.2 Culture Media:

12.2.1 Cooked Salt Meat medium

12.2.2 Baird-Parker agar

12.2.3 Blood agar

### 12.3 Procedure:

12.3.1 Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution

12.3.2 Filter the sample (requisite volume) through a sterile membrane filter (0.45µm pore size) and place the filter in 50 mL of Cooked Salt Meat medium. Incubate overnight at 37°C.

12.3.3 After incubation streak a loopful from Cooked Salt Meat medium on Baird-Parker agar for at least 30 hr at 37°C and overnight on Blood agar at 37°C.

### 12.4 Observation:

**12.4.1** *S. aureus* show shiny black colonies with or without grey-white margins on Baired-Parker agar

**12.4.2** Usually golden yellow colonies on Blood agar.

## **12.5 Identification:**

**12.5.1 Gram Staining:** If there are typical/suspected colonies, pick up the colonies and confirm by gram staining. *S. aureus* are Gram positive cocci which appear in clusters.

**12.5.2 Coagulase test:** The test may be performed using one of following method.

**12.5.2.1 Slide method:** Emulsify a portion of suspected colony in normal saline water on a clean slide. Mix it with a straight wire dipped in rabbit plasma. Coagulase positive staphylococci produce visible clumping immediately. Positive control with a known coagulase positive strain of *S. aureus* and a control of rabbit plasma without inoculum should be included in the test.

**12.5.2.2 Tube method:** Emulsify a portion of suspected colony from 24 hr growth on blood agar in 1 mL citrated rabbit plasma, diluted 1 in 5, 0.85% saline. Incubate at 37°C, preferably in a water bath. Observe every hour to observe clotting of plasma. Positive control with a known coagulase positive strain *S. aureus* and a control of rabbit plasma without inoculum should be included in the test. Tube method shall be preferred

## **12.6 Expression of results:**

Presence/Absence of *S. aureus* given after confirmation of colonies in the sample examined.

Test for *S. aureus*= Present/absent per X mL sample

## **12.7 References**

**12.7.1** IS 5887(Part-II) : 1976 (Reaffirmed 2005)

**12.7.2** IS 14543 : 2004

IS15188:2012

## 13. Detection of Viruses:

MS2 Phages are indicator of viral contamination in drinking water. MS2 phage shall be absent in 1 L of water when tested as per method USEPA 1602. If MS2 phages are detected then virological examination shall be done as per method given below:

**1.0 Scope:** This method cover detection of Hepatitis A virus (HAV) and enterovirus (EV) in water. Detection of Hepatitis A virus (HAV) and enterovirus (EV) RNA shall be considered as an indication of presence of viruses in water.

### 2.0 Principle:

The method involves the concentration of viruses from 100 litre of drinking water to 1ml by membrane filter technique. The concentrate is subjected to amplification using polymerase chain reaction (PCR) and primers based on highly conserved regions of viral genomes. This method can detect as low as 10 genome copies. Stringent precautions are needed to avoid contamination with amplified DNA products leading to false positive reactions. Steps involved include concentration of water, RNA extraction, complementary DNA (cDNA) synthesis and PCR.

### 3.0 Apparatus and Reagents:

#### 3.1 CONCENTRATION OF DRINKING WATER

##### 3.1.1 Apparatus

3.1.1.1 Pressure Pump

3.1.1.2 Membrane Filter Assembly with 144mm Diameter with Tripod Stand

3.1.1.3 Pressure Vessel (50 litre capacity) with Pressure Gauge

3.1.1.4 Inter-connecting Pressure Tubes

##### 3.1.2 Reagents

3.1.2.1 Autoclaved double distilled water

3.1.2.2 Aluminum Chloride

3.1.2.3 HCl/NaOH Urea (Extra Pure)

3.1.2.4 Disodium Hydrogen Phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ ) - 0.2M filter sterilized.

3.1.2.5 Sodium Dihydrogen Phosphate ( $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ ) - 0.2M filter sterilized.

3.1.2.6 Citric Acid - 0.1M filter sterilized.

3.1.2.7 L-Arginine - 0.5M filter Sterilized.

3.1.2.8 Urea-Arginine Phosphate Buffer (U-APB)- Mix 4.5 gm of urea with 2mL of 0.2M  $\text{NaH}_2\text{PO}_4$  and 2mL of 0.5 M L-Arginine and make up the volume to 50mL with sterile distilled water. The pH of the eluent shall be 9.0.

3.1.2.9 Magnesium Chloride ( $\text{MgCl}_2$ ) - 1M.

3.1.2.10 McIl Vaines Buffer (pH 5.0)- Mix 9.7 mL of 0.1 M citric acid with 10.3mL of 0.2M  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  under sterile conditions.

## 3.2 RNA EXTRACTION

### 3.2.1 Apparatus

3.2.1.1 Cooling Centrifuge

3.2.1.2 Deep Freezer ( $-20^\circ\text{C}$ )

3.2.1.3 Vortex Mixer

3.2.1.4 Pipette Man

### 3.2.2 Reagents

3.2.2.1 Cetyl Trimethyl Ammonium Bromide (CTAB) Buffer

CTAB : 1 percent

Sodium Dodecyl Sulphate (SDS) : 1 percent

EDTA : 20 mM

Sodium Chloride : 1 M

3.2.2.2 Phenol, Chloroform and Isoamylalcohol in the ratio of 25:24:1 (PCI)

3.2.2.3 Ethanol

3.2.2.4 TE Buffer (pH 8.0)

Tris base : 1M

EDTA : 0.5 M

3.2.2.5 Sodium Acetate : 3M.

## 3.3 COMPLEMENTARY DNA (c DNA) SYNTHESIS

### 3.3.1 Apparatus

3.3.1.1 PCR Machine

## 3.3.1.2 Deep Freezer (-20°C)

**3.3.2 Reagent**

## 3.3.2.1 cDNA synthesis kit

**3.4 PCR AMPLIFICATION****3.4.1 Apparatus**

## 3.4.1.1 PCR Machine

## 3.4.1.2 Deep Freezer (-20°C)

## 3.4.1.3 Micropipette

**3.4.2 Reagent**

## 3.4.2.1 Primers for EV and HAV

EV	sense primer	5' – TCC TCC GGC CCC TGA ATG CG – 3'
	antisense primer	5'- ATT GTC ACC ATA AGC AGC CA - 3'
HAV	sense primer	5' –GTTTT GCTCC TCTTT ATCAT GCTAT G- 3'
	antisense primer	5'- GGAAA TGTCT CAGGT ACTTT CTTTG-3'

## 3.4.2.2 PCR Master Mix

## 3.4.2.3 Mineral Oil

**3.5 AGROSE GEL ELECTROPHORESIS****3.5.1 Apparatus**

## 3.5.1.1 Micropipette

## 3.5.1.2 Electrophoresis Apparatus

## 3.5.1.3 Gel Documentation System

**3.5.2 Reagent**

## 3.5.2.1 Running Buffer – 50X TAE buffer

Tris base/ Tris buffer : 121.00 gm

Glacial acetic acid : 28.55 mL

0.5 M EDTA	: 50.00 mL
Distilled water	: 300.45 mL

Make the final volume upto 1000ml with deionised distilled water, sterilize and store at 4°C. The final concentration for the preparation of agarose gel and to run gel shall be 1X.

3.5.2.2 Tracking Dye – 6X bromophenol blue.

3.5.2.3 Ethidium Bromide – 0.5 µg/mL

#### **4.0 Procedure**

##### **4.1 CONCENTRATION OF DRINKING WATER:**

Filter 100 litre of drinking water sample through membrane filter assembly using either positively charged membrane of 144mm diameter or 0.22micron diameter pore size nitrocellulose membrane. For positively charged membrane the test water pH need not be adjusted. But for the 0.22micron nitrocellulose membrane adjust the pH to 3.5 after adding the aluminium chloride as a coagulant to a final concentration of 0.0005M. At lower pH pass the water through the membrane. The flow rate shall be 40lt/h approximately. After the completion of the filtration, elute the adsorbed particles using 100mL of urea-arginine phosphate buffer using 1ml of magnesium chloride (1M). Dissolve the resultant precipitate centrifuged out of the sample in 800 - 1.0mL of McIlvaines buffer. The processed sample can be stored at refrigerator until required.

##### **4.2 RNA EXTRACTION:**

Treat 300µL of concentrated water sample with equal volume of CTAB and 1/10<sup>th</sup> volume of PCI. Vortex and centrifuge at 5000 x gm for 30 min at 4°C. Add 1/10<sup>th</sup> volume of 3M sodium acetate and double the volume of cold ethanol to the aqueous layer. Keep the mixture at either -20°C for overnight or in liquid nitrogen for 2 – 5 min. Centrifuge at 10000 X gm for 30 min at 4°C. Discard the supernatant and air dry the pellet and dissolve it in 20 µL TE buffer.

##### **4.3 COMPLEMENTARY DNA (c DNA) SYNTHESIS:**

Suspend the extracted RNA in 20  $\mu\text{L}$  of cDNA reaction mixture, which consists of 4 $\mu\text{L}$  of 5X reverse transcriptase reaction buffer [250mM TRIS – HCl (pH 8.5), 40mM KCl, 150mM  $\text{MgCl}_2$ , 5mM dithiothreitol (DTT)], 0.5 $\mu\text{L}$  of 10 mM deoxynucleotide phosphate (dNTP), 2 $\mu\text{L}$  of hexa nucleotide mixture, 1 $\mu\text{L}$  of 25U of Maloney Murine Leukaemia Virus (M-MuLV) reverse transcriptase, 0.5 $\mu\text{L}$  of 20U of human placental RNase inhibitor. Heat the reaction mixture to 95 $^\circ\text{C}$  for 5 min and rapidly chill on ice this is followed by the additional of 1  $\mu\text{L}$  (25 U/ $\mu\text{L}$ ) of M-MuLV reverse transcriptase. Incubate the reaction mixture as given by the manufacturer of the kit quickly chill the reaction tube on ice.

#### 4.4 PCR Amplification:

##### 4.4.1 PCR Amplification for Hepatitis A Virus (HAV)

In 5 $\mu\text{L}$  of cDNA, add 95 $\mu\text{L}$  of a PCR Master Mix (10mM tris-HCL (pH8.3), 50mMKCl, 2.5mM  $\text{MgCl}_2$ , 0.01 percent gelatin (1X PCR buffer), 200 $\mu\text{M}$  of each dNTP, 1.5U of *Thermus Aquaticus* polymerase). Add 25 pico moles of sense and antisense oligonucleotide primers of HAV and overlay with mineral oil. Appropriate positive and negative controls shall be included with each run. Set the following reaction at thermo cycler:

Denaturation at 94 $^\circ\text{C}$  for 2 min

Denaturation for 1.0 min at 94 $^\circ\text{C}$	}	35 cycles
Annealing for 1.0 min at 57 $^\circ\text{C}$ ,		
Extension for 1.3 min at 72 $^\circ\text{C}$		
Final extension at 72 $^\circ\text{C}$ for 7 min		

##### 4.4.2 PCR Amplification for Enterovirus

In 5 $\mu\text{L}$  of cDNA, add 95 $\mu\text{L}$  of a PCR Master Mix (10 mM TRIS-HCL (pH 8.3), 50mM KCl, 2.5mM  $\text{MgCl}_2$ . 0.01 percent gelatin (1XPCR buffer), 200 $\mu\text{M}$  of each dNTP, 1.5 U of *Thermus aquaticus* polymerase). Add 25 picomoles of sense and antisense oligonucleotide primers of EV and overlay with mineral oil. Appropriate positive and negative controls shall be included with each run. Set the following reaction at thermo cycler:

Denaturation at 94°C for 2min

Denaturation for 1.0 min at 94°C  
Annealing for 1.0 min at 42°C,  
Extension for 2.0 min at 72°C  
Final extension at 72°C for 7 min.

} 35 cycles

#### 4.5 AGROSE GEL ELECTROPHORESIS:

**4.5.1** Run the PCR amplified product of EV and HAV on 1.5 percent agarose gel using 1X TAE buffer. Load 10µL of amplified product after mixing it with 1µL 10X loading Dye. Run the molecule weight marker along with the samples. Run the electrophoresis at 100V for 30 min. Stain the gel with ethidium bromide (0.5µL/mL) for 20 min. Wash it with distilled water and view under UV transilluminator and photograph the gel to analyse the band pattern. EV gives the band as 155 base pair and the HAV give band as 225 base pair.

#### 5.0 References:

5.1 IS 10500:2012

## 14. Detection of Giardia and Cryptosporidium

Detection of Giardia shall be carried out as per USEPA 1623 and detection of Cryptosporidium shall be carried out as per USEPA 1622 or 1623 method.

## CHAPTER II: TEST METHODS FOR WATER FOR PROCESSED FOOD INDUSTRY AS PER IS 4251

### 1. Standard Plate Count

#### 1.1 Principle :

Nutrient agar is a general purpose growth medium commonly used to assess "Total" or Viable bacterial growth of a water sample. The number of microorganisms per milliliter of sample is calculated from the number of colonies obtained on Nutrient agar (NA) plate from selected dilution. Poured plates are prepared using a specified culture medium and a specified quantity of the sample. The plates are incubated at 37°C for 48 hr

#### 1.2 Culture Media:

1.2.1 Nutrient agar

1.2.2 Quarter's Strength Ringer solution

#### 1.3 Procedure:

1.3.1 Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution.

1.3.2 Aseptically inoculate 1mL of sample or other suitable dilution of the water sample using sterile pipette into sterile petri plates in duplicate. The petri dishes should be labeled with the sample number, date and any other desired information.

1.3.3 Pour into each plate 15–18 mL of the molten sterilized Nutrient agar (cooled to 44–47°C). Avoid pouring of molten medium directly onto the inoculum. Mix the media and inoculum by swirling gently clockwise and anti-clockwise so as to obtain homogenous distribution of inoculum in the medium.

1.3.4 Allow to cool and solidify.

1.3.5 After complete solidification, invert the prepared petriplates and incubate at 37°C for 48 hr.

1.3.6 After specified incubation period count all colonies including pinpoint colonies using colony counter. Spreading colonies shall be considered as single colony. If less than one quarter of petriplates is overgrown by spreading, count the colonies on the unaffected part of the petriplate and calculate corresponding number of the entire petriplate. If more than one quarter is overgrown by spreading colonies discard the plate.

1.3.7 Consider the plates having count between 30 to 300. Report the results as the average of colonies in a particular dilution falling within limits.

#### **1.4 References:**

1.4.1 IS 4251 : 1967 (Reaffirmed 1992)

1.4.2 IS 1622 : 1981(Reaffirmed 2003)

## **2. Proteolytic Plate Count**

### **2.1 Principle:**

Nutrient agar (NA) with added Skimmed milk powder is used for the enumeration of Proteolytic organisms. The number of proteolytic microorganisms per milliliter of sample is calculated from the number of colonies with clearing zone of proteolysis from selected dilution.

### **2.2 Culture Media:**

2.2.1 Nutrient agar (NA) with Skimmed milk powder

2.2.2 Quarter's Strength Ringer solution

### **2.3 Procedure:**

2.3.1 Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution.

2.3.2 Aseptically inoculate 1ml of diluted or undiluted the water sample using sterile pipette into sterile petri plates in duplicate. The petri plates should be labeled with the sample number, date and any other desired information.

2.3.3 Add skimmed milk powder @5-6 mL/100mL in Nutrient agar.

2.3.4 Pour into each plate 15–18 mL of the molten sterilized NA + Skimmed milk powder media (cooled to 44–47°C). Avoid pouring of molten medium directly onto the inoculum. Mix the media and inoculum by swirling gently clockwise and anti-clockwise so as to obtain homogenous distribution of inoculums in the medium.

2.3.5 After complete solidification, invert the petri plates and incubate at 37°C for 48±3 hr.

2.3.6 After specified incubation period count all colonies having zone of proteolysis. Flood the plate with dilute Hydrochloric acid to confirm zone of proteolysis.

2.3.7 After determining the colony count, report the results as Proteolytic count/mL

## 2.4 References:

2.4.1 IS 4251 : 1967 (Reaffirmed 1992)

2.4.2 IS 1622 : 1981(Reaffirmed 2003)

## 3. Lipolytic Plate Count

**3.1 Principle:** Tributyrin agar is used for the enumeration of Lipolytic organisms. The number of Lipolytic microorganisms per milliliter of sample is calculated from the number of colonies with clearing zone from selected dilution

### Culture Media:

3.1.1 Tributyrin agar (Nutrient agar with Tributyrin)

3.1.2 Quarter's Strength Ringer solution

### 3.2 Procedure:

3.2.1 Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution.

3.2.2 Aseptically inoculate 1ml of diluted or undiluted sample using sterile pipette into sterile petri plates in duplicate. The petri plates should be labeled with the sample number, date and any other desired information.

3.2.3 Pour into each plate 15–18 mL of the molten sterilized Trybutyrin agar (cooled to 44–47°C). Avoid pouring of molten medium directly onto the inoculum. Mix the media and inoculum by swirling gently clockwise and anti-clockwise so as to obtain homogenous distribution of inoculum in the medium.

3.2.4 After complete solidification, invert the prepared plates and incubate at 30°C for 72±3 hr.

3.2.5 After specified incubation period count all colonies having well defined clearing zone extending at least 1 mm from the edge of colony.

3.2.6 After determining the colony count, report the results as Lipolytic count/mL

### 3.3 References:

3.3.1 IS 4251 : 1967 (Reaffirmed 1992)

## 4. Test for Coliform (MPN method)

The Coliform group includes all the aerobic and facultative anaerobic gram negative, non spore forming rod shaped bacteria which ferment lactose with gas formation within 48hr at 37°C

**4.1 Principle:** Multiple tube dilution test includes presumptive, confirmed & completed tests as total independent procedures. The results are actually estimate based on certain probability formula. The most satisfactory information is obtained when the largest portion examined shows no gas in all or majority of the tubes. The

Most probable number (MPN) value for a given sample is obtained by the use of MPN tables.

## **4.2 Culture Media:**

4.2.1 MacConkey broth

4.2.2 Brilliant green bile lactose broth

4.2.3 Nutrient agar

4.2.4 MacConkey agar

4.2.5 Lactose broth

## **4.3 Procedure:**

### **4.3.1 Presumptive Test:**

4.3.1.1 Inoculate a series of MacConkey broth tubes with appropriate measured quantities of water to be tested. (50mL x 1, 10mL x 5, 1mL x 5 and 0.1mL x 5)

4.3.1.2 Inoculate all the tubes at 37°C for 24-48 hr.

4.3.1.3 Examine each tube at the end of 24±2hr for gas production and if no gas has been formed, reincubate upto 48 hr.

4.3.1.4 Record the presence or absence of the gas at each examination of the tube regardless of the amount.

4.3.1.5 The absence of gas formation at the end of 48±3 hr in any amount in inner fermentation tube constitutes a negative test.

### **4.3.2 Confirmed Test:**

4.3.2.1 Transfer a loopful of culture from positive tubes of Macconkey broth to Brilliant Green broth (BGB). During such transfer gently shake the tube or mix by rotating.

4.3.2.2 Incubate the inoculated tubes at 37°C for 48±3hr

4.3.2.3 The formation of gas in any amount in the Durahm tube of BGB tube at any time within 48±3 hr constitute a positive confirmed test.

### 4.3.3 Completed Test:

4.3.3.1 Streak a loopful from positive BGB tube to MacConkey agar plates. Incubate at 37°C for 24±2 hr.

4.3.3.2 From each plate pick typical colony and inoculate in Lactose broth and Nutrient agar slants. Incubate at 37°C for 24 to 48 hr.

4.3.3.3 Pick up the strain from Nutrient agar and Gram stain. Coliform are gram negative, non-spore forming bacilli.

4.3.3.4 Observe Lactose broth for gas formation after incubation. Coliform shows gas production.

### 4.4 Special Precautions:

4.4.1 The concentration of nutritive ingredients in the mixture should be sufficient and as per requirements. 10 mL & above aliquots should be inoculated in double strength and 1 ml and its dilutions should be inoculated in single strength medium

### 4.5 References:

4.5.1 IS 1622:1981(Reaffirmed 2003)

4.5.2 IS 4251: 1967 (Reaffirmed 1992)

## 5 Thermophilic Bacterial Count

**5.1 Principle:** Thermophilic bacteria grow at relatively high temperature. Nutrient agar is a general purpose growth medium commonly used to assess "Total" or "Viable" bacterial growth of a water sample. The number of microorganisms per milliliter of sample is calculated from the number of colonies obtained on Nutrient agar (NA) plate from selected dilution. Poured plates are prepared using a specified culture medium and a specified quantity of the sample. The plates are aerobically incubated at 55°C for 48 hr.

## 5.2 Culture Media:

5.2.1 Nutrient agar

5.2.2 Quarter's Strength Ringer solution

## 5.3 Procedure:

5.3.1 Disinfect the surface of the bottle/pouch/cups containing sample with 70% ethanol. Thoroughly mix the sample by vigorous shaking to achieve uniform distribution.

5.3.2 Aseptically inoculate 1mL of sample or other suitable dilution of the water sample using sterile pipette into sterile petri plates in duplicate. The petri dishes should be labeled with the sample number, date and any other desired information.

5.3.3 Pour into each plate 15–18 mL of the molten sterilized Nutrient agar (cooled to 44–47°C). Avoid pouring of molten medium directly onto the inoculum. Mix the media and inoculum by swirling gently clockwise and anti-clockwise so as to obtain homogenous distribution of inoculums in the medium.

5.3.4 Allow to cool and solidify.

5.3.5 After complete solidification, invert the prepared plates and incubate at 55°C for 48±3 hr.

5.3.6 After specified incubation period count all colonies including pinpoint colonies using colony counter. Spreading colonies shall be considered as single colony. If less than one quarter of dish is overgrown by spreading colonies, count the colonies on the unaffected part of the dish and calculate number of colonies in the entire petri plate. If more than one quarter is overgrown by spreading colonies discard the plate.

5.3.7 Consider the plates having count between 30-300. Report the results as the average of colonies in a particular dilution falling within limits.

## 5.4 References:

5.4.1 IS 4251: 1967 (Reaffirmed 1992)

5.4.2 IS 1622: 1981 (Reaffirmed 2003)

## 6. Slime Forming Bacteria

Slime forming bacteria are bacteria producing slime which will adhere to structures.

**6.1. Principle:** Detection of slime forming bacteria is based on staining with Lugol's Solution and Gram stain followed by microscopic examination.

**6.2. Reagents:**

6.2.1. Lugol's Iodine solution

6.2.2. Gram stain reagents

**6.3. Procedure:**

6.3.1. Place on clear slide a small amount of sample and spread it evenly. Cover with a cover glass and examine under the low power of the microscope for large forms, such as algae and moulds and record.

6.3.2. Prepare another mount as above and stain with Lugol's Iodine solution. Examine under high power of the microscope for filamentous bacteria.

6.3.3. Dry and fix the smear, stain by the Gram's method and observe under the oil immersion. A variety of organisms may be observed of which one or two types may be prominent. Record the observation.

**6.4. References:**

6.4.1. IS 4251 : 1967 (Reaffirmed 1992)

6.4.2. IS 1622 : 1981(Reaffirmed 2003)

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## APPENDIX II

### EQUIPMENT, MATERIAL & GLASSWARE

List of equipment/labware required for microbiological analysis is given below-

1. Biosafety Cabinet/ Laminar Air Flow Chamber
2. Membrane Filtration assembly/ apparatus with Membrane Filters with pore size 0.45  $\mu\text{m}$
3. BOD Incubators/Universal Incubator(s)
4. Autoclave(s)
5. Weighing Balance (s)
6. pH Meter(s)
7. Hot Plate/Heating Mantle(s)
8. Water bath(s)
9. Drying Oven (Hot air Oven(s))
10. UV Cabinet
11. Colony Counter
12. Microscope(s)
13. Vacuum pump(s)
14. Petri plates
15. Inoculation Loop of 3 mm diameter
16. Forcep(s)
17. Inoculating Loop(s)
18. Autopipettes (100-1000  $\mu\text{l}$ , 1-10 ml)

19. Anaerobic jar(s)
20. Durahm tubes

## APPENDIX -III

### MEDIA PREPARATION

Use dehydrated complete medium or constituents of uniform quality and recognized analytical grade for uniformity of results. Weigh the required amount of media as mentioned or recommended by the manufacturer and dissolve the media in required amount of distilled water. Use distilled water/Milli-Q water or water of equivalent grade for preparation of media.

Different media along with compositions and preparation details are listed below:

Sr. No.	Media										
1.	<p><b>Andrade Peptone Water</b></p> <table style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="text-align: left; width: 80%;">Ingredients</th> <th style="text-align: right; width: 20%;">g/L</th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.000</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.000</td> </tr> <tr> <td>Andrade indicator</td> <td style="text-align: right;">: 0.100</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.5±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the media ingredients completely. Adjust the pH and sterilize by autoclaving at 10 lbs pressure (115°C) for 20 min. Cool to room temperature and aseptically add sterile stock solution of carbohydrate to a final concentration of 1.0% (w/v). Distribute in sterile test tubes.</p>	Ingredients	g/L	Peptic digest of animal tissue	: 10.000	Sodium chloride	: 5.000	Andrade indicator	: 0.100	Final pH ( at 25°C) 7.5±0.2	
Ingredients	g/L										
Peptic digest of animal tissue	: 10.000										
Sodium chloride	: 5.000										
Andrade indicator	: 0.100										
Final pH ( at 25°C) 7.5±0.2											
2.	<p><b>Alkaline Peptone Water</b></p> <table style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="text-align: left; width: 80%;">Ingredients</th> <th style="text-align: right; width: 20%;">g/L</th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 8.2±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>	Ingredients	g/L	Peptic digest of animal tissue	: 10.0	Sodium chloride	: 5.0	Final pH ( at 25°C) 8.2±0.2			
Ingredients	g/L										
Peptic digest of animal tissue	: 10.0										
Sodium chloride	: 5.0										
Final pH ( at 25°C) 8.2±0.2											

3.	<p><b>Asparagine Proline Broth</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients g/L</th> <th style="text-align: left;">Single strength</th> <th style="text-align: left;">Concentrated</th> </tr> </thead> <tbody> <tr> <td>DL-Asparagine</td> <td>: 2.0</td> <td>3.2</td> </tr> <tr> <td>L-Proline</td> <td>: 1.0</td> <td>1.6</td> </tr> <tr> <td>Dipotassium phosphate</td> <td>: 1.0</td> <td>1.6</td> </tr> <tr> <td>Magnesium sulphate</td> <td>: 0.5</td> <td>0.8</td> </tr> <tr> <td>Potassium sulphate</td> <td>: 10.0</td> <td>16.0</td> </tr> <tr> <td colspan="3">Final pH ( at 25°C) 7.2±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water containing 25 mL or 40 mL ethanol respectively. Heat to boiling to dissolve the medium completely. Distribute as desired in screw-capped bottles. Close the caps so that the seal in the lid just touches the lip of the bottle. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Tighten the caps of the bottles immediately after removal from the autoclave to prevent loss of ethanol by evaporation. It is not advisable to use polypropylene caps without seals. Alternatively, ethanol may be sterilized separately by filtration and then added aseptically to the sterile cooled medium.</p>	Ingredients g/L	Single strength	Concentrated	DL-Asparagine	: 2.0	3.2	L-Proline	: 1.0	1.6	Dipotassium phosphate	: 1.0	1.6	Magnesium sulphate	: 0.5	0.8	Potassium sulphate	: 10.0	16.0	Final pH ( at 25°C) 7.2±0.2		
Ingredients g/L	Single strength	Concentrated																				
DL-Asparagine	: 2.0	3.2																				
L-Proline	: 1.0	1.6																				
Dipotassium phosphate	: 1.0	1.6																				
Magnesium sulphate	: 0.5	0.8																				
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Final pH ( at 25°C) 7.2±0.2																						
4.	<p><b>Baird Parker Agar Base</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: left;">g/L</th> </tr> </thead> <tbody> <tr> <td>Casein enzymic hydrolysate</td> <td>: 10.0</td> </tr> <tr> <td>Meat extract</td> <td>: 5.0</td> </tr> <tr> <td>Yeast extract</td> <td>: 1.0</td> </tr> <tr> <td>Glycine</td> <td>: 12.0</td> </tr> <tr> <td>Sodium pyruvate</td> <td>: 12.0</td> </tr> <tr> <td>Lithium chloride</td> <td>: 5.0</td> </tr> <tr> <td>Agar</td> <td>: 20.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.0±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 950 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by</p>	Ingredients	g/L	Casein enzymic hydrolysate	: 10.0	Meat extract	: 5.0	Yeast extract	: 1.0	Glycine	: 12.0	Sodium pyruvate	: 12.0	Lithium chloride	: 5.0	Agar	: 20.0	Final pH ( at 25°C) 7.0±0.2				
Ingredients	g/L																					
Casein enzymic hydrolysate	: 10.0																					
Meat extract	: 5.0																					
Yeast extract	: 1.0																					
Glycine	: 12.0																					
Sodium pyruvate	: 12.0																					
Lithium chloride	: 5.0																					
Agar	: 20.0																					
Final pH ( at 25°C) 7.0±0.2																						

	<p>autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 50°C and aseptically add 50 mL concentrated Egg Yolk Tellurite Emulsion (commercially available). Mix well and pour into sterile Petri plates.</p> <p><b><i>Warning: Lithium chloride is harmful. Avoid bodily contact and inhalation of vapours. On contact with skin wash with plenty of water immediately</i></b></p>
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5.	<p><b>Bile Esculin Azide Agar</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Casein enzymic hydrolysate</td> <td style="text-align: right;">: 17.0</td> </tr> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Oxgall</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Esculin</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Ferric ammonium citrate</td> <td style="text-align: right;">: 0.5</td> </tr> <tr> <td>Sodium azide</td> <td style="text-align: right;">: 0.15</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">7.1±0.1</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.</p> <p><b>Caution:</b> <i>Sodium azide has a tendency to form explosive metal azides with plumbing materials. It is advisable to use enough water to flush off the disposables.</i></p>	Ingredients	g/L	Casein enzymic hydrolysate	: 17.0	Peptic digest of animal tissue	: 3.0	Yeast extract	: 5.0	Oxgall	: 10.0	Sodium chloride	: 5.0	Esculin	: 1.0	Ferric ammonium citrate	: 0.5	Sodium azide	: 0.15	Agar	: 15.0	Final pH ( at 25°C)	7.1±0.1
Ingredients	g/L																						
Casein enzymic hydrolysate	: 17.0																						
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Yeast extract	: 5.0																						
Oxgall	: 10.0																						
Sodium chloride	: 5.0																						
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Ferric ammonium citrate	: 0.5																						
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Agar	: 15.0																						
Final pH ( at 25°C)	7.1±0.1																						
6.	<p><b>Bile Salts Agar</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Meat extract</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Sodium taurocholate</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">8.5±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by</p>	Ingredients	g/L	Peptic digest of animal tissue	: 10.0	Meat extract	: 5.0	Sodium chloride	: 5.0	Sodium taurocholate	: 5.0	Agar	: 15.0	Final pH ( at 25°C)	8.5±0.2								
Ingredients	g/L																						
Peptic digest of animal tissue	: 10.0																						
Meat extract	: 5.0																						
Sodium chloride	: 5.0																						
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Agar	: 15.0																						
Final pH ( at 25°C)	8.5±0.2																						

	autoclaving at 15 lbs pressure (121°C) for 15 minutes. Pour into sterile Petri plates.
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7.	<p><b>Bismuth Sulphite Agar</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Beef extract</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Dextrose</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Disodium phosphate</td> <td style="text-align: right;">: 4.0</td> </tr> <tr> <td>Ferrous sulphate</td> <td style="text-align: right;">: 0.3</td> </tr> <tr> <td>Bismuth sulphite indicator</td> <td style="text-align: right;">: 8.0</td> </tr> <tr> <td>Brilliant green</td> <td style="text-align: right;">: 0.025</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 20.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.6±0.1</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and <b>DO NOT STERILIZE IN AUTOCLAVE</b> or by fractional sterilization since overheating may destroy the selectivity of the medium.</p> <p>The sensitivity of the medium depends largely upon uniform dispersion of precipitated bismuth sulphite in the final gel, which should be dispersed before pouring into sterile Petri plates.</p>	<b>Ingredients</b>	<b>g/L</b>	Peptic digest of animal tissue	: 10.0	Beef extract	: 5.0	Dextrose	: 5.0	Disodium phosphate	: 4.0	Ferrous sulphate	: 0.3	Bismuth sulphite indicator	: 8.0	Brilliant green	: 0.025	Agar	: 20.0	Final pH ( at 25°C) 7.6±0.1	
<b>Ingredients</b>	<b>g/L</b>																				
Peptic digest of animal tissue	: 10.0																				
Beef extract	: 5.0																				
Dextrose	: 5.0																				
Disodium phosphate	: 4.0																				
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Agar	: 20.0																				
Final pH ( at 25°C) 7.6±0.1																					
8.	<p><b>Blood Agar</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Meat Extract</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.4±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add 10% sterile sheep blood. Mix well and pour into sterile Petri</p>	<b>Ingredients</b>	<b>g/L</b>	Peptic digest of animal tissue	: 10.0	Meat Extract	: 10.0	Sodium chloride	: 5.0	Agar	: 15.0	Final pH ( at 25°C) 7.4±0.2									
<b>Ingredients</b>	<b>g/L</b>																				
Peptic digest of animal tissue	: 10.0																				
Meat Extract	: 10.0																				
Sodium chloride	: 5.0																				
Agar	: 15.0																				
Final pH ( at 25°C) 7.4±0.2																					

	plates.
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<p>9.</p>	<p><b>Brilliant Green Agar</b></p> <table data-bbox="446 268 1069 873"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Meat extract</td> <td style="text-align: right;">5.0</td> </tr> <tr> <td>Peptone</td> <td style="text-align: right;">5.0</td> </tr> <tr> <td>Disodium hydrogen phosphate</td> <td style="text-align: right;">1.0</td> </tr> <tr> <td>Sodium dihydrogen phosphate</td> <td style="text-align: right;">0.6</td> </tr> <tr> <td>Lactose</td> <td style="text-align: right;">10.000</td> </tr> <tr> <td>Sucrose</td> <td style="text-align: right;">10.000</td> </tr> <tr> <td>Phenol red</td> <td style="text-align: right;">0.090</td> </tr> <tr> <td>Brilliant green</td> <td style="text-align: right;">0.005</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">15.000</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.0±0.1</td> </tr> </tbody> </table> <p><b>Directions:</b> Prepare media as per manufacture instructions. Heat with occasional agitation and bring just to the boil to dissolve the medium completely. Adjust the pH and <b>DO NOT AUTOCLAVE</b>. Mix well before pouring into sterile Petri plates.</p>	Ingredients	g/L	Meat extract	5.0	Peptone	5.0	Disodium hydrogen phosphate	1.0	Sodium dihydrogen phosphate	0.6	Lactose	10.000	Sucrose	10.000	Phenol red	0.090	Brilliant green	0.005	Agar	15.000	Final pH ( at 25°C) 7.0±0.1	
Ingredients	g/L																						
Meat extract	5.0																						
Peptone	5.0																						
Disodium hydrogen phosphate	1.0																						
Sodium dihydrogen phosphate	0.6																						
Lactose	10.000																						
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Phenol red	0.090																						
Brilliant green	0.005																						
Agar	15.000																						
Final pH ( at 25°C) 7.0±0.1																							
<p>10.</p>	<p><b>Brilliant Green Bile Lactose Broth</b></p> <table data-bbox="446 1299 1069 1635"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">10.0</td> </tr> <tr> <td>Lactose</td> <td style="text-align: right;">10.0</td> </tr> <tr> <td>Oxgall</td> <td style="text-align: right;">20.0</td> </tr> <tr> <td>Brilliant green</td> <td style="text-align: right;">0.0133</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.2±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and distribute in fermentation tubes containing inverted Durhams tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>	Ingredients	g/L	Peptic digest of animal tissue	10.0	Lactose	10.0	Oxgall	20.0	Brilliant green	0.0133	Final pH ( at 25°C) 7.2±0.2											
Ingredients	g/L																						
Peptic digest of animal tissue	10.0																						
Lactose	10.0																						
Oxgall	20.0																						
Brilliant green	0.0133																						
Final pH ( at 25°C) 7.2±0.2																							

11.	<p><b>Buffered Peptone Water</b></p> <table data-bbox="502 248 1236 571"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Casein enzymic hydrolysate</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Disodium hydrogen phosphate.12H<sub>2</sub>O</td> <td style="text-align: right;">: 9.0</td> </tr> <tr> <td>Monopotassium hydrogen phosphate</td> <td style="text-align: right;">: 1.5</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">7.1±0.1</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and dispense as desired and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>	Ingredients	g/L	Casein enzymic hydrolysate	: 10.0	Sodium chloride	: 5.0	Disodium hydrogen phosphate.12H <sub>2</sub> O	: 9.0	Monopotassium hydrogen phosphate	: 1.5	Final pH ( at 25°C)	7.1±0.1		
Ingredients	g/L														
Casein enzymic hydrolysate	: 10.0														
Sodium chloride	: 5.0														
Disodium hydrogen phosphate.12H <sub>2</sub> O	: 9.0														
Monopotassium hydrogen phosphate	: 1.5														
Final pH ( at 25°C)	7.1±0.1														
12.	<p><b>Cetrimide Agar</b></p> <table data-bbox="502 898 1125 1272"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Pancreatic digest of gelatin</td> <td style="text-align: right;">: 20.0</td> </tr> <tr> <td>Magnesium chloride</td> <td style="text-align: right;">: 1.4</td> </tr> <tr> <td>Dipotassium sulphate</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Cetrimide</td> <td style="text-align: right;">: 0.3</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 13.6</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">7.2±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL purified/distilled water containing 10 mL glycerin/glycerol. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes or as per validated cycle. Mix well before pouring into sterile Petri plates.</p>	Ingredients	g/L	Pancreatic digest of gelatin	: 20.0	Magnesium chloride	: 1.4	Dipotassium sulphate	: 10.0	Cetrimide	: 0.3	Agar	: 13.6	Final pH ( at 25°C)	7.2±0.2
Ingredients	g/L														
Pancreatic digest of gelatin	: 20.0														
Magnesium chloride	: 1.4														
Dipotassium sulphate	: 10.0														
Cetrimide	: 0.3														
Agar	: 13.6														
Final pH ( at 25°C)	7.2±0.2														
13.	<p><b>Chloramphenicol Yeast Glucose Agar</b></p> <table data-bbox="502 1713 1077 1973"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Dextrose</td> <td style="text-align: right;">: 20.0</td> </tr> <tr> <td>Chloramphenicol</td> <td style="text-align: right;">: 0.1</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> </tbody> </table>	Ingredients	g/L	Yeast extract	: 5.0	Dextrose	: 20.0	Chloramphenicol	: 0.1	Agar	: 15.0				
Ingredients	g/L														
Yeast extract	: 5.0														
Dextrose	: 20.0														
Chloramphenicol	: 0.1														
Agar	: 15.0														

	<p>Final pH ( at 25°C) 6.6±0.2</p> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.</p>
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14.	<p><b>Cooked Meat Salt Medium</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Beef heart, solids</td> <td style="text-align: right;">98.0</td> </tr> <tr> <td>Proteose peptone</td> <td style="text-align: right;">20.0</td> </tr> <tr> <td>Dextrose</td> <td style="text-align: right;">2.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">5.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.2±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend 12.5 gm of dehydrated media of above composition in 100 mL distilled water (or suspend 1.25 gm in 10 mL distilled water in test tubes). Mix thoroughly and allow to stand for 15 minutes until all the particles are thoroughly wetted. Add 10% Sodium Chloride to the broth, Adjust pH to 7.8 and use it as Cooked salt meat medium.</p>	<b>Ingredients</b>	<b>g/L</b>	Beef heart, solids	98.0	Proteose peptone	20.0	Dextrose	2.0	Sodium chloride	5.0	Final pH ( at 25°C) 7.2±0.2											
<b>Ingredients</b>	<b>g/L</b>																						
Beef heart, solids	98.0																						
Proteose peptone	20.0																						
Dextrose	2.0																						
Sodium chloride	5.0																						
Final pH ( at 25°C) 7.2±0.2																							
15.	<p><b>Deoxycholate Citrate Agar Medium</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">5.0</td> </tr> <tr> <td>Beef extract</td> <td style="text-align: right;">5.0</td> </tr> <tr> <td>Lactose</td> <td style="text-align: right;">10.0</td> </tr> <tr> <td>Sodium citrate</td> <td style="text-align: right;">8.5</td> </tr> <tr> <td>Ferric citrate</td> <td style="text-align: right;">1.0</td> </tr> <tr> <td>Sodium deoxycholate</td> <td style="text-align: right;">5.0</td> </tr> <tr> <td>Sodium thiosulphate</td> <td style="text-align: right;">5.4</td> </tr> <tr> <td>Neutral red</td> <td style="text-align: right;">0.020</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">12.000</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.3±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and <b>DO NOT AUTOCLAVE</b>. Avoid excessive heating as it is detrimental to the medium.</p>	<b>Ingredients</b>	<b>g/L</b>	Peptic digest of animal tissue	5.0	Beef extract	5.0	Lactose	10.0	Sodium citrate	8.5	Ferric citrate	1.0	Sodium deoxycholate	5.0	Sodium thiosulphate	5.4	Neutral red	0.020	Agar	12.000	Final pH ( at 25°C) 7.3±0.2	
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16.	<p><b>Differential Reinforced Clostridial medium</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Tryptose</td> <td style="text-align: right;">10.0</td> </tr> <tr> <td>Meat extract</td> <td style="text-align: right;">10.0</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">1.50</td> </tr> <tr> <td>Sodium acetate, hydrated</td> <td style="text-align: right;">5.0</td> </tr> <tr> <td>Starch</td> <td style="text-align: right;">1.0</td> </tr> <tr> <td>Glucose</td> <td style="text-align: right;">1.0</td> </tr> <tr> <td>L-Cysteine hydrochloride</td> <td style="text-align: right;">0.5</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">7.2±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Just before use add 0.5mL filter sterilized solution prepared by mixing equal volumes of 4% w/v solution of sodium sulphite and 7% w/v ferric citrate, to 25 mL of single strength medium or 0.4 mL and 2 mL to 10 mL and 50 mL of double strength medium respectively. Mix well.</p>	Ingredients	g/L	Tryptose	10.0	Meat extract	10.0	Yeast extract	1.50	Sodium acetate, hydrated	5.0	Starch	1.0	Glucose	1.0	L-Cysteine hydrochloride	0.5	Final pH ( at 25°C)	7.2±0.2
Ingredients	g/L																		
Tryptose	10.0																		
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17.	<p><b>Ethyl Violet Azide Dextrose Agar</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Tryptose</td> <td style="text-align: right;">20.000</td> </tr> <tr> <td>Dextrose</td> <td style="text-align: right;">5.000</td> </tr> <tr> <td>Dipotassium phosphate</td> <td style="text-align: right;">2.700</td> </tr> <tr> <td>Monopotassium phosphate</td> <td style="text-align: right;">2.700</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">5.000</td> </tr> <tr> <td>Sodium azide</td> <td style="text-align: right;">0.400</td> </tr> <tr> <td>Ethyl violet</td> <td style="text-align: right;">0.00083</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">7.0±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs</p>	Ingredients	g/L	Tryptose	20.000	Dextrose	5.000	Dipotassium phosphate	2.700	Monopotassium phosphate	2.700	Sodium chloride	5.000	Sodium azide	0.400	Ethyl violet	0.00083	Final pH ( at 25°C)	7.0±0.2
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Tryptose	20.000																		
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	pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates
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<b>18.</b>	<p><b>Gelatin Agar</b></p> <table border="0" style="width: 100%;"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Gelatin</td> <td style="text-align: right;">: 120.0</td> </tr> <tr> <td>Peptone</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Meat Extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 30.0</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.0±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Distribute in Sterilized Test Tubes.</p>	<b>Ingredients</b>	<b>g/L</b>	Gelatin	: 120.0	Peptone	: 5.0	Meat Extract	: 3.0	Sodium chloride	: 30.0	Agar	: 15.0	Final pH ( at 25°C) 7.0±0.2							
<b>Ingredients</b>	<b>g/L</b>																				
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<b>19.</b>	<p><b>Glucose Salt Teepol Broth</b></p> <table border="0" style="width: 100%;"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td colspan="2"><b>Part A -</b></td> </tr> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Beef extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 30.0</td> </tr> <tr> <td>Glucose</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Methyl violet</td> <td style="text-align: right;">: 0.002</td> </tr> <tr> <td colspan="2"><b>Part B -</b></td> </tr> <tr> <td>Teepol</td> <td style="text-align: right;">: 4.000</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 8.8±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients of Part A in 1000 mL distilled water containing 4.0 mL of Part B. Heat gently to dissolve the medium completely. Dispense in Bottles/Flask as desired. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>	<b>Ingredients</b>	<b>g/L</b>	<b>Part A -</b>		Peptic digest of animal tissue	: 10.0	Beef extract	: 3.0	Sodium chloride	: 30.0	Glucose	: 5.0	Methyl violet	: 0.002	<b>Part B -</b>		Teepol	: 4.000	Final pH ( at 25°C) 8.8±0.2	
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20.	<p><b>Hugh Leifson Medium</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 2.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Dipotassium phosphate</td> <td style="text-align: right;">: 0.3</td> </tr> <tr> <td>Glucose</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Bromo thymol blue</td> <td style="text-align: right;">: 0.03</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Final pH (at 25°C)</td> <td style="text-align: right;">7.1±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat with frequent stirring. Boil to dissolve the medium completely. Adjust the pH and dispense in tubes in duplicate for aerobic and anaerobic fermentations. Sterilize by autoclaving at 10 lbs pressure (115°C) for 20 minutes. Cool the tubes with medium in an upright position.</p> <p><i>Note:: In an additional set of tubes 5mm paraffin oil may be layered on surface of medium for the differentiation of oxidative &amp; fermentative organisms.</i></p>	Ingredients	g/L	Peptic digest of animal tissue	: 2.0	Sodium chloride	: 5.0	Dipotassium phosphate	: 0.3	Glucose	: 10.0	Bromo thymol blue	: 0.03	Agar	: 3.0	Final pH (at 25°C)	7.1±0.2
Ingredients	g/L																
Peptic digest of animal tissue	: 2.0																
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21.	<p><b>Kauffman Mueller's Tetrathionate Broth Base</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Peptone</td> <td style="text-align: right;">: 9.0</td> </tr> <tr> <td>Meat extract</td> <td style="text-align: right;">: 9.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 4.5</td> </tr> <tr> <td>Calcium carbonate</td> <td style="text-align: right;">: 50.0</td> </tr> <tr> <td>Sodium thiosulphate</td> <td style="text-align: right;">: 50.0</td> </tr> <tr> <td>Oxbile</td> <td style="text-align: right;">: 10.0</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat just to boiling. <b>DO NOT AUTOCLAVE</b>. Cool and just before use aseptically add 19 mL of iodine solution.(20 gm iodine and 25 gm Potassium iodide in 100 mL sterile distilled water) and 9.5 mL of 0.1 % Brilliant green solution. Mix well before</p>	Ingredients	g/L	Peptone	: 9.0	Meat extract	: 9.0	Sodium chloride	: 4.5	Calcium carbonate	: 50.0	Sodium thiosulphate	: 50.0	Oxbile	: 10.0		
Ingredients	g/L																
Peptone	: 9.0																
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Sodium chloride	: 4.5																
Calcium carbonate	: 50.0																
Sodium thiosulphate	: 50.0																
Oxbile	: 10.0																

	<p>dispensing in the sterile tubes to dispense Calcium carbonate uniformly.</p> <p><b><i>Note: Due to presence of Calcium carbonate the prepared medium forms opalescent solution with white precipitate</i></b></p>
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22.	<p><b>Kligler Iron Agar</b></p> <table data-bbox="502 248 1129 907"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Beef extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Casein enzymic hydrolysate</td> <td style="text-align: right;">: 20.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Lactose</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Glucose anhydrous</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Ferrous ammonium sulphate, 6H<sub>2</sub>O</td> <td style="text-align: right;">: 0.5</td> </tr> <tr> <td>Sodium thiosulphate pentahydrate</td> <td style="text-align: right;">: 0.5</td> </tr> <tr> <td>Phenol red</td> <td style="text-align: right;">: 0.025</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">7.4±0.1</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Mix well and distribute into tubes. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow the tubes to cool in slanted position to form slopes with about 1 inch butts. Best reactions are obtained on freshly prepared medium.</p>	Ingredients	g/L	Beef extract	: 3.0	Yeast extract	: 3.0	Casein enzymic hydrolysate	: 20.0	Sodium chloride	: 5.0	Lactose	: 10.0	Glucose anhydrous	: 1.0	Ferrous ammonium sulphate, 6H <sub>2</sub> O	: 0.5	Sodium thiosulphate pentahydrate	: 0.5	Phenol red	: 0.025	Agar	: 15.0	Final pH ( at 25°C)	7.4±0.1
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Beef extract	: 3.0																								
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Agar	: 15.0																								
Final pH ( at 25°C)	7.4±0.1																								
23.	<p><b>Lactose Broth</b></p> <table data-bbox="478 1429 1136 1697"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Beef extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Lactose</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">6.9±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and distribute into tubes with inverted Durhams tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool the tubes before inoculation</p>	Ingredients	g/L	Peptic digest of animal tissue	: 5.0	Beef extract	: 3.0	Lactose	: 5.0	Final pH ( at 25°C)	6.9±0.2														
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24.	<p><b>Lysine Decarboxylase Broth</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Dextrose</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>L-Lysine hydrochloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Bromocresol purple</td> <td style="text-align: right;">: 0.02</td> </tr> <tr> <td colspan="2">Final pH (at 25°C) 6.8±0.1</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat, if necessary to dissolve the medium completely. Adjust the pH and dispense 5 mL amount into screw-capped test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>	<b>Ingredients</b>	<b>g/L</b>	Peptic digest of animal tissue	: 5.0	Yeast extract	: 3.0	Dextrose	: 1.0	L-Lysine hydrochloride	: 5.0	Bromocresol purple	: 0.02	Final pH (at 25°C) 6.8±0.1			
<b>Ingredients</b>	<b>g/L</b>																
Peptic digest of animal tissue	: 5.0																
Yeast extract	: 3.0																
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25.	<p><b>MacConkey Agar</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 20.0</td> </tr> <tr> <td>Lactose</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Bile salts</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Neutral red</td> <td style="text-align: right;">: 0.07</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.5±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling with gentle swirling to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Avoid overheating. Cool to 45-50°C and pour into sterile Petri plates. The surface of the medium should be dry when inoculated.</p>	<b>Ingredients</b>	<b>g/L</b>	Peptic digest of animal tissue	: 20.0	Lactose	: 10.0	Bile salts	: 5.0	Sodium chloride	: 5.0	Neutral red	: 0.07	Agar	: 15.0	Final pH ( at 25°C) 7.5±0.2	
<b>Ingredients</b>	<b>g/L</b>																
Peptic digest of animal tissue	: 20.0																
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26.	<p><b>MacConkey Broth w/ Neutral Red</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/l</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 20.0</td> </tr> </tbody> </table>	<b>Ingredients</b>	<b>g/l</b>	Peptic digest of animal tissue	: 20.0												
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Peptic digest of animal tissue	: 20.0																

	Lactose	:	10.0
	Bile salts	:	5.0
	Sodium chloride	:	5.0
	Neutral red	:	0.075
	Final pH ( at 25°C)		7.4±0.2
	<b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and distribute into tubes with inverted Durhams tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool the tubes before inoculation		

27.	<p><b>Malonate Broth</b></p> <table data-bbox="438 280 1077 772"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Ammonium sulphate</td> <td style="text-align: right;">: 2.0</td> </tr> <tr> <td>Dipotassium phosphate</td> <td style="text-align: right;">: 0.6</td> </tr> <tr> <td>Monopotassium phosphate</td> <td style="text-align: right;">: 0.4</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 2.0</td> </tr> <tr> <td>Sodium malonate</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Dextrose</td> <td style="text-align: right;">: 0.25</td> </tr> <tr> <td>Bromothymol blue</td> <td style="text-align: right;">: 0.025</td> </tr> </tbody> </table> <p>Final pH ( at 25°C) 6.7±0.1</p> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and distribute into tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool the tubes before inoculation.</p>	Ingredients	g/L	Yeast extract	: 1.0	Ammonium sulphate	: 2.0	Dipotassium phosphate	: 0.6	Monopotassium phosphate	: 0.4	Sodium chloride	: 2.0	Sodium malonate	: 3.0	Dextrose	: 0.25	Bromothymol blue	: 0.025
Ingredients	g/L																		
Yeast extract	: 1.0																		
Ammonium sulphate	: 2.0																		
Dipotassium phosphate	: 0.6																		
Monopotassium phosphate	: 0.4																		
Sodium chloride	: 2.0																		
Sodium malonate	: 3.0																		
Dextrose	: 0.25																		
Bromothymol blue	: 0.025																		
28.	<p><b>Medium for dihydrolase and decarboxylase activity</b></p> <table data-bbox="502 1265 1101 1534"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Glucose</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Bromocresol Purple</td> <td style="text-align: right;">: 0.2</td> </tr> </tbody> </table> <p>Final pH ( at 25°C) 6.7±0.2</p> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and divide in four aliquots. Add Arginine, lysine and ornithine separately (@ 1% of l-form, or as 2% of dl-form) and sterilize by autoclaving at 10 lbs pressure (115°C) for 20 minutes.</p>	Ingredients	g/L	Glucose	: 1.0	Peptic digest of animal tissue	: 5.0	Yeast extract	: 3.0	Bromocresol Purple	: 0.2								
Ingredients	g/L																		
Glucose	: 1.0																		
Peptic digest of animal tissue	: 5.0																		
Yeast extract	: 3.0																		
Bromocresol Purple	: 0.2																		

29.	<p><b>Milk Agar with Cetrimide</b></p> <table border="0" style="width: 100%;"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td colspan="2"><b>Part A -</b></td> </tr> <tr> <td>Skim milk powder</td> <td style="text-align: right;">: 100.0</td> </tr> <tr> <td colspan="2"><b>Part B -</b></td> </tr> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Cetrimide</td> <td style="text-align: right;">: 0.4</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.3±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above composition of Part B in 250 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 20 minutes. Suspend Part A in 750 mL of distilled water and sterilize by autoclaving at 15 lbs pressure (121°C) for 5 minutes. After autoclaving mix Part A and B and pour into sterile Petri plates.</p>	Ingredients	g/L	<b>Part A -</b>		Skim milk powder	: 100.0	<b>Part B -</b>		Peptic digest of animal tissue	: 10.0	Sodium chloride	: 5.0	Yeast extract	: 3.0	Cetrimide	: 0.4	Agar	: 15.0	Final pH ( at 25°C) 7.3±0.2	
Ingredients	g/L																				
<b>Part A -</b>																					
Skim milk powder	: 100.0																				
<b>Part B -</b>																					
Peptic digest of animal tissue	: 10.0																				
Sodium chloride	: 5.0																				
Yeast extract	: 3.0																				
Cetrimide	: 0.4																				
Agar	: 15.0																				
Final pH ( at 25°C) 7.3±0.2																					
30.	<p><b>MR-VP Medium (Glucose Phosphate Broth)</b></p> <table border="0" style="width: 100%;"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/l</th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Dextrose</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Dipotassium hydrogen phosphate</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.5±0.1</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Adjust the pH and distribute in test tubes in 3 mL amounts or as desired. And sterilize by autoclaving at 15 lbs pressure (115°C) for 10 minutes.</p>	Ingredients	g/l	Peptic digest of animal tissue	: 5.0	Dextrose	: 5.0	Dipotassium hydrogen phosphate	: 5.0	Final pH ( at 25°C) 7.5±0.1											
Ingredients	g/l																				
Peptic digest of animal tissue	: 5.0																				
Dextrose	: 5.0																				
Dipotassium hydrogen phosphate	: 5.0																				
Final pH ( at 25°C) 7.5±0.1																					

31.	<p><b>Nitrate Broth</b></p> <table data-bbox="446 280 1037 548"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Meat extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Potassium nitrate</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">7.0±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and dispense 5 mL in tubes. And sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>	<b>Ingredients</b>	<b>g/L</b>	Peptic digest of animal tissue	: 5.0	Meat extract	: 3.0	Potassium nitrate	: 1.0	Final pH ( at 25°C)	7.0±0.2		
<b>Ingredients</b>	<b>g/L</b>												
Peptic digest of animal tissue	: 5.0												
Meat extract	: 3.0												
Potassium nitrate	: 1.0												
Final pH ( at 25°C)	7.0±0.2												
32.	<p><b>Nutrient Agar</b></p> <table data-bbox="502 896 1157 1220"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Glucose</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Beef extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">7.2±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>	<b>Ingredients</b>	<b>g/L</b>	Glucose	: 1.0	Peptic digest of animal tissue	: 5.0	Beef extract	: 3.0	Agar	: 15.0	Final pH ( at 25°C)	7.2±0.2
<b>Ingredients</b>	<b>g/L</b>												
Glucose	: 1.0												
Peptic digest of animal tissue	: 5.0												
Beef extract	: 3.0												
Agar	: 15.0												
Final pH ( at 25°C)	7.2±0.2												
33.	<p><b>Nutrient Agar No 2</b></p> <table data-bbox="502 1601 1093 1915"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Beef extract</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">7.5±0.2</td> </tr> </tbody> </table>	<b>Ingredients</b>	<b>g/L</b>	Peptic digest of animal tissue	: 10.0	Beef extract	: 10.0	Sodium chloride	: 5.0	Agar	: 15.0	Final pH ( at 25°C)	7.5±0.2
<b>Ingredients</b>	<b>g/L</b>												
Peptic digest of animal tissue	: 10.0												
Beef extract	: 10.0												
Sodium chloride	: 5.0												
Agar	: 15.0												
Final pH ( at 25°C)	7.5±0.2												

	<b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.
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34.	<p><b>Nutrient Agar for motility Test</b></p> <table data-bbox="502 309 1098 627"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Beef extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 4.5</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.6±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and dispense in test tubes and add a glass tube having both open ends in each tube. sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>	<b>Ingredients</b>	<b>g/L</b>	Peptic digest of animal tissue	: 10.0	Beef extract	: 3.0	Sodium chloride	: 5.0	Agar	: 4.5	Final pH ( at 25°C) 7.6±0.2	
<b>Ingredients</b>	<b>g/L</b>												
Peptic digest of animal tissue	: 10.0												
Beef extract	: 3.0												
Sodium chloride	: 5.0												
Agar	: 4.5												
Final pH ( at 25°C) 7.6±0.2													
35.	<p><b>Nutrient Broth</b></p> <table data-bbox="502 1041 1098 1332"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Beef extract</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.4±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes</p>	<b>Ingredients</b>	<b>g/L</b>	Peptic digest of animal tissue	: 10.0	Beef extract	: 10.0	Sodium chloride	: 5.0	Final pH ( at 25°C) 7.4±0.2			
<b>Ingredients</b>	<b>g/L</b>												
Peptic digest of animal tissue	: 10.0												
Beef extract	: 10.0												
Sodium chloride	: 5.0												
Final pH ( at 25°C) 7.4±0.2													
36.	<p><b>Overlay Agar Medium</b></p> <table data-bbox="502 1736 1098 1971"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Agar</td> <td style="text-align: right;">: 12-18gm</td> </tr> <tr> <td>Water</td> <td style="text-align: right;">: 1000 mL</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.0±0.2</td> </tr> </tbody> </table>	<b>Ingredients</b>	<b>g/L</b>	Agar	: 12-18gm	Water	: 1000 mL	Final pH ( at 25°C) 7.0±0.2					
<b>Ingredients</b>	<b>g/L</b>												
Agar	: 12-18gm												
Water	: 1000 mL												
Final pH ( at 25°C) 7.0±0.2													

	<p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes</p>
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37.	<p><b>Peptone Water</b></p> <table border="0" style="width: 100%;"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 20.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.4±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and dispense in test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes</p>	Ingredients	g/L	Peptic digest of animal tissue	: 20.0	Sodium chloride	: 5.0	Final pH ( at 25°C) 7.4±0.2							
Ingredients	g/L														
Peptic digest of animal tissue	: 20.0														
Sodium chloride	: 5.0														
Final pH ( at 25°C) 7.4±0.2															
38.	<p><b>Plate Count Agar (Standard Methods Agar)</b></p> <table border="0" style="width: 100%;"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Casein enzymic hydrolysate</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 2.5</td> </tr> <tr> <td>Dextrose</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td colspan="2">Final pH (at 25°C) 7.0±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and dispense in test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.</p>	Ingredients	g/L	Casein enzymic hydrolysate	: 5.0	Yeast extract	: 2.5	Dextrose	: 1.0	Agar	: 15.0	Final pH (at 25°C) 7.0±0.2			
Ingredients	g/L														
Casein enzymic hydrolysate	: 5.0														
Yeast extract	: 2.5														
Dextrose	: 1.0														
Agar	: 15.0														
Final pH (at 25°C) 7.0±0.2															
39.	<p><b>Rappaport Vassiliadis Soya Broth (RVS Broth)</b></p> <table border="0" style="width: 100%;"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Peptone from soyabeans</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 4.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 8.0</td> </tr> <tr> <td>Dipotassium hydrogen phosphatetrihydrate</td> <td style="text-align: right;">: 0.4</td> </tr> <tr> <td>Potassium dihydrogen phosphate</td> <td style="text-align: right;">: 0.6</td> </tr> <tr> <td>Magnesium chloride, 6H<sub>2</sub>O</td> <td style="text-align: right;">: 31.7</td> </tr> </tbody> </table>	Ingredients	g/L	Peptone from soyabeans	: 1.0	Peptic digest of animal tissue	: 4.0	Sodium chloride	: 8.0	Dipotassium hydrogen phosphatetrihydrate	: 0.4	Potassium dihydrogen phosphate	: 0.6	Magnesium chloride, 6H <sub>2</sub> O	: 31.7
Ingredients	g/L														
Peptone from soyabeans	: 1.0														
Peptic digest of animal tissue	: 4.0														
Sodium chloride	: 8.0														
Dipotassium hydrogen phosphatetrihydrate	: 0.4														
Potassium dihydrogen phosphate	: 0.6														
Magnesium chloride, 6H <sub>2</sub> O	: 31.7														

	<p>Malachite green : 0.04</p> <p>Final pH ( at 25°C) 5.2±0.1</p> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and dispense in test tubes. Sterilize by autoclaving at 115°C for 15 mins.</p>
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40.	<p><b>Selenite F Broth (Twin Pack) -</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td colspan="2"><b>Part A -</b></td> </tr> <tr> <td>Peptone</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Lactose</td> <td style="text-align: right;">: 4.0</td> </tr> <tr> <td>Disodium hydrogen phosphate</td> <td style="text-align: right;">: 9.5</td> </tr> <tr> <td>Sodium dihydrogen phosphate</td> <td style="text-align: right;">: 0.5</td> </tr> <tr> <td colspan="2"><b>Part B -</b></td> </tr> <tr> <td>Sodium hydrogen selenite</td> <td style="text-align: right;">: 4.0</td> </tr> <tr> <td>Final pH (at 25°C)</td> <td style="text-align: right;">7.1±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend 4.0 gm of dehydrated media of Part-B composition in 1000 mL purified/ distilled water. Add 19.0 gm of dehydrated media of Part – A ingredients. Mix well. Warm to dissolve the medium completely. Distribute in sterile test tubes. Sterilize in a boiling water bath or free flowing steam for 30 minutes.</p> <p><b>DO NOT AUTOCLAVE.</b> Excessive heating is detrimental. Discard the prepared medium if large amount of selenite is reduced (indicated by red precipitate at the bottom of tube/bottle).</p> <p><b>Caution: Sodium hydrogen selenite (Sodium biselenite) is very toxic and corrosive agent and causes teratogenicity. Handle with great care. If there is contact with skin, wash immediately with lot of water</b></p>	Ingredients	g/L	<b>Part A -</b>		Peptone	: 5.0	Lactose	: 4.0	Disodium hydrogen phosphate	: 9.5	Sodium dihydrogen phosphate	: 0.5	<b>Part B -</b>		Sodium hydrogen selenite	: 4.0	Final pH (at 25°C)	7.1±0.2
Ingredients	g/L																		
<b>Part A -</b>																			
Peptone	: 5.0																		
Lactose	: 4.0																		
Disodium hydrogen phosphate	: 9.5																		
Sodium dihydrogen phosphate	: 0.5																		
<b>Part B -</b>																			
Sodium hydrogen selenite	: 4.0																		
Final pH (at 25°C)	7.1±0.2																		
41.	<p><b>Simmons Citrate Agar</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Magnesium sulphate</td> <td style="text-align: right;">: 0.2</td> </tr> <tr> <td>Ammonium dihydrogen phosphate:</td> <td style="text-align: right;">1.0</td> </tr> <tr> <td>Dipotassium phosphate</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Sodium citrate</td> <td style="text-align: right;">: 2.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Bromothymol blue</td> <td style="text-align: right;">: 0.08</td> </tr> </tbody> </table>	Ingredients	g/L	Magnesium sulphate	: 0.2	Ammonium dihydrogen phosphate:	1.0	Dipotassium phosphate	: 1.0	Sodium citrate	: 2.0	Sodium chloride	: 5.0	Bromothymol blue	: 0.08				
Ingredients	g/L																		
Magnesium sulphate	: 0.2																		
Ammonium dihydrogen phosphate:	1.0																		
Dipotassium phosphate	: 1.0																		
Sodium citrate	: 2.0																		
Sodium chloride	: 5.0																		
Bromothymol blue	: 0.08																		

	<p>Agar : 15.0</p> <p>Final pH ( at 25°C) 6.8±0.2</p> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and dispense in test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>
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42.	<p><b>Slanetz and Bartley Medium</b></p> <table border="0" style="width: 100%;"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Tryptose</td> <td style="text-align: right;">: 20.0</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Dextrose</td> <td style="text-align: right;">: 2.0</td> </tr> <tr> <td>Disodium phosphate</td> <td style="text-align: right;">: 4.0</td> </tr> <tr> <td>Sodium azide</td> <td style="text-align: right;">: 0.4</td> </tr> <tr> <td>2,3,5-Triphenyl tetrazolium chloride</td> <td style="text-align: right;">: 0.1</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.2±0.1</td> </tr> </tbody> </table> <p><b>Directions</b></p> <p>Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH. Mix well and pour into sterile Petri plates.</p> <p><b><i>DO NOT AUTOCLAVE OR OVERHEAT. Excessive heating is detrimental.</i></b></p> <p><b><i>Warning: Sodium azide has a tendency to form explosive metal-azides with plumbing materials. It is advisable to use enough water to flush off the disposables.</i></b></p>	Ingredients	g/L	Tryptose	: 20.0	Yeast extract	: 5.0	Dextrose	: 2.0	Disodium phosphate	: 4.0	Sodium azide	: 0.4	2,3,5-Triphenyl tetrazolium chloride	: 0.1	Agar	: 15.0	Final pH ( at 25°C) 7.2±0.1	
Ingredients	g/L																		
Tryptose	: 20.0																		
Yeast extract	: 5.0																		
Dextrose	: 2.0																		
Disodium phosphate	: 4.0																		
Sodium azide	: 0.4																		
2,3,5-Triphenyl tetrazolium chloride	: 0.1																		
Agar	: 15.0																		
Final pH ( at 25°C) 7.2±0.1																			
43.	<p><b>Tryptone Soya Agar(Soyabean Casein Digest Agar)</b></p> <table border="0" style="width: 100%;"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Pancreatic digest of casein</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td>Papaic digest of soyabean meal</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td colspan="2">Final pH (at 25°C) 7.2±0.1</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and sterilize by</p>	Ingredients	g/L	Pancreatic digest of casein	: 15.0	Papaic digest of soyabean meal	: 5.0	Sodium chloride	: 5.0	Agar	: 15.0	Final pH (at 25°C) 7.2±0.1							
Ingredients	g/L																		
Pancreatic digest of casein	: 15.0																		
Papaic digest of soyabean meal	: 5.0																		
Sodium chloride	: 5.0																		
Agar	: 15.0																		
Final pH (at 25°C) 7.2±0.1																			

	autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well and pour into sterile Petri plates.
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44.	<p><b>TCBS Agar</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sodium citrate</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sodium thiosulphate</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sodium cholate</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Oxgall</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Sucrose</td> <td style="text-align: right;">: 20.0</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Ferric citrate</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Bromo thymol blue</td> <td style="text-align: right;">: 0.04</td> </tr> <tr> <td>Thymol blue</td> <td style="text-align: right;">: 0.04</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 8.6±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and <b>DO NOT AUTOCLAVE</b>. Cool to 50°C and pour into sterile Petri plates</p>	<b>Ingredients</b>	<b>g/L</b>	Yeast extract	: 5.0	Peptic digest of animal tissue	: 10.0	Sodium citrate	: 10.0	Sodium thiosulphate	: 10.0	Sodium cholate	: 3.0	Oxgall	: 5.0	Sucrose	: 20.0	Sodium chloride	: 10.0	Ferric citrate	: 1.0	Bromo thymol blue	: 0.04	Thymol blue	: 0.04	Agar	: 15.0	Final pH ( at 25°C) 8.6±0.2	
<b>Ingredients</b>	<b>g/L</b>																												
Yeast extract	: 5.0																												
Peptic digest of animal tissue	: 10.0																												
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Thymol blue	: 0.04																												
Agar	: 15.0																												
Final pH ( at 25°C) 8.6±0.2																													
45.	<p><b>Triple Sugar Iron Agar</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;"><b>Ingredients</b></th> <th style="text-align: right;"><b>g/L</b></th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 20.0</td> </tr> <tr> <td>Meat extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Lactose</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Sucrose</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td>Glucose</td> <td style="text-align: right;">: 1.0</td> </tr> <tr> <td>Ferric citrate</td> <td style="text-align: right;">: 0.3</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Sodium thiosulphate, pentahydrate</td> <td style="text-align: right;">: 0.3</td> </tr> <tr> <td>Phenol red</td> <td style="text-align: right;">: 0.024</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 12.0</td> </tr> </tbody> </table>	<b>Ingredients</b>	<b>g/L</b>	Peptic digest of animal tissue	: 20.0	Meat extract	: 3.0	Yeast extract	: 3.0	Lactose	: 10.0	Sucrose	: 10.0	Glucose	: 1.0	Ferric citrate	: 0.3	Sodium chloride	: 5.0	Sodium thiosulphate, pentahydrate	: 0.3	Phenol red	: 0.024	Agar	: 12.0				
<b>Ingredients</b>	<b>g/L</b>																												
Peptic digest of animal tissue	: 20.0																												
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Sodium chloride	: 5.0																												
Sodium thiosulphate, pentahydrate	: 0.3																												
Phenol red	: 0.024																												
Agar	: 12.0																												

Final pH (at 25°C) 7.4±0.2

**Directions:** Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and distribute into test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Allow the medium to set in sloped form with a butt about 1 inch long.

46.	<p><b>Tributyryn Agar Base w/o Tributyrin</b></p> <table data-bbox="459 280 1050 539"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Peptic digest of animal tissue</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">: 3.0</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.5±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Add 2gm of Tributyrin. Heat if necessary to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Shake the flask and individual plate so as to maintain uniform turbidity.</p>	Ingredients	g/L	Peptic digest of animal tissue	: 5.0	Yeast extract	: 3.0	Agar	: 15.0	Final pH ( at 25°C) 7.5±0.2					
Ingredients	g/L														
Peptic digest of animal tissue	: 5.0														
Yeast extract	: 3.0														
Agar	: 15.0														
Final pH ( at 25°C) 7.5±0.2															
47.	<p><b>Tryticase-soy-sodium Chloride Broth</b></p> <table data-bbox="459 1010 1050 1382"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Tryptone</td> <td style="text-align: right;">: 15.0</td> </tr> <tr> <td>Phytone</td> <td style="text-align: right;">: 5.0</td> </tr> <tr> <td>Dipotassium Phosphate</td> <td style="text-align: right;">: 2.5</td> </tr> <tr> <td>Sodium Chloride</td> <td style="text-align: right;">: 30.0</td> </tr> <tr> <td>Glucose</td> <td style="text-align: right;">: 2.5</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.3±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and dispense into sterilized test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>	Ingredients	g/L	Tryptone	: 15.0	Phytone	: 5.0	Dipotassium Phosphate	: 2.5	Sodium Chloride	: 30.0	Glucose	: 2.5	Final pH ( at 25°C) 7.3±0.2	
Ingredients	g/L														
Tryptone	: 15.0														
Phytone	: 5.0														
Dipotassium Phosphate	: 2.5														
Sodium Chloride	: 30.0														
Glucose	: 2.5														
Final pH ( at 25°C) 7.3±0.2															
48.	<p><b>Tryptone Broth</b></p> <table data-bbox="459 1821 1098 2000"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/L</th> </tr> </thead> <tbody> <tr> <td>Tryptone</td> <td style="text-align: right;">: 10.0</td> </tr> <tr> <td colspan="2">Final pH ( at 25°C) 7.5±0.2</td> </tr> </tbody> </table>	Ingredients	g/L	Tryptone	: 10.0	Final pH ( at 25°C) 7.5±0.2									
Ingredients	g/L														
Tryptone	: 10.0														
Final pH ( at 25°C) 7.5±0.2															

	<p><b>Directions:</b> Suspend 10 gm of tryptone in 1000 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and dispense into tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.</p>
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49.	<p><b>Urea Agar Base, Christensen</b></p> <table data-bbox="502 248 1165 705"> <thead> <tr> <th data-bbox="502 248 981 291">Ingredients</th> <th data-bbox="981 248 1165 291">g/L</th> </tr> </thead> <tbody> <tr> <td data-bbox="502 331 981 374">Peptic digest of animal tissue</td> <td data-bbox="981 331 1165 374">: 1.0</td> </tr> <tr> <td data-bbox="502 389 981 432">Dextrose</td> <td data-bbox="981 389 1165 432">: 1.0</td> </tr> <tr> <td data-bbox="502 448 981 490">Sodium chloride</td> <td data-bbox="981 448 1165 490">: 5.0</td> </tr> <tr> <td data-bbox="502 506 981 548">Monopotassium phosphate</td> <td data-bbox="981 506 1165 548">: 2.0</td> </tr> <tr> <td data-bbox="502 564 981 607">Phenol red</td> <td data-bbox="981 564 1165 607">: 0.012</td> </tr> <tr> <td data-bbox="502 622 981 665">Agar</td> <td data-bbox="981 622 1165 665">: 15.0</td> </tr> <tr> <td colspan="2" data-bbox="502 680 1165 705">Final pH ( at 25°C) 6.8±0.1</td> </tr> </tbody> </table> <p data-bbox="311 784 1407 1097"><b>Directions:</b> Suspend above ingredients in 950 mL distilled water. Heat if necessary to dissolve the medium completely. Adjust the pH and sterilize by autoclaving at 10 lbs pressure (115°C) for 15 minutes. Cool to 50°C and aseptically add 50 mL of sterile 40% Urea Solution and mix well. Dispense into sterile tubes and allow to set in a slanting position. Do not overheat or reheat the medium as urea decomposes very easily.</p>	Ingredients	g/L	Peptic digest of animal tissue	: 1.0	Dextrose	: 1.0	Sodium chloride	: 5.0	Monopotassium phosphate	: 2.0	Phenol red	: 0.012	Agar	: 15.0	Final pH ( at 25°C) 6.8±0.1					
Ingredients	g/L																				
Peptic digest of animal tissue	: 1.0																				
Dextrose	: 1.0																				
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Monopotassium phosphate	: 2.0																				
Phenol red	: 0.012																				
Agar	: 15.0																				
Final pH ( at 25°C) 6.8±0.1																					
50.	<p><b>Violet Red Bile Lactose Agar (VRBL Agar)</b></p> <table data-bbox="311 1288 1085 1892"> <thead> <tr> <th data-bbox="311 1288 981 1330">Ingredients</th> <th data-bbox="981 1288 1085 1330">g/L</th> </tr> </thead> <tbody> <tr> <td data-bbox="311 1400 981 1442">Peptic digest of animal tissue</td> <td data-bbox="981 1400 1085 1442">7.000</td> </tr> <tr> <td data-bbox="311 1458 981 1500">Yeast Extract</td> <td data-bbox="981 1458 1085 1500">3.000</td> </tr> <tr> <td data-bbox="311 1516 981 1559">Lactose</td> <td data-bbox="981 1516 1085 1559">10.000</td> </tr> <tr> <td data-bbox="311 1574 981 1617">Bile salts mixture</td> <td data-bbox="981 1574 1085 1617">1.500</td> </tr> <tr> <td data-bbox="311 1632 981 1675">Sodium chloride</td> <td data-bbox="981 1632 1085 1675">5.000</td> </tr> <tr> <td data-bbox="311 1691 981 1733">Neutral red</td> <td data-bbox="981 1691 1085 1733">0.030</td> </tr> <tr> <td data-bbox="311 1749 981 1792">Crystal violet</td> <td data-bbox="981 1749 1085 1792">0.002</td> </tr> <tr> <td data-bbox="311 1807 981 1850">Agar</td> <td data-bbox="981 1807 1085 1850">15.000</td> </tr> <tr> <td colspan="2" data-bbox="311 1865 1085 1890">Final pH ( at 25°C) 7.4±0.1</td> </tr> </tbody> </table> <p data-bbox="311 1960 1407 2002"><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat with</p>	Ingredients	g/L	Peptic digest of animal tissue	7.000	Yeast Extract	3.000	Lactose	10.000	Bile salts mixture	1.500	Sodium chloride	5.000	Neutral red	0.030	Crystal violet	0.002	Agar	15.000	Final pH ( at 25°C) 7.4±0.1	
Ingredients	g/L																				
Peptic digest of animal tissue	7.000																				
Yeast Extract	3.000																				
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Neutral red	0.030																				
Crystal violet	0.002																				
Agar	15.000																				
Final pH ( at 25°C) 7.4±0.1																					

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	stirring to boiling to dissolve the medium completely. DO NOT AUTOCLAVE. Cool to 45°C and pour into sterile Petri plates. Overheating is detrimental to the medium.
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51.	<p><b>Wagatsuma Agar Base</b></p> <table data-bbox="459 309 1082 853"> <thead> <tr> <th data-bbox="459 309 922 342">Ingredients</th> <th data-bbox="975 309 1034 342">g/L</th> </tr> </thead> <tbody> <tr> <td data-bbox="459 421 922 454">Peptic digest of animal tissue</td> <td data-bbox="975 421 1034 454">: 10.0</td> </tr> <tr> <td data-bbox="459 477 922 510">Yeast extract</td> <td data-bbox="975 477 1034 510">: 3.0</td> </tr> <tr> <td data-bbox="459 533 922 566">Sodium chloride</td> <td data-bbox="975 533 1034 566">: 70.0</td> </tr> <tr> <td data-bbox="459 589 922 622">Dipotassium phosphate</td> <td data-bbox="975 589 1034 622">: 5.0</td> </tr> <tr> <td data-bbox="459 645 922 678">Mannitol</td> <td data-bbox="975 645 1034 678">: 10.0</td> </tr> <tr> <td data-bbox="459 701 922 734">Crystal violet</td> <td data-bbox="975 701 1034 734">: 0.001</td> </tr> <tr> <td data-bbox="459 757 922 790">Agar</td> <td data-bbox="975 757 1034 790">: 15.0</td> </tr> <tr> <td colspan="2" data-bbox="459 813 1034 846">Final pH ( at 25°C) 8.0±0.2</td> </tr> </tbody> </table> <p data-bbox="312 925 1406 1182"><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and steam for 30 min and cool to 50°C. Add 100 mL of 20% suspension of freshly drawn citrated human red cells (previously washed three times in sterilized physiological saline). Mix well and pour into sterile petridishes</p>	Ingredients	g/L	Peptic digest of animal tissue	: 10.0	Yeast extract	: 3.0	Sodium chloride	: 70.0	Dipotassium phosphate	: 5.0	Mannitol	: 10.0	Crystal violet	: 0.001	Agar	: 15.0	Final pH ( at 25°C) 8.0±0.2							
Ingredients	g/L																								
Peptic digest of animal tissue	: 10.0																								
Yeast extract	: 3.0																								
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Mannitol	: 10.0																								
Crystal violet	: 0.001																								
Agar	: 15.0																								
Final pH ( at 25°C) 8.0±0.2																									
52.	<p><b>Xylose-Lysine Deoxycholate Agar (XLD Agar)</b></p> <table data-bbox="504 1317 1050 2000"> <thead> <tr> <th data-bbox="504 1317 882 1350">Ingredients</th> <th data-bbox="951 1317 1010 1350">g/L</th> </tr> </thead> <tbody> <tr> <td data-bbox="504 1406 882 1440">Yeast extract</td> <td data-bbox="983 1406 1034 1440">: 3.0</td> </tr> <tr> <td data-bbox="504 1462 882 1496">L-Lysine</td> <td data-bbox="983 1462 1034 1496">: 5.0</td> </tr> <tr> <td data-bbox="504 1518 882 1552">Lactose</td> <td data-bbox="983 1518 1034 1552">: 7.5</td> </tr> <tr> <td data-bbox="504 1574 882 1608">Sucrose</td> <td data-bbox="983 1574 1034 1608">: 7.5</td> </tr> <tr> <td data-bbox="504 1630 882 1664">Xylose</td> <td data-bbox="983 1630 1034 1664">: 3.5</td> </tr> <tr> <td data-bbox="504 1686 882 1720">Sodium chloride</td> <td data-bbox="983 1686 1034 1720">: 5.0</td> </tr> <tr> <td data-bbox="504 1742 882 1776">Sodium deoxycholate</td> <td data-bbox="983 1742 1034 1776">: 2.5</td> </tr> <tr> <td data-bbox="504 1798 882 1832">Sodium thiosulphate</td> <td data-bbox="983 1798 1034 1832">: 6.8</td> </tr> <tr> <td data-bbox="504 1854 882 1888">Ferric ammonium citrate</td> <td data-bbox="983 1854 1034 1888">: 0.8</td> </tr> <tr> <td data-bbox="504 1910 882 1944">Phenol red</td> <td data-bbox="983 1910 1034 1944">: 0.08</td> </tr> <tr> <td data-bbox="504 1966 882 2000">Agar</td> <td data-bbox="983 1966 1034 2000">: 15.0</td> </tr> </tbody> </table>	Ingredients	g/L	Yeast extract	: 3.0	L-Lysine	: 5.0	Lactose	: 7.5	Sucrose	: 7.5	Xylose	: 3.5	Sodium chloride	: 5.0	Sodium deoxycholate	: 2.5	Sodium thiosulphate	: 6.8	Ferric ammonium citrate	: 0.8	Phenol red	: 0.08	Agar	: 15.0
Ingredients	g/L																								
Yeast extract	: 3.0																								
L-Lysine	: 5.0																								
Lactose	: 7.5																								
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Ferric ammonium citrate	: 0.8																								
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Agar	: 15.0																								

	<p>Final pH ( at 25°C) 7.4±0.1</p> <p><b>Directions:</b> Suspend above ingredients in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. Adjust the pH and <b>DO NOT AUTOCLAVE OR OVERHEAT</b>. Avoid excessive heating as it is detrimental to the medium. Mix well and pour into sterile Petri plates. It is advisable not to prepare large volumes that will require prolonged heating.</p>																														
53.	<p><b>Chromogenic coliform Agar (CCA)</b></p> <table border="0"> <thead> <tr> <th style="text-align: left;">Ingredients</th> <th style="text-align: right;">g/l</th> </tr> </thead> <tbody> <tr> <td>Enzymatic digest of casein</td> <td style="text-align: right;">1.000</td> </tr> <tr> <td>Yeast extract</td> <td style="text-align: right;">2.000</td> </tr> <tr> <td>Sodium chloride</td> <td style="text-align: right;">5.000</td> </tr> <tr> <td>Sodium dihydrogen phosphate, 2H<sub>2</sub>O</td> <td style="text-align: right;">2.200</td> </tr> <tr> <td>Disodium hydrogen phosphate</td> <td style="text-align: right;">2.700</td> </tr> <tr> <td>Sodium pyruvate</td> <td style="text-align: right;">1.000</td> </tr> <tr> <td>Sorbitol</td> <td style="text-align: right;">1.000</td> </tr> <tr> <td>Tryptophan</td> <td style="text-align: right;">1.000</td> </tr> <tr> <td>Tergitol-7</td> <td style="text-align: right;">0.150</td> </tr> <tr> <td>6-chloro-3-indoxyl β-D-galactopyranoside</td> <td style="text-align: right;">0.200</td> </tr> <tr> <td>5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid cyclohexamine ammonium salt, monohydrate</td> <td style="text-align: right;">0.100</td> </tr> <tr> <td>IPTG (Isopropyl-β-D-thiogalactopyranoside)</td> <td style="text-align: right;">0.100</td> </tr> <tr> <td>Agar</td> <td style="text-align: right;">15.000</td> </tr> <tr> <td>Final pH ( at 25°C)</td> <td style="text-align: right;">6.8±0.2</td> </tr> </tbody> </table> <p><b>Directions:</b> Suspend 30.92 gm (the equivalent weight of dehydrated medium per litre) in 1000 mL distilled water. Heat to boiling to dissolve the medium completely. DO NOT AUTOCLAVE. DO NOT OVERHEAT. Cool to 45-50°C. Mix well and pour into sterile Petri plates</p>	Ingredients	g/l	Enzymatic digest of casein	1.000	Yeast extract	2.000	Sodium chloride	5.000	Sodium dihydrogen phosphate, 2H <sub>2</sub> O	2.200	Disodium hydrogen phosphate	2.700	Sodium pyruvate	1.000	Sorbitol	1.000	Tryptophan	1.000	Tergitol-7	0.150	6-chloro-3-indoxyl β-D-galactopyranoside	0.200	5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid cyclohexamine ammonium salt, monohydrate	0.100	IPTG (Isopropyl-β-D-thiogalactopyranoside)	0.100	Agar	15.000	Final pH ( at 25°C)	6.8±0.2
Ingredients	g/l																														
Enzymatic digest of casein	1.000																														
Yeast extract	2.000																														
Sodium chloride	5.000																														
Sodium dihydrogen phosphate, 2H <sub>2</sub> O	2.200																														
Disodium hydrogen phosphate	2.700																														
Sodium pyruvate	1.000																														
Sorbitol	1.000																														
Tryptophan	1.000																														
Tergitol-7	0.150																														
6-chloro-3-indoxyl β-D-galactopyranoside	0.200																														
5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid cyclohexamine ammonium salt, monohydrate	0.100																														
IPTG (Isopropyl-β-D-thiogalactopyranoside)	0.100																														
Agar	15.000																														
Final pH ( at 25°C)	6.8±0.2																														

**APPENDIX -IV**

**MPN Table**

IS : 1622 - 1981

TABLE 3 MOST PROBABLE NUMBER (MPN) OF ORGANISMS PRESENT PER 100 ml OF SAMPLE AND CONFIDENCE LIMITS USING 5 TUBES OF 10 ml, 5 TUBES OF 1 ml AND 5 TUBES OF 0.1 ml

NUMBER OF POSITIVE TUBES			MOST PROBABLE NUMBER (MPN) PER 100 ml	LIMITS WITHIN WHICH MPN PER 100 ml CAN LIE	
10-ml Tubes	1-ml Tubes	0.1-ml Tubes		Lower Limit	Upper Limit
(1)	(2)	(3)	(4)	(5)	(6)
0	0	1	2	<0.5	7
0	0	2	4	<0.5	11
0	1	0	2	<0.5	7
0	1	1	4	<0.5	11
0	1	2	6	<0.5	15
0	2	0	4	<0.5	11
0	2	1	6	<0.5	15
0	3	0	6	<0.5	15
1	0	0	2	<0.5	7
1	0	1	4	<0.5	11
1	0	2	6	<0.5	15
1	0	3	8	1	19
1	1	0	4	<0.5	11
1	1	1	6	<0.5	15
1	1	2	8	1	19
1	2	0	6	<0.5	15
1	2	1	8	1	19
1	2	2	10	2	23
1	3	0	8	1	19
1	3	1	10	2	23
1	4	0	11	2	25
2	0	0	5	<0.5	13
2	0	1	7	1	17
2	0	2	9	2	21
2	0	3	12	3	28
2	1	0	7	1	17
2	1	1	9	2	21
2	1	2	12	3	28
2	2	0	9	2	21
2	2	1	12	3	28
2	2	2	14	4	34
2	3	0	12	3	28
2	3	1	14	4	34
2	4	0	15	4	37
3	0	0	8	1	19
3	0	1	11	2	25
3	0	2	13	3	31
3	1	0	11	2	25
3	1	1	14	4	34
3	1	2	17	5	46
3	1	3	20	6	60
3	2	0	14	4	34
3	2	1	17	5	46
3	2	2	20	6	60
3	3	0	17	5	46
3	3	1	21	7	63
3	4	0	21	7	63
3	4	1	24	8	72
3	5	0	25	8	75
4	0	0	13	3	31
4	0	1	17	5	46
4	0	2	21	7	63
4	0	3	25	8	75

(Continued)

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TABLE 3 MOST PROBABLE NUMBER (MPN) OF ORGANISMS PRESENT PER 100 ml OF SAMPLE AND CONFIDENCE LIMITS USING 5 TUBES OF 10 ml, 5 TUBES OF 1 ml AND 5 TUBES OF 0.1 ml — Contd

NUMBER OF POSITIVE TUBES			MOST PROBABLE NUMBER (MPN) PER 100 ml	LIMITS WITHIN WHICH MPN PER 100 ml CAN LIE	
10-ml Tubes	1-ml Tubes	0.1-ml Tubes		Lower Limit	Upper Limit
(1)	(2)	(3)	(4)	(5)	(6)
4	1	0	17	5	46
4	1	1	21	7	63
4	1	2	26	9	78
4	2	0	22	7	67
4	2	1	26	9	78
4	2	2	32	11	91
4	3	0	27	9	80
4	3	1	33	11	93
4	3	2	39	13	106
4	4	0	34	12	96
4	4	1	40	14	108
4	5	0	41	14	110
4	5	1	48	16	124
5	0	0	23	7	70
5	0	1	31	11	89
5	0	2	43	15	114
5	0	3	58	19	144
5	0	4	76	24	180
5	1	0	33	11	93
5	1	1	46	16	120
5	1	2	63	21	154
5	1	3	84	26	197
5	2	0	49	17	126
5	2	1	70	23	168
5	2	2	94	28	219
5	2	3	120	33	281
5	2	4	148	38	366
5	2	5	177	44	515
5	3	0	79	25	187
5	3	1	109	31	253
5	3	2	141	37	343
5	3	3	175	44	503
5	3	4	212	53	609
5	3	5	253	77	788
5	4	0	130	35	302
5	4	1	172	43	486
5	4	2	221	57	698
5	4	3	278	90	849
5	4	4	345	117	999
5	4	5	426	145	1161
5	5	0	240	68	754
5	5	1	348	118	1005
5	5	2	542	180	1405

TABLE 4 MOST PROBABLE NUMBER (MPN) OF ORGANISMS PRESENT PER 100 ml OF SAMPLE AND CONFIDENCE LIMITS USING 5 TUBES OF 10 ml, 5 TUBES OF 1 ml AND 5 TUBES OF 0.1 ml  
( Clause 3.3.1.3 )

COMBINATION OF POSITIVES	MPN INDEX/ 100 ML	95 PERCENT CONFIDENCE LIMITS		COMBINATION OF POSITIVES	MPN INDEX/ 100 ML	95 PERCENT CONFIDENCE LIMITS	
		Lower	Upper			Lower	Upper
0-0-0	< 2	—	—	4-2-0	22	9.0	56
0-0-1	2	1.0	10	4-2-1	26	12	65
0-1-0	2	1.0	10	4-3-0	27	12	67
0-2-0	2	1.0	13	4-3-1	33	15	77
				4-4-0	34	16	80
1-0-0	2	1.0	11	5-0-0	23	9.0	86
1-0-1	4	1.0	15	5-0-1	30	10	110
1-1-0	4	1.0	15	5-0-2	40	20	140
1-1-1	6	2.0	18	5-1-0	30	10	120
1-2-0	6	2.0	18	5-1-1	50	20	150
				5-1-2	60	30	180
2-0-0	4	1.0	17	5-2-0	50	20	170
2-0-1	7	2.0	20	5-2-1	70	30	210
2-1-0	7	2.0	21	5-2-2	90	40	250
2-1-1	9	3.0	24	5-3-0	80	30	250
2-2-0	9	3.0	25	5-3-1	110	40	300
2-3-0	12	5.0	29	5-3-2	140	60	360
3-0-0	8	3.0	24	5-3-3	170	80	410
3-0-1	11	4.0	29	5-4-0	130	50	390
3-1-0	11	4.0	29	5-4-1	170	70	480
3-1-1	14	6.0	35	5-4-2	220	100	580
3-2-0	14	6.0	35	5-4-3	280	120	690
3-2-1	17	7.0	40	5-4-4	350	160	820
				5-5-0	240	100	940
4-0-0	13	5.0	38	5-5-1	300	100	1 300
4-0-1	17	7.0	45	5-5-2	500	200	2 000
4-1-0	17	7.0	46	5-5-3	900	300	2 900
4-1-1	21	9.0	55	5-5-4	1 600	600	5 300
4-1-2	26	12	63	5-5-5	1 600	—	—

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