APPENDICES

APPENDIX 1

Colour

The colour of the sample was visually observed.

APPENDIX 2

Odour

The odour of the sample was noted by directly smelling the sample.

APPENDIX 3

Temperature

Temperature was measured using mercury filled centigrade thermometer (0º C to 50º C). The readings were made by dipping the thermometer in samples for 2 minutes before constant readings were obtained.

APPENDIX 4

Determination of pH

Principle

A glass surface in contact with hydrogen ions of the solution under test, acquires an electrical potential which depends on the concentration of H^+ions. A measure of the electrical potential (emf), gives H^+ion concentration or pH of the solution.

Procedure

The pH meter was first standardized using buffer solutions of pH 7.0 and pH 9.2. The contents were stirred intermittently using glass rod and after 20 minutes the electrodes were washed with distilled water, wiped with filter paper and the electrodes were immersed in the sampleand readings were recorded.

APPENDIX 5

Electrical conductivity

The Electrical conductivity was estimated using conductivity bridge and expressed in (μmhos/cm).

APPENDIX 6

Determination of total suspended solids

Principle

A well-mixed sample is filtered through a weighed standard glass-fibre filter and the residues retained on the filter are dried to a constant weight at 103°C – 105°C. The increase in weight of the filter represented the total suspended solids. If the suspended material clogs the filter and prolongs filtration, it may be necessary to increase the diameter of the filter or decrease the sample volume.

Procedure

Suspended solids of the sample were estimated by centrifugation method. 50 ml of the sample was centrifuged and after centrifugation the residue was washed with distilled water, recentrifuged and the suspended solids in the centrifuge tube was transferred to a pre weighed silica dish and dried at 105º C. The increase in weight was equal to the amount of suspended solids. The suspended solids present in the sample were calculated by using the formula.

$$\text{Total suspended solids in mg/l} = \frac{\text{Final wt.} - \text{Initial wt. of the crucible}}{\text{Volume of the sample}} \times 1000$$

APPENDIX 7

Determination of total dissolved solids (Filtration Method)

Principle

A well-mixed sample is filtered through a standard glass fiber filter and the filtrate is evaporated to dryness in a weighed dish and dried to constant weight at 180^{o}C. The increase in dish weight represents the total dissolved solids.

Procedure

250ml of the sample was filtered through a glass microfiber filter paper and the filtrate was evaporated in a tared porcelain dish which was preheated at 105^{o}C and then at 550^{o}C for one hour in a muffle furnace. The porcelain dish was cooled and brought to

constant weight. The dish was kept at 180^{o}C for 1 hour, cooled and weighed. The increase in weight denoted the amount of total dissolved solids. The results were expressed as mg total dissolved solids per litre of the sample. The total dissolved solids present in the sample was calculated by using the following formula

$$\text{Total dissolved solids in mg/l} = \frac{\text{Final wt.} - \text{Initial wt. of the crucible}}{\text{Volume of the sample}} \times 1000$$

APPENDIX 8

Total solids

Calculation

Mg/l total solids (TS) = mg/l total suspended solids (TSS)+ mg/L total dissolved solids(TDS)

APPENDIX 9

Estimation of total hardness (EDTA-Titrimetric Method)

Principle

Ethylene diamine tetra acetic acid and its sodium salts (EDTA) form a chelated soluble complex when added to a solution of certain metal cations. If a small amount of dye such as Eriochrome Black – T or calmagite is added to an aqueous solution containing calcium and magnesium ions at a pH of 10, the solution becomes wine red. If EDTA is added as a titrant, the calcium and magnesium was complexed and the solution turns from wine red to blue, marking the end point of the titration.

Reagents

1. Calcium standard solution: 1 g of pure calcium carbonate was dissolved in 1 litre of distilled water using 20.5 ml of HCl and the contents were warmed. 1 ml = 1 mg $CaCO_3$.
2. Standard EDTA titrant (0.02 N): 3.723 g disodium ethylene diamine tetra acetate dihydrate was dissolved in 1 litre of water. It was standardized against standard calcium solution. One ml of 0.02 N EDTA ≡ 1 mg of $CaCO_3$.

3. Eriochrome Black – T indicator: 0.5 g of the dye was mixed with 100g of sodium chloride to obtain a dry powder mixture.

Procedure

50 ml of the sample was placed in a conical flask, diluted to 50 ml and added. One millilitre of buffer solution was added per 50 ml volume of the sample. The pH of the titre should be 10.0 ± 0.1. A pinch of the indicator was added and titrated with standard EDTA, until a reddish tinge appeared. The end point was the appearance of blue colour.

Calculation

If EDTA titrant is exactly 0.02 N,

$$\text{mg / Total Hardness (as } CaCO_3\text{)} = \frac{\text{ml EDTA titrant} \times 1 \times 1000}{\text{ml sample taken for estimation}}$$

APPENDIX 10

Estimation of total alkalinity (Titrimetric Method)

Principle

When a sample containing carbonate and bicarbonate are titrated against the standard sulphuric acid, phenolphthalein loses its pink colour when half of the carbonate is converted to bicarbonate. Twice this value is a measure of carbonates present in the sample.

To the colourless solution, a few drops of methyl orange is added and titrated against sulphuric acid till straw yellow colour changes to pinkish red colour. This value gives the amount of acid required to neutralize the bicarbonate originally present and that from the carbonates. By subtracting the first titre value from the second one, acid required to neutralise the bicarbonate originally present in the sample is obtained.

Reagents

1. Sodium carbonate solution 1N: 13.25 g was dissolved in 250 ml water.
2. Sulphuric acid 1N: 28 ml of concentrated sulphuric acid was made up to a litre with distilled water.

3. Sulphuric acid 0.02 N: Diluted approximate volumes of 1N sulphuric acid to prepare 0.02 N sulphuric acid ≡ 1.0 mg $CaCO_3$.
4. Phenolphthalein indicator: 500 mg of phenolphthalein was dissolved in 50 ml of ethyl alcohol and 50 ml of distilled water. 0.02N sodium hydroxide solution was added dropwise until a faint pink colour appeared.
5. Mixed indicator solution: 20 mg of methyl red and 100 mg of bromocresol green were dissolved in 100 ml of 95% isopropyl alcohol.

Procedure

To 25 ml of the sample equal volume of distilled water and a pinch of phenolphthalein indicator solution was added in a 250ml conical flask. Pink colour appeared which was then titrated with 0.02N sulphuric acid until the solution became colourless. Three drops of mixed indicator solution was added to the solution in which phenolphthalein alkalinity had been determined and titrated against 0.02N sulphuric acid. The colour was changed from emerald green to light pink. (If no pink colouration occurred, it indicated nil phenolphthalein alkalinity).

Calculation

$$\text{Total alkalinity as } CaCO_3\text{(mg/l)} = \frac{2 \text{ ml of } 0.02N\ H_2SO_4 \text{ for total alkalinity end point} \times 50 \times 0.02 \times 1000}{2 \text{ ml sample taken for titration}}$$

APPENDIX 11

Estimation of dissolved oxygen

Dissolved oxygen of the water sample was estimated by Winkler's method.

Reagents:

1. Manganese sulphate: 480g of manganous sulphate tetrahydrate is dissolved and made up to 1000ml with distilled water (Discarded if it changes colour with starch).

2. Alkaline iodide-azide reagent: 500g of sodium hydroxide and 150g of potassium iodide along with 10g of sodium azide (NaN3) is dissolved and made up to 1000ml with distilled water.
3. Conc. sulphuric acid
4. Starch indicator: 0.5g of starch is dissolved in distilled water and boiled for few minutes.
5. Stock sodium thiosulphate: 24.82g of sodium thiosulphate pentahydrate (Na2S202. 5H2O) is dissolved in distilled water and made up to 1000ml.
6. Standard sodium thiosulphate (0.025N): 250ml of the stock sodium thiosulphate pentahydrate is made up to 1000ml with distilled water to give 0.025N.

Procedure:

The samples are collected in BOD bottles, to which 2ml of manganous sulphate and 2ml of potassium iodide are added and sealed. This is mixed well and the precipitate allowed settling down. At this stage 2ml of conc. sulphuric acid is added, and mixed well until all the precipitate dissolves. 203ml of the sample is measured into the conical flask and titrated against 0.025N sodium thiosulphate using starch as an indicator. The end point is the change of colour from blue to colourless.

Calculations

203ml because (200) (300)/ (200-4) = 203ml.

1ml of 0.025N Sodium thiosulphate = 0.2mg of Oxygen

$$\text{DO (MG/l)} = \frac{(0.2)\ (1000 \text{ ml of Sodium thiosulphate})}{200}$$

APPENDIX 12

Estimation of Biochemical Oxygen Demand (Winkler's Iodometric Method)

Principle

BOD determination involves the measurement of dissolved oxygen content of the sample, before and after 5 days incubation at 20°C. The reduction in oxygen content is due to the demand exerted by the microbiological population and it is a measure of oxidisable organic matter in the sample.

When manganous sulphate is added to the sample containing potassium iodide, manganous hydroxide is formed, which is oxidized by the dissolved oxygen of the sample to basic manganic oxide. On addition of sulphuric acid, the basic manganic oxide liberates iodine, equivalent to that of dissolved oxygen originally present in the sample. The liberated iodine is titrated with a standard solution of sodium thiosulphate using starch as indicator.

Reagents for the preparation of dilution water

1. Calcium chloride solution : 27.5 g was dissolved in one litre of distilled water.
2. Magnesium sulphate solution: 25 g was dissolved in one litre of distilled water.
3. Ferric chloride solution: 0.25 g was dissolved in one litre of distilled water.
4. Phosphate buffer (pH 7.2) : 21.75 g dipotassium hydrogen phosphate, 33.4 g disodium hydrogen phosphate heptahydrate and 1.7 g ammonium chloride were taken in one litre standard flask, dissolved in distilled water and made up to the mark. The buffer was stored in a refrigerator to prevent mold growth.

Reagents for the estimation of dissolved oxygen

1. Manganous sulfate solution: 91 9 manganous sulfate monohydrate was dissolved and diluted to 250 ml with distilled water.
2. Alkali - iodide - azide reagent: Reagent A – 175 g potassium hydroxide and 37.5 potassium iodide were dissolved in 250 ml of water. Reagent B – 2.5 g sodium azide was dissolved in 10.0 ml of water. Reagent A and B were mixed.
3. Concentration sulphuric acid.
4. Phosphoric acid : 85 – 90%.
5. Sodium thiosulphate solution (0.1 N) : 24.82 g was dissolved in distilled water and made up to one litre.
6. Sodium thiosulphate solution, 0.025 N : 250 ml of 0.1 N sodium thiosulphate solution was diluted to 1000 ml with distilled water. 1 ml of 0.025 N sodium thiosulphate ≡ 0.2 mg dissolved oxygen.
7. Starch solution - 1%

Procedure

Preparation of dilution water: 1 ml each of calcium chloride, magnesium sulphate, ferric chloride and phosphate buffer solution was added to one litre of aerated distilled water and mixed thoroughly. This is the standard dilution water prepared freshly every time.

Seeding of the dilution water: It is essential to seed the dilution water. The seeding material generally used is freshly settled raw sewage. 2 ml of raw sewage was added to one litre of dilution water.

Dilution of the samples: The test water samples were diluted with seeded dilution water sample (1%, 5% and 10%) in dilution mixture for the water sample. Each dilution sample was taken in a set of two BOD bottles.

Determination of dissolved oxygen (DO) before and after 5 days incubation : In one set of flasks DO was determined immediately while the other set was dept for incubation at 20º C for 5 days. After 5 days, DO for the incubated sample was determined. Determination of DO is as follows:

To the contents of the BOD bottle 2 ml of manganous sulphate solution and 2 ml of alkali - iodide - azide solution was added the bottle was stoppered and mixed thoroughly. A brown precipitate of basic manganic oxide was formed, which was allowed to settle completely leaving a clear supernatant liquid. Then 2 ml of conc. sulphuric acid was added along the sides of the bottle, stoppered and mixed for complete dissolution. The contents were transferred to a 500 ml conical flask and titrated immediately against 0.025 N sodium thiosulphate using starch as an indicator.

Calculation for DO

Volume of 0.025 N thiosulpnate used up in the titration = DO in mg / l

DO at 0º C and 760 mm pressure = DO x 0.07 mg / l

Calculation for BOD

$$\text{BOD (5 days at 20º C)} = \frac{(DO_0 - DO_5 - BC) \times 100}{\text{Percent sample}}$$

DO_0 = Initial DO

DO_5 = DO after 20º C incubation for 5 days

BC = Blank Correction ie, Difference in DO of blank on the initial day and after 5 days incubation.

APPENDIX 13

Estimation of chemical oxygen demand (Titrimetric Method)

Principle

Chemical Oxygen Demand (COD) is defined as the amount of a specified oxidant that reacts with the sample under controlled conditions. The quantity of oxidant consumed is expressed in terms of its oxygen equivalence. Because of its unique chemical properties, the dichromate ion ($Cr_2 O_7^{2-}$), the specified oxidant is reduced to the chromic ion (Cr^{3+}).

COD often is used as a measurement of pollutants in waste water and natural waters. Most types of organic matter are oxidized by boiling the mixture of chromic and sulfuric acids. A sample is refluxed in strongly acid solution with a known excess of potassium dichromate. After digestion, the remaining unreduced potassium dichromate is titrated with ferrous ammonium sulfate to determine the amount of potassium dichromate consumed and the oxidizable matter is calculated in terms of oxygen equivalent.

Reagents

1. Mercuric sulphate crystals.
2. Sulphuric acid – silver sulphate reagent: Dissolved 10.1 g of silver sulphate in 1 litre of concentrated sulphuric acid and allowed the solution to stand for two days for complete dissolution.
3. Potassium dichromate solution 0.125N: Dissolved 0.129 g of potassium dichromate in distilled water and made up to 1 litre. 1 ml of 0.125N potassium dichromate ≡ 1 mg of oxygen.
4. Ferroin indicator solution: Dissolved 95 mg of ferrous sulphate in 500ml of distilled water, added 1.485g of 1, 10 phenanthroline monohydrate and mixed thoroughly.

5. Ferrous ammonium sulphate solution 0.125 N **:** 40g of ferrous ammonium sulphate was dissolved in distilled water, 20ml of concentrated sulphuric acid was added and made up to one litre with water. The contents were standardized with 0.125 N potassium dichromate.

Procedure

A refluxing flask of 250 ml capacity was used with a ground glass 24/40 neck fitted with a 300 mm double surface condenser to which a glass cap was fitted. 50 ml of the sample was placed in the flask and a suitable quantity of mercuric sulphate was added so that the ratio of chloride content of the sample to mercuric sulphate was 1: 10 (For this, chloride content of the sample was estimated). Five ml of sulphuric acid – silver sulphate reagent was added to dissolve the mercuric sulphate and cooled in cold water while mixing. 25ml of 0.125 N potassium dichromate was pipetted into the flask and mixed well. 70 ml of sulphuric acid - silver sulphate reagent was added very carefully and mixed. A few porcelain bits were added and the condenser was attached to the refluxing flask. Water was circulated and refluxed for two hours. The flask was cooled after removing from the flask. The contents of the flask were transferred and diluted to 350ml with distilled water. About 2 to 3 drops of ferroin indicator was added and titrated against 0.125N ferrous ammonium sulphate solution. The end point was the sharp colour change from blue-green to reddish brown. A blank was conducted using 50ml of distilled water instead of the sample.

Calculation

$$\text{COD in mg / 1} = \frac{(\text{Blank titre value} - \text{sample titre value}) \times 0.125 \times 1000 \times 8}{\text{Volume of sample taken}}$$

APPENDIX 14

Estimation of Chlorides (Titrimetric Method)

Principle

Silver nitrate reacts with chloride ions to form silver chloride. The completion of reaction is indicated by the red colour produced by the reaction of silver nitrate with potassium chromate solution which is added as an indicator.

Reagents

Chloride free double distilled water was used for all reagents.

1. Standard silver nitrate titrant, 0.0282N: 4.791 g of silver nitrate was dissolved in 1 litre of distilled water. Standardized it against 0.0282 N sodium chloride solution. 1 ml of exactly 0.0282 N $AgNO_3$ ≡ 1.0 mg chloride.
2. Standard sodium chloride titrant, 0.0282 N **:** 1.648 g of sodium chloride was dissolved in 1 litre of distilled water. 1.0 ml ≡ mg chloride.
3. Potassium chromate indicator solution: 25 g of potassium chromate was dissolved in 100ml of distilled water and silver nitrate solution was added dropwise until a slight red precipitate was formed. The contents were allowed to stand for 12 hours, filtered and made up to 500ml with distilled water.
4. Aluminium hydroxide suspension: 100 g of aluminium ammonium sulphate was dissolved in 1000 ml distilled water and warmed to 60^{o}C by stirring 55 ml of conc. ammonia solution. The precipitate was allowed to settle for an hour and washed with distilled water to make the precipitate free from chloride. The decantate was checked by treating a portion of it with silver nitrate solution until the precipitate was free from chloride and diluted it to 1000 ml with distilled water.

Procedure

To 50 ml of the sample, 3 ml of aluminium hydroxide was added, stirred well, allowed to settle and filtered. The precipitate was washed with chloride free distilled water, the filtrate and washings were combined. 100ml of the samples were pipetted into a porcelain dish and the pH was adjusted in the range of 7 - 9.5. One ml of potassium chromate indicator solution was added and titrated it against standard silver nitrate solution with constant stirring until a slight precipitable reddish colouration persisted. A blank was measured by placing 100 ml chloride – free distilled water instead of sample.

Calculations

If the silver nitrate solution is exactly 0.0282 N,

$$\text{Chloride (mg per litre)} = \frac{(\text{ml } AgNO_3 \text{ for sample} - \text{ml } AgNO_3 \text{ for blank}) \times 1000}{\text{ml sample taken for estimation}}$$

If the silver nitrate solution is not exactly 0.0282N,

$$\text{fChloride (mg per litre)} = \frac{(\text{ml } AgNO_3 \text{ for sample} - \text{ml } AgNO_3 \text{ for blank}) \times \text{Normality of } AgNO_3 \times 35.45 \times 1000}{\text{ml sample taken for estimation}}$$

APPENDIX 15

Estimation of phosphates

The amount of phosphate was estimated by stannous chloride method.

Principle

Ammonium molybdate reacts with phosphate to form molybdophosphoric acid which is reduced to a blue coloured complex (molybdenum blue) by the addition of stannous chloride.

Reagents

1. Phenolphthalein indicator solution: 500 mg of phenolphthalein was dissolved in 50 ml of ethyl alcohol and 50 ml of distilled water was added.
2. Sulphuric acid – nitric acid solution: 75 ml Conc. H_2SO_4 was added to about 150 ml. distilled water and cooled. 1 ml conc. HNO_3 was added and diluted to 250 ml with distilled water.
3. Ammonium molybdate solution: 2.5 g ammonium molybdate was dissolved in about 200 ml. distilled water.280 ml conc. H_2SO_4 was added to 400 ml distilled water and cooled. Molybdate solution was added to the diluted acid and dilute to 1000 ml.
4. Stannous chloride solution: 2.5 g fresh stannous chloride was dissolved in 100 ml glycerol and heated in a water bath.
5. Phosphate stock solution: 439 mg potassium dihydrogen phosphate was dissolved in distilled water and made up to 1000 ml in a volumetric flask. Two drops of toluene was added as a preservative.

6. Phosphate standard solution: 10 ml phosphate stock solution was pipetted into a 1000ml volumetric flask and made up to the mark with distilled water and should be prepared freshly.1.0 ml ≡ 1 mg P

Procedure

1. 50 ml of the sample was taken in a Nessler tube and 1 drop of phenolphthalein indicator was adeed. The pink colour developed was destroyed by adding one or two drops of Sulphuric – nitric acid solution.

2. Phosphate working solution was pipetted in to a series of 100 ml Nessler tubes covering the range up to 20 µg P and made up to 100 ml with distilled water. A Nessler tube containing 100 ml distilled water was kept as a blank.

3. To the blank, standards and sample 4 ml ammonium molybdate solution and 0.5 ml stannous chloride solution were added.

4. Between 10 – 12 minutes the colour developed was measured at 690nm against the reagent blank using a spectrophotometer.

5. A calibration curve was prepared and amount of phosphate equivalent to the observed optical density was calculated and the result was expressed as mg phosphate per litre of sample.

APPENDIX 16

Estimation of Sulphates (Turbidimetric Method)

Principle

Sulphate ions are precipitated as barium sulphate crystals of uniform size in acid medium. Light absorbed by the precipitate is measured using a spectrophotometer.

Reagents

1. Conditioning reagent: 75 g of sodium chloride was dissolved in 300 ml distilled water, added 30 ml conc. HCl and 100 ml of 95% ethyl alcohol (or isopropyl alcohol). To the above, 50 ml of glycerol was added and mixed well.

2. Barium chloride solution: 100 g of barium chloride was dissolved in 1000 ml of distilled water and was filtered through Whatman No.1 filter paper.

3. Standard sulphate solution: 147 mg anhydrous sodium sulphate was dissolved in distilled water and made up to 1000ml. 1 ml = 100 μg SO_4^{2-}

Procedure

Standards

In a 250 ml conical flask 5, 10, 15, 20, 25, 30, 35 and 40 ml of standard sulphate solution was measured separately and diluted to 100 ml. 5 ml of conditioning reagent was added and mixed well using magnetic stirrer. The speed of stirring should be the same for both standards and samples. While stirring, 0.5 g of barium chloride crystals was added and stirring was continued for one minute. Immediately after one minute, an aliquot of the solution was taken in an absorption cell and measured the optical density at a wavelength of 420 nm. A blank was carried out and a graph was plotted relating optical density to μg of SO_4.

Samples

To a 250 ml conical flask, 50 ml of the sample was measured and diluted to 100 ml and preceded from steps as for standards. From the calibration graph, the mg of sulphate equivalent to the optical density was calculated.

Calculation

$$\text{mg/l sulphate as } SO_4^2 = \frac{\text{mg } SO_4 \text{ X } 1000}{\text{ml sample taken for estimation}}$$

APPENDIX 17

Estimation of Nitrates

Reagents

Nitrate stock solution

722 mg potassium nitrate was dissolved in distilled water and made up to 1000 ml in a volumetric flask.

Nitrate standard solution

100 ml nitrate stock solution was pipetted in to a 1000 ml volumetric flask and made up to the mark with distilled water.

Brucine – Sulphanilic acid solution

1 g brucine sulphate and 100 mg sulphanilic acid was dissolved in 70 ml hot distilled water 3 ml conc. HCl was added cooled and diluted to 100 ml with distilled water.

Sulphuric acid solution

500 ml conc. H_2SO_4 was added to 75 ml distilled water and cooled to room temperature.

Procedure

1. 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 ml of nitrate standard solution were taken in a series of 50 ml beakers and diluted to 5 ml with distilled water.

2. A beaker containing 5 ml of distilled water was used as a blank.

3. 2 ml of the sample was taken in a 50 ml beaker and diluted to 5 ml with distilled water. 1 ml of brucine sulphanilic acid solution was added to the blank, standards and sample are mixed well.

4. 10 ml of Sulphuric acid solution was taken in a second series of 50 ml beakers. The contents of the first series of beakers were poured in to each of the second series of beakers and mixed well. Beakers were kept in the dark for 10 minutes.

5. 10 ml of distilled water was added to all the beakers. Beakers were allowed to cool for 20 – 30 minutes. The colour development was read in a colorimeter against 510 nm. Using the calibration curve the mg. equivalent of nitrate nitrogen in the sample was found out.

Calculation

$$\text{Nitrate (mg/l)} = \frac{\text{mg. Nitrate X 1000}}{\text{ml. sample taken for estimation}}$$

APPENDIX 18

Estimation of lead

Principle

Lead can be determined at a wavelength of 283.3 nm by AAS with aspiration of the sample into the oxidising air-acetylene flame. When the aqueous sample is aspirated, the sensitivity for 1% absorption is 0.5 mg/L and the detection limit is 0.05 mg/L.

Reagents

1. Ammonia solution, approximately 0.5N: Dilute 3.5ml ammonia solution to 100ml with water.
2. Dithizone stock solution, 0.1% (in chloroform): keep it in an amber bottle and store in a refrigerator.
3. Dithizone working solution (in water): Transfer 12ml of stock dithizone solution into a 100ml sepersting funnel. Add 20ml 0.5N. ammonia solution and shake well. Allow the phases to separate and reject the lower chloroform layer. Filter the aqueous layer through a wetted filter paper (to remove droplets of chloroform) in to a 50ml amber bottle.
4. Sodium hexameta phosphate solution 10%: Dissolve 10g sodium hexameta phosphate in 100ml distilled water. Remove traces of lead by extraction with stock dithizone solution (0.1% chloroform solution) after adjusting the pH to 9 with conc. Ammonia solution. Acidify the aqueous layer in the separating funnel using 1+1 HCl and then extract with chloroform to remove free dithizone until the chloroform layer becomes colourless. Reject the chloroform layer. Adjust the pH of the aqueous solution to 9.5.
5. Hydroxyl- amine hydrochloride solution 1%: Dissolve 1g. of NH_2 OH. HCl in 100ml distilled water.
6. Alkaline cyanide solution: prepare a mixture of solution containing 340ml. ammonia (sp. gr 0.88) and 680ml water. Dissolve 3.0g sodium sulfite $Na_2 SO_3$. $7H_2O$ in the mixture. Add 30ml 1% potassium cyanide solution and mix well. Keep it in a tightly stoppered bottle.

7. Lead stock solution: Dissolve 1.599g lead nitrate, Pb $(NO_3)_2$ in small amount of water. Add 10ml conc. Nitric acid and make up to 1000ml. 1ml= 1.0mg. Pb
8. Lead intermediate solution: dilute 10ml of the above lead stock solution to 1000ml. with distilled water (prepare freshly). 1.0ml= 10µg. Pb
9. Lead working solution: Dilute 10ml of the lead intermediate solution to 100ml. with distilled water (prepare freshly). 1.0ml= 1.0µg. Pb

Procedure

Standards

1. Place 50ml. of lead free distilled water in to a series of short stem separating funnels. Pipet 0.0, 1.0, 2.0, 3.0, 4.0 10.0ml of lead working solution in to them.

2. Add the following reagents in order with shaking after each ccondition, (i) 1.0ml sodium hexametaphosfate solution, (ii) 1.0ml hydroxylamine hydrochloride solution, (iii) 30ml alkaline cyanide solution, (iv) 0.5ml dithizone working solution and (v) 10ml chloroform.

3. Shake the funnel vigorously and allow the layers to separate. Dry the stem of the funnel with filter paper strips. Draw the chloroform layer in the optical cell.

4. Measure the optical densities using a spectrophotometer at 540nm or filterphotometer with a green filter using a 10mm cell, plot a calibration curve.

APPENDIX 19

Estimation of nickel

The amount of nickel was estimated by dimethyl glyoxime method.

Principle

Nickel reacts with dimethyl glyoxime in the presence of an alkaline oxidising agent to form a characteristic red colour complex which can either be measured visually or photometrically.

Interferences

Iron, manganese and copper interfere and if they are present in concentration thrice that of nickel, their interference is suppressed by the addition of sodium citrate. If they are

present in excess concentrations, nickel dimethyl glyoxime complex alone is separated by extraction with chloroform and preceded further.

Reagents

Nickel stock solution

447.9 mg Nickel sulfate (NiSO4.6H2O) was dissolved in distilled water and the volume was made upto 1000 ml. in volumetric water (1.00 ml = 100μg Ni).

Nickel working solution

10.0 ml of nickel stock solution was pipetted into a 100 ml volumetric flask and made upto the mark with distilled water (1.00 ml = 10 μg Ni).

0.5N Hydrochloric acid

50ml conc. HCl was diluted to 1000 ml with distilled water

Sodium citrate solution

125g sodium citrate was dissolved in 500 ml distilled water.

0.05 N Iodine solutions

20g potassium iodide, was dissolved in 5 ml. distilled water. 6.4 g iodine was dissolved in this solution, and the solution was diluted to 1000 ml.

Dimethyl glyoxime solution

1g dimethyl glyoxime was dissolved in 100 ml of concentrated ammonia solution. 100 ml distilled water was added and filtered if necessary.

Additional reagents for the removal of interferences

Dilute ammonia solution - 10 ml. concentrated ammonia solution was diluted to 500ml with distilled water.

Chloroform

Procedure

1. Appropriate volumes of nickel working solution covering the range up to 100 μg was taken in a series of 50ml Nessler tubes. 50 ml Nessler tube with distilled water was kept as the blank.

2. A suitable aliquot of the neutralised (acid digested sample containing not more than 100 µg nickel) was taken in a 50ml Nessler tube.

3. To the blank, standards and sample 20 ml of 0.5 N. HCl was added.

4. Then following reagents were added in order with mixing after each addition:

(i) 10 ml. sodium citrate solution

(ii) 2 ml. iodine solution and

(iii) 4 ml. dimethyl glyoxime solution

5. The volume in all the flasks were made upto 50 ml. with distilled water and allowed to stand for 20 minutes.

6. Optical density was measured in a spectrophotometer at 470 nm against the reagent blank. A calibration curve was prepared and the microgram of nickel equivalent to the observed optical density was determined. The result was expressed as mg nickel per litre of the sample.

APPENDIX 20

Estimation of zinc

Principle

Zinc reacts with dithizone to form a co-ordinate compound which when extracted with carbon tetra chloride is red in colour and is used to measure the zinc content.

Reagents

1. Sodium citrate solution: dissolve 50g sodium citrate in 450ml distilled water.
2. Zinc stock solution: dissolve 100mg. zinc metal (30 mesh) in about 1ml + 1 HCL, make up to 1000ml in a volumetric flask using distilled water. 1.0 ml= 100µg zinc

3. Zinc working solution: pipette 10ml zinc stock solution into a 1000ml volumetric flask and make up to the mark with distilled water. 1.0ml= 1µg zinc
4. Methyl red indicator solution: dissolve 100mg methyl red in distilled water and dilute to 1000ml.
5. Conc. Ammonia solution
6. Potassium cyanide solution, 5%: dissolve 5g potassium cyanide in 100ml distilled water.
7. Con. Acetic acid
8. Carbon tetra chloride
9. Bis (2- hydroxyl ethyl) dithio carbamate solution: mix 4.0g. diethanol amine and 1ml carbon disulphide with 40ml methyl alcohol. Prepare freshly.
10. Dithizone solution, 0.005%: dissolve 5mg dithizone thoroughly in alittle carbon tetra chloride an dilute to 100ml with CCl_4.
11. Sodium sulfide solution I: dissolve 3.0g $Na_2S.H_2O$ or 1.65g $Na_2S.3H_2O$ in 100ml distilled water.
12. Sodium sulfide solution II: dilute 4.0ml of sodium sulfide solution I to 100ml with distilled water. Prepare just before use.

Procedure

1. Pipette 1.0, 2.0…….10.0ml of zinc working solution into a series of 125ml separating funnels and dilute to 20ml with distilled water.
2. Include a separating funnel containing 20ml distilled water as the blank.
3. Place in a separating funnel an aliquot of the sample containing not more than 10µg Zn and dilute to 20ml with distilled water.
4. To the blank, standards and sample, add 2 drops of methyl red indicator solution and 2ml sodium citrate solution. If the colour is not yellow at this point, add conc. ammonia solution drop wise to the separating funnels, until the indicator just turns yellow.
5. Add 1ml potassium cyanide solution and then conc. acetic acid solution until the solution becomes neutral peach in colour.

6. Add 5ml carbon tetrachloride to each separating funnel to extract the methyl red and discard CCl_4 layer.
7. Add 1ml of bis (2- hydroxyl ethyl) dithio carbamate solution. Mix well by shaking, and then add 10ml dithizone solution. Shake well and draw off the CCl_4 layer into another separating funnel. Extract the aqueous solution in the first separating funnel with 5ml portions of dithizone solution until the last CCl_4 extract remains green. Combine all the CCl_4 extracts in the second separating funnel.
8. To the combined extracts in the second separating funnel, add 10ml sodium sulfide solution II. Shake well and discard the aqueous layer. Wash the CCl_4 layer with further 10ml of sodium sulfide solution II until the unreacted dithizone has completely been removed as shown by the colour of the aqueous layer either colourless or very pale yellow.
9. Draw off the CCl_4 layer into a 50ml volumetric flask after removing the water adhering to thje stem of the funnel using a cotton swab. Make up to the mark with CCl_4.
10. Determine the absorbance of the blank, standards and sample at 535nm. Plot a calibration curve. Find out µg equivalent of zinc to the optical density.

APPENDIX 21

Estimation of chromium

Principle

Under acidic conditions, hexavalent chromium reacts with s- diphenyl carbazide to form a reddish violet colored complex which can be determined either visually or photometrically.

Reagents

1. Chromium stock solution: Dissolve 283mg dried potassium dichromate in distilled water and make up to the mark with distilled water.

   1.0ml= 100µg Cr

2. Chromium working solution: pipette 10ml chromium working solution into a 500ml volumetric flask and make up to the mark with distilled water. 1.0ml= 2.0µg Cr

3. Sulfuric acid: 5%- carefully add 50ml conc. H_2SO_4 to 950ml distilled water and cool.

4. Phosphoric acid, 85%

5. Diphenyl carbazide solution: Dissolve 500mg s- diphenyl carbazide and 8g phthalic anhydride in 200ml. 95% ethyl alcohol. Keep it in a refrigerator.

Procedure

1. Place appropriate volumes of chromium working solution in 50ml volumetric flasks or nesslers tube containing 25ml distilled water as the blank.

2. Place 25ml or an aliquot (containing not more than 50µg. Cr) of the neutralized sample in a volumetric flasks or nesslers tube.

3. To the blank, standards and sample add the following reagents were added in order with mixing after each addition:

   (i) 10ml. 5% sulfuric acid
   (ii) 0.4ml. Phosphoric acid and
   (iil. i) 4ml Diphenyl carbazide solution.

4. Dilute to the mark with distilled water, mix well and set aside for 5minutes.

5. Measure the optimal densities of the blank, standards and sample using a spectrophotometer at a wavelength of 540nm taking water as the reference.

6. Prepare a calibration curve. Find out the mg. Cr equivalent to the observed optical density.

APPENDIX 22

Estimation of copper

Principle

Copper is reduced with hydroxyl- amine hydrochloride and the pH is adjusted to 4.0 to 6.0. The cuprous ions react with neocuproin to form a yellow coloured complex which is extracted with chloroform and measured colorimetrically.

Reagents

1. Conc. HCL

2. Hydroxylamine- hydrochloride solution, 20%: Dissolve 50g Hydroxylamine- hydrochloride, NH_2 OH.HCl in re- distilled water and dilute to 250ml.

3. Neo cuproine solution: Dissolve 200mg. neo cuproine (2, 9 dimethyl- 1, 10 phenanthroline hemihydrate) in 200ml methyl alcohol. Keep it in a refrigerator. The reagent is stable under ordinary storage conditions for a month or more.

4. Sodium citrate solution, 25%: Dissolve 125g sodium citrate dehydrate in re- distilled water and dilute to 500ml. transfer to a 1000ml separating funnel, add 10ml hydroxylamine- hydrochloride solution and 10ml neo cuproine solution. Extract with 50ml chloroform to remove copper impurities and discard the chloroform layer.

5. Copper stock solution: place 100.0mg polished electrolytic copper in a conical flask. Add 10ml re- distilled water and 3ml conc. nitric acid. After the reaction is subsided, warm gently for complete dis-solution and then boil to expel oxides of nitrogen. Cool and add about 50ml re- distilled water. Transfer quantitatively to a 1000ml volumetric flask and make up to the mark with re-distilled water. 1.0ml=100µg Cu

6. Copper working solution: pipette 50.0ml of stock solution and make up to 1000ml in a volumetric flask.

Procedure

1. Into a series of 125ml separating funnels, pipette 1.0, 2.0, 4.0, 10.0ml of copper working solution. Dilute to 50ml with re- distilled water. Include a separating funnel containing 50ml re- distilled water as the blank.

2. Place an appropriate aliquot of the sample and dilute to re- distilled water if necessary.

3. Add 0.1ml conc. HCl to each separating funnel and mix well.

4. Add the following reagents in the order with shaking after each addition.

(i) 5ml hydroxylamine- hydrochloride solution

(ii) 10ml sodium citrate solution

(iii) 10ml neocuproin solution

5. Add 20ml chloroform and shake the funnel vigorously. Allow the phases to separate and drain the chloroform layer into 50ml nesslers tubes if photometric comparison is made. Extract with another 20ml portion of chloroform and combine with the first chloroform extract. Make up to 50ml with isopropyl alcohol.

6. Compare the colour of the sample with that of the standards visually or measure the absorbance of standards and samples using blank as the reference at 457nm using a light path of 1cm. Find out the mg equivalent of the sample to the observed optical density. Express the result as mg/l copper (Cu).

APPENDIX 23

Oil and Grease

Principle

Oil and grease are dissolved in a suitable solvent and extracted from the aqueous phase. The solvent layer is then evaporated and the residue is weighed as oil and grease.

Reagents

1. Magnesium sulphate ($MgSO_4$. $7H_2O$)- 1% aqueous solution
2. Milk of lime - 2% aqueous solution
3. Dilute HCL – 25ml of conc. HCL was added to 75ml of distilled water and mixed well.
4. Petroleum ether- Boiling range 40º C to 60º C.
5. Anhydrous sodium sulphate

Procedure

1. 500 ml of well mixed sample was taken in a beaker and 5 ml of magnesium sulphate solution was added. Small amounts of milk of lime were added with continuous stirring until flocculation occurs.

2. The precipitate was dissolved in dilute hydrochloric acid and transferred to the separating funnel. The beaker was washed with 50 ml of petroleum ether and poured into the separating funnel.

3. The aqueous layer was transferred to another separating funnel and again extracted with 50 ml of petroleum ether.

4. Two ether extracts were taken in a 200 ml beaker 2 g of anhydrous sodium sulphate was added. The beaker was covered with a watch glass and the contents were mixed at frequent intervals for about 30 minutes.

5. The contents were filtered into an evaporating dish using Whatmann No. 42 filter paper containing sodium sulphate in its cone, moistened with the solvent. The beaker was washed with two 20 ml portions of petroleum ether and filtered through the same filter paper.

6. Petroleum ether was evaporated by keeping it on a water bath. The dish was weighed. The difference in weight corresponded to the amount of oil and grease present in the aliquot of the sample.

APPENDIX 24

Estimation of total nitrogen (Titrimetric method)

Reagents

1. Mixed indicator solution – dissolved 200mg of methyl red indicator in 100ml 95% ethyl alcohol. Dissolve 100mg methyelene blue indicator in 100ml 95% ethyl alcohol

2. Standard sulphuric acid titrant 0.02N

Procedure

Titrated ammonia in distillate with standard 0.02N H_2SO_4 titrant until indicator turns a pale lavender colour.

Calculation

Mg nitrogen/L = (A-B)X 280ml sample

A= volume of titrated H_2SO_4 for sample (ml)

B= volume of titrated H_2SO_4 for blank (ml)

APPENDIX 25

Estimation of total phosphorus (Calculation)

Material

Sulphuric acid – dilute 2.8ml of concentrated sulphuric acid 1l by adding distilled water. Take 20ml of it and dilute to 1l with distilled water to get 0.002N sulphuric acid

Method

1. Air dried soil sample and take 1g of it in a 500ml flask. Add 200ml of sulphuric acid to it and shake for about half an hr.
2. Filter the suspension through a filter paper. Determine the phosphate content in the filtrate following the method described for inorganic phosphorus in water

Calculation

$$PO4\text{-}P = (mg/l) = \frac{PS \times V}{1000 \times W}$$

APPENDIX 26

Determination of Potassium

Principle

In flame photometry, the solution under test is passed under carefully controlled conditions as a very fine spray in the air supply to a burner. In the flame, the solution evaporates and the salt dissociates to given neutral atoms. A very small proportion of this move into a higher energy state. When these excited atoms fall back to the ground state, the light emitted is of characteristic wavelength which is measured.

Reagents

Triple acid 9:2:1 ratio of concentrated nitric acid: concentrated sulphuric acid and concentrated perchloric acid.

Procedure

1. For potassium estimation 1.0ml of the soil sample was taken in the microkjeldahl flask and 12ml of triple acid was added and the samples were digested over heated stand bath made up to 50ml with distilled water.
2. The potassium content was fed directly to the flame photometer after adjusting the flame photometer to zero with blank and standardizing with 100ppm of potassium solution with 100 galvanometer readings.

3. The galvanometer readings were noted. From the standard curve drawn the corresponding ppm was read. From the ppm, the percentage of sodium of sodium and potassium was calculated

Calculation

Na content (%) = ppm X 100X100

$$\text{Potassium content (\%)} = \text{ppm} \times \frac{100}{10^6} \times \frac{100}{1} \times 100$$

Weight of the sample taken = 'W' (g)

Volume made upto = 'V' (ml)

Content of K or Na in sample material

$$\text{With reference to standard graph} = \frac{\text{Ppm}}{10^6} \times \frac{100}{1} \times 100$$

$$\text{The percentage of K or Na} = \frac{\text{Ppm}}{10^6} \times \frac{100}{W}$$

APPENDIX 27

Estimation of sodium (Calculation)

Material: Ethyl alcohol, ammonium acetate

Method

Prepare the soil extract as described for calcium. Determine the sodium content in extract following the method of sodium determination

Calculation

$$\text{Sodium (mg/g)} = \frac{A \times V}{W \times 1000}$$

APPENDIX 28

Estimation of calcium (Calculation)

Material

Ethyl alcohol, ammonium acetate

Method

1. Preparation of soil extract – air dry the soil and take 50g of it a flask. Add 100ml of 40% of ethyl alcohol, shake well, wait for about ethyl alcohol and filter the suspension through filter paper

2. Further wash the soil residue on filter paper with 40% ethyl alcohol and family with absolute ethyl alcohol and finally with absolute ethyl alcohol

3. Transfer the residue to a beaker, add 100ml of ammonium acetate solution, stir, and allow to stand overnight.

4. Filter the supernatant through filter paper and collect the filtrate.

Calculation

$$\text{Calcium (mg/g)} = \frac{T \times 400.4 \times V_1}{V_2 \times W \times 1000}$$

APPENDIX 29

Estimation of iron (Calculation)

Method

1. Take 10g of air dry soil and add 100ml of distilled water to make 1:10 w/v suspension

2. Filter it through a filter paper and determine the iron in filterate following the method used for iron estimation in water

3. Analysis a fraction of soil sample for moisture content as described earlier

Calculation

$$\text{Iron (mg/g)} = \frac{F}{10} \times \frac{V}{W} \times \frac{1}{(1000 - M)}$$

APPENDIX 30

Estimation of copper, zinc, chromium, cadmium, nickel and manganese (Atomic absorption spectrophotometric method)

Principle

The technique involves determination of metals by the measurement of an element. When the radiation characteristic to a particular element passes through the atomic vapour of the element, absorption, of radiation occurs in proportion to the concentration of the atoms in the light path. The source of characteristic radiation is a hollow cathode lamp, the cathode being made of the element desired to be estimated.

Reagents

Air cleaned and dried through a suitable filter to remove oil, water and other foreign substances. The source may be a compressor or commercially bottled gas.

1. Acetylene, standard commercial grade
2. Metal free water
3. Calcium solution: Dissolved 630mg calcium carbonate in 50ml of 1 + hydrochloric acid and diluted to 1000ml with water
4. Hydrochloric acid 1%, 10% and 20%
5. Lanthanum solution: Dissolved 58.65g lanthanum oxide in 250ml concentrated hydrochloric acid added and slowly until the material was dissolved and diluted to 1000ml with water.
6. Hydrogen peroxide (30%)
7. Nitric aid (2%)
8. Standard metal solution: prepared a series of standard metal solution in the optimum concentration range by appropriate dilution of the following stock solutions with water containing 1.5ml concentrated nitric acid/L
9. Copper: Dissolved 0.1g copper metal in 2ml of concentrated nitric acid and diluted with water 1ml - 100µg copper

10. Zinc: Dissolve 100mg, 30-mesh zinc metal in a slight excess of 1_1 HCl, dilute to 1000ml with water 1ml - 100µg zn
11. Chromium: Dissolved 0.1923g CrO_3in water acidified with 10ml concentrated nitric acid and diluted to 1000ml with water. 1ml = 100µg chromium
12. Cadmium: weight 100mg pure Cd metal and HCl use heat to assist metal dissolution, transfer quantitatively to a one liter volumetric flask and dilute to 1000m
13. Nickel: Dissolve 447.9mg nickel sulphate in 1000ml distilled water
14. Manganese: Dissolve 10mg of manganese into a volumetric flask with 10ml concentrated HCl and dilute to 1000ml with water.

Sample preparation

10g of the soil was taken in 100ml kjeldahi flasks. Added 25ml of 3:2:1 triple acid mixture (concentrated nitric acid: concentrated perchloric acid: concentrated sulphuric acid) and left a side for 3-4hrs in a fume cupboard. Then heat for 30min until the initial vigorous reaction has subsided. Heated more strongly for 4hrs until the nitrous fumes were removed and white fumes of perchloric acid were with 3-4 washings of deionised water to 10ml volumetric flask and made up to the mark with water.

Procedure

Selected at least there concentrations of each standard metal solution to find out the expected metal concentration of a sample. Then aspirated each standard in turn into flame and recorded the absorbance.

Prepared a calibration curve by plotting the absorbance of standards verses their concentrations. Plotted calibration curve for chromium based on original concentration of standard before the addition of hydrogen peroxide. Rinsed nebulizer by aspirating water containing 1.5ml concentrated nitric acid. Aspirated the sample and determined its absorbance against blank. The estimation of chromium, copper, zinc, cadmium, nickel and manganese were done at the wavelengths of 357.9, 324.7, 248.3, 213.9, 345.6 and 3.09.5nm respectively.

Calculation

Calculated the concentration of each metal ion in milligrams per liter by referring to the appropriate calibration curve.

APPENDIX 31

Haematological parameters

Red blood corpuscle (RBC) count

RBC count was made with a Neubauer crystalline counting chamber as described by Davidson and Henry (1969). The blood from the control fish and each group of treated samples were collected in a vial containing 2% ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. The blood was drawn up to 0.5 marks in RBC pipette and immediately the diluting fluid was drawn up to the mark 101 (thus the dilution is 1:200). The solution was mixed well by shaking gently. It was allowed to stand for 2 or 3 minutes. The counting chamber and cover glass were cleansed and the cover glass was placed over the ruled area. Again the solution was mixed gently and stem full of solution was expelled and a drop of fluid was allowed to flow under the cover slip holding the pipette at an angle of 40°, it was allowed to stand for 2 to 3 minutes to allow RBC to settle. Afterwards the ruled area of the counting chamber was focused under the microscope and the number of RBC's were counted in five small squares of the RBC column under high power and the number of RBC per cu mm were calculated accordingly.

$$\frac{\text{No. of cells X dilution factor X depth factor}}{\text{Area conuted}}$$

White blood corpuscles (WBC) count

Blood is drawn from the vial into WBC pipette up to 0.5 marks and immediately the diluting fluid is drawn up to 11 marks. The solution is mixed thoroughly by shaking gently. The rest of the procedure is the same as described by Davidson and Henry (1969) for RBC count. In case of WBC, count was made in bigger squares of the chamber. The WBC count was expressed in cu mm.

Estimation of haemoglobin (Hb)

The hemoglobin concentration was estimated by Acid - haematin method (Sahli, 1962). N/10 hydrochloric acid was taken up to 20 marks in a graduated tube. Blood was collected directly from the eyeball up to 20 cu mm in the Hb pipette and the outer side was wiped out and this was transferred into the graduated tube containing N/10 hydrochloric acid. Pipette was rinsed two or three times with dilute hydrochloric acid. It was allowed to stand for 10 to 20 minutes after thorough mixing. Then N/10 HC1 was added drop by drop, mixing between each addition until the blood color matched with the standard color. And then the results were read from the scale on the graduated tube and the Hb concentration was expressed in grams percent.

Hematocrit (Packed Cell Volume)

Hematocrit was estimated by employing micro hematocrit (capillary) method by sodium heparinized micro hematocrit capillaries as described by Nelson and Morris (1989) using RM 12°C micro centrifuge and a micro hematocrit reader.

Principle

When anticoagulant whole blood is centrifuged at a constant speed, erythrocytes (RBC), which are heavier than white cells, platelets and plasma, are settled at the bottom. This red cell column is called hematocrit or packed cell volume which is expressed as fraction of the whole blood (level of plasma).In microhematocrit method, the anticoagulated blood is centrifuged in a sealed capillary tube and with help of a special hematocrit reader the volume of packed red cells and percentage of the whole blood (level of plasma) are determined.

Procedure

Two sodium heparinized capillary microhematocrit tubes each having length about 7 cm long with a uniform bore of about 1mm were taken. Then the blood from control and experimental groups kept in their respective vials were filled up to 5 cm by capillary movement. The sucking end of each tubes were sealed with modeling clay and the filled tubes were placed in radial grooves of the microhematocrit centrifuge head with the sealed end away from the centre and speed at 10,000 rpm for 5 min, using RM 12°C micro centrifuge. The tubes were then taken and the length of the whole column including

plasma and that of the red cell column alone was measured in a millimeter rule of the microhematocrit reader. The concentration of the red cells were taken as the hematocrit value which is expressed in percentage.

APPENDIX 32

Biochemical analyses

Estimation of plasma protein

Plasma protein estimation was done according to the method of Lowry et al. (1951).

Principle

The final blue color of protein is produced by the reaction of carbamyl groups of protein molecules in the sample with alkaline copper and potassium of the reagent. This complex together with tyrosine and tryptophan of the sample is produced with phosphomolybdate of the Folin phenol reagent.

Reagents

Solution A: 2.00 gm of sodium carbonate was dissolved in 100.00 ml of 0.1N NaOH.

Solution B: 500.00 mg of copper sulphate was dissolved in 100.00 ml of 1% sodium potassium tartarate solution.

Solution C: 50.00 ml of solution A was mixed with 1 ml of solution – B.

Folin – phenol reagent : 1.0 ml of Folin – phenol reagent was mixed with 1.0 ml of double distilled water.

Procedure

Five test tubes were taken and marked as Blank (B), Test (UT), Test (T), Control (C) and Standard (S). 0.10 ml of plasma from control and methyl parathion treated fish was taken in respective tubes (Control and Test tubes). Then 0.90 ml of distilled water was added. 1 ml of distilled water was taken in 'Blank' tube. They were treated with 5.0 ml of Solution – C for 10 minutes, and then 0.5 ml of Folin-Phenol reagent was added to each tube. The colour intensity (O.D) of Control (C) and Test (UT), Test (T) against Blank (B) was read after 15 min at 720 nm by using UV Spectrophotometer. For the preparation of

'Standard'(S) 1.0 mg of bovine serum albumin (Sigma- Chemical company, USA). Biochemical parameters 69 was added to 10.0 ml of IN NaOH and made upto 100.0 ml in a solution standard flask. From this, 1.0 ml of solution was taken in 'Standard' tube and mixed with 0.5 ml of Solution C, kept for 10 min, then 0.5 ml of Folin Phenol reagent was added. The optical density of the 'Standard' (S) was read as mentioned above.

Calculation

$$\frac{\text{OD of Unknown}}{\text{OD of Known}} \times \text{Concentration of Standard} = \mu g \text{ of protein in ml of plasma}$$

Estimation of plasma glucose

Plasma glucose was estimated by O-Toluidine method (Cooper and McDaniel, 1970).

Principle

Glucose reacts with O-Toluidine in presence of acetic acid to form a green colour derivative which is measured at 630 nm by using UV Spectrophotometer.

Reagent utilized

Reagent 1 : O-Toluidine colour reagent

Reagent 2 : Glucose standard, 100 mg%

Procedure

Five test tubes were taken and marked as Blank (B), Control (C), Test (UT), Test (T) and Standard (S). To each test tube 5 ml of Reagent-1 (O-Toluidine colour reagent) was added. Then 0.1 ml of distilled water was added to the test tube marked B (Blank). Similarly, 0.1 ml of plasma from control and methyl parathion treated fish was added to the respective tubes (Control and Test tubes). Then, 0.1 ml of Reagent -2 (Glucose standard) was added to the test tube marked as S (Standard). The contents in all the tubes were mixed well and heated in boiling water for 10 minutes. Then, the test tubes were cooled under running tap water for 5 minutes and the optical density of the test samples were measured at 630 nm within 30 minutes against blank using UV Spectrophotometer

Calculation

$$\text{Plasma glucose in mg/100 ml} = \frac{\text{O.D. of the test}}{\text{O.D. of the Standard}} \times 100$$

APPENDIX 33

Enzyme analyses

1. Estimation of Plasma Glutamic oxaloacetate transaminase (GOT)

GOT activity was estimated by 2, 4-DNPH method (Reitman and Franckel, 1957).

Principle

GOT catalyses the transamination of L- Aspartate and α- ketoglutarate (α-KG) to form Oxaloacetate and L- Glutamate. Oxaloacetate so formed is coupled with 2, 4-Dinitrophenyl hydrazine (2, 4-DNPH) to form a corresponding hydrazone, a brown coloured complex in alkaline medium and this can be measured colorimetrically.

ά- Ketoglutarate +L-Aspartate ↔ L-Glutamate+ Oxaloacetate

Oxaloacetate + 2, 4-DNPH ↔ Corresponding Hydrazone (brown colour)

Reagents

Reagent 1: Buffered Aspartate ά-ketoglutarate substrate, pH 7.4

Reagent 2: 2, 4-DNPH colour reagent

Reagent 3: Sodium hydroxide, 4N

Reagent 4: Working Pyruvate standard, 2mm.

Preparation of working solution

Solution 1: Dilute 1ml of Reagent 3 to 10ml with purified water.

Procedure

Five test tubes were taken and marked as Blank (B), Standard (S), Test (UT), Test (T) and Control (C). To each test tube 0.25 ml of Reagent 1 (Buffered Aspartate

ά-ketoglutarate substrate) was added. Then 0.05 ml of sample from untreated, treated fish and Standard (Reagent 4) were added to respective 'Test' and 'Standard' tube, respectively, mixed well and incubated at 37ºC for 60 minutes. Then 0.25 ml of Reagent-2 (2, 4-DNPH colour reagent) was added to all tubes and mixed well. After that 0.05 ml of deionised water and sample were added to the test tubes 'Blank' and 'Control', respectively and mixed well. All test tubes were allowed to stand at room temperature for 20 minutes. Then to all the test tubes about 2.5 ml of Solution-1 was added, mixed well and allow to stand at room temperature for 10 minutes. The O.D values were measured against distilled water using UV Spectrophotometer at 505 nm. The same procedure was repeated for control sample.

Calculation

$$\text{GOT activity IU/L} = \frac{\text{Absorbance of Test- Absorbance of Control}}{\text{Absorbance of Standard- Absorbance of Blank}} \times \text{Conc. of Standard}$$

2. Estimation of Plasma Glutamic pyruvate transaminase (GPT)

GPT activity was estimated by 2, 4-DNPH method (Reitman and Franckel, 1957).

Principle

GPT catalyses the transamination of L- Alanine and ά-Ketoglutarate to form pyruvate and L- Glutamate. Pyruvate so formed is coupled with 2, 4- Dinitrophenyl Hydrazine to form a corresponding hydrazone, a brown coloured complex in alkaline medium and this can we measured calorimetrically.

ά-Ketoglutarate +L-Alanine ↔ L-Glutamate+ Pyruvate

Pyruvate +2, 4- DNPH ↔ Corresponding Hydrazone (brown colour)

Reagents

Reagent 1: Buffered Alanine ά- KG substrate, PH 7.4

Reagent 2: DNPH color Reagent Reagent 3: Sodium Hydroxide, 4N

Reagent 3: Working pyruvate standard, 2mM.

Preparation of working solution

Solution I: Dilution 1ml of Reagent 3 to 10 ml with purified water.

Procedure

Five test tubes were taken and marked as Blank (B), Standard (S), Test (UT), Test (T) and Control (C). To each test tube 0.25 ml of Reagent 1 (Buffered Alanine ά- KG substrate) was added. Then 0.05 ml of sample from untreated, treated treated fish and Standand (Reagent – IV) were added to respective 'Test' and 'Standard' tubes, respectively, mixed well and incubated at 37ºC for 30 minutes. Then 0.25 ml of Reagent-2 (DNPH color reagent) was added to all tubes and mixed well. After that 0.05 ml of deionised water and sample were added to the test tubes 'Blank' and 'Control', respectively and mixed well. All test tubes were allowed to stand at room temperature for 20 minutes. Then to all the test tubes about 2.5 ml of Solution-1 was added, mixed well and allow to stand at room temperature for 10 minutes. The O.D values were measured against distilled water using UV Spectrophotometer at 505 nm. The same procedure was repeated for control sample.

Calculation

$$\text{GPT activity (IU/L)} = \frac{\text{Absorbance of Test- Absorbance of Control}}{\text{Absorbance of Standard- Absorbance of Blank}} \times \text{Conc. of Standard}$$

3. Estimation of Plasma Lactate Dehydrogenase (LDH)

LDH activity in plasma was estimated by 2,4-DNPH method (King, 1959) using Diagnostic Reagent Kit supplied by Span Diagnostics Pvt. Ltd., Surat, India.

Principle

Lactate dehydrogenase catalyses the following reaction:

LDH Lactate + NAD ^====* Pyruvate + NADH

Products so formed are coupled with 2,4- Dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydrazone, which gives brown colour in alkaline medium.

Reagents

Reagent 1 Reagent 3 Reagent 4 Reagent 5 Reagent 6

Buffered Lactate Substrate, pH 10.0

Reagent 2(A)

NAD for Test

Reagent 2(B)

NAD for Graph DNPH Colour Reagent

NADH

Sodium hydroxide, 4N

Working Pyruvate Standard, 1 mM 143

Preparation of Working Solutions

Solution I (A) : Just before use each vial of Reagent - 2(A) (NAD for Test) was reconstituted with 0.3 ml of distilled water.

Solution I (B) : Just before use, each vial of Reagent - 2(B) (NAD for Graph) was reconstituted with 1.4 ml of distilled water.

Solution II : Just before use, each vial of Reagent - 4 (NADH) was reconstituted with 1.2 ml of Reagent-1 (Buffered Substrate).

Solution III : Reagent - 5 (Sodium hydroxide, 4N) was diluted 1 to 10 with distilled water

Preparation of Standard Curve

Seven test tubes were taken and serially numbered from 1 to 7. They represented 0, 167, 333, 500, 667, 833 and 1000 lU/litre of enzyme activity. Prescribed volumes of Reagent - 6, Solution - II, Reagent - 1, Solution I (B) , distilled water and Reagent - 3 were added to each of the tubes, mixed well and incubated at 37°C for 5 min. Then 5.0 ml of Solution -III was added to each of the tubes. The contents were mixed well by inversion

and allowed to stand at room temperature for 5 min. The O.D. of the contents of the tubes from 2 to 7 were measured against that of tube 1 (Blank) at 440 nm using spectrophotometer (Spectronic - 20, Baush and Lomb, USA). The standard curve was plotted by taking enzyme activity (IU/litre) on' the X-axis and O.D. on the Y-axis.

Procedure

For the estimation of LDH in plasma, two test tubes marked as "Control" (C) and "Test" (T) were taken. To the 'Control' and 'Test' tubes, 0.5 ml of Reagent - l was added. To the tube marked 'Test' 0.05 ml diluted plasma was added and to the tube marked 'Control', 0.01 ml of distilled water was added. The contents in the tubes were mixed well and incubated at 37°C for 5 min. Then 0.1 ml of Solution -I (A) was added to the 'Test' tube, mixed well and both the 'Control' and 'Test' tubes wereincubated for 37°C for 15 min. The tubes were taken out and 0.5 ml of Reagent - 3 was added to both the tubes. Immediately 0.05 ml of diluted plasma was added to the 'Control' tube alone. Once again the contents in both the tubes were mixed well and incubated at 37°C for 15 min., after which the tubes were taken out and 5.0 ml of Solution -III was added to both the tubes. The contents were mixed well by inversion and allowed to stand at room temperature for 5 min. The O.D. of 'Control' and 'Test' were measured against distilled water using spectrophotometer (Spectronic - 20, Baush and Lomb, USA). Calculation The nett O.D. of the Test = O.D. Test - O.D. Control The nett O.D. of the test was marked on Y-axis of the standard curve and it was extrapolated to the corresponding enzyme activity on the X - axis.

4. Estimation of Reduced Glutathione (GSH)

Reduced glutathione was determined by the method of Ellman (1959)

Principle

Reduced glutathione on reaction with DTNB (5, 5'-dithiobis nitro benzoic acid) produces a yellow coloured product that absorbs at 412nm.

Reagents

1. TCA (5%)
2. Sodium phosphate buffer (0.2M, pH 8.0)

3. DTNB (0.6mM in 0.2M phosphate buffer)

4. Standard GSH (10nmoles/ml of 5% TCA)

Enzyme extract

0.5ml of sample homogenate was pipette out and precipitated with 2.0ml of 5% TCA. The precipitated sample was centrifuged at 1000rpm for 10 minutes. The supernatant (0.1ml) was used for the estimation of GSH.

Procedure

The supernatant (0.1ml) was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0). Standard GSH corresponding to concentrations ranging between 2 and 10 nmoles were also prepared. Two ml of freshly prepared DTNB solution was added and the intensity of the yellow colour developed was measured in a Spectrophotometer at 412nm after 10 minutes. The values are expressed as n moles GSH/µm sample.

APPENDIX 34

Histological Studies

For histological studies the methods of Pearse (1968), Roberts (1978) and Humason (1979) were followed.

Fixation

Gill, liver and kidney of fish Labeo rohita were carefully dissected out from control and treated groups and they were cut into bits of 1 to 2 cm in diameter. The sections were immediately put in Bouin‘s fluid fixative and kept for a period of 24 h (to prevent post-mortem changes and shrinkage during dehydration, embedding and sectioning processes).

Washing

After 24 h fixation in Bouin‘s fluid fixative, the sections were taken and the excessive fixative was removed by transferring the sections to 50% alcohol (to prevent interference with subsequent process). In the present study washing with running water is avoided to prevent the loss of soluble picrates.

Dehydration

After the removal of the excessive fixatives, the sections were put in alcohol series (30 %, 50 %, 70 %, 90 % and 100 %) for a period of 30 minutes in each alcohol series. A minimum of two or three changes were given in absolute alcohol (100 %) for the complete removal of water from the sections.

Clearing

During clearing, the alcohol from the sections was removed by using xylene as a clearing agent. The sections were kept in xylene for 30 minutes to 1 h until they became transparent.

Infiltration

During infiltration, xylene from the sections was replaced by using paraffin wax. The sections were kept in a paraffin embedding bath which contained metal cups filled with paraffin at 58º-60ºC. A minimum of three changes were given in paraffin wax with 30 minutes duration in each.

Embedding

The organ/tissues were embedded in L‘ blocks filled with molten paraffin wax with proper orientation. Then, blocks were kept in water overnight to ensure complete solidification and the blocks were removed for sectioning.

Sectioning

For sectioning, organ/tissues were cut with 7µ thickness by using rotary microtome. The sections were spread on a glass slide using egg albumin as an adhesive and were slightly heated in a spirit lamp. After complete spreading, the sections were placed in an oven overnight at 37ºC and the sections were taken for staining.

Staining

Paraffin wax from the sections was removed by using xylene as dewaxing agent. Then the sections were hydrated by immersing in descending grades of alcohol (absolute alcohol, 90%, 70%, 50%, 30%) for about 1-2 minutes in each alcohol series. The sections were stained in Heidenhain‘s iron haematoxylin stain for 2 to 5 min and they were washed in tap water until the sections became bluish black in colour and then stained in 1% Eosin.

For destaining, 1% Iron alum was used. The sections were dehydrated through ascending grades of alcohol (30%, 50%, 70%, 90%, absolute alcohol) for about 5 seconds in each alcohol series. Then the sections were cleared in xylene and mounted permanently in a glass slide using DPX mountant with cover glass. The histopathological changes of gill, liver and kidney of fish from control and treated groups were given as photomicrographs in appropriate places in the text.

APPENDIX 35

Bacterial toxicity (agar - well diffusion method)

Principle

The anitimicrobials present in the sample was allowed to diffuse out into the medium and interact in a plate freshly seeded with the test organisms. The resulting zones of inhibition will be uniformly circular as there will be confluent lawn of growth. The diameter of zone of inhibition can be measured in millimeter.

Reagents

1. Muller Hinton Agar Medium (1 L)

The medium was prepared by dissolving 38g of the commercially available Muller Hinton Agar Medium (HiMedia) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure 121° C for 15 minutes. The autoclaved medium was mixed well and poured onto 100mm petriplates (25 – 30 ml/ plate) while still molten.

2. Nutrient broth (1 L)

One litre of nutrient broth was prepared by dissolving 13 g of commercially available nutrient broth medium (HiMedia) in 1000 ml of distilled water and boiled to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure at 121° C for 15 minutes.

Procedure

Petriplates containing 20 ml Muller Hinton medium were seeded with 24 hours culture of bacterial strains separately. Wells were cut and 10 µl of the sample (untreated, treated and control) were added. The plates were then incubated at 37° C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well (NCCLS, 1993).

APPENDIX 36

Fungal toxicity (Agar plug method)

The activity of the sample on various fungal strains was assayed by agar plug method.

Principle

The fungicidal effect of the sample can be assessed by the inhibition of mycelial growth of the fungus and was observed as a zone of inhibition near the wells.

Reagents

1. Rose Bengal Chloramphenical Agar medium (1 L)

   The commercially available (HiMedia) Rose Bengal Chloramphenical agar medium (32.15 g) was suspended in 1000ml of distilled water. The medium was dissolved completely by boiling and was then autoclaved at 15 lbs pressure (121° C) for 15 minutes.

Procedure

Rose Bengal Chloramphenical Agar medium was prepared and poured on to the petriplates. A fungal plug was placed in the center of the plate. Wells were cut and 10 µl of the sample (untreated, treated and control) were added.